Molecular analysis of inflammation, oxidative stress, and gastric carcinogenesis: Signal transduction and gene expression changes.

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Molecular Analysis of Inflammation, Oxidative Stress, and Gastric Carcinogenesis:

Signal Transduction and Gene Expression Changes

By

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A thesis submitted for the partial fulfilment of the requirement for the degree of Doctor of Philosophy

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Declaration

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Summary

Long term gastric inflammation (chronic gastritis) and its coupled tumourigenic inflammatory milieu (Reactive Oxygen/ Nitrogen Species (RO/NS), cytokines, etc.) is seen to impact cells at several levels, and as such is seen to be a major driving force of gastric carcinogenesis.

The goal of this investigation was to examine the impact of inflammation, with a special focus on the oxidative stress component, on signal transduction and gene expression changes in gastric epithelial cells at the levels of Mitogen Activated Protein Kinase (MAPK) and Nuclear Factor Kappa-B (NFκB) pathways and downstream gene expression targets. Both *in vitro* and *in vivo* studies were performed, and a combination of microarray, real-time PCR, and western blot methodologies were employed to evaluate signalling and gene expression changes.

Two *in vitro* models were utilised, comprising an initial chemically induced oxidative stress model, in which cells were exposed to hydrogen peroxide (H_2O_2), and a more elaborate inflammatory model whereby cells were exposed to leukocytes optimised to undergo an oxidative burst response. In both cases, MAPK and NFκB signal transduction pathways were seen to be affected, at the levels of the pathways themselves, and downstream gene expression targets, with over-expression of c-fos and interleukin – 8 (IL-8) being the most consistently observed changes.

Analysis of pre-malignant gastric biopsy specimens brought to light the potential importance of aberrant MAPK signalling and c-fos over-expression in the earliest stages of disease pathogenesis, the changes being observed most commonly in chronic inflammation/ gastritis tissues, so translating *in vitro* findings to a more clinically relevant setting.

Overall, the findings provide strength to the notion that oxidative stress is a key player in gastric carcinogenesis, seen here at the levels of signalling and gene expression changes. Oxidative stress and the MAPK and NFκB pathways emerge as potential therapeutic targets for the management of gastric cancer.

Abbreviations

- AP-1 Activator Protein - 1
- APS Ammonium persulphate
- bp Base pairs
- cDNA Copy DNA
- CDK Cyclin dependent kinase
- C_T Threshold cycle
- DAPI 4, 6-diamidino-2-phenylindole
- DMSO Dimethyl sulfoxide
- DNA Deoxyribonucleic acid
- dNTP Deoxynucleotide triphosphate
- EDTA Ethylenediaminetetraacetic acid
- ELISA Enzyme – Linked Immuno – Sorbent Assay
- ERK Extracellular Signal Regulated Kinase
- FISH Fluorescence *in situ* hybridisation
- FITC Fluorescein isothiocyanate
- fMLP N-Formyl-L-methionyl-L-leucyl-L-phenylalanine
- GFP Green Fluorescent Protein
- HRP Horseradish peroxidase
- IL-8 Interleukin – 8
- IκB Inhibitor of Kappa – B
- IKK Inhibitor of Kappa – B Kinase
- LOH Loss of heterozygosity
- LPS Lipopolysaccharide
- MAPK Mitogen Activated Protein Kinase
- mRNA Messenger RNA
- MTS 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt

- NFκB Nuclear Factor Kappa – B
- NIK NFκB Inducing Kinase
- PBS Phosphate buffered saline
- PCR Polymerase chain reaction
- RNA Ribonuclei acids
- RNS Reactive Nitrogen Species
- ROS Reactive Oxygen Species
- RO/NS Reactive Oxygen and/ or Nitrogen Species
- RT-PCR Real Time – polymerase chain reaction
- SDS Sodium dodecylsulphate
- SSC Saline sodium citrate
- TEMED NNN'N'-tetramethylethyl-1, 2-diaminoethane
- T_M Melting temperature
- TNF Tumour Necrosis Factor
- TNFR Tumour Necrosis Factor Receptor
- Trafs Tumour Necrosis Factor Receptor – Associated Factors
- v/v Volume per volume
- VEGF Vascular Endothelial Growth Factor
- w/v Weight per volume

Special Note: Throughout this thesis highly reactive oxygen/ nitrogen centred species and their derivatives are collectively referred to as ROS, RNS, or RO/NS and the terms are often used interchangeably since it is difficult to determine which species are important in a given tissue setting due to their high reactivity and transient nature, with several different species being present at any given time.

Contents

Chapter 1

General Introduction

1.1 Cancer

Cancer is a major health burden and cause of morbidity in the UK, more than 270,000 new cases were registered in the year 2000 alone, over half of these cases being fatal if past trends continue (Working Group on Diet and Cancer, 1998). More than one in three people will be diagnosed with cancer in their lifetime, and one in four people will die from the disease.

Cancer is a complex disease. The first historical description of the condition was in relation to breast carcinoma (discovered in Egypt and dates back to approximately 1600 B.C. The Edwin Smith Papyrus, thought the term *cancer* was not used). The term 'cancer' now encompasses over 200 different forms of the disease, characterised by uncontrolled, abnormal growth of cells that have the potential to invade surrounding tissues and spread locally or throughout the body via the bloodstream and lymphatics. Almost every tissue in the human body is susceptible to the development of cancer and cancers at the different sites are considered different forms of the disease, each with different aetiology, pathogenesis, clinical manifestations, prognosis, and treatment regimes. Cancer is the result of aberrant changes in cells leading to abnormal cellular proliferation whereby cells divide uncontrollably and gain the potential to invade and destroy surrounding normal tissues.

The disease has a recognised genetic basis. In fact this notion has held strong since 1914 when Theadore Boveri hypothesised that clonal chromosomal changes were responsible for cancer development (Cowell, 2001). However, unlike other types of genetic disorder, for most cancers the genetic changes leading to the disease are not inherited but rather arise in somatic cells as a result of environmental exposures/ factors. Hence, genes and environment play important roles in carcinogenesis. In addition, cancer, unlike conventional genetic diseases, is usually the end result of an accumulation of multiple genetic defects giving cells a growth advantage driving clonal expansion and ultimately progression to a neoplasm. Advances in molecular

cancer research have provided an explosion of evidence supporting the link between genetic instability and malignancy. This instability manifests itself in the form of tiny aberrations such as point mutations through to gross chromosomal abnormalities. Genomic aberrations frequently cited in the expanding body of literature connecting cancer development with genetics include:-

- Presence of damaged and/ or aberrant numbers of chromosomes in tumour cells.
- Defective DNA repair mechanisms eliciting increased susceptibility to cancer development.
- DNA sequence alterations which may be caused by mutagenic compounds that have hence been deemed carcinogenic.
- Inheritance of defective genes that cause hereditary syndromes that predispose to various types of cancer (Bishop, 1987).
- Gene expression changes in malignant cells.

On these grounds, cancer is considered a phenotype, resulting from the accumulation of multiple molecular defects that progressively drive multi-step tumourigenesis from a normal cell, through pre-malignant foci (benign tumours) to localised tumours (*in situ* carcinomas) and ultimately truly malignant invasive and metastatic cancers (Loeb, 1991).

Most of the factors that cause cancer act by damaging genes through induction of genetic mutations, either directly or indirectly (Bishop & Weinberg, 1996). The molecular changes that drive cancer development include gene mutations (point mutations, deletions, insertions); chromosomal aberrations (aneuploidy, structural rearrangements); and gene and protein expression changes.

1.1.1 Multi-step nature of Carcinogenesis

It is now widely accepted that malignant tumours develop through a multi-step mechanism resulting from accumulation of numerous genetic errors in critical regulatory pathways. The detectable presence of multiple genetic alterations in tumour cells strongly implicated and supported the idea that the molecular abnormalities accumulate in cells in a step-wise fashion during neoplastic progression

(Yokota, 2000). This concept is supported by both molecular and clinical observations. From a clinical view-point, the step-wise nature of cancer development is reflected in the several types of morphological and histological pre-malignant lesions, such as hyperplasia, metaplasia, and dysplasia detected on the road to malignant tumour development in many diverse tissues. This is exemplified by the progression of colorectal carcinogenesis (Vogelstein and Kinzler, 1993). At the molecular level, comparative molecular analyses between pre-malignant, and early and late stage tumours frequently show an increase in frequency of genetic aberrations at the later stages (Yokota, 2000). Again this verifies the multi-step accumulation of genetic changes that accompany cancer formation. Further support comes from epidemiological studies, which show that, with the exception of some rare childhood cancers, cancer is generally a disease of old age (King, 1996; Vogelstein and Kinzler, 1993).

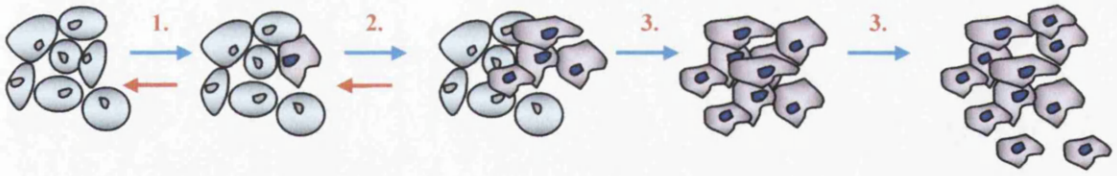
Cells are seen to undergo marked changes in behaviour and morphology during cancer development and this is considered to be a consequence of the build-up of sequential genetic changes. On the whole, the tumours that develop from these cells go on to become more and more aggressive and malignant in time. This phenomenon is termed tumour progression (Nowell, 1986), following on from initiation and promotion, all together comprising the three recognised stages in multi-step carcinogenesis (summarised in table 1.1 and fig. 1.1) Progression is possibly the most critical stage of carcinogenesis, as at this time, cells within localised/ *in situ* tumours acquire the capacity to invade surrounding tissues (by detaching from neighbouring tumour cells and cells of the resident tissue) and metastasise to secondary, often distant sites in the body (by migrating along nerves, vessels, and lymphatics (McKinnell *et al.* 1998)). These two acquired characteristics define malignancy, and often lead to morbidity and mortality. It is thus fundamental from a clinical perspective to gain further understanding of carcinogenesis in order to be able to potentially intervene at earlier stages, hence putting brakes on, or preventing malignancy.

In the present research gastric cancer and its pre-malignant stages are of particular interest.

Table 1.1 The three stages of carcinogenesis.

Stage	Description
Initiation	The stage at which an initial molecular change is introduced into a cell giving it potential to develop further into a pre-malignant population of cells. Initiating events are normally induced by endogenous (e.g. hormonal stimuli, endogenous metabolic processes leading to free radical generation and damage to critical cellular macromolecules) or exogenous (environmental exposures/ insults) carcinogens.
Promotion	An event stimulating proliferation of an initiated transformed cell giving rise to a pre-neoplastic/ neoplastic population of cells. May occur as a result of exposure to promoting agents or due to the acquisition of further random genetic changes in the initiated cell(s).
Progression	The final step in the conversion of benign to fully malignant cells. At this stage cells show all of the classic attributes of cancer cells. Cells exhibit autonomous growth, often show more rapid growth, invasiveness, metastasis, and increased genetic instability.

Figure 1.1 Multi-step nature of carcinogenesis. Step 1 – initiation (induction of an oncogenic mutation, reversible step DNA damage vs. repair), 2 – promotion (stimulated proliferation of initiated transformed cell, reversible step since cell cycle homeostasis can be normalised by removal of promoting stimulus), and 3 – progression – adoption of a truly malignant phenotype (irreversible).



Normal cell population. Any tissue may be affected

Transformed pre-neoplastic cell (lilac) due to genetic insult. Dysregulated cell growth

Neoplastic population of cells. ↑ proliferative potential. ↑ aggressive growth

Local/ in situ tumour mass. Further mutation and epigenetic changes

Increased malignancy, invasiveness & metastasis.

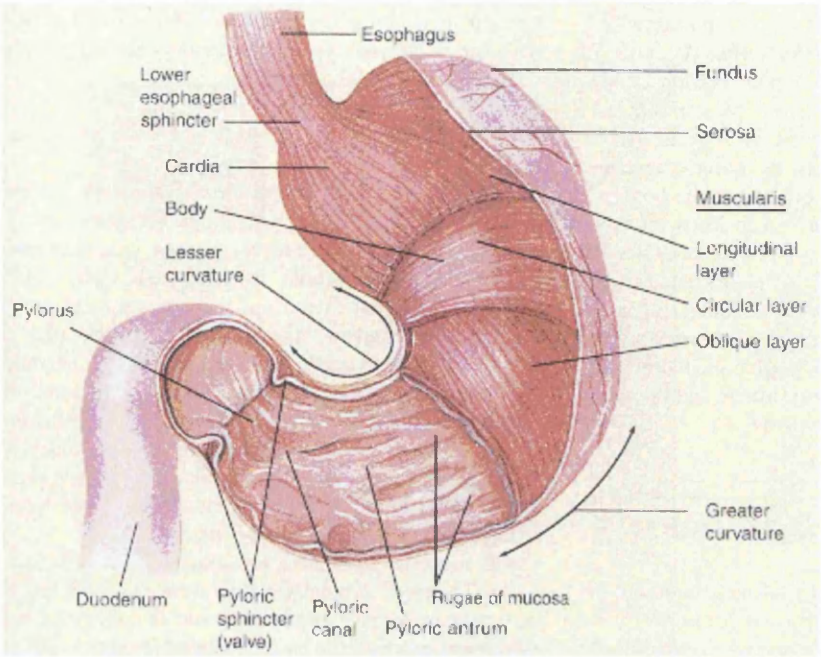
1.2 The Stomach

The stomach is a dilated muscular portion of the gastrointestinal (GI) tract, the major function of which is storage and partial digestion of fragmented food entering via the oesophagus. Anatomically, the organ lies in the upper left of the abdomen just under the diaphragm, and is between the oesophagus and duodenum (the first part of the small intestine). The primary roles of the stomach are to retain fragmented food while it is partially broken down and digested, to begin digestion of proteins, to aid in the absorption of vitamin B12, and to store food prior to its passage to the small intestine. The stomach is well adapted to its roles. Unlike the two muscular layers found in the rest of the gastrointestinal tract, the stomach has three muscular layers in its wall, – circular, longitudinal, and oblique, contractions of which cause the stomach wall to begin powerful wave-like contractions that assist in the mixing up and breaking down of food into a semi-liquid paste, or chyme. The muscular contractions mix the food up with the secretions of the gastric mucosa (stomach juices) containing hydrochloric acid (HCl) and pepsin. HCl has antibacterial properties and helps to optimise the pH for stomach enzymes, whilst pepsin digests proteins.

At the gross anatomical level, the stomach has different areas, in which the stomach lining varies at the histological level. These are the: cardia, fundus, antrum, and pylorus (fig. 1.2). The gastric epithelium in the different areas contains specialised cells that produce various secretions including mucus (produced by mucus cells), HCl (produced by oxyntic or parietal cells), digestive enzymes (produced by chief or peptic cells), and hormones (produced by enteroendocrine cells). The major functions of the gastric epithelium are to secrete acid and digestive enzymes. It also secretes mucus to protect the stomach lining from the corrosive effects of the acid and enzymes, as well as to lubricate ingested food.

The stomach tissues, like most other tissues in the body are susceptible to malignancy (Weinberg, 1997), with cancers of the gastric epithelium being the most common.

Figure 1.2 External and internal anatomy of the human stomach (taken from Tortora and Grabowski, 1996).



1.2.1 Cancer of the Stomach

Over the past few decades the overall worldwide incidence and mortality rates of gastric cancer, or cancer of the stomach, have declined (Parkin *et al.,* 2002; Muñoz and Franceschi, 1997; Parkin *et al.,* 1993). Despite this decrease in incidence, gastric cancer is still one of the most commonly diagnosed malignancies and remains the second largest cause of cancer related death worldwide (Hohenberger *et al,* 2003; Peek and Blaser, 2002; Chan *et al.,* 1999) with approximately 649,000 people dying from this malignancy each year (Correa, 2004). Gastric cancer is recognised as an important form of malignant disease, with clear and significant socio-economic, ethnic, and geographical differences in distribution. In the UK alone, gastric cancer accounts for 6% of all cancers in men, and 2% of all cancer cases in women. Stomach cancer is the 5th most common cancer in men and the 9th most common cancer in women in the UK. Each year, there are nearly 6,300 new cases of stomach cancer in men and nearly 3,500 cases in women. The incidence rate for the UK in 1999 was 12.3 (age standardized rate (ASR) per 100,000), with the highest rates found in Scotland and Wales (data from **www.cancerresearchuk.org**). In 1998 the ASR for

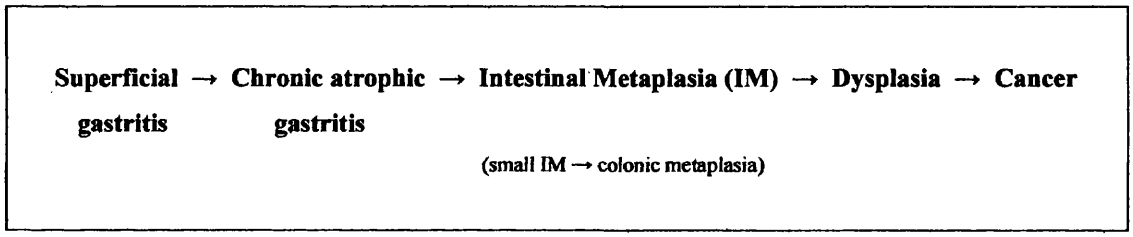
gastric cancer incidence for both sexes was 12.15, and the ASR for mortality rates for both sexes in the same year was 8.97 (Ferlay *et al.*, 1998). It is thus estimated that in the UK, around 12,000 new cases of gastric cancer are diagnosed each year (**www.nchod.nhs.uk**; MacDonald and Ford, 1997), and over half of these cases will be fatal. It is clear from such data that gastric cancer is a significant cause of morbidity and mortality in the UK and the rest of the world, constituting a clear health burden. Gastric cancer typically invades the muscularis propria (the major muscle wall surrounding the stomach) prior to diagnosis and 5-year survival rates are poor, for example, less than 15% in the United States (Correa, 2004).

Malignant tumours of the stomach can be classified based on gross morphological and histo-pathological features. It has been reported based on pathological studies/ examinations that more than 90% of stomach cancers are adenocarcinomas (i.e. epithelial in origin), the remainder being non-Hodgkin's lymphomas or leiomyosarcomas (Fuchs and Mayer, 1995). The prognosis and treatment of the different types of cancer differs, thus distinction between them is critical. For more than a century, morphological observations of the human stomach have accumulated and this has led to the classification system of Laurén (1965). This classification recognizes that gastric carcinomas can be divided into two subtypes: an intestinal type and a diffuse type.

The intestinal (or well-differentiated) type of tumour is characterized by large distinct neoplastic cells with large and irregular nuclei. These cells are cohesive and from gland-like tubular structures (Stadtländer and Waterbor, 1999; Fuchs and Mayer, 1995). The diffuse (or undifferentiated) type of gastric cancer is a poorly differentiated tumour. Malignant cells are discohesive and instead infiltrate the stomach wall as individual small solitary cells leading to thickening of the stomach wall (Faraji and Frank, 2002; Stadtländer and Waterbor, 1999; Fuchs and Mayer, 1995). The two types appear to evolve through different pathways, have a different prognosis, and preferentially affect different age and population groups. The recent decline in gastric cancer is largely due to a decline in the incidence of the intestinal subtype (Correa and Chen, 1994). Intestinal type gastric cancer more frequently affects elderly individuals, with males having a greater tendency to develop the disease. This type predominates in high risk areas and is preceded by a long precancerous process characterized by successive tissular changes.

In the Correa model, the currently accepted model of human gastric carcinogenesis based on epidemiological, pathological, and clinical observations (Correa and Shiao, 1994; Correa, 1992; Correa, 1988; Correa *et al.*, 1975), the development of intestinal type gastric cancer is preceded by a prolonged multi-step precancerous process. This precancerous process involves a progression through distinct precursor lesions involving superficial gastritis (inflammation of the gastric mucosa), chronic atrophic gastritis, intestinal metaplasia (IM) (Muñoz and Matko, 1972), and dysplasia before evolving into invasive neoplasia. The proposed progression sequence for the development of the intestinal type of human gastric cancer is shown in figure 1.3.

Figure 1.3 Pathway for Human Gastric Carcinogenesis for Intestinal type Gastric Cancer.



Studies of various markers of cellular differentiation led to the proposal of the neoplastic progression sequence. Changes in levels, nature, or presence/ absence of digestive enzymes, mucins, antigens, and oncogenes led to the speculation that gastritis is followed by IM whereby cells express phenotypic markers of 'mature' intestinal cells. These mature cells then 'de-differentiate' giving rise to primitive cells in dysplasia that express phenotypic markers reminiscent of gastric stem cells or multipotential neck cells (Correa, 1988). This would then lead to the suggestion that the precancerous process may activate dormant cellular capacities, e.g. alleviate repression of expression of certain genes, and in doing so, drive the evolution to neoplasia. Abnormal phenotypic expression of markers can also be used to identify high-risk subjects for neoplastic transformation (Correa, 1992). Epidemiological studies continue to provide data on gastric cancer risk factors and preventative factors, some of which will be discussed later (section 1.4). These factors can effectively trigger a cascade of changes in the gastric mucosa that eventually lead to the development of intestinal-type gastric cancer.

In contrast to the intestinal type, the diffuse type of gastric cancer usually presents at an earlier age and its incidence is similarly high in most populations (Stadtländer and Waterbor, 1999). The prognosis of the diffuse type is less favourable than for the intestinal type (Fuchs and Mayer, 1995). Unlike intestinal subtype, the diffuse type has no clearly defined precursor lesions (Hohenberger and Gretschel, 2003).

The present studies focus on gastric cancer of the intestinal type since this subtype has been more extensively studied and develops through pre-malignant stages, the progression of which is believed to be largely driven by chronic inflammation of the gastric mucosa (Merchant, 2005; De Luca and Iaquinto, 2004; Oshima *et al.*, 2003; Ernst, 1999; Pignatelli *et al.*, 1998; Crabtree, 1996).

1.3 Precursor gastric lesions

As previously detailed, gastric cancer of the intestinal subtype develops through a series of pre-malignant precursor gastric lesions, starting with superficial gastritis and progressing through atrophic gastritis, intestinal metaplasia, dysplasia, and ultimately gastric cancer collectively referred to as Correa's pathway/ cascade to gastric cancer (Correa, 1988; Correa *et al.*, 1975). The sections that follow detail the pre-malignant lesions.

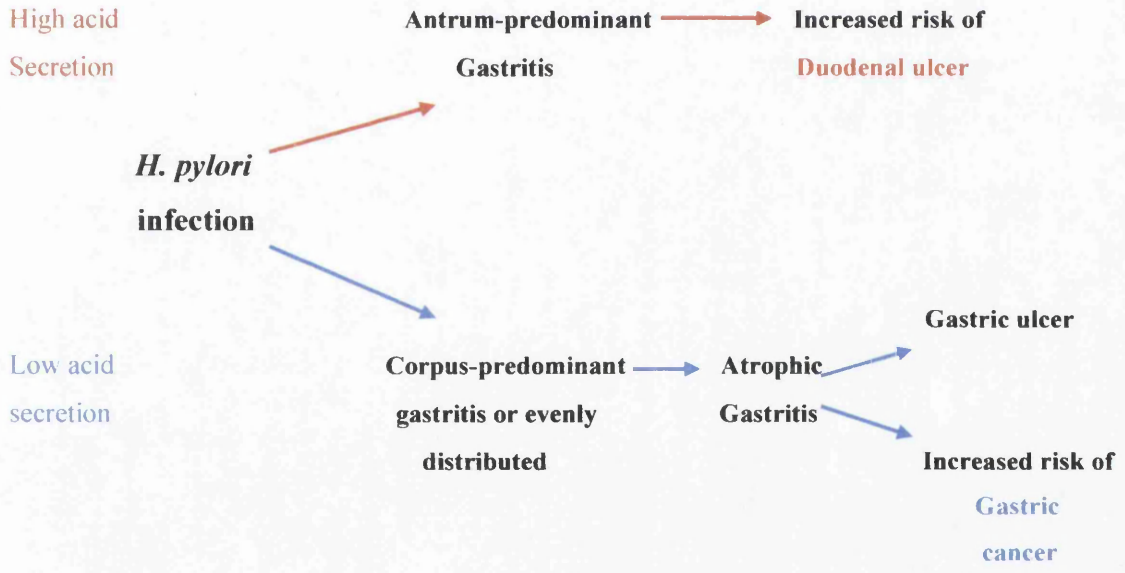
1.3.1 Gastritis

A number of morphological changes have been observed in the progression sequence, notably inflammation, atrophy, and loss of cellular differentiation. The inflammatory changes effectively constitute gastritis, inflammation of the gastric mucosa. Gastritis can be acute or chronic. Acute gastritis is an inflammatory response in the gastric mucosa resulting in extensive infiltration of neutrophils at the surface epithelium (Lee, 1997). Chronic gastritis is a long-term inflammatory condition that can persist in an individual for decades, and usually begins in the antrum and progresses in a proximal manner with age. The severity of chronic gastritis also increases with age (Siurala *et al.*, 1985; Morson *et al.*, 1980). Chronic gastritis can be non-atrophic or atrophic. The pathology of non-atrophic gastritis is essentially

inflammation, and can be classified as superficial gastritis (also known as antral-predominant gastritis) or pangastritis (also known as non-ulcer gastritis).

Interestingly, the major causative factor of chronic gastritis is chronic *Helicobacter pylori* (*H. pylori*) infection (Marshall, 1986). It is seen that virtually all patients in whom infection persists into older ages develop chronic gastritis. The variable clinical outcomes of the *H. pylori* – induced gastritis are dependent on a number of factors, a key factor being level of stomach acid secretion (Suerbaum and Michetti, 2002). This is summarised in figure 1.4. The different types of gastritis also predispose affected individuals to development of other GI disorders, including duodenal ulcer, gastric ulcer, and gastric neoplasia.

Figure 1.4 Possible clinical outcomes of *H. pylori* infection depending upon the background gastric acid levels.



It is clear from figure 1.4 that the greatest concern with regard to precursor lesions to gastric cancer is atrophic gastritis. This type of chronic gastritis increases the risk of gastric cancer by approximately six-fold and also increases the risk of gastric ulcer disease. In fact, atrophic gastritis predominates in populations at high

risk of intestinal type gastric cancer (Faraji and Frank, 2002; Correa *et al.,* 1990). The condition is characterised by chronic inflammation of the gastric mucosa in the antrum and body with accompanying gland loss (atrophy) and thinning of the mucosa believed to be a result of enhanced apoptosis in gastritis tissues (Mannick *et al.,* 1996). This results in a decrease in gastric acid secretion and a change in the gastric environment, most likely to one favouring carcinogenesis. It is vital for the clinician to distinguish between non-atrophic and atrophic gastritis (Rugge *et al.,* 2002) since only the latter has been linked to subsequent development of intestinal type gastric cancer, following progression through IM and dysplasia (Faraji and Frank, 2002; Genta, 1997). The aetiology of this gastric lesion involves a combination of *H. pylori* infection (Asaka *et al.,* 1996) and environmental and host genetic factors, all of which may trigger the development of atrophic gastritis and/ or its progression to gastric cancer in some individuals.

1.3.2 Intestinal Metaplasia

Intestinal metaplasia (IM) is defined as the replacement of normal gastric mucosa with cell types not formal for the tissue. In this case the metaplastic cells are intestinal-type cells like goblet cells, columnar cells and Paneth cells (Leung and Sung, 2002). IM results when atrophic gastritis is accompanied by de-differentiation of gastric mucosal cells. The gastric epithelial cells are effectively replaced by intestinal-type cells, resulting from a change in cell population due to altered selective pressures and/ or microenvironment (Slack, 1986). Not all individuals with atrophic gastritis develop IM. Increased pH due to loss of glands, subsequent increase in bacterial growth and increased generation of N/O mutagens has been implicated in driving progression to IM. It is believed that this stage in the progression sequence involves successive genetic changes, which lead to loss of cellular differentiation and acquisition of an altered phenotype. Mutagens generated in the stomach may cause these genetic changes.

IM is clearly recognized as a pre-malignant lesion in the Correa model of gastric carcinogenesis (Correa, 1988; Correa *et al.,* 1975). It is frequently seen as an intermediate step prior to dysplasia and adenocarcinoma (Faraji and Frank, 2002; Leung and Sung, 2002; Correa, 1988). A ten-fold increased risk of gastric cancer in individuals with IM has been predicted on epidemiological grounds compared to

individuals without IM (Leung and Sung, 2002; Filipe *et al.*, 1994). Despite this, the majority of individuals with IM never progress further. This may relate to exposures to environmental factors, variation in host genetics, or to there being histological subtypes of IM that are associated with variable cancer risk. In the most widely used classification system for IM, proposed by Jass and Filipe (1980) there are two classes of IM: *complete* and *incomplete*. Type I or the complete type resembles the small intestine phenotype and hence is also referred to as small intestinal metaplasia (SIM) and is generally associated with a low risk of progression to gastric cancer (Leung and Sung, 2002). Incomplete metaplasia (which encompasses types II and III) resembles the large intestine and hence is known as Colonic metaplasia (CM). There is a progressive series of changes from type I through to type III IM, with type III IM possessing the highest malignant potential (Leung and Sung, 2002; Celso *et al.*, 1999; Wu *et al.*, 1998; Filipe *et al.*, 1994; Rokkas *et al.*, 1991; Silva *et al.*, 1990), individuals with type III IM having a four-fold increased risk of gastric cancer compared to individuals with type I.

In a subset of individuals with IM, the metaplastic tissues undergo further genomic and phenotypic alterations leading to progression to dysplasia.

1.3.3 Dysplasia

Dysplastic tissues show loss of organization due to the presence of undifferentiated or partially differentiated cells interspersed amongst differentiated ones (McKinnell *et al.*, 1998). Gastric dysplasia characteristically shows disordered polarity, nuclear stratification, disappearance of goblet and Paneth cells and glandular overcrowding. The lesions can be polypoid, ulcerative, or flat and indistinctive (Faraji and Frank, 2002). Dysplasia can progress further to intestinal-type gastric cancer (neoplasia). This progression is slow and can take several months to years (Fengolio-Preiser *et al.*, 1989).

In the presence of the appropriate stimuli or etiological forces dysplasia will progress to carcinoma. Interestingly, some researchers have reported that upon removal of the causative stimulus, atrophic gastritis, IM and dysplasia may disappear (Dixon, 2001; Correa *et al.*, 2000; Oberhuber *et al.*, 1998); this however remains controversial (Leung and Sung, 2002; McKinnell *et al.*, 1998). If this is true, then identification and removal of the stimuli may prevent development of gastric cancer

and hence dramatically decrease the risk of intestinal type gastric cancer. Hence it is important to identify risk factors that may trigger or drive gastric cancer development.

1.4 Gastric Cancer Risk Factors

Extensive research over the past few decades, in particular from epidemiological studies has helped to identify risk factors or forces implicated in gastric cancer aetiology. Factors can be subdivided into a number of categories, namely precursor conditions, genetics, and environmental factors.

1.4.1 Precursor Conditions and Genetics

Precursor conditions that predispose individuals to gastric cancer are atrophic gastritis and IM as already discussed. Several investigators have demonstrated an apparent correlation between incidence of atrophic gastritis, IM and gastric cancer, the precursor lesions having the highest prevalence in regions of the world where gastric cancer rates are high (Nardone *et al.*, 2007; Weck and Brenner, 2006; Chen *et al.*, 2001; You *et al.*, 1993; Correa *et al.*, 1990). These observations suggest that atrophic gastritis and IM are important precursor lesions to endemic intestinal-type gastric cancer. However, the fact that both lesions have been reported in otherwise healthy individuals who do not go on to develop gastric cancer indicates that these factors alone are insufficient to cause gastric cancer. On these grounds gastric cancer is believed to be a complex, multifactorial disease, its development being influenced by a complex interaction between precursor conditions, genetic predisposition, infection, and environmental factors in particular diet (Hohenberger and Gretschel, 2003).

Other precursor conditions documented to predispose to gastric cancer include pernicious anaemia (Hsing *et al.*, 1993), partial gastrectomy (Tersmette *et al.*, 1990), Ménétrier's disease, and gastric adenomatous polyps (Fuchs and Mayer, 1995).

The importance of genetic factors in the pathogenesis of gastric cancer is well documented. For example, family members of an affected individual have two- to three- times the risk of developing gastric cancer compared with the general population (Nomura, 1982), indicating a genetically mediated susceptibility to gastric cancer.

Host genetic variability can act as a background on which other risk factors can act. For example, El-Omar *et al.* (2000) reported that genetic polymorphisms in *Interleukin- (IL-) 1β* can increase susceptibility of gastric cancer by enhancing the inflammatory response against endogenous or exogenous factors such as *H. pylori* infection, a well-known risk factor for gastric cancer. The biological basis for this observation is that IL-1β is a proinflammatory cytokine and is also a potent inhibitor of gastric acid secretion. *H. pylori* infection or other stimuli trigger an inflammatory response which is heightened in individuals with the *IL-1β* polymorphism, leading to atrophic gastritis of the gastric body and concomitant hypochlorhydia (reduced gastric acid), both of which markedly increase the risk of gastric cancer. Similarly, absence of the DQA1*0102 allele of the *HLA-DQA1* gene (a component of human MHC (major histocompatability complex)) has been linked to increased risk of *H. pylori* gastritis, and hence, intestinal-type gastric cancer (Azuma *et al.*, 1998). Such observations may help to explain why not all individuals with precursor lesions go on to develop gastric cancer.

1.4.2 Age, Sex, and Socio-economic status

Age and sex are also recognized risk factors for gastric carcinoma. For the majority of cases, gastric cancer incidence and mortality rates increase with age, and are higher in males than in females (Parkin *et al.*, 2002; Hass and Schottenfeld, 1975).

Gastric cancers are generally more frequently found in people of socio-economically deprived classes (Sugimura and Sasako, 1997; Sitas *et al.*, 1992). This is reflected in the observed sharp decline in gastric cancer mortality rates over the past decades, particularly in developed countries where improved living conditions are found. In addition, the risk of distal gastric cancer throughout the world is inversely associated with socio-economic status, the lower the socio-economic status, the greater the risk of distal gastric cancer (Fuchs and Mayer, 1995).

1.4.3 Environmental and Geographic factors

Interestingly, a pronounced worldwide/ geographical variation in gastric cancer incidence and mortality rates has been well documented (Parkin *et al.*, 2002; Stadtländer and Waterbor, 1999; Whelan *et al.*, 1993). This well documented

geographic variation of rates is a known feature of the disease (Yamiguchi and Kakizoe, 2001). Worldwide, the highest incidence rates are found in Japan, China, South America (Chile, Costa Rica), the Russian Federation, and the former Soviet Union. The lowest rates occur in the United States, the UK (Northern Europe), Canada, Australia, and parts of Africa (e.g. Uganda) (Faraji and Frank, 2002; Holcombe, 1992). Recently the incidence of gastric cancer has been declining on an annual basis in the United States and other industrialized countries. However, even within the US there exist sub-groups, which can be classed as high-risk groups due to reports of high incidences of gastric cancer in these areas (Wiggins *et al.,* 1989; Haenszel, 1958). It is through epidemiological studies that these geographical and ethnic differences in incidence rates and gastric tumour types have been discovered. Some of the most interesting epidemiological observations come from migrant studies (McMicheal *et al.,* 1980; Haenszel *et al.,* 1972). Individuals/ populations migrating from high risk to low risk areas of gastric cancer may still display a high risk in the new location, despite a change in lifestyle. An example of such a study is provided by the work of Haenszel *et al.* (1972). Japanese immigrants in Hawaii were studied and it was found that the individuals from the high risk population (Japan) still had a high risk of developing gastric cancer despite having adopted a Western lifestyle. Interestingly, a decrease in cancer risk is apparent in their second and third generation offspring. This indicates that environmental factors play an important part in the aetiology of gastric cancers at an early stage. Early exposures to environmental factors can imprint a high gastric cancer risk that remains even after a change of environment. Similarly, in America it has been noted that Hispanics, American Indians, blacks, & immigrants from Latin America, Asia, and Northern Europe are at higher risk of developing gastric cancer than that of Native white Americans (Neuget *et al.*, 1996; Correa, 1992; Wiggins *et al.,* 1989). Such data on dramatic geographical variation in disease incidence lead one to speculate that gastric cancer is influenced greatly by environmental factors (Chan *et al.*, 1999; Fuchs and Mayer, 1995), and that there are geographical differences in causative factors. Interactions between genetic and environmental risk factors may help to explain the worldwide variation in gastric cancer rates.

The decrease in gastric cancer throughout the world over the last 50 years is largely due to a decrease in cancer of the distal stomach (gastric body and antrum, see

fig. 1.2) (Fuchs and Mayer, 1995). It has been speculated that this decrease can be attributed to increased consumption of fresh fruit and vegetables (Palli, 2000; World Cancer Research Fund (WCRF) panel, 1997), decreased consumption of salty and smoked foods, and improved methods for preserving food such as refrigeration (Hohenberger and Gretschel, 2003; Terry *et al.*, 2002).

The described geographic variation in gastric cancer rates may be accounted for by geographic variation in environmental influences/ exposures, and interaction of environmental factors with genetic variations. One such important environmental factor is diet.

1.4.4 Diet

Diet plays a significant role in gastric carcinogenesis (Stadtländer and Waterbor, 1999). Dietary irritants such as salt (NaCl) have been, through epidemiological observations, linked to higher gastric cancer risk (Chen *et al.*, 1990a; Joosens and Geboers, 1981), and diets rich in salted, smoked, or pickled foods have been linked to an increased risk of gastric cancer (Ramon *et al.*, 1993). This may explain the high risk of gastric cancer in Eastern countries, like Japan, where diets are rich in salted, smoked, and dried foods. The recent decline in deaths from gastric cancer in Japan has been correlated with a parallel decrease in consumption of salty foods. In experimental models of gastric cancer, NaCl is seen to potentiate the effects of chemical carcinogens such as methyl N'-nitro-N-nitrosoguanidine (MNNG) (Watanabe *et al.*, 1992; Takahashi *et al.*, 1983). Salt may thus act as a tumour promoter.

Excessively salty diets have been shown to induce gastric atrophy in laboratory mice (Kodoma *et al.*, 1984). Salt may induce gastritis by causing irritation to the gastric mucosa by damaging the protective mucous layer and underlying epithelial cells, leading to an inflammatory response. More significantly, high intake of salt has been shown to increase cellular replication (Correa, 1992), and in doing so makes cells more susceptible to mutagenesis and carcinogenesis (Charnley and Tannenbaum, 1985) since DNA damage can be converted into fixed mutations more readily (Ames and Gold, 1990), increasing the endogenous mutation rate. Consumption of excessively salty foods may therefore, over time, lead to atrophic

gastritis and so increase the risk of gastric cancer development (Fuchs and Mayer, 1999).

Foods rich in nitrates, nitrites, and secondary amines have also been shown to be a cause for concern, as these compounds may form N-nitroso compounds, potent carcinogens shown to induce gastric tumours in animals (Correa *et al.*, 1975). N-nitroso compounds are found naturally in cured meat and fish, and fried or grilled meat (Yamaguchi and Kakizoe, 2001). In addition, N-nitroso compounds can be generated in the gastric lumen, in the presence of dietary nitrates, by bacteria, which can flourish in the gut of patients with hypochlorhydia (reduced acid secretion). This may be a particular problem in regions of the world where dietary components are rich in nitrates (Sierra *et al.*, 1991). Another problem is that nitrates in the gut can combine with mutagenic precursors in foods to give rise to potent mutagens (Correa, 1992; Chen *et al.*, 1990b; Wakabayashi *et al.*, 1985; Yang *et al.*, 1984). In addition, nitrite, nitrate, and nitrosating agents can also be synthesized by infiltrating macrophages and neutrophils during gastric inflammation.

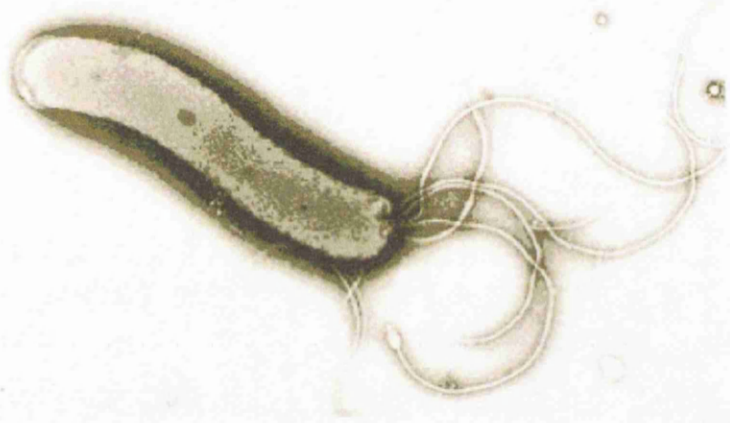
The great interest in diet and cancer emanates from the hope that dietary changes can be made to reduce the incidence and mortality rates of gastric cancer (Correa *et al.*, 2003; Correa *et al.*, 2000; Palli, 2000; Correa, 1992). One approach is to reduce intake of excessively salty, pickled, and smoked foods. An increasing body of evidence suggests that increasing intake of fresh fruits and vegetables can decrease gastric cancer risk (Hohenberger and Gretschel, 2003; Stadtländer and Waterbor, 1999). Regular consumption of fresh fruits and vegetables provides the necessary vitamins and antioxidants to maintain a healthy nutritional status. Most research into dietary cancer preventative agents in the diet has focused on antioxidants (Kobayashi *et al.*, 2002; Serafini *et al.*, 2002; Su *et al.*, 2000; Correa *et al.*, 1998; Correa, 1995; Correa, 1992).

Antioxidants include ascorbate (Vitamin C), β-carotene (a component of vitamin A), and α-tocopherol (vitamin E), all of which are abundant in fresh fruit and vegetables and the levels of such micronutrients in the serum of patients has been related to gastric cancer risk (You *et al.*, 2000; Ruiz *et al.*, 1994; Haenszel *et al.*, 1985). Antioxidants inhibit the reduction of nitrate to nitrite (Correa, 1992) and it has also been shown that the mutagenicity of nitric oxide (NO, an inflammatory mediator/ radical produced by macrophages which may be present in inflamed mucosa) can be inhibited by β-carotene and α-tocopherol (Arroyo *et al.*, 1992). Ascorbate is thought

to be protective against gastric cancer as it is a scavenger of reactive oxygen species (ROS) produced in the gastric mucosa and heavily implicated in gastric carcinogenesis (Stadtländer and Waterbor, 1999; Leaf *et al.*, 1991; Stemmerman and Mower, 1981). ROS induce DNA damage and therefore increase the risk of mutations, which can lead to cancer (see section 1.6.1).

1.4.5 Helicobacter pylori

Figure 1.5 The bacterium *Helicobacter pylori* (*H. pylori*) a major causative factor in gastric carcinogenesis.



'Microbial pathogens contribute to the genesis of a substantial number of malignancies worldwide and nearly 1.2 million cases of cancer per year are attributable to infectious agents' (Peek and Crabtree, 2006; Coussens and Werb, 2002).

Epidemiologic studies consistently demonstrate a link between gastric cancer risk and *H. pylori* infection (Correa, 2004; Peek and Blaser, 2002; *Helicobacter* and Cancer collaborative group, 2001; Danesh, 1999; Watanabe *et al.*, 1997; Correa, 1996; O'Conner *et al.*, 1996; Webb and Forman, 1995; Hu *et al.*, 1994; IARC, 1994; Sipponen *et al.*, 1992; Forman *et al.*, 1991; Parsonnet *et al.*, 1991). *H. pylori* is likely to be the most significant etiological factor in gastric carcinogenesis. Several studies suggest that there are very few patients with gastric cancer who are not infected with *H. pylori* (Uemura *et al.*, 2001). Data from the Eurogast study group indicates an

international association between *H. pylori* infection and gastric cancer, the prevalence of *H. pylori* infection correlating with the incidence of gastric cancer in 13 countries (The Eurogast study group, 1993) possibly accounting for the geographic variation in the prevalence of the disease (Hohenberger and Gretschel, 2003; Parkin *et al.*, 2002).

H. pylori is a spiral-/ helicoidal-shaped, microaerophic, gram negative bacterium (fig. 1.5), and is a well known gastric pathogen (Suerbaum and Michetti, 2002) selectively colonising the gastric mucosa (Peek and Crabtree, 2006). *H. pylori* infection is causally linked to several gastric conditions. The bacterium was isolated for the first time in 1982 from gastritis and peptic ulcer biopsy specimens (Marshall and Warren, 1984). Since then, several studies have linked persistent *H. pylori* infection with chronic atrophic gastritis, a known inflammatory precursor of gastric adenocarcinoma (section 1.3.1) (Uemura *et al.*, 2001; Sipponen and Marshall, 2000; IARC, 1994; Nomura *et al.*, 1991; Parsonnet *et al.*, 1991; Marshall, 1986). Indeed virtually all infected individuals develop a co-existing gastritis which can exist for decades.

Several serological studies in different populations have established a strong correlation between gastric cancer and infection/ prior infection with *H. pylori* (Tatsuta *et al.*, 1993; Forman *et al.*, 1991; Karnes *et al.*, 1991; Nomura *et al.*, 1991; Parsonnet *et al.*, 1991; Correa *et al.*, 1990). All studies led to the conclusion that infection with *H. pylori* increased the risk of developing gastric cancer. In fact, based on several reports, it has been estimated that individuals infected/ previously infected with *H. pylori* have a three- to eight- fold higher risk of gastric cancer compared to non-infected counterparts (Rokkas, 2000; Fuchs and Mayer, 1995) with an average of approximately six-fold increased risk (*Helicobacter* and Cancer collaborative group, 2001; Eurogast study group, 1993; Parsonnet *et al.*, 1991).

H. pylori infection is thus seen as an aetiological factor in the development of gastric cancer; both adenocarcinomas and gastric mucosa associated lymphoid tissue (MALT) lymphomas (Versalovic, 2003). The *H. pylori* – gastric cancer link seems to be restricted to cancers of the distal stomach as opposed to cancers of the gastroesophageal junction and gastric cardia (Parsonnet *et al.*, 1991). *H. pylori* infection in particular appears to increase the risk of intestinal-type gastric cancer by inducing gastritis and atrophic changes. There are also some reports that *H. pylori*

infection can also influence development of diffuse-type gastric cancer (Solcia *et al.*, 1996; Kikuchi *et al.*, 1995; Parsonnet *et al.*, 1991).

A consequence of this epidemiological data is that *H. pylori* has been classified as a definite human carcinogen by the International Agency for Research on Cancer (IARC, 1994) (the first bacterium to be classified in this manner). As a result there has been an explosion in *H. pylori* research in recent years. *H. pylori* has been detected in populations throughout the world and may be acquired in early childhood by transmission via the oral-oral and faecal-oral routes (Blaser, 1998; IARC, 1994). The infection in adults is usually chronic and persists throughout life unless appropriate therapy is provided. Interestingly, the incidence and prevalence of *H. pylori* infection shows geographic variation (Suerbaum and Michetti, 2002), and is related is to socio-economic conditions, with infection being more prevalent in low socio-economic classes where poor housing and hygiene are problems (IARC, 1994). The infection is acquired via oral ingestion of the bacterium (which has been detected in drinking water), and is transmitted within families in early childhood. Familial clustering of gastric cancer may therefore be associated with a familial clustering of *H. pylori* infection (Brenner *et al.*, 2000; IARC, 1994). The prevalence of the bacterium declines with improved hygiene and food preservation and this may help to account for the decreasing incidence of gastric cancer over the last fifty years.

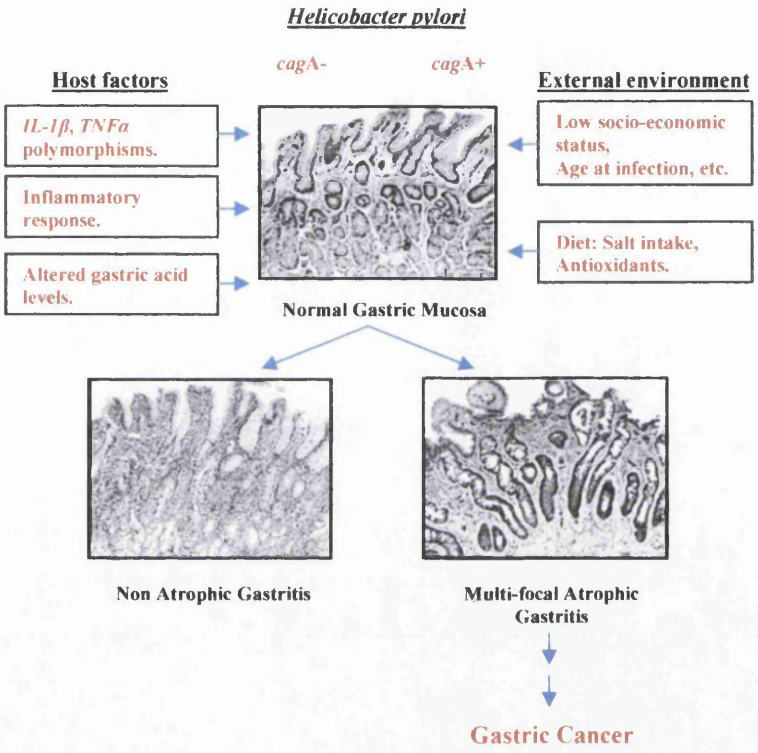
H. pylori research has brought to light several interesting observations regarding the pathogenesis of *H. pylori* associated conditions. Infection with the bacterium is associated with several clinical outcomes including gastritis, duodenal/ gastric ulcer disease and gastric cancer (Hohenberger and Gretschel, 2003). *H. pylori* infection is the most common cause of chronic gastritis (Torres *et al.*, 2000) which involves extensive infiltration of inflammatory polymorphonuclear (PMN) leukocytes and consequent inflammation. It is believed that the extent of this inflammatory response may determine whether gastritis progresses to atrophy and possibly even to cancer. This may vary from individual to individual due to environmental and host genetic factors. For example, as stated in section 1.4.1, individuals with an *IL-1β* polymorphism exhibit a heightened inflammatory response to *H. pylori* infection and hence may have a greater risk of neoplastic progression than individuals without the polymorphism (El-Omar *et al.*, 2000). Also, several reports indicate that high salt diets may enhance *H. pylori* – induced gastric carcinogenesis, possibly by enhancing *H. pylori* colonization (Fox *et al.*, 1999) or adding to gastric irritation and

inflammation. Thus *H. pylori* does not appear to act alone to increase the risk of gastric cancer development. Genetic and environmental co-factors are also required and only a subset of *H. pylori* infected individuals will develop gastric cancer. In fact, only 1% of *H. pylori* infections are estimated to lead to gastric cancer (Farthing, 1998).

H. pylori infection is virtually always accompanied by inflammation of the gastric mucosa (Dixon 1991). The severity of the inflammatory response to infection, and the associated risk of precancerous and cancerous changes in the gastric mucosa appear to depend on the strain of *H. pylori.* There is marked genetic variability in *H. pylori* strains resulting in differing degrees of virulence (Israel *et al.,* 2001). Two disease related alleles have been studied extensively, being the *cag*A, and *vac*A genes (Peek and Crabtree, 2006; Akopypants *et al.,* 1998; Censini *et al.,* 1996; Fox *et al.,* 1992). The *cag*A gene is part of a segment of the genome termed the *cag* pathogenicity island (PAI). It has been seen that whilst all *H. pylori* strains can induce the development of gastritis (albeit to differing degrees), cag^+ strains tend to result in a more severe gastritis accompanied by a heightened inflammatory response and increased risk of atrophic changes, IM and cancer development (Nogueria *et al.,* 2001; Webb *et al.,* 1999; Queiroz *et al.,* 1998; Shimoyama *et al.,* 1998; Torres *et al.,* 1998; Parsonnet *et al.,* 1997; Rudi *et al.,* 1997; Beales *et al.,* 1996; Crabtree *et al.,* 1996; Blaser *et al.,* 1995; Crabtree *et al.,* 1995; Kuipers *et al.,* 1995; Peek *et al.,* 1995; Crabtree *et al.,* 1993; Crabtree *et al.,* 1991). In a review paper published in 2006, Peek and Crabtree conclude that differential host inflammatory responses to the bacterium, and hence, clinical outcome, are a consequence of substantial genotypic diversity between isolates (Peek and Crabtree, 2006). It is important to note however, that carcinogenesis is ultimately driven by a synergy of host and bacterial factors, host genetic diversity, particularly involving polymorphisms in immune response genes such as *IL-1β* and *TNF-α*, can increase the risk of an individual developing intestinal type gastric cancer (Garza-Gonzalez *et al.,* 2005; Ohyauchi *et al.,* 2005; Chen *et al.,* 2004; El Omar *et al.,* 2003; Rad *et al.,* 2003; Zeng *et al.,* 2003; Machado *et al.,* 2001; El Omar *et al.,* 2000). As such it is believed that the outcome of chronic *H. pylori* infection is largely dependent upon a combination of host and bacterial factors (summarised in fig. 1.6), and it has been suggested that genotyping of host polymorphisms and bacterial variation, in conjunction with endoscopic surveillance may help to identify individuals at heightened risk of developing gastric cancer (Peek

and Crabtree, 2006; Correa and Schneider, 2005; Correa *et al.*, 2004; Figueiredo *et al.*, 2002).

Figure 1.6 Host and bacterial factors affecting the outcome of infection. Adapted from Correa *et al.* (2004).



1.4.5.1 Possible mechanism(s) of *H. pylori* – associated gastric pathogenesis

H. pylori infection of the gastric mucosa sets in motion a well co-ordinated inflammatory response. It is believed that the bacterium is capable of breaking down the protective mucous barrier in the stomach (possibly by impairing the secretion of mucins (Correa and Miller, 1998)) and damaging the underlying gastric epithelial cells so instigating an inflammatory response. This response is characterised by extensive infiltration of inflammatory polymorphonuclear (PMN) leukocytes, primarily neutrophils at first (Naito and Yoshikawa, 2002), later being accompanied by monocytes and tissue macrophages, which are recruited to the mucosa by chemotactic factors (such as cytokines) produced by the gastric epithelium and by the

bacterium itself. The leukocytes attempt to deal with the infection by phagocytosing bacteria at the surface of the epithelium and employing bactericidal factors such as ROS to kill *H. pylori*. Unfortunately for the host, the bacterium has evolved mechanisms of evading the host response (Baldari *et al.*, 2005), for example, by the production of detoxifying enzymes that protect against the effects of ROS (Bernard *et al.*, 2004). The consequence of this is a sustained inflammatory response, since the stimulus (*H. pylori*) remains at the site, leading to further recruitment and activation of leukocytes. The infection has been reported to induce a Th1 type inflammatory response with respect to the array of cytokines and chemokines that are detected at the infection site, being characterised by IFNγ (as oppose to IL-4 in a Th2 response) (Crabtree *et al.*, 2004; Smythies *et al.*, 2000; Bamford *et al.*, 1998; Crabtree, 1998) as well as TNFα, IL-1β, IL-6, and IL-8 that originate from leukocytes and gastric epithelium (in particular IL-1β, IL-6, and IL-8) (Lindholm *et al.*, 1998; Messa *et al.*, 1996; Mohammadi *et al.*, 1996; Weigert *et al.*, 1996; Crabtree *et al.*, 1995; Karttunen *et al*, 1995; Crabtree *et al.*, 1994a; Crabtree *et al.*, 1994b; Moss *et al.*, 1994; Noach *et al.*, 1994). These pro-inflammatory mediators may contribute to *H. pylori* induced pathologies (Ernst *et al.*, 1999) by recruitment and activation of more inflammatory cells and driving the generation of RO/NS (e.g. TNFα and IL-8 (Naito and Yoshikawa, 2002; Garcia-Ruiz *et al.*, 1997; Lo and Cruz, 1995)). Neutrophils generate bactericidal RO/NS, such as hydrogen peroxide (H_2O_2), superoxide radical ($O_2^{\cdot -}$), and nitric oxide (NO) (Peek and Blaser, 2002), in an attempt to destroy infection (Crabtree, 1996a; Crabtree, 1996b). Since *H. pylori* can evade this, the RO/NS cause damage to host epithelial cells instead, damaging vital macromolecules such as lipids, proteins and DNA.

It is clear then that infection with *H. pylori* results in an inflammatory response, which can often be long-term leading to changes in the gastric microenvironment, driving physiological and molecular changes in the gastric mucosa which could ultimately favour gastric carcinogenesis. Extensive review of the large body of literature brings attention to several components of the infection and its associated inflammatory response that may underlie the pathogenesis of gastric cancer. These include:-

- Disruption of proliferation – apoptosis balance.

The bacterium has been shown to increase the rate of cellular proliferation of gastric epithelial cells, inducing hyperproliferation of the gastric epithelium as well as alterations in apoptosis (Correa and Miller, 1998; Jones *et al.*, 1997; Peek *et al.*, 1997; Cahill *et al.*, 1996; Lynch *et al.*, 1995; IARC, 1994; Cahill *et al.*, 1993; Buset *et al.*, 1992). *H. pylori* has been shown to induce apoptosis in the gastric epithelium (Yoshimura *et al.*, 2000), both directly, through bacterial cytotoxins, and indirectly via the cytokines and RO/NS produced in the immune response to infection (Jones *et al.*, 1997). The increase in apoptosis results in an increase in cellular proliferation in order to balance cell kinetics (Leung and Sung, 2002; Xia *et al.*, 2001). Unfortunately the balance can be lost and this can lead to cancer through cellular accumulation and loss of gastric integrity (Pritchard and Crabtree, 2006; Piotrowiski *et al.*, 1997; Bechi *et al.*, 1996; Fan *et al.*, 1996; Moss *et al.*, 1996). In particular, infection with *cag*A^+ *H. pylori* strains has been reported to result in increased proliferation and apoptosis of the gastric epithelium (Le'Negrate *et al.*, 2001; Moss *et al.*, 2001; Rokkas *et al.*, 1999). Oxidative stress is a favoured hypothetical explanation for the alterations in the proliferation – apoptosis balance associated with infection (Correa and Miller, 1998).

- Chronic Inflammatory response and infiltration of leukocytes.

It is well documented that the chronic gastric inflammation associated with infection greatly and significantly increases the risk of developing gastric cancer (Correa, 2004; Peek and Blaser, 2002; *Helicobacter* and Cancer Collaborative Group, 2001; Jaiswal and LaRusso, 2002; Correa, 1996; Forman *et al.* 1991). Inflammatory responses result in the release of large amounts of inflammatory mediators such as RO/NS from PMN leukocytes and macrophages. The bacterium causes the activation of neutrophils (Unemo *et al.*, 2005) and disrupts their release of ROS (mainly superoxide) such that instead of being released into the phagosome (that forms within cells upon phagocytosis) and killing engulfed *H. pylori*, the metabolites are released extracellularly (Allen *et al.*, 2005). A consequence of this is increased risk of gastric carcinogenesis via induction of oxidative DNA damage in adjacent cells (Correa, 1997). Indeed the greater the severity of inflammation the greater the risk of malignancy perhaps owing to increased generation of RO/NS and so a greater degree

of DNA damage (Farinati *et al.*, 2003; Yoshimura *et al.*, 2000; Correa and Miller, 1995) and peroxidative damage (Danese *et al.*, 2001; Baik *et al.*, 1996; Farinati *et al.*, 1996; Davies *et al.*, 1994). The accumulation of oxidative DNA damage may lead to gene modifications and an ultimate cancer endpoint (Nishibayashi *et al.*, 2003; Ernst, 1999).

- Oxidative DNA damage.

In vivo studies indicate that *H. pylori* may induce increased synthesis of RO/NS in the gastric mucosa of infected patients (Obst *et al.*, 2000; Suzuki *et al.*, 1996; Davies *et al.*, 1994). Oxidative stress is frequently observed in infected individuals (Felley *et al.*, 2002). Strong evidence suggests that RO/NS play an important role in carcinogenesis, and are heavily implicated in the aetiology of gastric carcinogenesis. RO/NS have a knock on effect on DNA synthesis, mutation, and apoptosis. *H. pylori* can thus act as an initiator of gastric carcinogenesis via the induction of oxidative DNA damage (Yoshimura *et al.*, 2000). Oxidative DNA damage usually results in the induction of DNA repair pathways or apoptosis in order to avoid mutation. Unfortunately if the levels of DNA damage are overwhelming and DNA repair pathways are inhibited (nitric oxide has been reported to inhibit DNA repair (Laval and Wink, 1994)) and the cells fail to execute apoptosis the result can be mutated transformed cells (Yoshimura *et al.*, 2000). Indeed *H. pylori* infection has been reported to induce mutations in the big blue transgenic mouse model (Jenks *et al.*, 2003; Touati *et al.*, 2003) and interestingly the mutation frequency was strongly related to the degree of inflammatory cell infiltrate suggesting that the inflammatory leukocytes may play an active role. Gastric biopsies from *H. pylori* – infected individuals show elevated levels of ROS compared to uninfected counterparts, and gastric cancer patients are seen to have higher levels of oxidative DNA damage in tumour tissues compared to normal tissues (Lee *et al.*, 1998). Thus inflammation and its associated RO/NS are likely key players in *H. pylori* associated gastric carcinogenesis (Jenks *et al.*, 2003).

- Decreased levels of antioxidants.

Ascorbate (Vitamin C) acts as a potent free radical scavenger and as such is seen to be protective against gastric cancer (Drake *et al.*, 1996). *H. pylori* has been

reported to cause decreased levels of ascorbate in the gastric juice of infected subjects (Hohenberger and Gretschel, 2003; IARC, 1994; Ruiz *et al.,* 1994). This results in a marked decrease in radical scavengers, and hence accumulation of generated oxygen and nitrogen radicals. This can result in enhanced levels of oxidative DNA damage. Vitamin C supplementation coupled with eradication of *H. pylori* has been reported to result in significantly reduced levels of DNA damage (Correa *et al.,* 2000; Rokkas *et al.,* 1995) and apoptosis, as well as prevention of cancer cell growth (Zhang *et al.,* 2002).

- Altered Gastric Acidity.

Chronic infection with *H. pylori* can disrupt gastric physiology at the level of the pH of the gastric juice. Inflammatory cytokines are important here, for example, both IL-1β and TNFα released at sites of infection are potent inhibitors of gastric acid secretion (Beales *et al.,* 1998). Certain polymorphisms in inflammatory genes, e.g. *IL-1β,* can lead to both a heightened inflammatory response to infection and decreased acid levels (hypochlorydia), resulting in an increased risk of gastric cancer (El-Omar *et al.,* 2000). In addition, atrophic changes induced by *H. pylori* can lead to hypochlorydia. The consequence of this is an altered gastric microenvironment which can favour carcinogenesis (Dockray *et al.,* 2001; Peek *et al.,* 2000; Yoshimura *et al.,* 2000).

- Mitogenic signalling and oncogenic gene expression changes.

H. pylori can cause molecular changes in the gastric epithelium which predispose to cancer development. Infection has been reported to induce mitogenic signalling in gastric epithelial cells by way of mitogen activated protein kinase (MAPK) signalling (Keates *et al.,* 2005; Zhu *et al.,* 2005; Wallasch *et al.,* 2002; Hirata *et al.,* 2001; Meyer-ter-Vehn *et al.,* 2000; Stein *et al.,* 2000; Wessler *et al.,* 2000; Keates *et al.,* 1999; Segal *et al.,* 1999), as well as the induction of transcription factors implicated in inflammation and oncogenesis such as nuclear factor kappa-B (NFκB) (Sharma *et al.,* 1998; Keates *et al.,* 1997; Munzenmaier *et al.,* 1997) and AP-1 (a downstream transcription factor target of MAPK signalling) (Naumann *et al.,* 2001; Naumann *et al.,* 1999), and the expression of potentially oncogenic genes including *c-FOS* (a component of the AP-1 transcription factor complex) (Meyer-ter-

Vehn *et al.*, 2000) and *IL-8* (Fischer *et al.*, 2001; Meyer-ter-Vehn *et al.*, 2000; Crabtree *et al.*, 1999; Li *et al.*, 1999; Glocker *et al.*, 1998; Shimoyama *et al.*, 1998; Aihara *et al.*, 1997; Crabtree *et al.*, 1995; Crabtree *et al.*, 1994a). The effect of infection on intracellular signalling and gene expression is further detailed in section 3.1.4. The RO/NS produced by leukocytes and gastric epithelial cells in response to infection have also been implicated in the induction of signalling and gene expression changes via the activation of redox-sensitive transcription factors which may adversely affect cellular proliferation (Teshima *et al.*, 2000).

Clearly the link between *H. pylori* infection and gastric cancer is complex, with several factors impacting disease pathogenesis, often in an interrelated (synergistic) manner. It is important to gain insight into the pathogenesis of *H. pylori*-induced gastritis and gastric cancer at all of these levels. Identification of molecular changes caused by *H. pylori* could provide a better understanding of the mechanistic basis underlying the pathogenesis of gastric cancer.

1.5 Molecular Biology of Gastric Cancer

In the past ten years much has been learnt about the molecular changes in gastric cancer and much continues to be learnt. Gastric cancer is effectively the end result of progressive genome destabilization. Like all cancers, it is characterised by dynamic changes in the genome. Several genetic changes have been detected and at least 4-7 genetic 'hits' are required to drive gastric carcinogenesis. As a result of these changes, cancer cells generally show six 'hallmarks', being – disregard for differentiation signals, insensitivity to anti-proliferative signals, capacity for sustained proliferation, evasion of apoptosis, tissue invasion and metastasis, and sustained angiogenesis (Hanahan and Weinberg, 2000). Numerous genetic alterations have been identified due to vast research efforts in this area, and table 1.2 summarises a handful of the changes along with the aspects of cellular physiology that they impact.

Despite advances, a clear-cut genetic basis of gastric cancer remains to be defined (Correa and Shiao, 1994). Marked variation in genetic changes is often seen between tumours as well as within a tumour mass. This leads one to speculate that the molecular pathogenesis of gastric cancer is influenced by a combination of factors

(section 1.4.5.1), *H. pylori* being the most significant. Large scale studies have identified several genes that can be impacted in *H. pylori* – associated gastric carcinogenesis throughout the pre-malignant stages. These include - up-regulation of inflammatory response genes e.g. *IL-8*, (Wallasch *et al.*, 2002; Maeda *et al.*, 2001; Crabtree *et al.*, 1995; Sharma *et al.*, 1995; Crabtree *et al.*, 1994a; Crabtree *et al.*, 1994b); up-regulation of *IκBα*, (Maeda *et al.*, 2001); AP-1 activation via up-regulation of c-*JUN*, *JUN-B*, *c-FOS* (Sepulveda *et al.*, 2002); up-regulation of cyclin D1 (*CCND1*) (Sepulveda *et al.*, 2002); EGF receptor activation (Wallasch *et al.*, 2002); decreased expression of E-cadherin (*CDH1*) (Sepulveda *et al.*, 2002; Terres *et al.*, 1998); up-regulation of certain growth factors (GFs), e.g. transforming growth factor α (*TGFα*), hepatocyte growth factor (*HGF*), and gastrin *(GAST)* (Konturek *et al.*, 2001; Beppu *et al.*, 2000; Choi *et al.*, 1999); increased *SURVIVIN* expression levels (Kania *et al.*, 2003); decreased caspase-3 (*CASP3*) expression (Kania *et al.*, 2003); over-expression of matrix metalloproteinase-7 (*MMP-7*) (Bebb *et al.*, 2003). This is just a 'snapshot' of the gene expression changes detected in *H. pylori* – associated gastric carcinogenesis. The changes drive disease pathogenesis by impacting cellular biochemistry and physiology at the levels of cellular proliferation (usually increases), apoptosis, mitogenic signalling, angiogenesis, and capacity for invasion and metastasis.

In recent years the involvement of inflammatory processes and their related generation of RO/NS (and resultant oxidative and nitrosative stress) in gastric carcinogenesis have received special attention. A recent study by Ding *et al.* (2007) demonstrated that *H. pylori* infection or H_2O_2 exposure to gastric epithelial cells could induce very similar cellular and molecular changes at the levels of increased ROS generation (which could be enhanced by pre-treatment with cytokines), and apoptosis, both of which could be inhibited by the addition of antioxidants. Such studies emphasise the importance of the inflammatory response and oxidative stress in carcinogenesis.

1.6 Historical Perspectives of Free Radicals

A free radical is a molecule possessing one or more unpaired electrons in its outer orbital. These molecules tend to be highly unstable due to their propensity to

Table 1.2 Summary of a 'snapshot' of some of the molecular alterations in intestinal type gastric cancer and its pre-malignant precursor lesions reported in the literature with respect to the aspects of cellular physiology that they impact. For further details refer to the cited references. LOH = loss of heterozygosity; PMN = polymorphonuclear.

Cell Physiology Affected	***Molecular Alteration***	***References***
Proliferation – Apoptosis balance (cell cycle and apoptosis)	*CYCLIN E* amplification/ over-expression	Nardone (2003); Tahara (1995a); Akama *et al.* (1995).
	LOH of cell cycle inhibitors e.g. $p16^{INK4}$ (*CDKN2A*), $p21^{waf}$ (*CDKN1A*), p27 (*CDKN1B*), and *FHIT*	Nardone (2003); Sard *et al.* (1999); Tahara (1995b).
	CYCLIN D overexpression	Sepulveda *et al.* (2002).
	LOH of anti-apoptotic *BCL-2*	Nardone (2003); Ayhan *et al.* (1994).
	H. pylori-induced over-expression of cyclo-oxygenase 2 (*COX-2*)	Leung and Sung (2002); Chan *et al.* (2001).
	LOH of *FAS* (cell death signal)	Park *et al.* (2001).
	LOH of *TP53*, mutation of *TP53* (often (oxidative due to PMN leukocytes)	Morgan *et* al. (2003); Ranzani *et al.* (1995); Correa and Shiao (1994); Sano *et al.* (1991).
	Mutations in *k-RAS* oncogene leading to activation in absence of signalling	Watari *et al.* (2007); Gong *et al.* (1999); Tahara (1995b).
	c-ERB-2 gene amplification/ over-expression (self sufficiency in mitogenic signalling)	Tahara (1995b); Uchino *et al.* (1993); Yonemura *et al.* (1991).

Table 1.2 cont.

Cell Physiology Affected	*Molecular Alteration*	*References*
Cell signalling	*VEGF* over-expression	Nardone (2003); Tahara (1995a).
	EGFR, *EGF*, and *TGFα* over-expression	Werner (2001).
	Nuclear Factor Kappa B (*NFκB*)	Maeda *et al.* (2000).
	IκBα up-regulation	Maeda *et al.* (2001).
	c-JUN, *JUN-B*, *c-FOS* up-regulation (AP-1)	Sepulveda *et al.* (2002)
Invasion and Metastasis	LOH of *APC*, mutation of *APC*	Nardone (2003); Tahara (1995a); Sano *et al.* (1991).
	β-catenin (*CTNNBIP1*) over-expression (also leads to enhanced proliferation)	Nardone (2003); Ougolkov *et al.*, (2001); El-Rifai *et al.* (2000).
	LOH of *DCC*	Nardone (2003); Tahara (1995b).
	Mutation and/ or reduced expression of E-cadherin (*CDH1*)	Sepulveda *et al.* (2002); Terres *et al.* (1998).
	MMP-7 over-expression	Bebb *et al.* (2003).
	IL-8 over-expression	Macrí *et al.* (2006); Kido *et al.* (2001); Yamaoka *et al.* (2001).

react with adjacent molecules. Many of these molecules are oxygen or nitrogen centred, hence Reactive Oxygen/ Nitrogen Species (RO/NS) [throughout this thesis the free radicals and their less reactive derivative molecules are collectively termed ROS, RNS, or RO/NS]. Under normal circumstances, free radicals are generated in cells as a consequence of normal metabolic processes. Their levels in the cell are very finely controlled by antioxidant systems – both enzymatic (e.g. catalase, superoxide dismutase (SOD)), and non-enzymatic (e.g. vitamins C and E), which act as free radical scavengers, effectively 'soaking up' excess free radicals.

Historically, the presence of free radicals in biological materials was first cited more than fifty years ago by Commoner *et al.* (1954), and the concept that free radicals/ reactive oxygen or nitrogen species (RO/NS) play an important role in biology and medicine was proposed in 1969, by Fridovich and McCord who cited the existence of a common, nearly ubiquitous cellular protein (now known to be Superoxide Dismutase (SOD)) that served to function as a free radical scavenger (McCord and Fridovich, 1969). This finding indicated that cells had evolved a defence mechanism against endogenously generated RO/NS. This notion was ridiculed at the time, giving rise to much scientific controversy, many scientists arguing that the sheer toxicity and reactivity of molecules like superoxide radical ($O_2^{\bullet-}$) would not allow their existence in living things. However, the explosion of research into the field of free radicals later proved their findings to be correct (Scanalios, 2002), and prompted further investigations into oxidative damage caused by free radicals to DNA, protein, lipids and other cellular constituents (Beckman and Ames, 1998). The discovery of SOD finally provided mechanistic evidence for Harman's free radical theory of ageing, proposed in 1956, in which it was speculated that cumulative cellular damage induced by radicals may contribute to the ageing process (Harman, 1956).

Indeed, since the evolution of aerobic life, all living organisms have been constantly exposed to both endogenously generated RO/NS as well as exogenous insults, and have evolved mechanisms to defend against toxicity (the aforementioned antioxidant systems), and mechanisms to exploit the potential benefits of the metabolites. Obvious positive uses of ROS include the conversion of food to energy, and defence against microorganisms. More recent findings show that RO/NS act as signal transducing molecules, having significant roles in the activation of transcription factors leading to gene expression (Ahmad *et al.*, 2006; Droge, 2001; Karin *et al.*,

2001; Sen and Packer, 1996; Storz and Polla, 1996; Schenk, 1994; Whiteside and Goodbourn, 1993; Kerr *et al.*, 1992).

Intracellular defence mechanisms deal very efficiently with the removal of excess RO/NS, thus preventing toxicity and cellular damage. Table 1.3 summarises the sources of endogenous and exogenous free radicals, as well as the mechanisms of their removal. Figure 1.7 further elaborates on intracellular generation of free radicals.

Table 1.3 Summary of the Generation and Removal of Reactive Oxygen and Nitrogen Species (RO/NS) in the cell.

Cellular oxidants	**Source**	**Oxidative species**
Endogenous	Mitochondria	$O_2^{\bullet -}$, H_2O_2, $^{\bullet}OH$
	Cytochrome P450	$O_2^{\bullet -}$, H_2O_2
	Macrophage/inflammatory cells	$O_2^{\bullet -}$, H_2O_2, $^{\bullet}NO$,OCl^-
	Peroxisomes	H_2O_2
Exogenous	Redox cycling compounds	$O_2^{\bullet -}$
	Metals (Fenton reaction)	$^{\bullet}OH$
	Radiation	$^{\bullet}OH$
Cellular antioxidants		
Enzymatic	***Nonenzymatic***	
Superoxide dismutase	Vitamin E	
Catalase	Glutathione	
Glutathione peroxidase	Vitamin C	
Glutaredoxin	Catechins	
Thioredoxin	β-carotene	

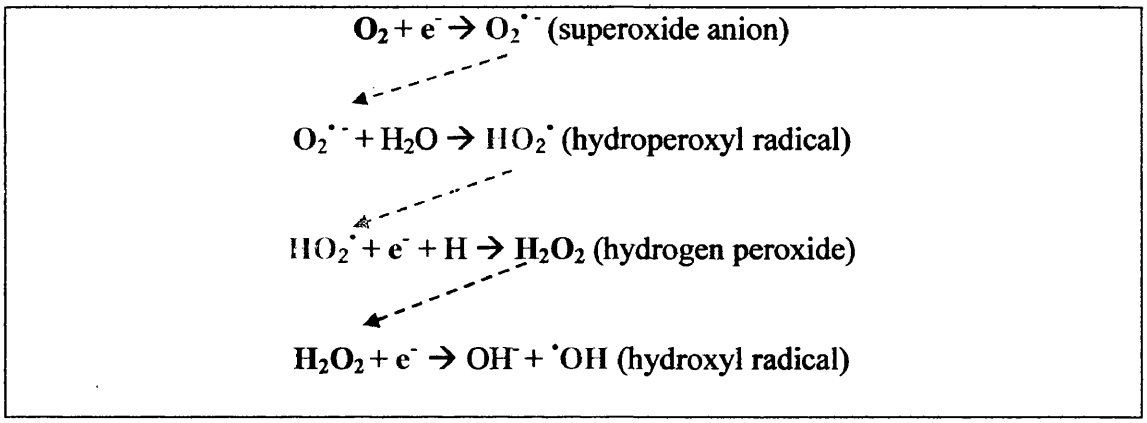
1.6.1 Oxidative Stress at the cellular level

Within the eukaryotic cell, most RO/NS originate from mitochondrial oxidative metabolism/ phosphorylation due to the leakage of RO/NS from the electron transport chain (ETC). Extra – mitochondrial sources also exist, including the endoplasmic reticulum (ER), the plasma membrane, peroxisomes, and the cytosol, where RO/NS are formed enzymatically via xanthine oxidase, cytochrome P450, reduced Nicotinamide Adenine Dinucleotide Phosphate (NADPH) oxidases, nitric oxide synthase (NOS), as well as other enzymes including those involved in

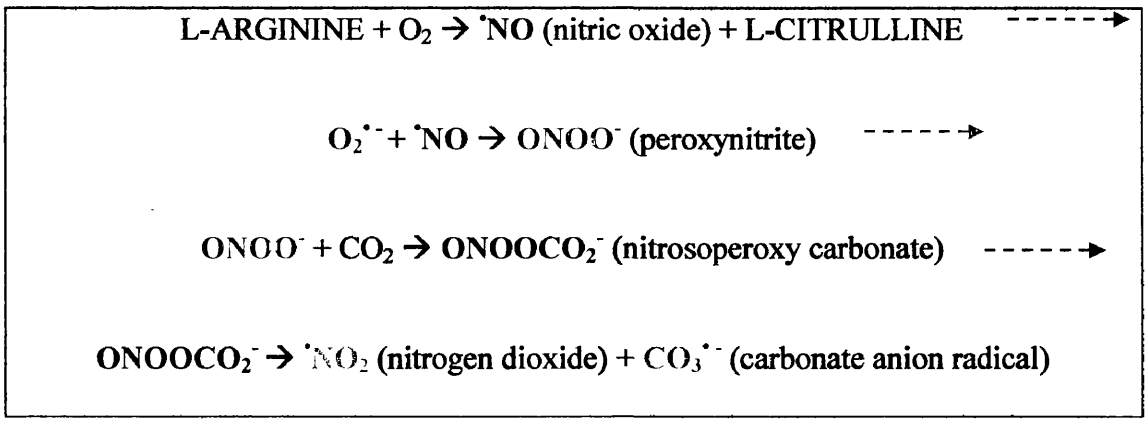
arachidonic acid metabolism such as cyclooxygenases (COXs and lipoxygenases (LOXs)) (Balaban *et al.*, 2005; Curtin *et al.*, 2002; Storz, 2006).

Figure 1.7 The formation of cellular RO/NS.

a. Generation of reactive oxygen species via reduction of molecular oxygen



b. Production of reactive nitrogen species



c. Fenton reaction

Research now dictates that the most biologically relevant radicals are superoxide ($O_2^{\bullet -}$), hydroxyl radical (·OH, considered the ultimate reactive metabolite (Henle and Linn, 1997)), and Nitric oxide (NO). These radicals have endogenous enzymatic origins, $O_2^{\bullet -}$ being generated by NAD(P)H oxidase (and xanthine oxidase), and NO arising as a consequence of activities of the various Nitric oxide synthase

(NOS) isoforms that exist (Dröge, 2002), whilst ·OH can be formed as a derivative of other species by non-enzymatic means. Figure 1.7 illustrates the generation of various radicals. Superoxide anion is formed by the univalent reduction of molecular oxygen. This process can be mediated by enzymes, as stated and also non-enzymatically by redox reactive compounds (e.g. the semi-ubiquinone compound of the mitochondrial electron transport chain). Superoxide can be converted into hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD), and can also be converted, non-enzymatically into H_2O_2 and singlet oxygen ($^1O_2$). The highly reactive hydroxyl radical (·OH) may also be generated from H_2O_2 in the presence of reduced transition metals (e.g. ferrous or cuprous ions) via the Fenton reaction (Fig. 1.7(c)). Likewise, several derivative species can be generated from NO (as summarised in Fig. 1.7(b)). Many of the physiological effects of the principle free radicals are brought about through their intermediates and by-products. The fact that so many species can co-exist in a given cellular environment at any time, makes it very challenging to pinpoint which agents are responsible for a given biological effect (Dröge, 2002).

RO/NS generally exist in cells at low but measurable concentrations, their levels being dependent upon the underlying balance between their rate of production (and/ or exposure) and their rate of removal by antioxidant enzymatic and non-enzymatic systems. Antioxidants have been defined in the literature as substances that are able to compete with other oxidisable substrates, even at relatively low concentrations, hence delaying significantly, oxidation of cellular targets (Halliwell and Gutteridge, 1989). This definition includes the non-enzymatic compounds such as ascorbate (Vitamin C), α-tocopherol (Vitamin E), β-carotene, and glutathione; and the enzymes, SOD, catalase, glutathione peroxidase (GPx), glutaredoxin and thioredoxin (table 1.3). When a redox imbalance exists in the cellular environment, the cell/ tissue is said to be in a state of *oxidative stress*. This is usually tightly balanced by the aforementioned antioxidant systems, in addition to other cellular components such as free amino acids (albeit at high molar concentrations) (Dröge, 2002), and often by the RO/NS themselves. A state of increased RO/NS concentrations can elicit negative feedback pathways that serve to lower markedly elevated levels. More interestingly, a major mechanism in redox homeostasis is now seen to be RO/NS-mediated induction of redox sensitive signal cascades that lead to increased expression of antioxidant enzymes, and other cellular antioxidant systems. Thus it is clear that living cells have

evolved several mechanisms to restore redox balance after exposures to increased RO/NS concentrations.

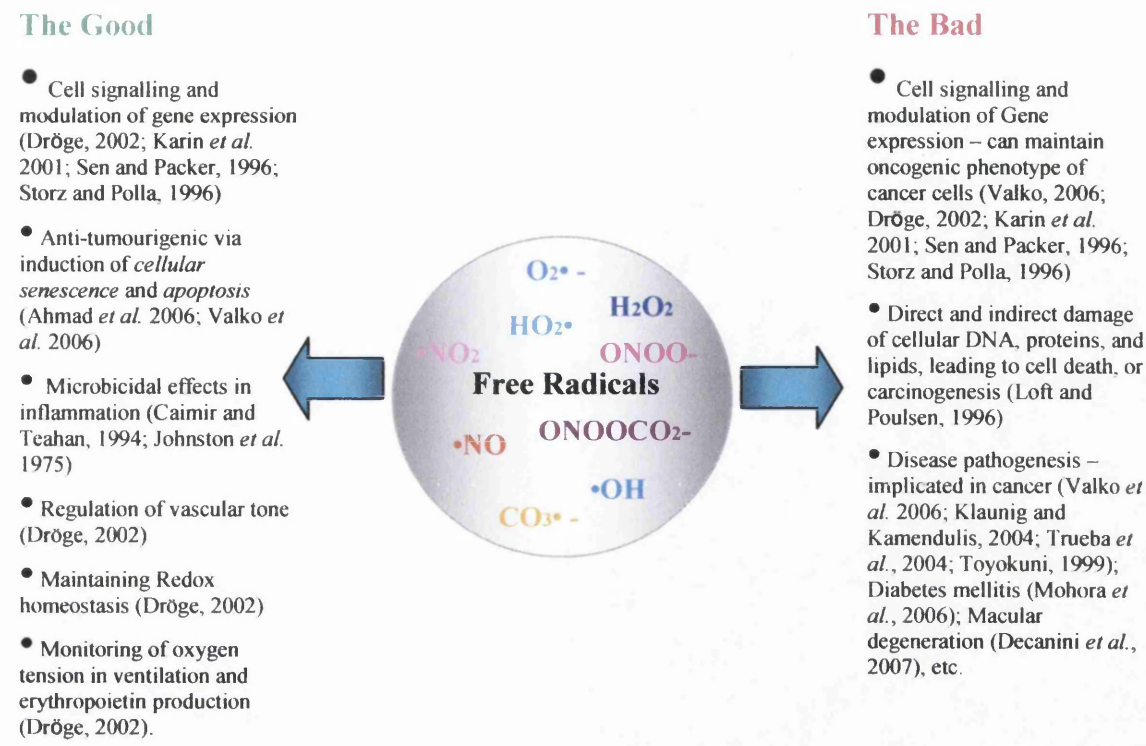
Unfortunately instances of oxidative stress can arise, as a result of environmental exposures to toxins, drugs, infection, etc., leading to shifts in the balance towards increased levels of RO/NS. This oxidative stress is often the underlying mechanism of several pathological conditions, such as chronic inflammation, rheumatoid arthritis, atherosclerosis, and cancer.

What impact does oxidative stress have at the cellular level?

Oxidative stress can have detrimental effects on cells, imposing oxidative damage on cellular macromolecules that can culminate in DNA damage, lipid peroxidation, protein oxidation, and changes in signal transduction and gene expression. The deleterious effects of RO/NS have long been known, the direct and indirect role these species play in DNA, cellular, and tissue damage, chronic tissue injury and disease is well documented (Mohora *et al.* 2006; Valko *et al.* 2004; Matteucci *et al.* 2000; Vendemiale *et al.* 1999; Trueba *et al.* 2004; Jenner, 1996; Knight, 1995; Stohs, 1995; Kehrer, 1993). Figure 1.8 summarises both the deleterious, and the beneficial effects of RO/NS.

The story of RO/NS remains one of a double-edged sword – on the good side, they can protect against oxidative stress, maintaining redox balance via redox homeostasis; they provide defence against infectious agents, being an integral part of the innate (non-specific) immune response. RO/NS are recognised as having an important role in infection and inflammation as bactericidal species (Casimir and Teahan, 1994; Johnston *et al.*, 1975); they can induce apoptosis and cellular senescence (hence may be anti-tumourigenic) (Valko *et al.*, 2006); and they can act as second messengers, leading to activation of pathways and genes that regulate cell growth. Ironically, this beneficial effect is also one that can lead to an oncogenic phenotype through enhanced mitogenesis (Valko *et al.*, 2007; Klaunig and Kamendulis, 2004; Dröge, 2002). In situations of chronic inflammation (e.g. as a consequence of persistent *H. pylori* infection) normal host cells in the vicinity can be continuously bombarded with RO/NS and their damaging effects, which can, in some instances, lead to the development of cancer.

Figure 1.8 Summary of the beneficial and deleterious effects of free radicals in biological systems.



1.6.2 Oxidative Stress and Cancer

As already discussed in section 1.6, RO/NS can induce DNA damage (and hence mutations), lipid peroxidation, changes to the cellular proteome, changes in apoptosis, cellular senescence and changes in the transduction of intracellular signalling cascades. All of these changes can result in alterations in a cells proliferative capacity, and hence drive and/ or maintain an oncogenic phenotype (Valko *et al.*, 2006). Redox imbalance resulting from oxidative stress has been observed in many cancer cells when compared to their normal counterparts (Halliwell *et al.*, 1992; Szatrowski and Nathan, 1991) and the importance of oxidative stress to carcinogenic processes has become increasingly well documented in the past decades (Fukuruma *et al.*, 2006; Ristow, 2006; Valko *et al.*, 2006; Waris and Ahsan, 2006; Klaunig and Kamendulis, 2004; Oshima *et al.*, 2003; Klauning *et al.*, 1998; Poulsen *et al.*, 1998; Wink *et al.*, 1998; Knight, 1995; Toyokuni *et al.*, 1995; Feig *et al.*, 1994;

Guyton and Kensler, 1993; Trush and Kensler, 1991; Breimer, 1990; Vuillaume, 1987; Ames, 1983).

One of the critical ways in which these molecules drive cancer development is via DNA mutation. In this way, RO/NS impact the initiation stages of carcinogenesis (Valko *et al.*, 2006). The continued presence of abnormal levels of RO/NS in initiated cells can then drive the promotion stage by firing off signal transduction pathways that promote cell growth and hence maintain an oncogenic phenotype. RO/NS are thus viewed as complete carcinogens since they can impact both the initiation and promotion stages of disease pathogenesis.

In a clinical context relevant to gastric carcinogenesis, oxidative stress is believed to be a key component of the chronic inflammatory processes that lie at the root of the molecular pathogenesis of the disease (Correa, 2006; Farinati *et al.*, 2003; Baik *et al.*, 1996; Davies *et al.*, 1994a).

1.7 Inflammation and Cancer

Chronic inflammation has been linked to several human cancers (Hagemann *et al.*, 2007; Balkwill *et al.*, 2005; Balkwill and Coussens, 2004; Coussens and Werb, 2002). The link between inflammation and cancer in fact traces back over two millennia to the Greek doctor Galen. In the 19th century Virchow hypothesised that tumours arise at sites of chronic inflammation (Coussens and Werb, 2002; Balkwill and Mantovani, 2001), and more recently tumours have been likened to 'wounds that do no heal' (Dvorak, 1986) really emphasising the importance of the relationship.

The underlying inflammation may be the result of chronic/ persistent exposures to infectious or physical agents, and indeed several infectious agents have now been classified as human carcinogens (Oshima *et al.*, 2003). Analysis of IARC monographs leads to the estimation that 18% of the worldwide cancer incidence was attributable to infectious agents in the year 2000 (compared to 16% in 1990), 4.5% being linked to *H. pylori,* which exemplifies the inflammation – cancer link (IARC, 1994). As already detailed, chronic infection with *H. pylori* leads to tissue inflammation in the stomach, manifest as chronic gastritis. In addition, inflammatory conditions can be caused by chemical and physical agents such as alcohol, tobacco

smoke, salt, etc. and consequently these are all recognised risk factors for various cancers. On these grounds it appears that a significant proportion of the global cancer burden can be causally linked to chronic infections and inflammation. The mechanistic nature of infection-/ inflammation-induced carcinogenesis is not fully elucidated. One hypothesis is that the inflammatory processes release excessive amounts of microbicidal reactive oxygen and nitrogen species (RO/NS) leading to a situation of oxidative stress, the consequences of which were outlined in section 1.6.1. The major source of these highly reactive molecular species in such instances, is the bodies defender cells – the leukocytes (Weitzman and Gordon, 1990) whose main purpose is to ward off invaders. Inflamed tissues are characterised by marked infiltration of inflammatory leukocytes, predominantly neutrophils and macrophages (Kozol, 1992), the innate immune responses effector cells, attempting to deal with the infection, and/ or tissue injury present at the affected site. The cells are recruited to the affected site and are activated to produce potent inflammatory mediators (including cytokines and chemokines) and microbicidal factors (e.g. RO/NS) primarily to attack and destroy invading microorganisms and foreign bodies. Inflammatory mediators released into the local tissue microenvironment serve to chemo-attract and/ or activate further inflammatory cells, leading to a heightened inflammatory response (de Visser and Coussens, 2006; Coussens and Werb, 2002a; Coussens and Werb, 2002b; Wilson and Balkwill, 2002; Torisu *et al.,* 2000). Typically, phagocytic cells (mainly neutrophils and macrophages) become highly active and release cytotoxic factors such as RO/NS including singlet oxygen, superoxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), and nitric oxide (NO). In addition, the inflammatory cells convert these into more highly reactive RO/NS, such as hydroxyl radicals ($^{\cdot}OH$), hypochlorous acid (HOCl), and peroxynitrite ($ONOO^-$) (D'Alessandro *et al.,* 2003; Oshima *et al.,* 2003). Under normal circumstances the inflammatory response is tightly controlled, so that upon eradication of the microorganism, foreign bodies, or irritant, the inflammation dampens down, such that the response is acute. If however, infection is not resolved rapidly, or if control of the response is not tightly regulated, the inflammation can become chronic.

Chronic inflammation is accompanied by complications that make an individual more susceptible to the development of various diseases, including cancer. A prolonged inflammatory response leads to the continual activation of inflammatory leukocytes and the concomitant release of RO/NS, proteolytic enzymes, and

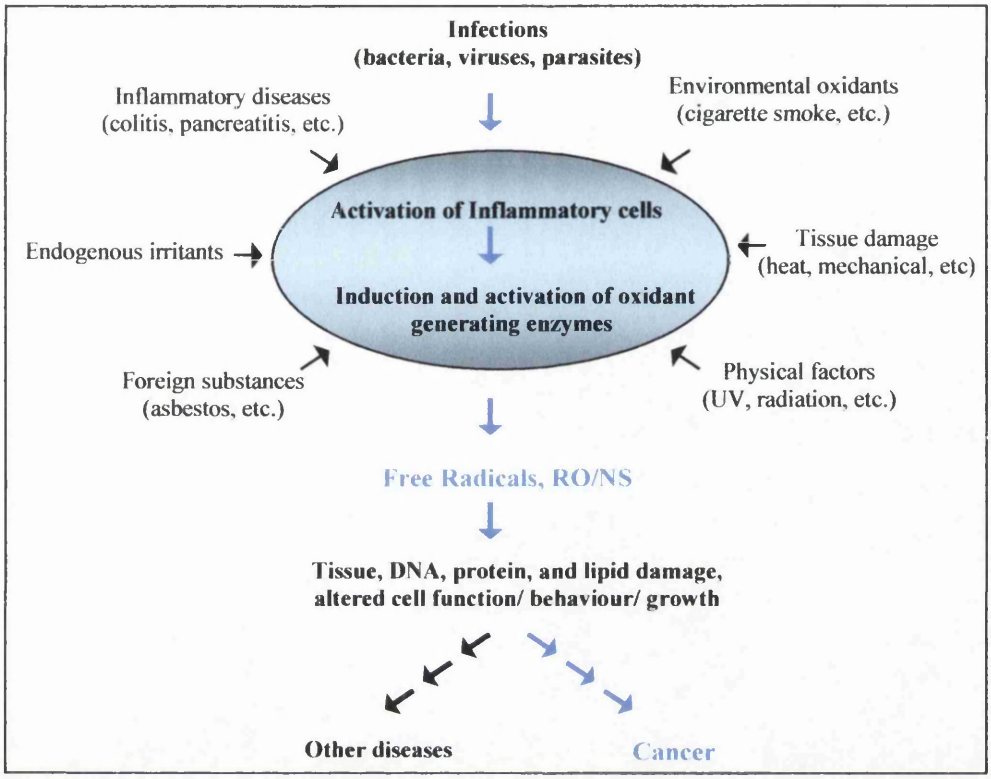
inflammatory mediators, creating a hostile microenvironment that can lead to tissue damage, and may favour the development of various pathological conditions, such as cataracts, macular degeneration (Decanini *et al.*, 2007); pancreatitis (Schulz *et al.*, 1999); diabetes (Mohora *et al.*, 2006; Dröge, 2002; Matteucci and Giampietro, 2000; Stohs, 1995); diabetic retinopathy (Stohs, 1995); diseases of old age such as atherosclerosis (Stohs, 1995); rheumatoid arthritis (Dröge, 2002); neurodegenerative diseases like Alzheimer's disease, Parkinson's disease, Huntington's disease, multiple sclerosis (MS), Friedreich's Ataxia (Calabrese *et al.*, 2005; Dröge, 2002; Borlongan *et al.*, 1996; Jenner, 1996); as well as neoplastic diseases (de Visser and Coussens, 2006; Balkwill and Coussens, 2004; Klaunig and Kamendulis, 2004; Coussens and Werb, 2002; Dröge, 2002; Balkwill and Mantovani, 2001; Robinson, 1998; Conner and Grisham, 1996). On these grounds chronic inflammation has been described as 'pathological inflammation' as oppose to acute inflammation which has been termed 'therapeutic inflammation' due to its ability to fairly rapidly resolve infection and injury with minimal damage to the host (Aggarwal *et al.*, 2006).

Activated phagocytes have long been documented to play roles in carcinogenesis by the induction of point mutations, chromosome damage, deregulated DNA methylation, and malignant transformation both *in vitro* and *in vivo* (Weitzman and Gordon, 1990; Weitzman *et al.*, 1989; Weitzman *et al.*, 1985; Weitberg *et al.*, 1983; Weitzman and Stossel, 1982; Weitzman and Stossel, 1981). So it has become apparent that, in some situations, inflammatory cells; in particular neutrophils and macrophages with the aim of dealing with the infection or injury at the site, can, in fact, make matters worse by generating and releasing RO/NS and cytokines, both of which can drive several aspects of cancer development (Valko *et al.*, 2006; Strieter, 2001).

This is often observed in gastric carcinogenesis, where chronic inflammation (an end result of *H. pylori* infection, dietary irritation, or other complications) is believed to be a major risk factor (Macarthur *et al.*, 2004; Shacter and Weitzman, 2002; Ernst, 1999; Oshima *et al.*, 1993). Figure 1.9 summarises the inflammation – cancer link.

In addition to impacting cells directly at the genetic level via the induction of DNA damage and thus mutations, chronic inflammation (and oxidative stress) also affects cells at the epigenetic level by causing changes in cellular signal transduction and gene expression, so driving changes in cell behaviour, function, and growth.

Figure 1.9 The inflammation – cancer link.



1.8 Signal Transduction and Cancer

Observations that cancer cells often display dysregulated behaviour in that they often proliferate and grow excessively when they should not, and do not die (via apoptosis) when they should, has prompted researchers to unravel the molecular mechanisms that underlie such changes in behaviour. This led to the realisation that in addition to the mutations and chromosomal aberrations frequently reported as common features of malignant disease, changes in signal transduction are also central. As such, cancer is more and more frequently being described as a disease of mis-regulated signal transduction (Arbiser, 2004; Eshel *et al.,* 2002; Radisky *et al.,* 2001; Fedi *et al.,* 1997; Hunter, 1997; Kohn *et al.,* 1992).

It is unsurprising that signalling pathways are often seen to be perturbed in tumour tissues as well as in pre-malignant stages of disease owing largely to the complexity of the pathways. One small change in a gene involved in a pathway or

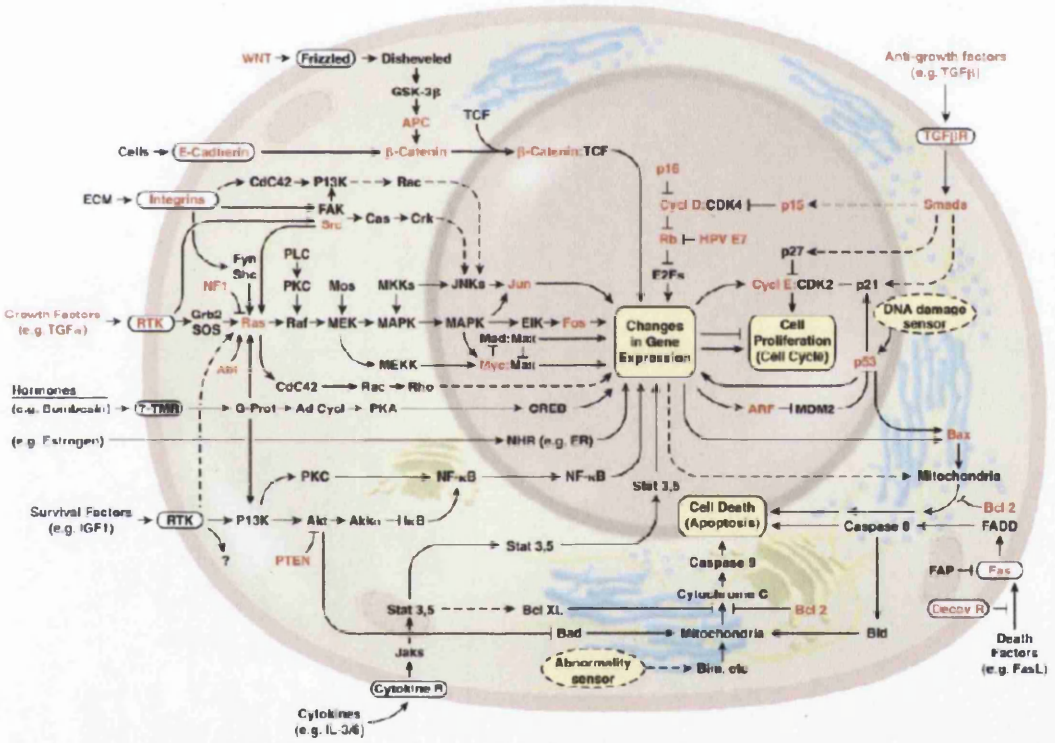
changes in external stimuli (as is often the case in the tumour microenvironment) could cause major signalling changes (Fedi *et al.,* 1997) – owing to the inherent complexity of pathways, overlap between pathways, and the signal amplification feature of cellular signalling cascades. Figure 1.10 illustrates the intricate networks of cellular signal transduction that have been delineated and highlights the complex interactions that exist both within and between pathways.

Of particular relevance to the present research are the effects of inflammatory processes and associated oxidative stress on signal transduction and gene expression changes, since such changes may lead to mitogenic and/ or oncogenic changes that lead to an ultimate cancer endpoint. Often the aberrant signalling detected in cancer results in enhanced cell survival and proliferation, and/ or reduced capacity for apoptosis (both hallmarks of cancer (Hanahan and Weinberg, 2000)), thereby causing an imbalance in cellular homeostasis, with a shift toward enhanced growth. Mitogen Activated Protein Kinase (MAPK) signalling (section 1.8.1) plays a key role in maintaining cellular homeostasis, and disruption of the pathway(s) is often manifest in many cancers (Dhillon *et al.,* 2007; Li *et al.,* 2003). The Nuclear factor - kappa B (NFκB) pathway (section 1.8.2) is also seen to play a pivotal role, mis-regulation of which can influence cell survival (Bubici *et al.,* 2006; Beg and Baltimore, 1996). Interestingly, both of these pathways have been reported to be affected in inflammation associated cancers (Balkwill and Coussens, 2004; Karin, 2004; Li and Verma, 2002; Dimitru *et al.,* 2000; Barnes and Karin, 1997; Karin, 1995) including *H. pylori* associated gastric cancer (Naumann and Crabtree, 2004; Maeda *et al.,* 2000; Meyer-Ter-Vehn *et al.,* 2000; van Der Brink *et al.,* 2000; Naumann *et al.,* 1999; Aihara *et al.,* 1997; Malinin *et al.,* 1997; Muller *et al.,* 1997). Moreover, both pathways can be induced by inflammatory mediators including cytokines, e.g. TNFα in the case of NFκB (Balkwill and Coussens, 2004; Greten *et al.,* 2004; Pikarsky *et al.,* 2004) and RO/NS (Gloire *et al.,* 2006; Takada *et al.,* 2003; Zhang *et al.,* 2001; Bowie and O'Neill, 2000; Manna *et al.,* 1998; Wang *et al.,* 1998; Aikawa *et al.,* 1997; Qin *et al.,* 1997; Guyton *et al.,* 1996; Rao,1996; Sen and Packer, 1996; Meyer *et al.,* 1993; Schreck *et al.,* 1991) since both pathways have redox sensitive components.

The major focus of the extensive research carried out here is inflammation and oxidative stress induced signal transduction and gene expression changes in gastric carcinogenesis, with a particular focus on MAPK and NFκB. As such, the pathways

and their relevance to cancer are detailed further in relevant sections throughout the thesis and are detailed briefly in sections 1.8.1 and 1.8.2 that follow.

Figure 1.10 The integrated networks of signal transduction pathways within a cell, which serve to relay extracellular signals through the cytoplasm and to the nucleus where an ultimate effect on gene expression and so cell behaviour/ physiology occurs. Amongst the complex pathways are the MAPK and NFκB pathways which are discussed in sections 1.8.1 and 1.8.2. Taken from Hanahan and Weinberg, 2000.



1.8.1 Mitogen Activated Protein Kinase (MAPK) pathway

The Mitogen Activated Protein Kinase (MAPK) pathway is an important signal transduction pathway consisting of (as its name suggests) a cascade of MAPK enzymes, each leading to the activation of the subsequent enzyme via a phosphorylation reaction (Su and Karin, 1996). These kinase enzymes are used

throughout evolution to control cellular responses to external signals such as stress, nutrient status, inductive signals and growth factors (Treisman, 1996), relaying messages from cell surface receptors to the nucleus (Turjanski *et al.*, 2007; Chang and Karin, 2001). These pathways are unique to eukaryotic cells, and affect many aspects of cellular regulation. In recent years it has become increasingly clear that MAPK pathways have knock on effects on almost all cellular process, ranging from gene expression to cell death (Chang and Karin, 2001; Graves *et al.*, 2000; Treisman, 1996). As a result these pathways can be seen as master controllers, and can influence whether a cell grows in a normal or aberrant fashion.

In mammalian systems at least four distinctly regulated groups of MAPK pathways have been identified, making up a MAPK superfamily. These include extracellular signal regulated kinase 1 (ERK1) and ERK2 (also known as p44(MAPK) and p42(MAPK)); Jun amino terminal kinases (JNK1/2/3); ERK5 (or BMK); and p38 MAPKs (p38α/β/γ/δ) (Chang and Karin, 2001; Gutkind, 2000). Each MAPK enzyme is inactive in the cell, unless an extracellular stimulus sets in motion the pathways that lead to their activation and their concomitant effects on cellular physiology.

It is fundamental that the MAPK enzymes and/ pathways have a high level of specificity, so that they can control several diverse cellular processes in response to a plethora of extracellular stimuli, particularly since this is achieved by all but a few specific MAPK enzymes (Chang and Karin, 2001; Schaffer *et al.*, 1998; Wasserman and Matthew,1998; Xia *et al.*, 1998). According to the literature, there is substantial evidence of the involvement of MAPK cascades in cellular processes including - regulation of gene expression, at both the levels of transcription, and translation; regulation of cell proliferation; control of cell cycle progression; regulation of cell survival; and influencing cell motility (Chang and Karin, 2001; Ichijo, 1999; Su and Karin, 1996; Treisman, 1996).

One of the most explored functions of MAPK signalling cascades is the regulation of gene expression in response to extracellular stimuli (Treisman, 1996). A key finding was that members of a family of transcription factors known as Activator Protein-1 (AP-1), which have been implicated in inflammation related changes in gene expression, are dependent on MAPK signalling pathways for their activity (Karin, 1995). AP-1 is seen as a 'master switch' in the control of cell life and death, mis-regulation of which can lead to oncogenic changes (Shaulian and Karin, 2001). The way in which MAPK pathways affect AP-1 activity is most commonly via the

phosphorylation (and hence activation) of Ets transcription factors which, in turn, lead to the induction of fos genes, the products of which dimerise with other FOS proteins, or JUN proteins to form AP-1 transcription factor complexes, with the c-FOS/ c-JUN dimer being the most commonly found in cells (Chang and Karin, 2001; Karin *et al.*, 1995; Angel and Karin, 1991; Chiu *et al.*, 1988). Further details of the link between MAPK signalling and AP-1 activation are outlined in chapter 3, section 3.1.2.1. From this well studied example, it is apparent that MAPK signalling modules can directly influence gene expression at the level of transcription factors.

Interestingly with respect to the present studies, aberrant MAPK signalling has been reported in several cancers (Dhillon *et al.*, 2007; Kohno and Pouyssegur, 2006; Dunn *et al.*, 2005; Calipel *et al.*, 2003; Li *et al.*, 2003) including gastric cancer (Watari *et al.*, 2007; Liang *et al.*, 2005; Moon *et al.*, 2005; Sepulveda *et al.*, 2002; Gong *et al.*, 1999; Barnard *et al.*, 1995; Tahara, 1995b), and can be induced by *H. pylori* infection (Du *et al.*, 2007; Keates *et al.*, 2005; Zhu *et al.*, 2005; Wallasch *et al.*, 2002; Hirata *et al.*, 2001; Meyer-ter-Vehn *et al.*, 2000; Stein *et al.*, 2000; Wessler *et al.*, 2000; Keates *et al.*, 1999; Segal *et al.*, 1999). As such MAPK pathways are viewed as a molecular link in the intimate relationship between inflammation and cancer (Karin, 2005). Likewise, NFκB signalling is key in this association.

1.8.2 Nuclear Factor Kappa-B (NFκB) pathway

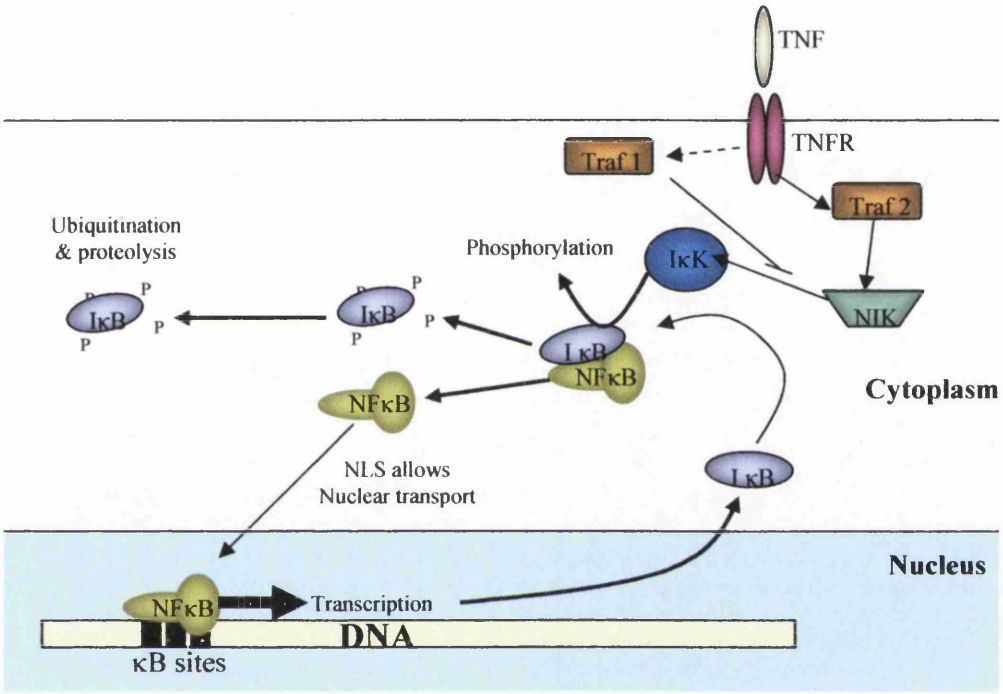
The NFκB family of transcription factors are critical components in the complex circuitry of cellular signal transduction and gene expression. The NFκB transcription factors are seen as master co-ordinators of diverse functions including immunity, inflammation, differentiation, and cell survival (Claudio *et al.*, 2006; Hayden and Ghosh, 2004). NFκB has received much attention in the scientific community since its discovery in 1986 (Gilmore and Temin, 1986; Sen and Baltimore, 1986) owing to its anti-apoptotic and pro-inflammatory capabilities which have frequently been linked to pathogenic states, in particular those associated with chronic inflammation, immunodeficiency, and cancer (Aggarwal *et al.*, 2006; Courtois and Gilmore, 2006; Dolcet *et al.*, 2005; Rayet and Gélinas, 1999). The importance of NFκB is highlighted in the existence of a website dedicated to NFκB research (**www.nf-kb.org**), with over 25,000 research/ review articles published to date (Gilmore, 2006).

At the level of signal transduction, the NFκB pathway serves as a paradigm of the transfer of signals from extracellular stimuli to the cells core at the nucleus (Hayden and Ghosh, 2004; Rothwarf and Karin, 1999). A critical step in this pathway is the activation of the NFκB transcription factor (comprised of dimers of proteins from the NFκB (e.g. p50, p52) and REL (e.g. RELB, RELA (p65)) superfamilies of NFκB proteins, with the p50 – RELA (i.e. p50-p65) dimer being the major form of NFκB transcription factor found in cells (Gilmore, 2006). Typically NFκB is constitutively expressed in eukaryotic cells in an inactive form, thus, activation of the transcription factor does not require *de novo* gene expression, control being achieved at the post-translational level. In its inactive state NFκB is present in the cytoplasm sequestered by an inhibitory protein - inhibitor of kappa-B (IκB) (Beg and Baldwin, 1993; Beg *et al.,* 1992; Baeurele and Baltimore, 1988). When cells are exposed to an activating stimulus signals are relayed from the cell surface membrane. Several upstream signalling processes have been described in NFκB signalling which vary depending upon the activating stimulus and cell type (Gilmore, 2006; Perkins, 2006) all of which converge at the activation of IκB kinase (IKK) complexes. IKK serves to phosphorylate IκB marking it for subsequent ubiquitination and degradation by the cellular proteasome. Degradation of IκB results in liberation of active NFκB which translocates to the nucleus where it drives the expression of NFκB regulated genes, including IκB (in a negative feedback manner), genes involved in inflammation, e.g. *IL-1*, *IL-6*, *IL-8*, *TNFA*, adhesion molecules, e.g. *VCAM*, *ICAM-1*, etc. (McKay and Cidlowski, 1999).

Diverse stimuli can lead to the activation of NFκB signalling including inflammatory cytokines, e.g. TNFα (Gilmore, 2006; Balkwill, 2004; McKay and Cidlowski, 1999; Schreck *et al.,* 1992), and RO/NS (detailed further in chapter 3 section 3.1.3) (Gloire *et al.,* 2006; Takada *et al.,* 2003; Zhang *et al.,* 2001; Bowie and O'Neill, 2000; Manna *et al.,* 1998; Wang *et al.,* 1998; Sen and Packer, 1996; Meyer *et al.,* 1993; Schreck *et al.,* 1991). Figure 1.11 illustrates one of the possible pathways for the activation of NFκB.

Aberrant NFκB signalling has been found to be a common feature of inflammation associated cancers (Aggarwal *et al.,* 2006; Courtois and Gilmore, 2006; Dolcet *et al.,* 2005; Balkwill and Coussens, 2004; Jenkins *et al.,* 2004; Karin, 2004; Li and Verma, 2002; Dimitru *et al.,* 2000; Rayet and Gélinas, 1999; Barnes and Karin, 1997; Karin, 1995) including gastric cancer.

Figure 1.11 Activation of the NFκB pathway by TNF. TNF activates NFκB by way of TNFR/Traf2/NIK/IKK signalling, which can be inhibited by Traf1. Ultimately IκB is degraded and active NFκB is free to enter the nucleus (removal of IκB results in exposure of nuclear localisation sequence (NLS) on NFκB so allowing nuclear translocation) where it can switch on target genes including *IκB*, which results in negative feedback as show. TNF = tumour necrosis factor; TNFR = tumour necrosis factor receptor; Trafs = tumour necrosis factor receptor – associated factors; NIK = NFκB inducing kinase. Figure adapted from Jenkins *et al.* (2004).



Interestingly *H. pylori* has been seen to rapidly induce the activation of NFκB signalling and the expression of target genes such as *IL-8* in gastric epithelial cells (Naumann and Crabtree, 2004; Maeda *et al.*, 2000; Meyer-Ter-Vehn *et al.*, 2000; Naumann *et al.*, 1999; Crabtree, 1998; Shimoyama *et al.*, 1998; Aihara *et al.*, 1997; Keates *et al.*, 1997; Malinin *et al.*, 1997; Muller *et al.*, 1997; Crabtree *et al.*, 1995). On these grounds the study of NFκB signalling in relation to inflammatory processes and oxidative stress in gastric cancer is warranted.

1.9 Aims of the Project

Upon review of the literature regarding potential pathogenic mechanisms that underlie gastric carcinogenesis it becomes quite clear that inflammation and oxidative

stress are critical components which can act either independently, or in conjunction with *H. pylori* infection to induce a multitude of molecular anomalies within cells.

The present work sets out to gain further insights into the involvement of inflammation and oxidative stress in gastric carcinogenesis at the level of signal transduction and gene expression changes using *in vitro* and *in vivo* models to test the hypothesis:-

Chronic gastric inflammation & accompanying generation of Reactive Oxygen Species (ROS) plays a significant role in gastric carcinogenesis by the induction of molecular changes (signal transduction, and gene and protein expression changes).

The broad aim of the studies was to identify signalling and gene expression changes that may be incurred in gastric cancer development, by inflammation and oxidative stress, which could potentially occur early on in the progression sequence (as early as gastritis). The work was carried out with the hope of identifying biomarkers - key molecular changes - that can be used in a clinical context to identify patients at high risk of gastric cancer development, enable early diagnosis, determination of prognosis, and monitor treatment in patients. The identification of signalling changes would also bring to light potential therapeutic targets, and recognition of the involvement of inflammation and oxidative stress in gastric cancer would add weight to the current prevention strategies employing anti-inflammatory drugs and/ or antioxidant supplementation (Correa *et al.*, 2004; Jones-Blackett *et al.*, 1999).

Two *in vitro* tissue culture models were employed in the studies – one in which cells were exposed to various doses of a model ROS, hydrogen peroxide (H_2O_2), for varying lengths of time in order to mimic the oxidative stress component of a gastric inflammatory response (chapter 3); and one in which gastric epithelial adenocarcinoma cells (HGC-27) were co-cultured with inflammatory leukocytes, so more realistically mimicking an *in vivo* inflammatory response (chapter 5). In the initial chemically induced model of oxidative stress (by way of H_2O_2 exposure) three cell lines were used - two gastric epithelial adenocarcinoma cell lines – AGS, derived from a primary gastric tumour; and HGC-27, derived from a lymph node metastasis of an advanced gastric tumour; and one normal fibroblast cell line - WILL1, derived from the foreskin of a one year old boy. The purpose of using three different cell lines

was to take into account any cell line differences in responsiveness between cancer cells and normal cells, and between cancer cells at different stages of the disease (AGS (early) vs. HGC-27 (late)). Obviously using cancer cell lines is not ideal for assessing changes in normal cells since the malignant cells are likely to respond differently. By using three different cell lines a clearer picture of changes consistently induced by ROS can be obtained. In the second *in vitro* model only the HGC-27 gastric cancer cell line was utilised due to time restraints and practical reasons. HGC-27 was co-cultured with inflammatory leukocytes derived from the HL-60 pro-myelocytic cell line optimised to generate ROS so that gastric epithelial cells are exposed to ROS from a more biologically relevant source in conjunction with other inflammatory mediators that may be released (e.g. cytokines and chemokines). In this way gastritis can be mimicked more closely to the *in vivo* situation. Following exposure of cells to H_2O_2 or inflammatory leukocytes, RNA and protein were extracted from cells for downstream signal transduction and gene expression analyses. Since the aim was to identify key signalling changes, initial studies involved the use of global microarray analysis of H_2O_2 treated cells in order to identify key pathways that are affected by oxidative stress. The findings from the experiments confirmed the involvement of MAPK and NFκB signalling in response to oxidative stress and helped in identifying downstream gene expression targets to analyse. Following on from this the pathways were explored in greater detail by assessing gene expression levels of the downstream targets – *c-FOS* and *VEGF* for MAPK, and *IL-8* and *IκB* for NFκB by way of quantitative (Q-)/ real time (RT-) PCR. Assessment of MAPK pathway activation was achieved via western blots for phosphorylated (and hence activated) MAPK proteins (phospho-ERK1/2 (p42/p44), phospho-p38). NFκB signalling was assessed further using a green-fluorescent protein (GFP) reporter construct in cells by way of transfection and subsequently visualising any cytoplasmic to nuclear transclocation of fluorescent, active NFκB protein following exposures by way of confocal microscopy. For the co-culture studies only *c-FOS* and *IL-8* gene expression were analysed by RT-PCR as markers for inflammation/ oxidative stress induced MAPK and NFκB signalling respectively, and MAPK signalling was further validated by way of western blots for phospho-ERK1/2 (p42/p44) (marker of ERK1/2 signalling).

In order to assess the clinical relevance of the *in vitro* studies, experimentation was subsequently translated to an *in vivo* study in which the signalling and gene

expression changes consistently observed in the cell culture studies were assessed in pre-malignant gastric biopsies in order to determine whether such changes may be truly important in early stages of gastric carcinogenesis (chapter 6). Pre-malignant biopsies (chronic inflammation/ gastritis, intestinal metaplasia, and *H. pylori* infected) were collected from consenting patients attending outpatient endoscopy clinic, RNA and protein extracted from the specimens, *c-FOS* expression analysed by RT-PCR and ERK signalling assessed by western blots for phospho-ERK. In this way the status of MAPK signalling and downstream gene expression in pre-malignant gastric tissues was assessed.

In brief, the findings from the studies highlight the involvement of oxidative stress in gastric carcinogenesis; at the levels of MAPK and NFκB signalling and downstream gene expression changes that favour cancer development; strengthening the notion that inflammation and oxidative stress are key players in driving the progression of cells to an ultimate cancer endpoint. This further emphasises the usefulness of therapeutic strategies that centre on blocking the generation of, and/ or sequestering excessive RO/NS in pre-malignant tissues such as antioxidant supplementation (Correa, 2004) and the use of anti-inflammatory drugs (Nardone and Rocco, 2004; Jones-Blackett *et al.,* 1999). In addition, the recognition of the involvement of signal transduction aberrations in disease pathogenesis brings forth the potential of targeted therapies. For instance, the identification of the involvement of MAPK signalling in gastric carcinogenesis supports the use of novel anti-inflammatory drugs that specifically target the pathways (Roberts and Der, 2007; Kaminska *et al.,* 2005; Karin, 2004) in gastric cancer therapy. Such approaches offer hope to aid tremendously in the management of this devastating disease.

Chapter 2

General Materials and Methods

Disposable gloves and laboratory coats were worn during all experimental manipulations for safety reasons and to avoid cross contamination.

Distilled water used in experiments was purified and deionised with the Milli-Q PF Ultrapure Water Purification System (Millipore, Watford, UK).

All general laboratory reagents/ chemicals used were from Sigma-Aldrich (Poole, UK) unless otherwise stated.

Specific materials and methods relevant to separate chapters are detailed in the materials and methods sections of the relevant chapters.

2.1 General Cell Culture

All cell culture manipulations were carried out using aseptic technique in class 2 Cytomat Pharmaceutical Safety cabinets (Medical Air Technology Ltd.). The cabinet, and all equipment going into it, were wiped down with 70% ethanol prior to culturing in order to maintain a good aseptic technique. All glassware and Pasteur pipettes used were autoclave sterilised at 121°C and 15psi for thirty minutes (Prior Clave Ltd). Disposable, sterile, plastic tissue culture flasks (NUNC, Fischer Scientific, Leicestershire, UK), pipettes, and centrifuge tubes (Sterilin, Stattfordshire, UK) were used for general culturing techniques. Cells were incubated in LEEC MK11 proportional temperature controller incubators at 37°C with an atmosphere of 5% CO_2. Cell culture medium was prepared freshly as and when required and was only used if a corresponding sterility test (section 2.1.1.2) was free from contamination. Only sterile solutions were used for cell culture and all solutions were pre-warmed in a 37°C water bath prior to use. On addition of culture medium to cells, the flasks were gassed briefly with CO_2 in order to adjust the pH of the medium.

2.1.1 Cell Lines

A total of four different cell lines were used in the present investigations, three attached cells lines – AGS, HGC-27, and WILL1, and one suspension cell line – HL-60.

2.1.1.1 Attached Cell Lines

The three attached cell lines employed in the experimentation were the gastric epithelial adenocarcinoma cell lines AGS and HGC-27, and the primary fibroblast cell line WILL1, and are described in section 3.2.1. Signal transduction and gene expression changes were examined in all three cell lines following treatment with hydrogen peroxide (H_2O_2). The purpose of using the three different cell lines was to establish if any cell line/ type differences existed in terms of response to exposures.

2.1.1.2 Cell Culture Medium

The growth medium used for the three attached cell lines was as follows:-

AGS – Ham's F12K (Gibco-BRL, Paisley, UK) plus 10% (v/v) Foetal Bovine Serum (FBS) (Gibco-BRL, Paisley, UK).
HGC-27 - Dulbecco's Modified Eagle's Medium (DMEM) (Gibco-BRL, Paisley, UK) supplemented with 10% (v/v) FBS and 1% non-essential amino acids (NEAA) (Gibco-BRL, Paisley, UK).
WILL1 - DMEM plus 10% (v/v) FBS.

Culture medium was prepared freshly as and when required following the aseptic techniques described in section 2.1. Each time fresh medium was prepared 10ml of the medium was placed in small 25cm^2 culture flasks, gassed with CO_2 and incubated at 37°C. This comprised a sterility test, and the medium in the flask was routinely monitored for cloudiness and particulate matter under the Nikon light microscope (Olympus Optical Co (UK) Ltd, London, UK) to check for contamination. Only medium that remained clear and free of contamination for at least 2 days in the sterility test was used for cell culture.

2.1.1.3 Initiation of Cell Lines

The cells were stored in freezing media (consisting of heat inactivated calf serum (HIS) + 10% Dimethyl Sulfoxide (DMSO)) in cryovials in liquid Nitrogen prior to use.

Cells were carefully removed from liquid Nitrogen and rapidly thawed by transferring to a 37°C water bath and gently agitating the cryovial. When completely thawed, the cryovials were cleaned with 70% ethanol and the cells were transferred to a centrifuge tube and centrifuged at 200 x g (1500rpm) for 8 min using an IEC Centra-3M centrifuge in order to pellet the cells. The DMSO was then removed by discarding the supernatant and 10ml of the appropriate pre-warmed medium was slowly added to the pellet to generate a cell suspension. The cell suspension was then transferred to a small 25cm^2 tissue culture flask and gassed with CO_2, in order to adjust the pH of the medium, and maintained in a 37°C incubator with an atmosphere of 5% CO_2 in air.

Cells were initially seeded at 2 – 4 x10^5 cells/ ml (for all three cell lines) into 25cm^2 flasks, incubated at 37°C, and checked regularly under the light microscope for inspection of cell growth and general cell health.

2.1.1.4 Sub-Culturing Attached Cell Lines

The three attached/ anchorage-dependent cell lines grew as monolayers attached to the bottom of the culture flasks. When cells reached confluency (generally between 70-80%) they were sub-cultured at a ratio of 1:4 for HGC-27, 1:3 for AGS, and 1:2 for WILL1 into medium 80cm^2 flasks as follows. Spent medium was removed from cells and the cell monolayer washed twice in 5ml pre-warmed Phosphate Buffered Saline (PBS) to remove extracellular serum proteins which act as trypsin inhibitors. The adherent cells were then detached from the flasks via 2 x 1min washes in ~ 2 - 3ml trypsin/EDTA (pre-warmed to 37°C) (Gibco-BRL). After checking under the light microscope that cells had begun to detach from the flasks (evident as cells appear round) the trypsin/EDTA was aspirated and the flasks gently agitated by tapping until the cells completely detached. The cells were then resuspended in 10ml complete medium and subdivided according to the sub-culturing ratios optimised for each cell line when carrying out routine sub-culturing. When accurate cell numbers

were required, e.g. for exposure experiments, the appropriate volume of cell suspension constituting 2 – 4 x 10^5 cells/ml or the cell concentration required for the particular experiment (counted using a Haemocytometer) was seeded into medium sized 80cm^2 flasks followed by the addition of cell culture medium (pre-warmed to 37°C) to a final volume of 15ml. The flasks were briefly gassed with CO_2 prior to placement in the 37°C incubator.

Subsequent sub-culturing was performed regularly as described, usually on an every other day basis to accommodate for the growth rate of the cells. Cells were either sub-cultured into medium-sized (80cm^2) or jumbo-sized flasks (175cm^2) depending on the amount of cells required for further experimental manipulations.

2.1.1.5 Freezing Down Attached Cell Lines

When cells were not in use they were stored in freezing media (consisting of heat inactivated calf serum (HIS) (Gibco-BRL, Paisley, UK) + 10% Dimethyl Sulfoxide (DMSO)) in cryovials in liquid Nitrogen. Cells at 70% confluence were trypsinised as described in section 2.1.1.4, and the resulting cell suspension centrifuged at 200 x g (1500rpm) for 8min. The resultant cell pellet was then resuspended in 3ml freezing media and divided equally amongst three 1.8ml cryovials. The cryovials were subsequently placed in a Biocell biofreezing vessel and placed in a -80°C Kelvinator overnight, allowing a slow reduction in temperature, before finally transferring the cryovials into a Cryomed liquid Nitrogen freezer for permanent storage until needed for experimentation.

2.1.1.6 Suspension Cell line: HL-60

The human promyeloblastic cell line HL-60 (derived from a 36-yr old Caucasian female with acute promyelocytic leukaemia) was obtained from the American Tissue Culture Collection (ATCC, Teddington, Middlesex, UK). The cell line was used in co-culture experimentation (chapter 5) in order to study the effects of inflammatory cells on gene expression and signal transduction changes in gastric epithelial cells after first optimising the induction of an inflammatory response in HL-60 (chapter 4). Further details about the cell line are provided in section 4.1.3.

The growth medium used for the culture of HL-60 was RPMI 1640 supplemented with 10% FBS, 2mM L-glutamine, Penicillin (60 units)/ Streptomycin (60μg) (all Gibco-BRL, Paisley, UK). The contents of the provided cryovials of cells were thawed by gentle agitation in a 37°C water bath for 2 minutes. When completely thawed, the cryovials were cleaned with 70% ethanol and the contents transferred to a 25ml centrifuge tube containing 9.0ml pre-warmed medium (37°C). After gentle mixing, the contents were centrifuged at 200 x g (1500rpm) for 5 minutes. Subsequently the supernatant was decanted and the cells re-suspended in 80cm^2 tissue culture flasks at a final cell concentration of 1x10^5 cells/ml in 15ml of complete growth media. The flasks were then gassed with 5% CO_2 in air and incubated at 37°C. After a 24hr incubation period the cells were examined under a light microscope to check for signs of contamination.

Cultures were maintained by the addition of fresh medium followed by gassing with CO_2, in order to dilute the cell concentration/ml and to provide nutrients, on the second or third day in culture depending on the cell densities observed. Alternatively, sub-culturing was performed by centrifugation of the cell suspension (following prior agitation to loosen cell aggregates) at 200 x g (1500rpm) for 5min, followed by removal of spent medium, resuspension of the cell pellet in fresh medium and dividing the cell suspension amongst several 80cm^2 cell culture flasks with the addition of growth medium to a final concentration of 1 x 10^5 cells/ml (cell counts determined using a haemocytometer). The cells were then gassed with 5% CO_2 and incubated at 37°C. Cell concentration was not allowed to exceed 1 x 10^6 cells/ml and the cell density was usually maintained between 1 x 10^5 and 1 x 10^6 viable cells/ml.

2.1.1.7 Freezing Down HL-60

When cells had grown to a concentration of 1 x 10^6 cells/ml and were not required for experimentation the cell suspension was centrifuged at 200 x g (1500rpm) for 5 minutes and the resultant cell pellet was resuspended in 6ml freezing medium (RPMI 1640 growth medium supplemented with 4.71% v/v DMSO). Subsequently 1.5ml of the suspension was pipetted into each of 4 x 1.8ml cryovials. The cryovials were next placed in a Biocell biofreezing vessel and stored at -80°C overnight so allowing a gradual freezing of the cell suspension prior to finally transferring the

cryovials into a Cryomed liquid nitrogen freezer for permanent storage until required for experimentation.

2.1.1.8 Cell Counting using the Haemocytometer and Trypan Blue Viability Assay

Total cell counts as well as the number of viable cells were regularly determined using a combination of haemocytometer counts and the Trypan Blue viability assay. Such information was required when sub-culturing cells in preparation for chemical treatments in order to ensure that cells were seeded into flasks at equal cell number so that cells reached the stage of sub-confluency in concert and that only cell populations with viability of 95% or more (unless otherwise stated) were used in subsequent experimentation.

Cells were detached from culture flasks by trypsinisation as described in section 2.1.1.4 and a cell suspension prepared in fresh growth medium. Next, 5ml of cell suspension was spun down by centrifugation at 200 x g (1500rpm) for 8 minutes to pellet the cells and the resultant cell pellet resuspended in 2ml Hank's balanced salt solution (HBSS) (Gibco-BRL, Paisley, UK). Subsequently 0.5ml of 0.4% (w/v) Trypan Blue solution was placed in a test tube and 0.3ml of HBSS and 0.2ml of the cell suspension were subsequently added (resulting in a cell dilution factor of 5). The solution was mixed thoroughly and left to stand at room temperature for no longer than 10-15 minutes. The haemocytometer was then set up by placing a clean glass cover-slip over the haemocytometer chambers, ensuring an air tight seal, and the chambers filled with cell suspension by carefully touching the glass cover-slip with a filled Pasteur pipette and allowing the cell suspension mixture to fill the chambers by capillary action. The numbers of cells in the four corner 1mm squares of the haemocytometer chamber were then counted. Cells counted were those within the squares and those touching the top and left perimeter of the squares only. Non-viable cells take up the trypan blue stain whilst viable cells do not. On this basis the number of viable and non-viable (blue) cells was scored as well as the total number of cells.

The number of cells per ml suspension was then calculated as follows:

Cells per ml = average count per square x dilution factor x 10^4

Total cells = cells per ml x original volume of fluid from which sample was removed

$$\text{Cell viability} = \frac{\text{Total viable cells (unstained)}}{\text{Total cells}} \times 100$$

Only cell suspensions with viability $\geq$ 95% were used for further experimental manipulations (unless otherwise stated), and the number of cells was adjusted according to requirements by either diluting with the further addition of medium or concentrating by harvesting cells via a centrifugation step and resuspending in a smaller volume of medium.

2.2 Chemically Induced Oxidative Stress in Attached Cell Cultures: Hydrogen Peroxide (H_2O_2) Exposure

Cells were seeded in 80cm^2 (for protein studies) or 175cm^2 (for RNA studies) cell culture flasks at 2 – 4 x10^5 cells/ ml from the previously established cultures (AGS, HGC-27, and WILL1) described in section 2.1.1.2 – 2.1.1.4 and grown until sub-confluent (~ 70%). Prior to dosing cells with hydrogen peroxide (H_2O_2) complete growth medium was aspirated, cells washed 2 X in pre-warmed PBS (37°C) and replaced with serum free medium. This was necessary in order to eliminate any interactions between H_2O_2 and serum proteins which would otherwise hinder full exposure of cells to H_2O_2 and its derivatives. In specially designated fume hoods for toxic handling, H_2O_2 (10 Molar) (Sigma-Aldrich, Poole, UK) diluted in 10mM TE buffer (Tris HCl pH 7.5 + 1mM EDTA) was added to culture flasks to make up a range of dosing concentrations. Cells were exposed to a dosing regimen consisting of 4, 8, or 24hr exposures to 0, 50, 150, 250, and 500μM H_2O_2. Doses were selected based on cytotoxicity studies previously carried out at the University of Wales Swansea (Morgan *et al.*, 2003). In these studies 200μM H_2O_2 was seen to produce a 50% survival rate, so lower concentrations of 50 and 150μM, and higher concentrations of 250 and 500μM were employed in this study in order to cover a wide dose range. The aim was to mimic chronic inflammation *in vitro*, and since the actual levels of ROS *in vivo* are unknown, a wide dose range was covered (detailed further in chapter 3). Also the lower concentrations in the range have previously been

shown to induce DNA damage (Jenkins *et al.,* 2001) as well as chromosomal damage. Cells were incubated at 37°C for either 4, 8, or 24hr before removal of H_2O_2 containing medium (into specially designated toxic disposal flasks) followed by two washes in pre-warmed PBS (37°C) prior to proceeding to RNA/ protein extraction (sections 2.4 and 2.6 respectively). Controls included in the experiments were exposed to 10mM TE and all treatments were carried out in duplicate.
All toxic waste (including solutions, pipettes, pipette tips) was double contained and disposed of accordingly in double lined toxic/ biohazard bags ready for incineration.

2.3 MTS Assay

The effect of chemical treatments on cell proliferation and viability in attached cell lines was assessed using the Promega CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (Southampton, UK) [MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) assay] as instructed by the manufacturer's protocol. Stock cultures of cells were maintained as described in section 2.1.1.3 – 2.1.1.4, and the cells used for the bioassay were stock cultures 2 days after the last sub-culture. Cells were trypsinised to detach cells from the flasks, and washed twice in pre-warmed serum free medium followed by centrifugation at 200 x g (1500rpm) for 8min and subsequent resuspension in serum free medium. The cell number and viability of cells was determined using the haemocytometer and trypan blue dye exclusion method (section 2.1.1.8), and the cells subsequently resuspended to a final concentration of 1 x 10^5/ml medium. Only cells with viability greater than 95% were used for testing. Next, 50µl of the resultant cell suspension (i.e. 5,000 cells) was dispensed into the appropriate wells of a sterile 96-well plate. The plate was then incubated overnight at 37°C in a 5% CO_2 incubator to allow the cells to attach to the wells and undergo approximately one cell cycle so ensuring adequate cell numbers for the assay. The appropriate concentrations of test chemical were then prepared by addition of the chemical to serum free medium. For H_2O_2 the doses made up were 0, 50, 150, 250, and 500µM. For each dose, 50µl was aliquotted into wells of a 96-well plate. Since the chemical was added to the cells at a 1:1 ratio, the doses were made up as 2X stocks to accommodate for the 1 in 2 dilution that occurs on addition of the chemical to the wells. For each dose, 2 wells were required, one for exposure of cells to the chemical, and one to serve as a control of

test chemical in the absence of cells to measure background absorbance. Each dose was carried out in triplicate. Cell-only and medium-only controls were also included in the plates. Three different plate assays were carried out, one for each time point used in the study (4, 8, or 24hrs). The cells were incubated at 37°C in an atmosphere of 5% CO_2 to make up the full exposure times. Subsequently 20µl of CellTiter 96® AQ_{ueous} One Solution Reagent was added to each well of the assay plate, and the plate incubated for 1 hour. Finally the absorbance at 490nm was determined using a 96-well plate reader (Anthos HTII plate reader, Labdesign, Sweden).

2.4 RNA Extraction from Cultured Cells

RNA was extracted from AGS, HGC-27 and WILL1 following dosing with H_2O_2 (chapter 3) or co-culture treatments (chapter 5). For each dose/ co-culture treatment a set of two treatments was carried out in duplicate. One set for protein extraction (carried out in 75cm^2 flasks) for protein expression studies, and the other set for RNA extraction (175cm^2 flasks required for good RNA yield).

All RNA extractions were undertaken in a Class II Lamina Flow Hood. Prior to RNA extraction the hood and all of the equipment to be used (pipettes, reagent bottles, filter tip and tube boxes) were thoroughly cleaned with RNase ZAP wipes (Ambion Ltd, Cambridgeshire, UK). RNase free filtered pipette tips were used for all pipetting and all microfuge tubes used were autoclave sterilised prior to use to remove any residual RNase, so minimising degradation of extracted RNA.

RNA was extracted from a minimum of 5 x 10^6 and a maximum of 1 x 10^8 cells using the QIAgen RNeasy Midi Kit (QIAgen, Crawley, West Sussex, UK) following the protocol for total RNA extraction from cultured mammalian cells with some modifications. Briefly, cells were harvested by detaching the cells from the culture vessels via trypsinisation (as described in section 2.1.1.4). The detached cells were resuspended in approximately 10ml of serum containing medium to inactivate the trypsin. The number of cells in each flask was then determined using a haemocytometer and the appropriate volumes (to give the desired cell numbers) were transferred to appropriately labelled centrifuge tubes and the cells spun down for 8min at 200 x g (1500rpm). The supernatant was decanted from the resultant cell pellets and any traces of medium were aspirated as the medium can interfere with later steps

in the protocol and hence reduce RNA yield. Approximately 1 x 10^8 cells were collected from each flask.

Cell pellets were then loosened by vortexing the tubes and 4ml buffer RLT (+ 1% β-mercaptoethanol) added in order to disrupt and lyse the cells. Subsequently the lysate was homogenised by vortexing the sample for 10sec and passing it through a sterile, disposable 18-gauge needle fitted to a sterile, disposable 1ml RNase free syringe 20-30 times. One volume (i.e. 4ml) 70% ethanol was next added to the homogenised lysates, and the solutions mixed thoroughly by vigorous shaking. The samples were then loaded onto RNeasy midi columns placed in the sterile 15ml RNase free centrifuge tubes provided in the kit. The tubes were closed gently and centrifuged at 3000 x g (4000rpm) for 5min using a Sanyo Centaur 2 centrifuge. All following centrifugation steps were also carried out at 3000 x g (4000rpm), and flow-through discarded after each centrifugation.

Subsequently, buffer RW1 was added to the columns at a volume of 4ml, and tubes centrifuged for 5min. Next, 2.5ml buffer RPE (+ 4 volumes ethanol) was applied to the columns and the tubes centrifuged for 2min to wash the columns. This wash step was repeated a second time, but tubes were spun for 5min the second time to ensure that no ethanol is carried over during the subsequent elution step. Finally, the bound RNA was eluted from the columns in RNase-free water using a modification of the manufacturer's protocol. Briefly, the RNeasy columns were transferred carefully into clean 15ml collection tubes provided and 75µl RNase-free water was applied directly onto the RNeasy silica-gel membrane. The tubes were left to stand for 1min and then centrifuged for 3min. This elution step was then repeated with a second 75µl volume of RNase free water to maximise RNA yield.

2.4.1 Post-extraction Processing of RNA

Following extraction of total RNA from cell lines (or from tissue biopsies (chapter 6)) the RNA was processed to remove any contaminating DNA, the concentrations quantified, and RNA quality assessed prior to downstream experimentation.

2.4.2 RNA Quantification

Following extraction of RNA from cells or tissues the RNA samples were coded accordingly and quantified spectrophotometrically by measuring the absorbance at 260nm (A_{260}) using a Beckman DU530 Spectrophotometer (Buckinghamshire, UK). The samples were diluted 25X to a total volume of 100µl (4µl RNA plus 96µl distilled water (dH_20)). The average of three A_{260} readings was taken for each sample and the value multiplied by 25 to give the RNA concentration in µg/ml. The 260/280nm ratio was also observed in order to obtain an idea of RNA purity and freedom of DNA contamination. dH_20 was used as the blank in all cases.

This initial quantification was carried out in order to determine whether or not the RNA extraction had been successful. Following this the RNA was subjected to in-solution DNase digestion (as described in section 2.4.3 that follows) to remove any contaminating genomic DNA which would otherwise hinder further analysis, including microarray analysis, and in particular Real-Time PCR analysis.

2.4.3 In-solution DNase treatment of RNA samples

In-solution DNase digestion of RNA samples to remove contaminating genomic DNA was carried out using the DNA-*free*™ kit from (Ambion Ltd, Cambridgeshire, UK) following manufacturer's protocol. This involved addition of 0.1 volumes 10X DNase I buffer (15µl to the 150µl RNA samples in 0.5ml microfuge tubes) and 1µl DNase I enzyme to the RNA. The solution was mixed gently and incubated at 37°C for 25min. Following this incubation the enzyme was removed from the RNA samples by the addition of 0.1 volumes (16.5µl) DNase inactivation reagent, flicking the tubes, and incubating the tubes for 2min at room temperature, ensuring that the inactivation reagent was evenly dispersed throughout by intermittent agitation. Finally, the tubes were centrifuged at 10,000 x g (13,000rpm) for 1min to pellet the inactivation reagent, the RNA solution (supernatant) removed from the pellet and transferred to clean tubes for long term storage. The RNA samples were sub-divided into 10µl aliquots in 0.2µl sterile microfuge tubes and stored at -80°C until required. Samples were allowed to go through two freeze – thaw cycles prior to discarding in order to maintain RNA integrity.

2.4.4 Post DNase RNA Quantification

RNA concentration was quantified spectrophotometrically as described in section 2.4.2. On average ~ 700µg/ml RNA was isolated per treatment. Again the 260/ 280nm ratio was assessed, and only samples with ratios ≥ 1.8 were considered free from DNA contamination and hence used for further analyses.

2.4.5 Reverse-Transcription PCR for assessment of RNA quality

The set up of all reactions described in this section was performed in dedicated Class II Lamina Flow PCR cabinet pre-cleaned (together with all equipment going into it – pipettes, filter tip boxes, tube racks, ice box) with 70% ethanol and exposed to UV light for 5-10 minutes prior to proceeding with reaction set up to avoid contamination. No tip/ PCR tube boxes were opened outside of the fume hood. Only autoclave sterilised pipette filter tips, microfuge tubes (Starlab, Milton Keynes, UK), and dedicated PCR pipettors were used for setting up PCR reactions. All reagents were thawed at room temperature, and enzymes, RNA, and reaction tubes kept on ice throughout.

2.4.5.1 cDNA synthesis

RNA was reverse transcribed into cDNA using the RETROscript kit (Ambion Ltd, Cambridgeshire, UK) following the 2-step reverse transcription-PCR protocol without heat denaturation of the RNA with some modifications.

Each reaction mix consisted of the following components: -

- 1µl oligo(dT),
- 1µl random decamers,
- 2ul 10X RT buffer,
- 4µl dNTP mix,
- 1µl MMLV-Reverse Transcriptase (100U),

+ 500ng RNA sample + nuclease-free H_2O to a 20µl final reaction volume.

When several reverse transcription reactions were being performed simultaneously, a master mix consisting of multiples of the above reagents (minus the RNA and H_2O) was prepared in order to ensure that the same component quantities were present in each reaction. The master mix was mixed well by pipetting and 9µl of the master mix was aliquotted into 0.2µl microfuge tubes. Volumes corresponding to 500ng RNA were then added to the reaction tubes, and the volumes made up to 20µl by the addition of nuclease-free H_2O. Reaction mixes were mixed thoroughly by pipetting and brief centrifugation using an MSE Micro Centaur centrifuge to collect reaction components to the bottom of the tubes. Tubes were then incubated at 44°C for 1hr using a PTC-225 Peltier Thermal Cycler (MJ Research, Essex, UK), followed by a 10min incubation at 90°C to inactivate the reverse transcriptase. Generated cDNA was then used in PCR reactions using primers for beta - (β-) actin (*ACTB*) (refer to table 3.2 for primer sequences) to assess DNA contamination, or stored at -20°C until required.

2.4.5.2 Polymerase Chain Reaction (PCR)

PCR amplification reaction tubes were set up to contain the following components: -

- 5µl Thermophilic DNA polymerase 10X buffer (10mM Tris-HCl pH 9 + 50mM KCl + 0.1% Triton X-100) (Promega, Southampton, UK),
- 4µl dNTP mix (containing 1.25mM of each dNTP, end concentration = 0.1mM each dNTP),
- 3µl 25mM Magnesium Chloride (Promega, Southampton, UK) (final concentration = 1.5mM),
- 1µl Forward primer (15pmol),
- 1µl Reverse primer (15pmol),
- 0.5µl Taq Polymerase (5U/µl, final = 2.5U) in storage buffer A (50mM Tris-HCl at pH 8, 100mM NaCl, 0.1mM EDTA, 1mM DTT, 50% glycerol, 1% Triton X-100) (Promega, Southampton, UK),
- 2µl sample (c)DNA (0.1µl/ml),
- 34µl sterilised dH_2O (to a total volume of 50µl).

Master mixes of the above reaction components were made (minus sample DNA) as required. The master mix was mixed by pipetting followed by brief centrifugation and 48µl aliquotted into labelled 0.2ml microfuge tubes. Finally 2µl of sample DNA was added to appropriate tubes and the contents mixed by vortexing and brief centrifugation. A negative control was included for each PCR reaction consisting of the same reaction components except for replacing the sample DNA with dH_2O. To check for DNA contamination in isolated RNA samples, controls were also included for each sample in which 5µl of RNA was included in the PCR reactions. Separate master mix was prepared for these controls in which 31µl dH_2O per reaction was included instead of 34µl. PCR product seen in these controls was taken as an indication of genomic DNA contamination in RNA samples, and such samples were excluded from further analysis.

Reaction tubes were placed in a PTC-225 Peltier Thermal Cycler (MJ Research, Essex, UK), and run on the appropriate program designed to suit the parameters and length of DNA to be amplified. Generally primers tended to be designed to anneal at 60°C, and so a program consisting of 94°C - 2 min, 94°C – 30sec (denaturation), 60°C – 20sec (primer annealing), 72°C – 30sec (extension), for 30 cycles was used. For analysing RNA quality, primers for β-actin (*ACTB*) were utilised, which have an annealing temperature of 60°C.

2.4.5.3 Gel Based Detection of PCR products

Required Reagents: -

- 100bp ladder (130ng/µl) (Promega, Southampton, UK) in 10mM Tris-HCl (pH 7.5) + 1mM EDTA,
- 10X TBE (0.89M Tris-base pH8, 0.89M boric acid, 0.02M EDTA),
- TEMED (Invitrogen, Paisley, UK),
- 10% Ammonium Persulphate (APS),
- Acrylamide: Bisacrylamide (37.5: 1) stock solution containing 30% (w/v) acrylamide (Severn Biotech Ltd, Worcestershire, UK),
- Gel loading buffer (50% (v/v) glycerol + 10% (v/v) 0.1M EDTA + 1% (w/v) Bromophenol Blue + 1% (w/v) SDS),

- 50X TAE buffer (1.5M Tris-base pH8, 0.05M EDTA, 5.7% (v/v) glacial acetic acid).

Polyacrylamide Gel Preparation: -

PCR products were analysed on 6% polyacrylamide gels prepared as follows. A 6% solution for 4 gels was made up consisting of 32ml dH_2O, 4.5ml 10X TBE buffer, and 8ml acrylamide: bisacrylamide solution combined in a Buchner flask. The solution was vacuum degassed for 5-10min before the addition of 45µl TEMED and 220µl 10% APS. The mixture was then quickly placed into 4 individual gel casters (Protean III gel system, Bio-Rad, Hertfordshire, UK) at approximately 10ml per cast using a syringe. Fifteen-toothed combs were then placed into each cast to form the sample wells in the gels. The gels were left to set at room temperature for 45min.

Polyacrylamide Gel Electrophoresis: -

Set polyacrylamide gels were placed in vertical electrophoresis tanks (Bio-Rad, Hertfordshire, UK). Subsequently 3µl loading buffer was mixed with 10µl of PCR product and samples were loaded into the set wells. A 100bp molecular marker was also included in the electrophoresis run (1µl 100bp ladder (Promega, Southampton, UK), 3µl loading buffer, 9µl dH_2O). Vertical electrophoresis was performed in 1X TBE buffer at a constant supply of 160V from a Bio-Rad Powerpack 300 power supply (Bio-Rad, Hertfordshire, UK). Electrophoresis was carried out until the blue loading dye was seen to reach the bottom of the gel plate (~ 30min).

Silver Staining of Polyacrylamide Gels: -

DNA bands in the gel were stained with silver nitrate (Sigma-Aldrich, Dorset, UK) by soaking the gels in a 0.1% silver nitrate solution for 10min, washing twice in dH_2O, followed by soaking in 0.375M sodium hydroxide solution plus 0.4% formaldehyde until PCR bands appeared.

Image Capture and Analysis: -

Silver stained gels were illuminated with white light, and the brightness, size, and focus manually adjusted in order to give the best image using the Bio-Rad gel documentation system Gel Doc 2000 (Bio-Rad, Hertfordshire, UK) in conjunction with the Quantity One version 4.0.3 software for image analysis.

2.5 Gene Expression Analysis

Gene expression levels and/ or changes in cells and tissues at the RNA level were examined using quantitative real-time (Q-/ RT-) PCR using primers specific to the genes of interest.

2.5.1 Real-Time PCR

Only RNA with a 260/280nm ratio of 1.8 or more following in solution DNase digestion (as described in section 2.4.3) and giving no PCR products in the test for genomic DNA contamination using primers for β-actin (*ACTB*) (section 2.4.5) was used in Real-Time PCR analysis.

cDNA was generated from sample RNA using a RETROscript reverse-transcription PCR kit (Ambion Ltd, Cambridgeshire, UK) as described in section 2.4.5.1.

2.5.1.1 Standard Curve Generation

cDNA was generated from various RNA sources seen to express the gene of interest e.g. *c-FOS*. For example, the source of RNA for β-actin (*ACTB*) and *c-FOS* standard curve generation was a pool of RNA from colorectal tumours and HGC-27 cells seen to express *c-FOS*. A 1 in 10 serial dilution of the cDNA was made up from 10^0 to 10^{-3} to allow for standard curve generation. In a real-time (RT) PCR reaction 2μl of each cDNA dilution was analysed with both primers for the *ACTB* housekeeping gene, for *ACTB* standard curve generation used as an internal control for normalisation, and primer pairs for the gene under analysis as described below. The same standard curve RNA was used for all repeats in a particular experimental

run. For example, in chapter 3, all plates carried out for analysis of *c-FOS* expression in AGS, HGC-27, and WILL1 included standard curves generated from the same RNA, so removing a possible source of variation in the final data.

2.5.1.2 The Reaction

RT-PCR reactions were set up in a laminar flow fume hood as for standard PCR using sterile nuclease free pipette filter tips and taking the precautions described in section 2.4.5.
Each reaction mix consisted of the following:

- 12.5µl iQ SYBR Green Supermix (Bio-Rad, Hertfordshire, UK),
- 1µl Forward primer (0.2µM final concentration),
- 1µl Reverse primer (0.2µM final concentration),
- 2µl sample cDNA,
- 8.5µl dH_2O (to a total volume of 25µl).

All reaction tubes were cooled on ice prior to set up, and the reactions made up on ice as a master mix containing SYBR Green Supermix, dH_2O, and appropriate primers prior to template addition to ensure that all reactions contained the same component quantities. Separate master mixes were prepared for each gene studied and the mixes vortexed and briefly centrifuged to ensure that they were homogeneous. Next, 2µl of appropriate sample cDNA was pipetted into appropriate wells of a sterile pre-chilled 96-well 0.2ml PCR plate (Starlab, Milton Keynes, UK) each dilution for the standard curves and each test sample occupying 3 wells in the plate as the reactions were each performed in triplicate. Subsequently 23µl of the appropriate master mix was aliquotted into the appropriate wells in the 96-well plate to make up the 25µl total reaction volumes. No template runs of nuclease free dH_2O were always included in each RT-PCR run for each primer pair to check for contamination. On completion of loading the reactions into wells, the plate was sealed with Optical Quality Sealing Tape (Bio-Rad, Hertfordshire, UK) and briefly centrifuged using an Allegra 21R centrifuge to collect all contents to the bottom of the wells. The set-up of a typical RT-PCR plate is illustrated in figure 2.1.

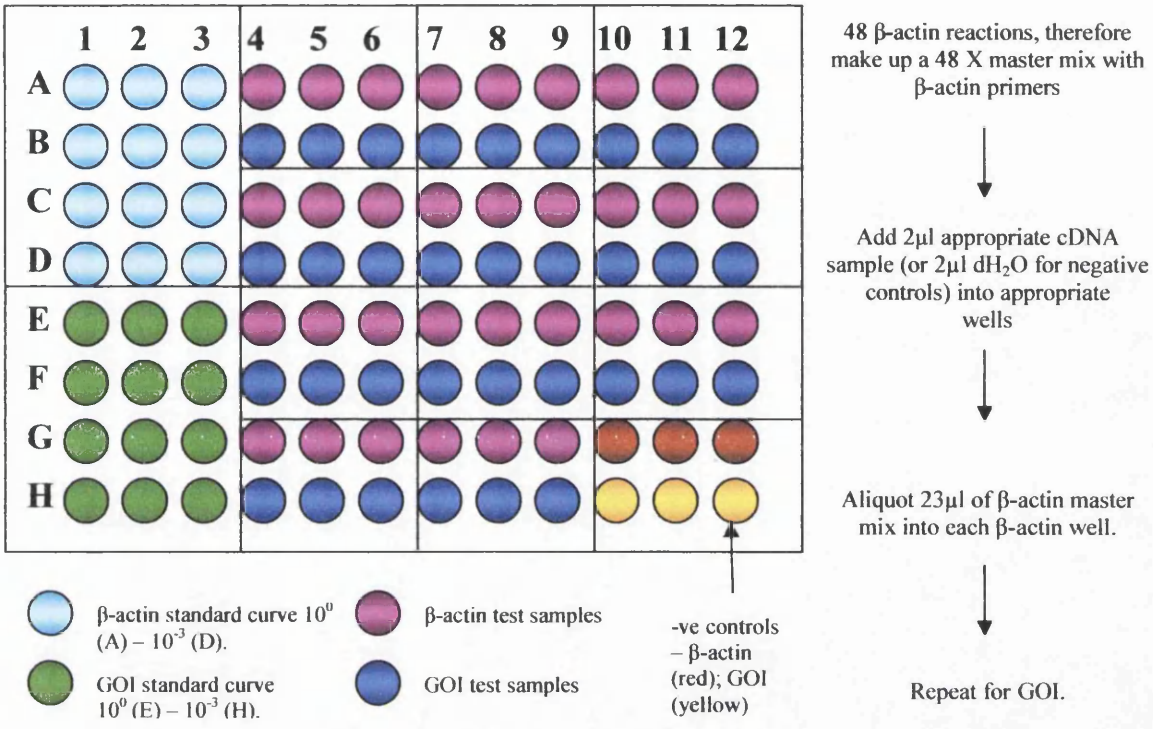
The sample plate was then placed in the iCycler iQ Thermal Cycler (Bio-Rad, Hertfordshire, UK) and run on the following program:

1. 95°C – 10sec
2. 60°C – 30sec } (repeat x 3)
3. 60°C – 45sec (repeat x 2)
4. 95°C – 3min
5. 94°C – 30sec
6. 60°C – 30sec
7. 72°C – 30sec (repeat x 40 cycles)
8. 55°C – 30sec
9. 95°C – 30sec
10. 10sec at each 1°C increase in temperature from 55°C to 95°C for melt curve generation.

Steps 1 – 3 of the protocol permitted well factor analysis, since well factors must be collected at the beginning of each experiment in order to compensate for any system or pipetting variations and hence optimise the quality and analysis of fluorescent data (from fluorescein in the SYBR Green Supermix). Well factors/ optical data collected at step 3 allowed normalisation of the fluorescent signals in each well for post-run analysis. Steps 4 – 7 constituted the PCR reaction, the fluorescent data for the amplification reaction was collected and analysed in real time at step 3. Steps 8 – 10 permitted melt-curve analysis, the fluorescent data collected and analysed in real-time at step 7 in this case.

Unless otherwise stated, all real time PCR reactions were repeated via intra-plate triplicates (as described) to account for pipetting errors, and via inter-plate triplicates using different batches of cDNA prepared on three different days to account for other possible sources of variation, such as the reverse transcription reaction, differences in machine efficiency, etc.

Figure 2.1 A typical Real-Time PCR plate and reaction set up for analysing Gene Of Interest (GOI).



2.5.1.3 Post-Run Data Analysis

Data analysis was performed individually for each sample using the iCycler iQ software version 2.3B. The specificity of the PCR reaction products generated was first addressed by analysis of the melt curves generated for the samples. Specific PCR should yield a single peak of a specific Tm (melting temperature) for each gene under analysis across all samples. The presence of more than one peak indicates the presence of non-specific PCR products and/ or primer-dimers in the sample reaction, hence samples without the correct Tm peak were omitted from subsequent analysis.

The relative amounts of initial cDNA template between controls and samples were calculated based on the concept of threshold cycles (C_T). The C_T is defined as the number of PCR cycles after which a fluorescence signal becomes clearly detectable above the background or baseline fluorescence signal seen in the amplification plots. The baseline was defined as the range of cycles during which no target amplification above the background signal was detectable. The range was set from the second cycle to the point two cycles earlier than the C_T value of the most

abundant sample – usually the reaction *with ACTB* primers and neat cDNA (10^0). A fixed fluorescence threshold level at a statistically significant point above the baseline was then set by the software 10 standard deviations from the baseline. Based on this set threshold, C_T values were calculated for each reaction. The C_T values for triplicates were examined and any outliers (values falling greater than 0.2 out from the mean of the triplicates), possibly resultant from experimental error, such as pipetting variation, removed from the analysis (leaving at least duplicate readings) as they may skew results. A standard curve could then be generated from the *ACTB* and gene of interest (GOI) dilution series data. These curves are invaluable for determining both the quality of the cDNA samples (from the *ACTB* standard curve) and the experimental success reflected in the PCR efficiency.

The PCR efficiency was determined by the following relationship:-

$$E = [10^{(-1/a)}] - 1$$

Where a = slope of the standard curve.

Real-time PCR data and results were only considered to be reliable, and so included in the final analyses, if the PCR efficiency lay within the range of 0.9 – 1.1 (i.e. between 90 – 110%).

2.6 Protein Extraction from Cultured Cells

Total cellular protein was extracted from cultured cells (AGS, HGC-27, WILL1) using a cell scraping and lysis methodology. Following chemical treatments or co-culture experiments, medium containing chemical/ cells was carefully removed (following the safety procedures outlined in section 2.2) and the attached cell monolayer washed 2 X with pre-warmed (37°C) PBS. After the 2nd PBS wash, all traces of PBS were removed by carefully aspirating any traces of solution from the flasks since residual salts from PBS may interfere with downstream protein extraction. Subsequently, 250µl modified RIPA buffer (50mM Tris-HCl pH7.4, 1mM EDTA, 1% (v/v) IGEPAL, 0.25% (w/v) Sodium Deoxycholate) with protease and phosphatase inhibitors added (protease inhibitors: 1mM AEBSF, 1µg/ml leupeptin; phosphatase inhibitors: 1mM Sodium Orthovanadate, 1mM Sodium Fluoride) was added to cell culture flasks and cells scraped into the buffer using sterile cell scrapers

(Fisher, Leicestershire, UK) resulting in lysis of cells. The lysate from each flask was next aspirated and placed in correspondingly labelled 1.5ml microfuge tubes, and 1 volume 2X western blot loading buffer (Lamelli buffer) (250mM Tris pH6.8, 10% (v/v) glycerol, 4% (w/v) SDS, 2% (v/v) β-mercaptoethanol, 0.006% (w/v) bromophenol blue) added to each sample and the samples mixed gently by pipetting up and down. Samples were subsequently boiled at 100°C for 5min in a heating block, prior to cooling on ice.

2.6.1 Protein Quantification

Protein concentration in extracted samples was determined using the 2-D Quant Kit (GE Lifesciences, Bucks, UK) as outlined in the manufacturer's protocol and all solutions used were provided in the kit unless otherwise stated. A standard curve was first prepared using a 2mg/ml bovine serum albumin (BSA) standard solution by setting up a dilution series (in dH_2O) in 2ml microfuge tubes consisting of 0, 10, 20, 30, 40, and 50μg protein, with the 0μg tube serving as the assay blank (i.e. dH_2O). Next 2ml microfuge tubes were set up to contain 15μl sample for quantification. Both the standard curve tubes and the sample tubes were set up in duplicate so that average readings could be obtained. Subsequently, 500μl precipitant solution was added to each tube, followed by vortexing and incubating at room temperature for 3min. An equal volume (500μl) of co-precipitant solution was next added to the tubes followed by brief mixing by inversion of tubes. The tubes were subsequently centrifuged at 10,000 x g (13,000rpm) for 5min to sediment the protein precipitates. Supernatants were then decanted from the tubes taking care not to disrupt the visible white pellets. To ensure complete removal of supernatant (which would otherwise interfere with accurate quantification) the tubes were quickly centrifuged again for a brief pulse to collect any remaining supernatant to the bottom of the tubes, after which any solution was carefully aspirated. Next 100μl copper solution and 400μl dH_2O were added to the tubes followed by vortexing to completely resuspend the pellets, after which 1ml working colour solution (100 parts colour solution A: 1 part colour solution B) was quickly added to the samples, the tubes mixed by inversion, and incubated at room temperature for 20min. The absorbance of each sample was then read using a spectrophotometer at 480nm (A_{480}) using dH_2O as the blank/ reference. Average readings were calculated for each tube and a standard curve

generated from which the concentration of each protein sample was determined. Subsequently the samples were aliquotted into 20µl aliquots and stored at -80°C until use. As for RNA, protein samples were used for up to two freeze-thaw cycles and subsequently discarded.

2.7 Protein Expression Analysis

Protein expression levels in samples were assessed by western blotting using antibodies to specific proteins of interest. The particular proteins studied were selected based on the results from RNA level gene expression data obtained from microarray studies (chapter 3).

2.7.1 Sodium Dodecyl Sulphate (SDS) Polyacrylamide Gel Preparation

Prior to western blotting, protein samples were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Sample volumes constituting 5µg protein were loaded into the wells of polyacrylamide gels consisting of an upper stacking layer and a lower resolving layer. When preparing the gels the lower resolving layer was prepared first, consisting of (for 2 gels): -

- 6.6ml acrylamide: bisacrylamide solution (Severn Biotech Ltd, Worcestershire, UK),
- 2.5ml 3M Tris pH8.9,
- 10.5ml dH_2O,
- 200µl 10% SDS,
- 60µl TEMED (Severn Biotech Ltd, Worcestershire, UK),
- 150µl 10% APS.

The mixture was then quickly placed into individual gel casters (Protean III gel system, Bio-Rad, Hertfordshire, UK) at approximately 10ml per cast using a syringe, leaving at least 1.5 inch at the top of the caster for the upper stacking gel. The resolving gel was allowed to set for 45min prior to the addition of the stacking layer which consisted of (for 2 gels): -

- 2.6ml acrylamide: bisacrylamide solution (Severn Biotech Ltd, Worcestershire, UK),
- 5ml 0.5M Tris pH6.8,
- 12ml dH_2O,
- 200µl 10% SDS,
- 60µl TEMED (Severn Biotech Ltd, Worcestershire, UK),
- 150µl APS.

The mixture was loaded on top of the pre-set resolving gel(s) using a syringe filling to the top of the caster. Fifteen-toothed combs were then placed into each cast to form the sample wells and the gels were left to completely set at room temperature for 45min. Up to four gels were produced at one time and any gels that were not immediately required were placed in an air tight container and stored at 4°C for up to three days.

2.7.2 SDS - Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Set polyacrylamide gels were placed in vertical electrophoresis tanks (Bio-Rad, Hertfordshire, UK) and samples were loaded into the set wells at volumes corresponding to 5µg protein for each sample. A molecular marker was also included in the electrophoresis run (Precision Plus Protein Dual Colour Standards (Bio-Rad, Hertfordshire, UK) loaded at 10µl in the first well in the gel. Vertical electrophoresis was performed in SDS running buffer (25M Tris Base pH8.3, 192mM Glycine, 0.1% (w/v) SDS) at a constant supply of 120V from a Bio-Rad Powerpack 300 power supply (Bio-Rad, Hertfordshire, UK). Electrophoresis was carried out until the dual colour molecular markers were seen to be clearly separated (1.5 – 2hr).

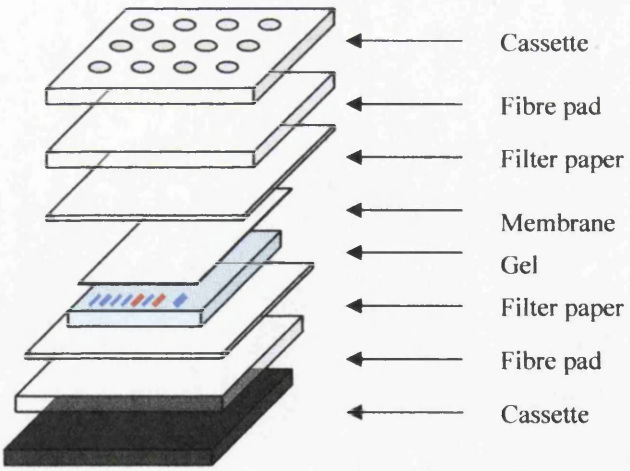
2.7.3 Western Blotting

Transfer of Proteins from SDS-polyacrylamide gel to Nitrocellulose Membrane

Following the separation of proteins by SDS-PAGE the proteins were transferred to nitrocellulose membranes (Bio-Rad, Hertfordshire, UK). This involved

an initial equilibration step whereby the gel(s), membranes, and any filter cards and fibre pads to be used in the transfer were incubated in ice-cold transfer buffer (50mM Tris Base pH8.1, 380mM Glycine, 0.1% (w/v) SDS, 20% (v/v) methanol) at 4°C for 20min. Following equilibration, transfer cassette(s), in which the gel was placed adjacent to a nitrocellulose membrane, were set up as depicted in figure 2.2. The cassettes were set up under transfer buffer to avoid the formation of bubbles which would interfere with the transfer process. Any bubbles that formed were carefully removed by applying a gentle even pressure, and horizontal electrophoresis was performed in transfer buffer whereby a constant supply of 400mA was applied for 1hr from the gel side of the cassette to the membrane side from a Bio-Rad Powerpack 300 power supply (Bio-Rad, Hertfordshire, UK) so ensuring proteins were transferred from gel to membrane. A transfer buffer ice-pack was placed in the buffer tank during electrophoresis to avoid overheating and loss of proteins. Upon completion of electrophoresis the successful transfer of proteins to membrane(s) could easily be determined as the dual protein colour markers could easily be seen.

Figure 2.2 Western blot transfer cassette set-up.



Western Blotting

Following successful transfer of proteins, the membrane was rinsed 2 X in TBS/ Tween (20mM Tris HCl pH8.9, 150mM NaCl, 0.5% (v/v) Tween) to remove all traces of transfer buffer. Next the membrane was incubated (protein side up

throughout) in 15ml blocking buffer (10% (w/v) skim milk in TBS/ Tween) in glass coplin jars for 1hr with gentle agitation at room temperature. After the blocking step, blocking buffer was removed and the membrane briefly rinsed in TBS/ Tween followed by the addition of 10ml western blot hybridisation buffer (3% BSA in TBS/ Tween) plus the appropriate dilution (1:1000) of specific primary antibody in order to perform primary antibody hybridisation overnight with gentle agitation at 4°C. The particular primary antibodies can be found in chapter specific materials and methods sections. In all cases the primary antibodies used were polyclonal antibodies raised in rabbit. Following the overnight primary antibody hybridisation step, hybridisation buffer was removed and the membrane washed via 4 X 5min washes in TBS/ Tween with gentle agitation at room temperature. Hybridisation with secondary antibody (goat anti-rabbit IgG – HRP conjugate (Abcam, Cambridge, UK)) at a dilution of 1:3000 in hybridisation buffer (3% BSA in TBS/ Tween) was then performed for 1hr with gentle agitation at room temperature. Upon completion of secondary antibody hybridisation, hybridisation buffer was removed and the membrane washed in TBS/ Tween 4 X 5min. Antibody binding was then visualised using the ECL kit (GE Lifesciences, Bucks, UK) following the manufacturer's protocol. Briefly, equal volumes of ECL detection solution 1 and 2 (provided in the kit) were mixed and 1ml applied to the protein side of the membrane ensuring that the entire surface of the membrane was covered. The membrane was left to incubate for 1min after which excess detection solution was allowed to drain from the membrane. The blot(s) was then placed protein side up on a fresh piece of clear acetate sheet, and a second sheet carefully placed on top taking care to remove all air bubbles. Horse Radish Peroxidase (HRP) conjugated to the secondary antibody catalyses the conversion of substrates in the ECL solution giving rise to chemiluminescence which can be detected using X-ray film. As such, the blot was placed in an X-ray film exposure cassette and, in a specialised dark room, a piece of hyperfilm ECL (GE Lifesciences, Bucks, UK) placed on top of the blot so allowing chemiluminescence exposure to the film. The initial exposure time was 5min, after which the film was developed using an AGFA Curix 60 X-ray film developer machine (Agfa UK, Middlesex, UK). If the bands were not clear enough or too intense following the initial 5min exposure, the exposure time was adjusted accordingly until the bands could be seen clearly enough with minimal background so permitting optimal downstream image analysis.

Typically in a western blot experiment two blots were performed on the same protein samples, one for the protein of interest and one for a loading control – typically β-actin (*ACTB*) to ensure that any observed differences were true and not due to loading differences between wells. If a phosphorylated protein (such as pERK1/2 (p42/p44)) was analysed, its un-phosphorylated counterpart (ERK1/2 (p42/p44)) served as the loading control. All Western blot experiments were performed in duplicate on different days to ensure reproducibility

Stripping Membranes for Re-use

Membranes could be stripped from bound antibody and re-hybridised up to 2 times. For complete removal of bound antibodies blots were washed in dH_2O for 5min, followed by a 5min wash in 0.2M NaOH, and a second 5min wash step in dH_2O all at room temperature with gentle agitation. The success of the stripping protocol was confirmed by carrying out ECL chemiluminscence visualisation (as previously described) to check for chemiluminescence from residual antibody binding. In the case of incomplete antibody removal the washes were repeated a second time, otherwise the membranes were stored in 5ml TBS/ Tween at 4°C until use (up to 2 weeks), or experimentation could proceed directly on to the western blotting blocking stage preparing the membrane for primary antibody binding, etc.

Image Analysis of Western Blots

Western blot films were scanned into JPEG format images which were subsequently analysed using the ImageJ software downloaded from the national institute of health (NIH) website (**http://rsb.info.nih.gov/ij/**). Images were opened using the software which permits selection of bands for analysis, generating pixel intensity data for each area selected. In an analysis it was ensured that the same area (cm^2) was analysed for each band so that when the pixel intensity was compared between bands any sources of variation other than true differences in intensity could be ruled out. As such, a small rectangle in centre of each band was analysed. Data was obtained for a particular protein of interest and its loading control and the intensity data for the protein of interest normalised against the loading control to rule out any variation introduced by loading differences between wells. Since all western blot

experiments were performed in duplicate, both images were analysed for a particular experiment, with the final results being the averages of the duplicate readings.

2.8 Data Analysis

All data analysis was performed using Microsoft Excel (Windows XP version) unless otherwise stated. Statistical analysis was carried out using the SPSS Version 13.0 software package.

Chapter 3

Reactive Oxygen Species (ROS)-induced Changes in Cellular Signal Transduction and Gene Expression: *MAPK and NFκB.*

3.1 Introduction

The origins, historical perspectives, and effects of free radicals or reactive oxygen/ nitrogen species (RO/NS) were discussed in detail in section 1.6. Molecular Oxygen is the primary biological electron acceptor, and its presence in the atmosphere drove the evolution of aerobic life forms, capable of harvesting vast amounts of energy through cellular respiration via oxidative phosphorylation (Storz, 2006; Balaban *et al.,* 2005). Aerobic life yields most of its energy through this process, which in eukaryotes takes place in the mitochondria. Unfortunately, this tremendous benefit of oxygen came at a price due to the inherent toxicity of the element. Coupled with oxygen metabolism comes the inadvertent formation of reactive oxygen species (ROS) such as superoxide ($O_2^{\cdot -}$, due to leakage of electrons from the electron transport chain), hydrogen peroxide (H_2O_2, generated from $O_2^{\cdot -}$), and hydroxyl radical ($^{\cdot}OH$, derived from H_2O_2 especially in the presence of transition metals such as iron (Storz, 2006; Balaban *et al.,* 2005; Curtin *et al.,* 2002)) which can pose serious, often lethal threats at the cellular level. As such, very early on in evolution, organisms were either made extinct by the presence of oxygen in the atmosphere, or evolved defence mechanisms to cope with its cytotoxicity associated with the generation of free radicals, which came in the form of both enzymatic and non-enzymatic anti-oxidant systems.

The generation of RO/NS in cells is an unavoidable by-product of cellular metabolism. Their levels in the cell are normally very finely controlled by the anti-oxidant systems, which serve to remove the reactive species at a rate that equals their generation in the cell. Unfortunately, this fine balance can be disrupted, leading to 'oxidative stress'. As detailed in section 1.6, this oxidative stress has been implicated in

several pathological conditions, such as chronic inflammation, rheumatoid arthritis, atherosclerosis, and cancer (Fukuruma *et al.,* 2006; Ristow, 2006; Waris and Ahsan, 2006; Klaunig and Kamendulis, 2004; Oshima *et al.,* 2003; Klaunig *et al.,* 1998; Poulsen *et al.,* 1998; Wink *et al.,* 1998; Knight, 1995; Toyokuni *et al.,* 1995; Feig *et al.,* 1994; Guyton and Kensler, 1993; Trush and Kensler, 1991; Breimer, 1990; Vuillaume, 1987; Ames, 1983).

With the exception of pathological states, under normal circumstances, not only have aerobic organisms evolved mechanisms for keeping RO/NS levels in check, they were also capable of evolving ways of exploiting the reactive derivatives for intracellular signal transduction and control of gene expression (Ahmad *et al.,* 2006; Gloire *et al.,* 2006; Storz, 2006; Hayden and Ghosh, 2004; Dröge, 2001; Karin *et al.,* 2001; Sen and Packer, 1996; Storz and Polla, 1996; Schenk, 1994; Whiteside and Goodbourn, 1993; Kerr *et al.,* 1992).

3.1.1 Effect of RO/NS on Cell Signalling

Signal transduction refers to the mechanisms by which cells sense and relay information in their environment to the inner core of the cell at the nucleus, where these signals can then elicit a cellular response at the levels of cellular physiology, growth, and development via alterations in gene expression. The signals typically (but not always) act at the cells surface through cell surface receptors, and classically include growth factors, hormones, cytokines, and other stimuli such as oxidative stress. On a very basic level, the signals/ environmental stimuli activate the cell surface receptors (for example via ligand binding), which go on to transmit a signal cascade through the cytoplasm (usually by a series of phosphorylation reactions) transmitting the information through signalling molecules (often kinases) and eventually to the nucleus, where changes in gene expression can occur, with concomitant changes in cellular behaviour. The general picture of signal transduction emerging from recent studies involves the sensing of a signal and its subsequent transduction to the transcription apparatus thereby affecting transcription initiation.

A number of key signalling pathways have been identified over the years, providing a wealth of knowledge into how cells respond to their microenvironment. To add further complexity, it has been seen that the signalling pathways are highly intricate and often overlapping and interrelated, demonstrating high specificity; in terms of which signals they respond to and which subsets of genes they activate; whilst at the same time having a certain level of diversity in the signals that can lead to their activation.

Cellular redox status has been seen to be an important factor in eliciting signal transduction activation and the subsequent changes in gene expression. In fact, research over the past decade has seen such an emphasis on oxidant, antioxidant, and redox state regulation of gene expression that it is now considered a sub-discipline in molecular biology (Sen and Packer, 1996). The fact that RO/NS are at the basis of many pathological conditions means that this link between RO/NS and gene expression (especially disease related/ specific gene expression) offers exciting therapeutic potential.

Gene expression changes are usually the major effect causing result of signal transduction, and several critical steps in a signal transduction cascade can be sensitive to redox status (governed by oxidant and antioxidant levels). In fact, the cellular redox state can affect very specific steps in a signalling pathway, as well as more general events such as protein phosphorylation and transcription factor binding to DNA (Sen and Packer, 1996). It is important to note that, while the focus on redox regulated signal transduction and gene expression is often in situations of cellular stress and pathogenic conditions, it is equally important in unperturbed and normal cells, functioning as a key control mechanism in cellular biochemistry. Indeed, direct exposure of cells to RO/NS activates a range of signalling pathways, but they are also seen to function as 'second messengers' in normal signalling independent of RO/NS exposure. The degree to which they act as classical second messengers however, is questionable. In some instances ligand binding (e.g. TNF-α) can result in an increase in intracellular ROS (Garcia-Ruiz *et al.*, 1997; Lo and Cruz, 1995) which then relay the signal to the nucleus by regulating redox sensitive signal transduction pathways (discussed in section 3.1.2 – 3.1.3). In this case the ROS may be viewed as classical second messengers of the primary signal. However, in other circumstances, for instance, when the primary signal from ligand binding cannot produce

enough phosphorylation to allow a full response, RO/NS may act to enhance phosphorylation (e.g. by transient inactivation of phosphatases), and can so be viewed as 'secondary messengers' to the primary signal (Nathan, 2003).

RO/NS from both extracellular and intracellular sources can affect gene expression. Extracellular RO/NS can instigate cellular signal transduction through generation of lipid peroxides within cell membranes (Suzuki *et al.,* 1997) or by activation of growth factor and cytokine receptors independent of receptor ligands (Huang *et al.,* 1996; Rao, 1996; Rosette and Karin, 1996; Nakashima *et al.,* 1994), for example, by triggering receptor autophosphorylation. Intracellular RO/NS generation has been seen to be induced by a variety of stimuli. For example, TNF-α provokes a rise in H_2O_2 production and release from mitochondria, as well as other ROS, by way of NADPH oxidase induction (Garcia-Ruiz *et al.,* 1997; Lo and Cruz, 1995). Transient increases in intracellular levels of H_2O_2 and other ROS have also been seen to occur through interaction of platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) with their receptors (DeYulia *et al.,* 2005; Jackson *et al.,* 2002). Nitrosative stress is also important since it can directly act on pathways regulating redox sensitive transcription factors like NFκB and AP-1, leading to either changes in their expression levels, activity, stability, or their accessibility to their promoter sites in DNA, hence impacting downstream gene expression (Krönke *et al.*, 2003). Interplay between ROS and RNS can also be fundamental in determining the extent of gene expression changes, since $NO^{\bullet}$ has in some instances been shown to directly inhibit cytokine mediated gene induction at the level of transcription (Peng *et al.,* 1995), as well as indirectly by scavenging $O_2^{\cdot -}$ in the process of peroxynitrite formation, hence modulating $O_2^{\cdot -}$ - dependent transcription.

Presently well over 100 mammalian genes have been recognized to be regulated by cellular redox state (Allen and Tresini, 2000; Adler *et al.,* 1999; Morel and Barouki, 1999), generally at the level of transcription, although alterations in mRNA stability (Chen *et al.,* 1998; De Kimpe *et al.,* 1998; Esposito *et al.,* 1997) and protein stability (Adler *et al.,* 1999; Iwai *et al.,* 1998; Shen *et al.,* 1995) have been reported.

The activity of many transcription factors can be modulated as a consequence of alterations in cellular redox and nitrosative conditions (Allen and Tresini, 2000; Belsham

and Mellon, 2000; Berendji *et al.,* 1999). This can occur either directly at the level of the redox sensitive transcription factors via oxidation/ reduction reactions, or indirectly at the level of various upstream signalling cascades. The pathways involved remain to be fully deciphered, owing to the fact that several of the pathways overlap and a host of stimuli can act within each pathway, at different levels. This goes back to the fine balance between signal transduction diversity and specificity which affords cells the ability to respond and adapt by different mechanisms, often in a tissue-/ cell-specific manner, to environmental changes. Whilst being highly beneficial to cells, this makes understanding the finer details of redox regulated signal transduction and gene expression very complex, although some general consensus findings are beginning to emerge. For example; the involvement of thiol groups in the regulation of gene expression; the existence of redox sensitive components of signal transduction pathways (e.g. protein kinases, protein phosphatases, phospholipases); the presence of an antioxidant (/electrophile) response element (ARE/ EpRE) in the promoter region of redox sensitive genes; the ability of oxidants to stimulate an increase in intracellular level of calcium ions, thereby affecting signal transduction, and can lead to the generation of more RO/NS; and the existence of transcription factors that exhibit redox sensitivity (Jackson *et al.,* 2002).

Considering that the scope of the work presented in this chapter focuses on the effects of RO/NS on signal transduction and gene expression, I will go on to discuss redox sensitive signal transduction pathways and transcription factors. Two of the best studied signalling pathways are the Mitogen Activated Protein Kinase (MAPK) pathways and the Nuclear Factor Kappa B (NFκB) pathways.

3.1.2 Mitogen Activated Protein Kinase (MAPK) pathway

The Mitogen Activated Protein Kinase (MAPK) pathway is a fundamental cellular signal transduction pathway consisting of a cascade of MAPK enzymes. These enzymes are one of the most extensively studied families of protein kinases (Kolch,

2000) that use ATP as a phosphate donor to phosphorylate downstream protein targets on either serine or threonine residues (Halliwell and Gutteridge, 2007).

As detailed in section 1.8.1 there are a number of major MAPK subfamilies – including ERK1/2 (p42/p44) [from here-on-in ERK1/2 (P42/P44) will be referred to as ERK], JNK, and p38. In all cases MAPK signalling is characterised by upstream signals feeding into a three-tiered cascade of core protein kinases. In the ERK pathway, for example, the upstream signal is co-ordinated by Ras (a G-protein), which then regulates the activity of a MAP kinase kinase kinase (termed MAPKKK, MAP3K or MEKK) which in turn activates a MAP kinase kinase (MAPKK, MAP2K, MKK, or MEK), which subsequently activates a MAP kinase (MAPK, for example, ERK1/2) via phosphorylation at each step. This phosphorylation cascade results in signal amplification and the potential for multiple levels of control. Through this phosphorylation cascade, MAPKs relay messages from cell surface receptors to the nucleus, hence mediating cellular responses to external signals such as stress, nutrient status, inductive signals and growth factors.

As their name suggests, the MAPKs arbitrate cellular responses to mitogens – stimulants of proliferation (mitosis). The pathways also orchestrate responses related to stress, differentiation, and gene expression. In this way, these pathways affect many aspects of cellular regulation, and impact almost all cellular processes, ranging from gene expression to cell death (Chang and Karin, 2001). As such MAPKs are viewed as master controllers.

The three major subfamilies of MAPKs (ERK, JNK, p38) are all activated by overlapping signalling cascades in which sequential phosphorylation is key. Multiple layers of complexity exist as inherent features of the pathways, lending ability to respond to a wide array of signals, whilst maintaining high levels of specificity and control. This is vital, since MAPK cascades can instigate a variety of cellular responses which may have detrimental consequences unless tightly controlled. Each pathway seems to be involved in particular cellular responses, the ERK pathway being fundamental for cell proliferation responses to growth factors, whilst the JNK and p38 pathways have more prominent roles in response to cellular stresses such as radiation, heat shock, oxidative

stress, cytokines (part of inflammatory response), etc. (Halliwell and Gutteridge, 2007).

Accumulating data in the literature is providing evidence that all three major MAPK subfamilies can be activated by RO/NS. For example, Lee and Esselman (2002) showed that phosphorylation of ERK, JNK, and p38 could be increased in Jurkat T cells (a lymphocyte cell line) by the addition of H_2O_2. Peroxynitrite ($ONOO^-$) has also been shown, in several different cell types, to cause activation of all three MAPK subfamilies (Klotz *et al.,* 2002). Several excellent reviews on oxidative stress related activation of MAPK pathways have been published (Genestra, 2007; McCubrey *et al.,* 2006; Jackson *et al.,* 2002, Sen and Packer, 1996). Interestingly, the outcome of MAPK activation can depend on cell type, and the amount of RO/NS added, for example, low levels of H_2O_2 added to alveolar type II cells stimulated proliferation via ERK activation (Sigaud *et al.,* 2005). In another study, H_2O_2 treatment of endothelial cells was shown to have a cytoprotective effect, preventing membrane blebbing through activation of ERK (Houle *et al.,* 2003). Generally speaking, the accumulating evidence suggests that ERK activation promotes cell growth and survival. However, ERK activation, whilst essential to neuronal cell development and plasticity, has been shown to have detrimental effects during oxidative neuronal injury (Chu *et al.,* 2004; Harper and Wilkie, 2003). Still, despite these observations, a very general, and perhaps oversimplified, picture is that ERK activation tends to promote cell survival, whereas prolonged JNK activation promotes cell death (e.g. via apoptosis). Since both pathways are usually simultaneously activated, finely controlled balance between the two is critical in determining cell fate, and redox mediated cross-talk between the two pathways may be important (Halliwell and Gutteridge, 2007).

In recent years it has become apparent that components of each of the MAPKs contain redox sensitive sites that provide the potential for redox modulation of the signalling. ROS have been observed to activate MAPKs via a Ras-dependent mechanism (Aikawa *et al.,* 1997; Rao, 1996). Indeed oxidative stress has been shown to activate Ras (Lander *et al.,* 1995), and accumulating evidence has shown $NO^{\bullet}$ to react with cysteine 118, on the surface of Ras, activating the G protein (Lander *et al.,* 1997; Lander *et al.,* 1995). Activated Ras can stimulate the small G protein Rac that subsequently activates

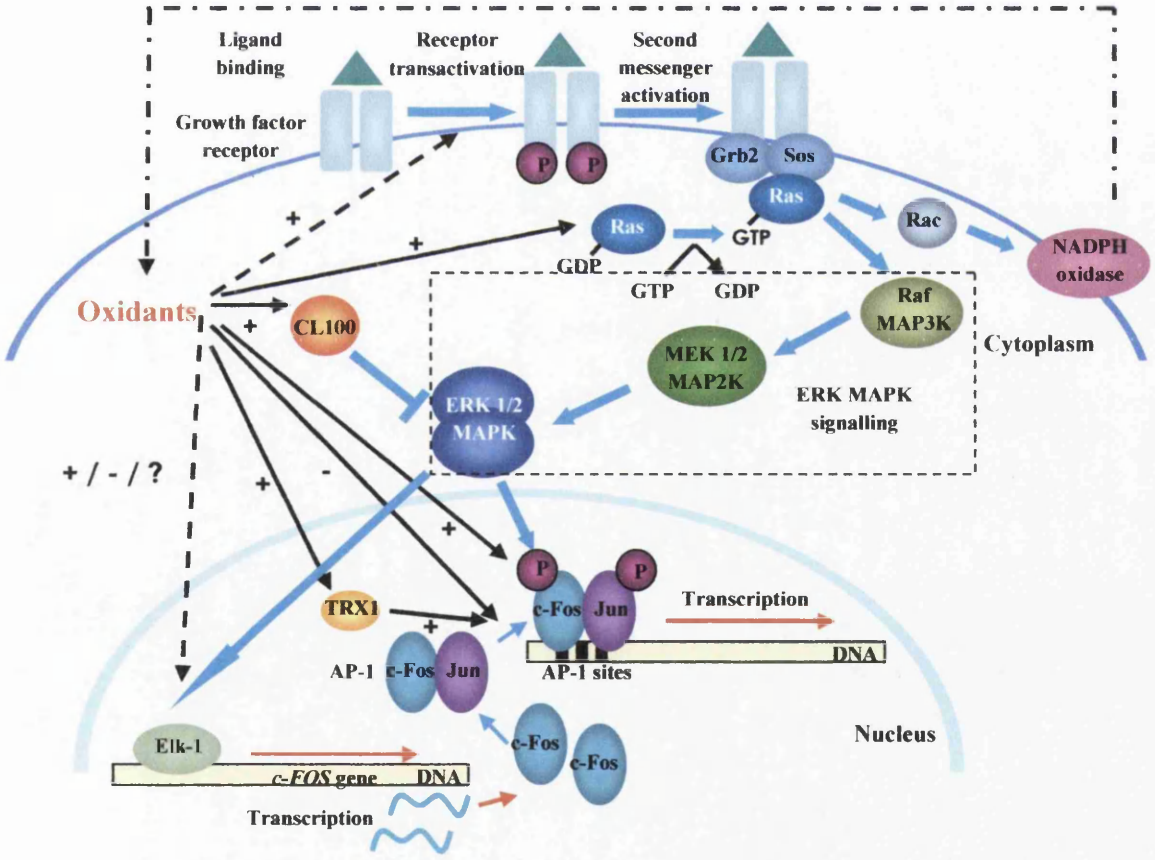
membrane bound NADPH oxidase, leading to the concomitant generation of ROS. RO/NS may also act at the level of dephosphorylation. For example, oxidative stress has been seen to be a potent inducer of expression of the protein phosphatase CL100 (Keyse and Emslie, 1992). This enzyme can dephosphorylate ERK, leading to its inactivation, representing a possible negative feedback loop for ROS induced ERK activation. Thus, it seems that redox regulation may modulate MAPK phosphorylation cascades at multiple levels (summarised in fig. 3.1), ultimately having profound effects on the cellular gene expression profile.

The link between signal transduction and regulation of gene expression has been very well documented (Karin, 1994; Karin and Smeal, 1992; Bohmann, 1990) and only in the past decade have the mechanisms by which signalling impacts gene regulation become apparent. In brief, extracellular signals regulate gene expression via activation of signal transduction cascades which convey the signal from the cell surface to the nucleus, where the activity of transcription factors is modulated. Most commonly this is achieved by changes in the phosphorylation state of the nuclear proteins (Karin, 1994; Karin and Smeal, 1992). So signal transduction pathways and transcription factors (as a major target) are intimately linked in the control of gene expression. This is exemplified by the ERK MAPK pathway, and its major downstream transcription factor target activator protein-1 (AP-1).

3.1.2.1 MAPK and AP-1

The involvement of MAPKs in the regulation of gene expression (in response to extracellular stimuli) has been highlighted in the past decade (Treisman, 1996; Su and Karin, 1996). A key finding was that members of the oncogenic AP-1 transcription factor family, which have been implicated in inflammation related changes in gene expression, are dependent upon MAPK signalling pathways for their activity (Karin, 1995). Regulation of the classical c-FOS/ c-JUN heterodimeric AP-1 protein is achieved either

Figure 3.1 Redox regulation of Mitogen Activated Protein Kinase (MAPK) cascades. Summary of the points at which cellular redox status (or in extreme conditions, oxidative stress) can modulate Extracellular signal Regulated Kinase 1/2 (ERK1/2 = p42/p44) signal transduction. Details connected by blue arrows show the normal sequence of events following triggering of the pathway, starting at the cell surface receptor, flowing through core kinase signalling (Raf – MEK1/2 – ERK1/2), and ending with gene expression. Black arrows highlight the points at which oxidants can impact the signalling, either positively (+), negatively (-), or as yet unclear (?). Refer to text for further details. (P = phosphorylated site; CL00 = phosphatase; Trx1 = thioredoxin; Elk-1 = a redox sensitive transcription factor).



the level of induced gene expression of components (*c-FOS* and *c-JUN*), or phosphorylation of nuclear c-FOS and c-JUN proteins (Xanthoudakis and Curran, 1996). This relies on upstream signalling pathways – notably the ERK1/2 (p42/p44) MAPK pathway (in the case of *c-FOS*), and the JNK MAPK pathway (in the case of *c-JUN*) (Jackson *et al.,* 2002; Karin, 1995; Deng and Karin, 1994).

In many cell types, *c-FOS* and *c-JUN* expression can be induced by a variety of stimuli including mitogenic signals and changes in cellular redox state (Angel and Karin, 1991; Abate *et al.,* 1990). The expression of *c-FOS* is induced through the ERK1/2 MAPK pathway via RAS (Deng and Karin, 1994), activated by diverse stimuli including RO/NS. Active RAS leads to activation of the ERK1/2 MAPK pathway, which culminates in the phosphorylation of the Elk-1/TCF transcription factor (Gille *et al.,* 1992) which subsequently drives the transcription of *c-FOS* (fig. 3.1). In this way, the ERK MAPK pathway can lead to increased AP-1 activity through increased levels of c-FOS, which combines with pre-existing c-JUN proteins forming a stable AP-1 complex (Smeal *et al.,* 1989). *c-JUN* is also controlled by an inducible promoter, but most cell types contain some c-JUN protein even in the absence of stimulation. Activation of the JNK MAPK pathway leads to phosphorylation of c-Jun protein, which may also drive increased *c-JUN* expression (Dérijard *et al.,* 1994). Signal transduction driven phosphorylation of c-FOS may also be important in the regulation of AP-1 activity, potentiating transcriptional activity (Sutherland *et al.,* 1992). This appears to be mediated by a distinct 88-kDa MAPK termed FRK (Deng and Karin, 1994). Like ERK and JNK proteins, FRK is an H-RAS activated proline-directed kinase, and it is believed that *c-FOS* transcriptional activity can be stimulated by FRK directed phosphorylation.

RO/NS can thus impact gene expression at the level of signal transduction pathways upstream of transcription factor targets, indirectly leading to changes in transcription factor activity, exemplified by the RAS-RAF-ERK1/2 pathway and AP-1. In other cases, RO/NS can directly modify transcription factor activity by mediating increased/ decreased turnover, expression, or nuclear translocation of transcription factors. This has been seen to occur in the case of AP-1 and NFκB (section 3.1.3). Rao *et al.* (1995) demonstrated that hydroperoxy fatty acids and H_2O_2 can lead to increased

c-FOS and *c-JUN* expression in rat aortic smooth muscle cells. RO/NS can also regulate AP-1 activity by influencing its ability to bind to promoter sites in DNA. A basic motif containing a conserved cysteine residue exists in both c-FOS and c-JUN proteins. The oxidation state of this residue (controlled by redox factor (REF)-1 and thioredoxin (TRX1)) is a critical determinant in the affinity of AP-1 for its consensus DNA binding sites, oxidation negatively interfering with DNA binding (Hirota *et al.,* 1997). Oxidative stress can lead to an increase in cellular TRX1 levels, which favours a reduced state of the conserved cysteine residue in AP-1 components, thus enhancing DNA binding and hence transcription factor activity (fig. 3.1). In this way oxidants can indirectly enhance AP-1 mediated gene expression.

The mechanisms by which RO/NS can impact MAPK and AP-1 activities is summarised in figure 3.1. The redox sensitive transcription factor nuclear factor kappa B (NFκB) is subject to similar mechanisms of control.

3.1.3 Nuclear Factor Kappa Beta (NFκB)

NFκB (discussed in detail in section 1.8.2) has long been seen as the paradigm model of a redox sensitive transcription factor (Li and Karin, 1999, Schreck *et al.,* 1992). There is no shortage of experimental data linking H_2O_2 treatment of cells to NFκB activation (Gloire *et al.,* 2006; Takada *et al.,* 2003; Zhang *et al.,* 2001; Bowie and O'Neill, 2000; Manna *et al.,* 1998; Sen and Packer, 1996; Meyer *et al.,* 1993; Schreck *et al.,* 1991), and in fact it was such experiments involving frank exposure of cells to H_2O_2 that first established a strong link between redox regulation and NFκB (Manna *et al.,* 1998; Schreck *et al,* 1991), such treatments being seen to trigger nuclear translocation of NFκB in certain cell types (Gloire *et al.,* 2006; Bowie and O'Neill, 2000). Also, it has been reported that intracellular ROS levels regulate NFκB activity in response to a variety of stimuli through mechanisms which are yet to be clearly defined (Sen and Packer, 1996; Anderson *et al.,* 1994; Schreck and Baeuerle, 1994; Israël *et al.,* 1992). One must be wary of such data however, since the outcome appears to be both cell type-,

and dose dependent, and there is some conflicting evidence in the literature (Hayakawa *et al.,* 2003; Bowie and O'Neill, 2000; Li and Karin, 1999; Bowie *et al.,* 1997; Anderson *et al.,* 1994), with some studies supporting a stance that in some cell types, ROS may behave as antagonists rather than agonists of NFκB activity (Gloire *et al.,* 2000; Garg and Aggarwal, 2002; Haddad, 2002). Other stimuli that also activate NFκB such as phorbol esters (e.g. PMA), proinflammatory cytokines, bacterial endotoxins, and bile acids may also act via a RO/NS dependent mechanism since treatment with these agents increases intracellular ROS levels (Jenkins *et al.,* 2007; Schreck *et al.,* 1992).

Further experimental data that supports the involvement of RO/NS in the induction and regulation of NFκB activity stem from indirect observations in which antioxidants, and antioxidant enzyme over-expression inhibit NFκB activation (Schreck *et al.,* 1992), as well as studies that link TNF, IL-1, PMA, LPS, UV light, and ionizing radiation (all known inducers of NFκB) to elevated cellular levels of ROS (Schreck *et al.,* 1992).

Based on these experimental observations it is now largely accepted that in certain cell types, but certainly not all, up-regulation of NFκB activity is part of the cellular response to oxidative stress (Li and Karin, 1999). The realisation that different cell types respond differently to direct oxidant exposure with respect to NFκB activation, coupled with the later finding that NFκB activity could still be regulated by intracellular redox status in cells that did not respond to the direct stimulation (e.g. Jurkat cells), led to the speculation that two distinct redox stimulated/ regulated mechanisms exist in the NFκB signal transduction pathway (Anderson *et al.,* 1994). One mechanism permits oxidants such as H_2O_2 to trigger signal transduction in responsive cell types, and the other allows intracellular redox levels to regulate the extent of activation; since oxidants can serve as essential messengers in downstream signalling; both converging on a common pathway that involves intracellular redox regulation. It has been hypothesised that intracellular levels of reduced glutathione (GSH), the major thiol and ROS scavenger in cells (Aruoma *et al.,* 1989) may be crucial for the cell type specific differences in NFκB activation, since levels can differ greatly from one cell type to another, and even in a particular cell type under different conditions (Li and Karin, 1999). Excellent experimental evidence

has been cited in the literature in support of this hypothesis, since induced increase of cellular GSH (by pre-incubation with N-acetyl-L-cysteine (NAC) – a precursor for GSH synthesis) was seen to abolish H_2O_2 induced NFκB activation (Schreck *et al.,* 1991), whilst depletion of cellular GSH resulted in clearly detectable H_2O_2 – induced NFκB activation in cells in which it was previously undetectable.

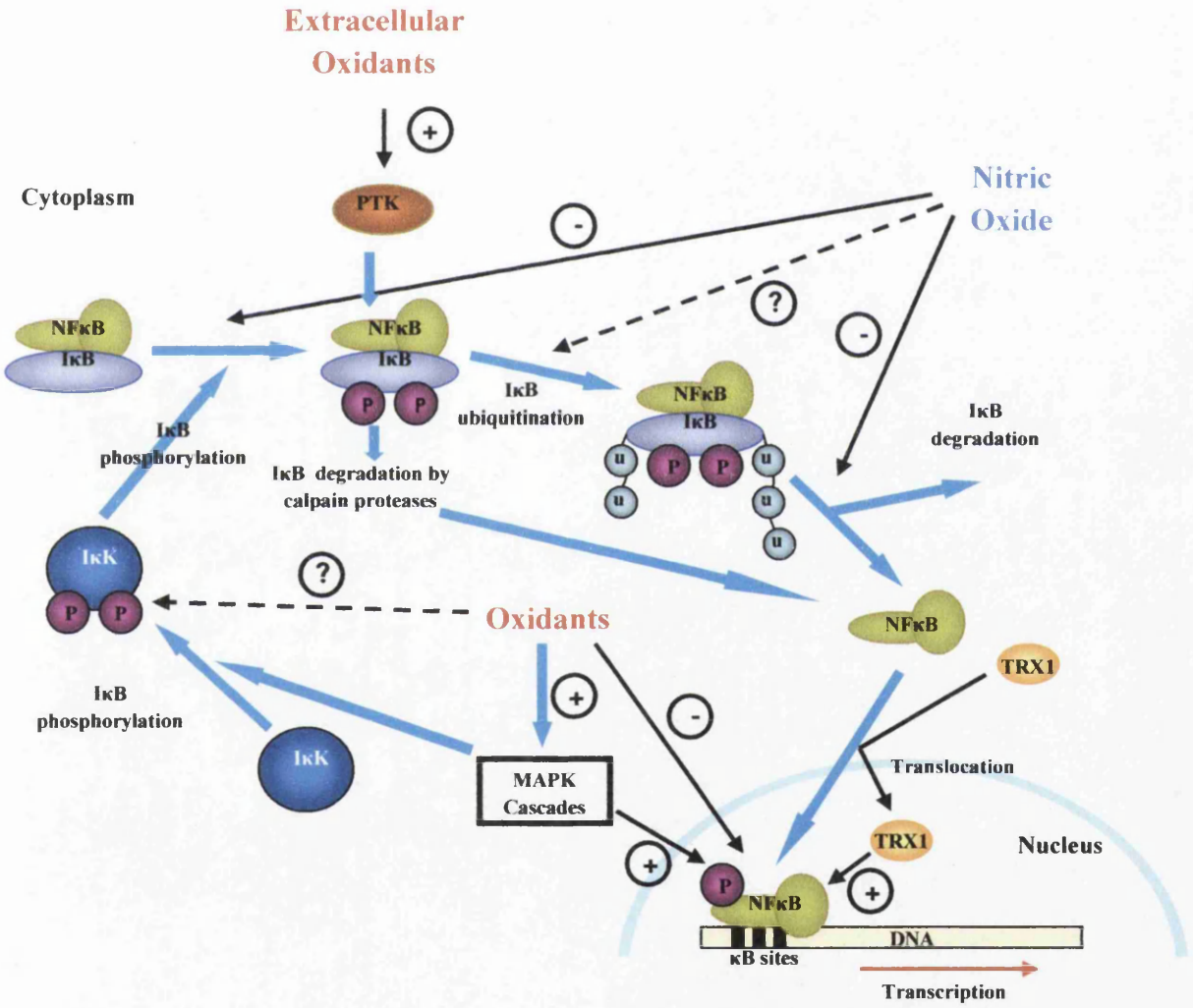
It is very difficult to decipher the exact mechanisms by which RO/NS regulate NFκB signalling and activity, since they appear to act at several levels, and in such short frames of time, and assays that are fast and sensitive enough to measure changes in intracellular levels of oxidants have yet to have been established.

It has been suggested that IκB phosphorylation and subsequent degradation may be the crucial step that is responsive to oxidative stress. Since IκB Kinase (IKK) – induced phosphorylation of IκB is the final step in NFκB activation which can be controlled before constitutively active components of the pathway come into play, it emerges that unless RO/NS are directly involved in this step, they are unlikely to serve a general signalling role in NFκB activation, and they may simply serve to regulate other redox sensitive components of the pathway. Undoubtedly the development of new more sophisticated methods aimed at the specific and highly sensitive detection of ROS in cells will help to clarify the steps at which such redox control occurs. In the meanwhile, speculations continue to be made based on the experimental evidence that continues to emerge. Hayden and Ghosh (2004) suggest that the ROS regulated step in cytokine mediated NFκB signalling may lie just downstream of IKK activation at the levels of IκBα-ubiquitin ligase, either affecting the enzymes activity, or the recognition of its phosphorylated-*IκB* target. Support for this mechanism is provided by studies which show that antioxidants and over-expression of peroxidase enzymes inhibit IκB degradation induced by a range of stimuli including TNFα, PMA, and LPS (Gloire *et al.,* 2006; Li and Karin 1999; Manna *et al.,* 1998). Some researchers in the field disagree with this model and speculate that the ROS – sensitive step in the NFκB activation pathways lies at the level of the IKK complex (Gloire *et al.,* 2006; Kretz-Remy *et al.,* 1996). Other notable observations that warrant consideration include documentation that whilst low doses of endogenous ROS may induce NFκB signalling, at higher doses (or above a

threshold level), they may have an inhibitory effect (Michiels *et al.*, 2002; Marshall *et al.*, 2000). Jaspers *et al.* (2001) showed that treatment of cells with high doses of H_2O_2 resulted in elevated levels of oxidised GSH which appeared to block proteolysis of IκB, and hence NFκB activation, by negatively impacting the activity of ubiquitin conjugating enzymes. There is also evidence to suggest that RO/NS may have an antagonistic effect on NFκB by more direct means, by interfering with the DNA binding activity of the transcription factor itself. Whilst the translocation of NFκB from nucleus to cytoplasm depends upon oxidising conditions in the cytoplasm, DNA-binding requires reducing conditions within the nucleus, supported by observations that reducing agents can enhance binding, whilst oxidising agents can inhibit it (Bowie and O'Neill, 2000). Indeed, the existence of a conserved redox-sensitive cysteine residue, Cys-62, in the Rel Homology Domain (RHD) of p50 that makes direct contact with DNA has been demonstrated (Brijelius-Flohe *et al.*, 2004; Haddad, 2002; Michiels *et al.*, 2002; Matthews *et al.*, 1992), and that oxidation of this residue may account for antagonistic effects of ROS. Hirota *et al.* (1999) cited evidence that Thioredoxin (Trx1) may be translocated into the nucleus concomitantly with NFκB, and that it reduces Cys-62 in the DNA binding loop, thereby enhancing DNA binding and transcriptional activity (Matthews *et al.*, 1992). Figure 3.2 summarises the outlined potential redox regulated steps in the NFκB pathway.

Several potential pathways exist for the activation of NFκB including amongst others, the classical/ canonical pathway and the alternative/ non-canonical pathway through which most well known mediators of NFκB act (Gilmore, 2006). Research in the past few years has brought to light the possibility that exposures to ROS like H_2O_2 may activate NFκB via an unconventional pathway involving phosphorylation of tyrosine (Tyr-42), threonine, and serine residues within the C-terminal of IκBα by protein tyrosine kinases (instead of at the canonical Ser-32/ Ser-36 sites by IKK), marking it for subsequent degradation by calpain proteases (Gloire *et al.*, 2006; Bowie and O'Neill, 2000; Schoonbroodt *et al.*, 2000) (fig. 3.2). The fine details of this pathway remain to be elucidated.

Figure 3.2 Summary of redox regulated steps in the NFκB signal transduction pathway. Details connected by blue arrows show the normal sequence of events following triggering of the pathway, starting with the *IκB*-bound NFκB complex (for more upstream events refer to section 1.8.2 and fig. 1.11) and ending with NFκB – mediated gene expression. Black arrows highlight the points at which RO/NS can impact the signalling, either positively (+), negatively (-), or as yet unclear (?). Refer to text for further details. (PTK = protein tyrosine kinase, Trx1 = thioredoxin, P = phosphorylated site, u = ubiquitinated site).



Interestingly oxidants may also activate NFκB signalling by way of MAPK pathway cross-talk (fig. 3.2), mediated by NFκB Inducing Kinase (NIK), a member of the MAPK family of enzymes, since they have been shown to induce NIK autophosphorylation, an event which can culminate in NFκB activation. Oxidant induced activation of RAS-RAF-ERK1/2 (p42/p44) MAPK signalling may also augment NFκB-dependent gene expression by way of MAPK cascade mediated phosphorylation of p65 (Jackson *et al.,* 2002). Whilst oxidants appear to have mainly positive effects on NFκB activity, $NO^{\cdot}$ can, at several levels – including reduction of IκB phosphorylation and ubiquitination, inhibit NFκB activation (Colasanti and Persichini, 2000) (fig. 3.2).

So, to date, the mechanisms by which RO/NS affect NFκB signalling and subsequent transcription factor activity are yet to be fully defined. What is clear, however, is that oxidative and nitrosative stress, and cellular redox status can impact NFκB activity in some way at several potential levels, the significance and impact being dose and cell type dependent. Figure 3.2 summarises the potential RO/NS sensitive steps of NFκB activation based on the evidence available to date.

The significance of redox regulated signal transduction and gene expression is apparent in several pathological states, exemplified by *Helicobacter pylori* – associated gastric carcinogenesis.

3.1.4. Involvement of RO/NS in *Helicobacter Pylori* - associated Gastric Carcinogenesis.

As discussed in section 1.4.5.1, excessive levels of RO/NS form a major component of *Helicobacter pylori* – associated gastric carcinogenesis, originating from the accumulation of activated inflammatory leukocytes at the vicinity of the infection, as well as enhanced generation of reactive metabolites by the gastric cells themselves (Ding *et al.*, 2007; Obst *et al.,* 2000; Bagchi *et al.,* 1996; Davies *et al.,* 1994a; Davies *et al.,* 1994b; Davies and Rampton, 1994; Weiss, 1989). Recently, Xu *et al.* (2004)

demonstrated that *H. pylori* resulted in enhanced H_2O_2 generation in AGS gastric epithelial cells via enhanced expression and activity of Spermine oxidase, resulting in H_2O_2 - induced apoptosis and DNA damage, providing a potential mechanistic link between *H. pylori* infection, oxidative stress, and gastric carcinogenesis. Enhanced apoptosis of gastric epithelial cells can result in increased epithelial permeability and mucosal damage, which drive a compensatory increase in cellular proliferation (Peek *et al.,* 2000; Peek *et al.,* 1999) resulting in inflammation, and potentially neoplastic development. The oxidative stress induced by infection may also lead to gene expression alterations via the redox sensitive AP-1 and NFκB transcription factors (Muller *et al.,* 1997). The increase in cellular proliferation in conjunction with redox sensitive gene expression alterations can provide a background upon which carcinogenesis is favoured.

With respect to how *H. pylori* impacts cellular signal transduction and gene expression, *H. pylori* – induced *IL-8* expression provides a good model. Upon direct contact of the bacterium with gastric epithelial cells, enhanced *IL-8* expression and release is induced (one of the most well studied genes in the disease pathology, amongst other gene expression changes), an effect which is enhanced by the presence of the *cag* pathogenicity island (PAI) in the infecting strain (Crabtree *et al.,* 1999; Crabtree *et al.,* 1995; Peek *et al.,* 1995; Crabtree *et al.,* 1994b), and is believed to be mediated by NFκB activation (Peek, 2001). The release of *IL-8* results in the infiltration of neutrophils and monocytes via a chemotactic gradient, resulting in an enhanced inflammatory response, which can result in chronic gastritis and potential progression to gastric neoplasia in a subset of individuals.

H. pylori has been demonstrated to rapidly activate NFκB and hence drive enhanced *IL-8* expression upon contact with gastric cells *in vitro* (Sharma *et al.,* 1998; Keates *et al.,* 1997), and the bacterium's capability to activate NFκB extends to the *in vivo* environment since activated NFκB is present within gastric epithelial cells of infected but not uninfected patients (Keates *et al.,* 1997). Experimentation by Maeda *et al.* (2000) helped in establishing a clearer understanding of the cellular signaling that drives *H. pylori* – induced NFκB activation. The hierarchical series of events involves *H. pylori* driven activation of NIK via upstream TRAF2 and TRAF6 (members of the TNF

receptor associated family (TRAF), serving as effectors of activated TNF and IL-1 receptors respectively) by mechanisms which remain elusive, that, in turn, phosphorylates and activates IKKs. Activated IKKs subsequently phosphorylate *IκB*, with concomitant proteasome-mediated degradation, culminating in the release and nuclear translocation of NFκB and induction of NFκB driven gene expression e.g. *IL-8* (Fig. 3.3).

More recently *H. pylori* has been seen to induce NFκB associated inflammatory signalling in gastric epithelial cells by way of a *cag* PAI – dependent mechanism involving intracellular nucleotide-binding oligomerisation domain protein (NOD1) receptor molecule (Viala *et al.,* 2004). NOD1 is part of a family of intra-cytoplasmic pathogen recognition proteins that recognise bacterial peptidoglycans. With respect to *H. pylori*, peptidoglycan, injected into gastric epithelial cells by way of the *cag* PAI type IV sectretion system, is recognised by NOD1, which then goes on to directly activate NFκB and hence NFκB – dependent gene expression.

MAPKs have also been implicated in *H. pylori* – induced signal transduction and gene expression changes. As detailed in section 3.1.3 MAPKs can directly influence activation of NFκB via NIK mediated activation of IKKs, and via ERK pathway mediated phosphorylation of p65 (Jackson *et al.,* 2002). In this way MAPKs can contribute to the enhanced expression of inflammatory cytokines (e.g. *IL-8*) by *H. pylori* infested gastric epithelium. In addition, MAPKs can directly activate transcription factors like AP-1 (section 3.1.2.1) which can lead to induction of *IL-8* expression (since the *IL-8* promoter contains both NFκB and AP-1 binding sites) (fig. 3.3).

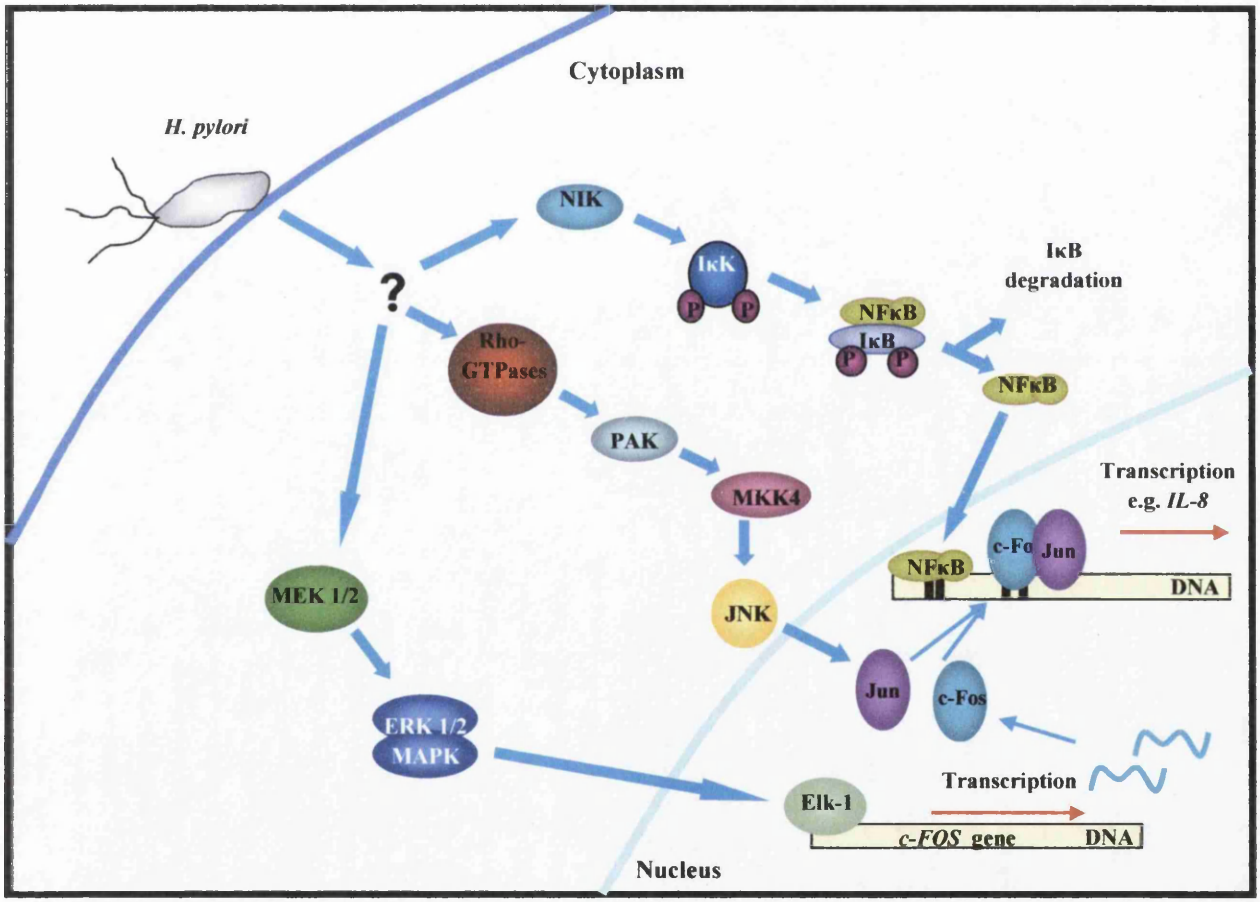
Dose-dependent activation of ERK, p38, and JNK MAPK in gastric epithelial cells by live *H. pylori* has been demonstrated (Keates *et al.,* 1999) which was seen to drive up-regulated *IL-8* expression. There has been some debate as to whether *H. pylori* – induced *IL-8* gene expression is dependent upon activation of NFκB, MAPK, or both (Meyer-Ter-Vehn *et al.,* 2000; Naumann *et al.,* 1999; Malinin *et al.,* 1997). Studies by Aihara *et al.* (1997) demonstrated that *H. pylori*-induced *IL-8* gene expression is dependent on both NFκB and AP-1 activation, and cross-talk between NFκB and MAPK pathways may be important. For example, MEKK1 and NIK each can directly activate

the IKK signalosome, thereby driving NFκB activation (Malinin *et al.,* 1997; Mercurio *et al.,* 1997). However, even though interplay between the two pathways may exist in *H. pylori* infected cells, no clear dependence seems to exist. For example, Keates *et al.* (1999) showed that whilst inhibition of ERK and p38 MAPK resulted in attenuation of *H. pylori* - induced *IL-8* expression, it did not affect NFκB activation. On these grounds it is believed that synergistic interactions between AP-1 and NFκB are required for maximal *H. pylori*-induced *IL-8* production. Other observations lead to the idea that there is considerable redundancy in the intracellular signalling pathways activated by *H. pylori* (Meyer-Ter-Vehn *et al.,* 2000; Naumann *et al.,* 1999).

What is clear is that by activating two prominent signal transduction pathways, *H. pylori* has been seen to induce gene expression alterations in infected cells, affecting a host of genes implicated in the carcinogenic process such as spermine oxidase (*SMO*) (Xu *et al.,* 2004), DNA damage response genes such as *GADD45A* (Myllykangas *et al.,* 2004), cell cycle genes such as cyclin D1 (*CCND1*) (Sepulveda *et al.,* 2002; Hirata *et al.,* 2001), cellular stress response genes such as heat shock proteins (*HSP*) (Myllykangas *et al.,* 2004), oxidative stress response genes e.g. superoxide dismutases (*SOD*) (Götz *et al.,* 1996), signalling molecules such as the serine threonine kinase *PIM-1* (Sepulveda *et al.,* 2002), growth factors and cytokines such as *VEGF* (Tucillo *et al.,* 2005; Strowski *et al.,* 2004; Caputo *et al.,* 2003), *IL-8* (Sharma *et al.,* 1998; Aihara *et al.,* 1997), and *TNFα* (Crabtree *et al.,* 1991), as well as transcription factors – notably the AP-1 transcription factor via up-regulation of *c-FOS* and *c-JUN* components (Sepulveda *et al.,* 2002; Chiou *et al.,* 2001; Mitsuno *et al.,* 2001; Meyer-Ter-Vehn *et al.,* 2000; Naumann *et al.,* 1999).

Whilst it is clear that *H. pylori* infection can lead to cellular signal transduction changes, it remains, as yet unclear exactly how this occurs. There appears to be some dependence on direct contact between bacterium and host cells for the induction of NFκB and MAPK pathways in gastric epithelial cells (Peek, 2001; Rieder *et al.,* 1997). Several reports, however, suggest that *H. pylori* can induce cytokine expression in various

Figure 3.3 Effects of *Helicobacter pylori* on cellular signal transduction and gene expression in gastric epithelial cells. By mechanisms that are yet to be elucidated, but are likely to involve bacterial factors and reactive oxygen/ nitrogen species (RO/NS), the bacterium triggers activation of NFκB (via several mechanisms including Nod1 – see text for further details), ERK MAPK, JNK MAPK, and p38 MAPK (not shown) pathways, all of which result in gene expression alterations via NFκB and AP-1 transcription factors, often leading to up-regulation of cytokine genes e.g. *IL-8*, *IL-6*, *TNFA*, and oncogenes e.g. *C-FOS*. For further details refer to text.



leukocyte types regardless of direct cellular contact, implicating soluble bacterial factors in the activation mechanism (Bhattacharyya *et al.,* 2002), which would obviously favour enhanced inflammation and a microenvironment that drives carcinogenesis. Clearly then, *H. pylori* can induce cellular signal transduction cascades by a number of means both via direct contact, which is likely to depend upon components of the *cag* PAI, and indirectly via soluble factors. In both cases, the early steps in signalling induction remain to be clearly defined. It is quite plausible that since *H. pylori* infection causes significant oxidative stress and enhanced generation of RO/NS in the vicinity of the infection (by both gastric epithelial cells and leukocytes), coupled with the importance of the redox sensitive NFκB and MAPK pathways in bacterium induced gene expression changes, that RO/NS may have a fundamental involvement (Baek *et al.,* 2004; Seo *et al.,* 2004; Chu *et al.,* 2003; Kim *et al.,* 2001; Lim *et al.,* 2001; Kim *et al.,* 2000).

3.1.5 Aims of the Chapter

On the basis of the literature reviewed there seems to be a strong grounds for the hypothesis that RO/NS play a key role in the pathogenesis of gastric cancer – being particularly significant to the *H. pylori* – associated and chronic inflammatory components of the pathogenesis of the disease. Persistent *H. pylori* infection and chronic inflammation go hand in hand, and together generate a hostile microenvironment within the gastric mucosa, characterised by infiltration of leukocytes, elevated levels of pro-inflammatory cytokines and enhanced generation of RO/NS from both leukocyte and gastric epithelial cell sources. In addition, *H. pylori* is well documented to induce activation of redox – sensitive transcription factors including NFκB and AP-1 via redox – sensitive signal transduction cascades including MAPKs, by way of infection associated oxidative stress amongst other mechanisms. This can lead to gene expression alterations that may have negative impacts on overall gastric health.

Based on these grounds one can speculate that *H. pylori* infection and its associated inflammation and generation of RO/NS may contribute to gastric

carcinogenesis at the molecular level through the induction of mutations, aberrant signal transduction, and gene expression alterations.

The work presented in this chapter aims to dissect the involvement of oxidative stress in gastric disease by exploring the effects of a model Reactive Oxygen Species (ROS), Hydrogen peroxide (H_2O_2) (so mimicking the oxidative stress induced by *H. pylori* infection), on human cell lines at the levels of gene and protein expression with a particular focus on signal transduction changes. H_2O_2 was selected on the basis that it is a diffusible ROS and so can readily diffuse into cells where it can impact signalling and gene expression, it can result in the generation of hydroxyl radical (·OH) which is considered the ultimate reactive metabolite (Henle and Linn, 1997), and has been used widely in oxidative stress research (Williams *et al.,* 2005; Jenkins *et al.,* 2001; Ruiz-Laguna and Pueyo, 1999; Wang *et al.,* 1998; Duthie *et al.,* 1997; Kleiman *et al.,* 1990; Moraes *et al.,* 1990). Moreover, tumour cells have been shown to constitutively generate H_2O_2 (Szatrowski and Nathan, 1991). Since the amount of ROS generated in inflamed gastric mucosa is not clearly defined and is subject to continuous change, a dose range was selected that fell around the LD_{50} of H_2O_2 seen in toxicity tests (50 – 500μM), is in line with dose ranges that have been employed in the literature (Williams *et al.,* 2005; Jenkins *et al.,* 2001; Duthie *et al.,* 1997; Kleiman *et al.,* 1990), and covers the concentration range that has been seen to be generated in tumour cell lines (Szatrowski and Nathan, 1991) (100 – 500μM/ 10^4 cells/ hr).

Three different cell lines were employed in the studies – WILL1 - a normal fibroblast cell line, AGS – a gastric cancer cell line derived from an early non-metastatic gastric tumour (generally considered to be a very good model of a primary gastric epithelial cell), and HGC-27 – a more advanced gastric cancer cell line derived from a metastatic stage of the disease. The importance of using the three cell lines is two-fold. It allows cell line and cell type specific comparisons to be made, as well as the effect of different stages of disease on responsiveness to oxidative stress, to be analysed.

Signal transduction and gene expression changes induced by H_2O_2 were analysed with the Extra-cellular signal-Regulated Kinase (ERK) Mitogen Activated Protein Kinase (MAPK) pathway being a central theme to the studies. The pathway was examined using

a variety of techniques (real-time (RT-)/ quantitative (Q-) PCR for downstream genes as well as western blots for active components of the pathway) since aberrant MAPK signalling is a common feature of cancer (Dhillon *et al.*, 2007), has been seen to be induced by *H. pylori* infection (Crabtree and Naumann, 2006), and has been detected in gastric tumours (Watari *et al.*, 2007; Sepulveda *et al.*, 2002; Gong *et al.*, 1999; Barnard *et al.*, 1995; Tahara, 1995b). The activity of the NFκB pathway was also studied by RT-PCR for *IL-8* and *IκB* genes, using the level of expression of the NFκB regulated genes as a surrogate marker for NFκB activity (Jenkins *et al.*, 2004).

3.2 Materials and Methods

3.2.1 Cell Culture

3.2.1.1 HGC-27

HGC-27, a human gastric adenocarcinoma permanent cell line derived from a lymph node metastasis of a Japanese patient with gastric adenocarcinoma of the undifferentiated sub-type, was obtained from the European Collection of Cell Cultures (ECACC, Salisbury, Wiltshire, UK, Catalogue number 94042256), and was cultured as described in section 2.1.1.

3.2.1.2 AGS

AGS, a hyperdiploid human cell line (modal chromosome number of 49), was derived from fragments of a primary gastric tumour from a 54 year old female Caucasian patient who had received no prior therapy. The cell line originates from a relatively poorly differentiated gastric cancer, and was obtained from the American Tissue Culture Collection (ATCC) (LGC Promochem, Teddington, Middlesex, UK, Catalogue number CRL-1739) and was cultured as described in section 2.1.1.

3.2.1.3 WILL 1

WILL1, a normal primary fibroblastic cell line derived from the foreskin of a one year old boy was grown as described in section 2.1.1.

3.2.2 Gene Expression Analysis

Cells were treated, RNA extracted and gene expression changes analysed using membrane cDNA arrays and real-time (RT) PCR.

3.2.2.1 Treatment of Cells

Cells of all three cell lines were treated with a variety of doses of Hydrogen Peroxide (H_2O_2) (Sigma-Aldrich, Poole, UK) as described in section 2.2. Prior to dosing, cell viability was assessed using the trypan blue cell viability assay as described in section 2.1.1.8, and only cell cultures with a viability of $\geq$ 95% were used in subsequent experimentation. MTS assays were also carried out as outlined in section 2.3 over a dose range of H_2O_2 for different time points in order to identify doses that may cause excessive toxicity.

The particular treatment regimens used for each cell line are summarised in table 3.1. All treatments were performed in duplicate, and the resultant RNA or protein extracted was pooled and analysed.

In addition, a preliminary experiment was performed in which cells were pre-treated for 30min with 10μM of the ERK MAPK inhibitor U0126 (Favata *et al.*, 1998) (Sigma-Aldrich, Poole, UK) prior to H_2O_2 dosing for 4hr in order to determine if a link between MAPK activity and H_2O_2 – induced *c-FOS* expression existed.

3.2.2.2 RNA Extraction

Total cellular RNA was extracted from dosed and control cells, and processed as described in section 2.4 – 2.4.4. It was fundamental that the RNA be of a high quality for downstream analysis, so RNA quality was assessed by spectrophotometric determination of the 260/280 ratio (only samples with a ratio $\geq$ 1.8 were used in subsequent analyses) and by reverse transcription-PCR as described in section 2.4.5. Briefly, RNA samples

Table 3.1 Summary of Hydrogen Peroxide treatment regimens carried out on cultured cells. All dosing was acute, with exposure times ranging from 5min to 24hr over a range of doses. Short burst treatments involved rapid exposures of cells to 150μM H_2O_2 in order to assess how rapidly the ROS can elicit MAPK pathway activation. Longer exposures were also carried out in order to look at downstream gene expression changes. * = Treatments analysed by western blot.

Cell Line	Treatment	
AGS, HGC-27, WILL1	*short burst*	*Long Exposures*
	- *EGF positive* *	- *4hr control* *
	- *Control* *	- *4hr 50μM H_2O_2*
	- *5min 150μM H_2O_2* *	- *4hr 150μM H_2O_2* *
	- *10min 150μM H_2O_2* *	- *4hr 250μM H_2O_2* *
	- *30min 150μM H_2O_2* *	- *4hr 500μM H_2O_2* *
		- *8hr control* *
		- *8hr 50μM H_2O_2*
		- *8hr 150μM H_2O_2* *
		- *8hr 250μM H_2O_2* *
		- *8hr 500μM H_2O_2* *
		- *24hr control* *
		- *24hr 50μM H_2O_2*
		- *24hr 150μM H_2O_2* *
		- *24hr 250μM H_2O_2* *
		- *24hr 500μM H_2O_2* *

(500-800ng – consistent for each cell line) were reverse transcribed for 1hr at 44°C using a Retroscript kit (Ambion, Warrington, UK), followed by PCR amplification of both cDNA and RNA using primers for β-actin (*ACTB*) (see table 3.2). Only samples that yielded one clear band following polyacrylamide gel visualisation were used in downstream experimentation.

3.2.2.3 Protein Extraction

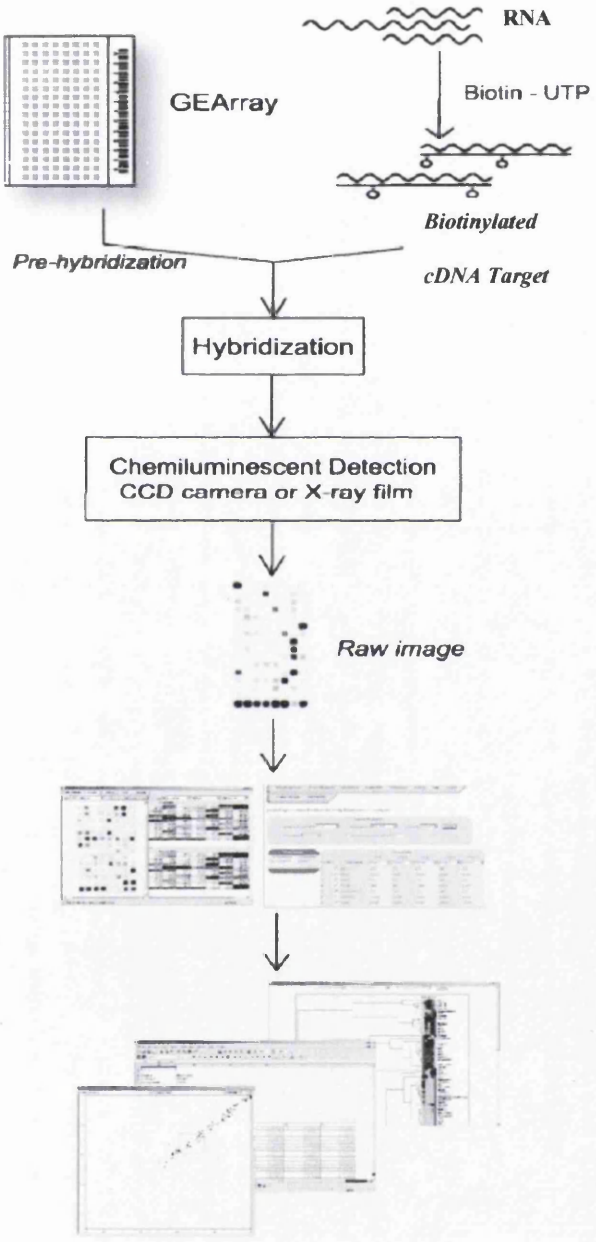
Total protein was extracted, processed, and quantified from dosed and control cells as described in section 2.6.

3.2.2.4a Membrane cDNA Arrays

For a 'global' gene expression analysis the GEArray Q series array kits from SuperArray (Cambridge, UK) were used as a cDNA gene expression profiling system. Array experiments were carried out as described in the manufacturer's protocol with some modifications as described below and summarised diagrammatically in figure 3.4. In this study, the Cancer Pathway Finder, and Nitric Oxide Arrays were used, since they cover a wide range of genes involved in cell signalling, apoptosis, proliferation, angiogenesis, and inflammation. The array gene tables are shown in Appendix I (sections AI.1 and AI.2 respectively).

All probe labelling reactions were carried out in Class II Lamina Flow cabinets following the same precautions as described for general PCR (section 2.5.4) and all RNA and enzymes used were kept on ice throughout. All incubations were carried out using an MJ Research Peltier Thermal Cycler (PTC)-2000 DNA Engine (Essex, UK). All incubations of microarrays were carried out in a Hybaid Rotating Hybridisation Incubator (Grant Instruments, Cambridgeshire, UK) with agitation.

Figure 3.4 Outline of Array Experimental Procedure, adapted from SuperArray Bioscience Corporation (Cambridge, UK) GEArray System User Manual (downloaded at **www.superarray.com**).



Target Synthesis and labelling

(1 - 2hr)

Overnight Hybridisation Step

Image Detection

(2 - 3hr)

Data Analysis

In all array experiments control arrays hybridised with cDNA generated from the RNA of untreated cells, and arrays hybridised with cDNA from treated cells were included. Only RNA from HGC-27 was used in this experimentation due to limitations on time and resources. RNA from cells that had been treated under normal and acidic pH (medium was acidified by the addition of hydrochloric acid (HCl) to pH5 (maximum acidity tolerable by the cells in culture) and then filter sterilised, treatments subsequently carried out as normal) were also included in the study, to assess if acidic conditions impact gene expression changes as may be the case in the stomach environment.

3.2.2.4b cDNA Probe Generation

RNA samples were converted into biotin labelled cDNA probes using the Ampolabelling Kit (Superarray, Cambridge, UK) following the Ampolabelling LPR protocol with modifications. For each RNA sample, the following reagents were mixed in sterile 0.2ml microfuge tubes to make up annealing mixes:-

- 1μg total RNA,
- 1μl buffer P (primer mix),
- RNase-free H_2O to a final reaction volume of 10μl.

The mixtures were then gently mixed by pipetting, and the tubes incubated at 70°C for 3min to allow for primer annealing, and subsequently cooled to 37°C for 10min. Whilst the annealing mix was incubating, an RT cocktail master mix was prepared consisting of appropriate multiples (depending on the number of reactions being carried out) of the following reagents:-

- 4μl buffer BN,
- 4μl RNase-free H_2O,
- 1μl RNase inhibitor,

- 1µl Reverse Transcriptase.

The cocktail was subsequently warmed to 37°C for 1min prior to adding 10µl of the mix to each annealing mixture. The resultant 20µl mixture was gently mixed with a pipettor and incubated at 37°C for 25min, followed by heating to 85°C in order to inactivate the reverse transcriptase and hydrolyse the RNA.

A linear polymerase chain reaction (LPR) mix was then prepared for each sample, using half the volumes indicated in the manufacturer's protocol, consisting of:-

- 9µl buffer L,
- 1µl Biotin-16-dUTP (Roche, East Sussex, UK),
- 4.5µl buffer AF (specific to each type of array),
- 0.5µl DNA Polymerase (LE).

The LPR mix was mixed well and 15µl was added to each RT reaction (to a final volume of 35µl) and the resultant mixture mixed well by pipetting. The sample was then heated to 85°C for 5 min before undergoing 30 cycles of 85°C – 1min; 50°C – 1min; 72°C – 1min in the thermal cycler. The resultant Biotin labelled cDNA target was used to probe the arrays.

3.2.2.4c Pre-Hybridisation Treatment of Arrays

During the 2 hour LPR reaction, the membrane arrays were prepared for overnight hybridisation with labelled cDNA probe by pre-hybridisation treatment. This consisted of wetting arrays (in the tubes provided) with dH_2O and then incubating the arrays in 2ml of GEAhyb solution (prepared by adding 300µg heat-denatured sheared salmon sperm DNA (final concentration 100µg/ml) (denatured at 100°C for 5 minutes in a heating block and then quickly quenched on ice) to 3ml GEAhyb hybridisation solution

(pre-warmed to 60°C in a water bath) per array) for 1 – 2hr at 60°C with continuous agitation in the hybridisation oven.

3.2.2.4d Array Hybridisation

After approximately 2 hours the LPR labelling reaction was immediately stopped by the addition of 5µl buffer C and chilling on ice. The labelled cDNA probe was then denatured by heating at 94°C for 2min and then quickly chilling on ice. The denatured probe (35µl per sample) was then added to 0.75ml of the prepared GEAhyb solution (described in section 3.2.2.4c) generating the hybridisation solution. The pre-hybridisation solution was then discarded from the tubes, after the 1 – 2hr pre-hybridisation step, and replaced with the 0.75ml hybridisation solution containing the labelled probe. The arrays were then hybridised overnight at 60°C with continuous agitation.

3.2.2.4e Post-Hybridisation

Post-hybridisation washes were performed in order to remove unbound probe and non-specifically bound probe in order to reduce background. All washes were performed at 60°C with continuous agitation in the hybridisation oven. Hybridisation solution was discarded and replaced with 5ml of pre-warmed wash solution 1 (2X SSC/ 1% SDS) and incubated for 15min. This wash step was repeated a second time, the wash solution discarded, and the wash procedure repeated with wash solution 2 (0.1X SSC/ 0.5% SDS). Subsequent steps were performed at room temperature with continuous agitation in the hybridisation oven. After the wash steps 2ml pre-warmed (37°C) GEAblocking solution Q was added to the hybridisation tubes and the arrays incubated for 40min in order to block the arrays. The arrays were then incubated in 2ml 1X buffer F/ 1:8000 alkaline

phosphatase (AP)-Streptavidin for 10min. The arrays were then subjected to four 5min washes in 4ml 1X buffer F, followed by 2 final rinses in 4ml AP assay buffer G.

3.2.2.4f Chemiluminescent Detection

In the earlier steps the arrays were incubated with AP-Streptavidin. This forms the basis of the chemiluminescence detection system as the Streptavidin binds to the biotin in the labelled cDNA probes that bound to the array gene spots, and the AP reacts with applied CPD star substrate to generate a detectable chemiluminescence signal.

The arrays were removed from their hybridisation tubes and placed on plastic acetate sheets cut to the size of the arrays. Subsequently, 0.75ml CPD-star substrate was applied to each array, the arrays covered with a second acetate sheet and left for 2min. CPD-star was then removed from the arrays by blotting the membranes, and the arrays transferred to fresh acetates. The arrays were then exposed to chemiluminescence film (Hyperfilm ECL) (GE lifesciences, Bucks, UK) for a period of 5min. The resultant film was developed in a dark room using the AGFA Currix 60 Film Processor (Agfa, Middlesex, UK). Depending upon the exposure result, re-exposure of film for shorter or longer periods was sometimes required until an adequate image was obtained.

3.2.2.4g Array Image Capture and Analysis

Approximately 1hr after arrays were exposed to CPD-star chemiluminescent substrate, the arrays were individually illuminated and an image captured (after manually positioning the array, zooming and focussing) using the Bio-Rad chemiluminescence documentation system Chemi Doc 2000 (Bio-Rad, Hertfordshire, UK) in conjunction with the Quantity One version 4.0.3 software for image analysis. The resultant image was converted into volume and density values for each gene spot using the volume array tool

and volume analysis options, which enable quantification of gene expression using the Superarray GEArray analyser (downloaded from **www.superarray.com**) following instructions in the user manual.

3.2.2.4h Stripping Arrays

Arrays were stripped and re-used up to 3 times. Stripping to remove bound probe was carried out by boiling the arrays in 0.5% SDS for 10min. They were then left to cool for 10min in the 0.5% SDS solution, rinsed in 2X SSC, and air dried. Arrays were subsequently exposed to chemiluminescence film to ensure complete removal of bound probe. The stripped arrays were stored at -20°C until required.

3.2.2.5a Real-Time PCR

All real-time (RT-) PCR experiments were carried out as described in detail in section 2.5.1. Specific genes analysed are summarised in table 3.2 together with the forward and reverse primer sequences used. Standard curves were generated from pooled RNA from cell culture and gastric biopsy specimens. The same standard curve RNA was used in each plate of an experimental run (refer to section 2.5.1.1 for further details).

3.2.2.5b Primer Design

Primers were designed based on sequences obtained from the NCBI website (www.ncbi.nlm.nih.gov). In this case, the *VEGF* gene sequence was obtained from GenBank

Table 3.2 Real-Time PCR primer sequences.

Gene	Primer Sequence	Source
ACTB		
- *Forward*	5' – GATGGCCACGGCTGCTTC – 3'	From Jenkins *et al.* (2004).
- *Reverse*	5' – TGCCTCAGGGCAGCGGAA – 3'	
c-FOS		
- *Forward*	5' – CGAGCCCTTTGATGACTTCCT – 3'	From Gan *et al.* (2000).
- *Reverse*	5' – GGAGCGGGCTGTCTCAGA – 3'	
VEGF		
- *Forward*	5' – GATCCGCAGACGTGTAAATG – 3'	Designed by eye from VEGF gene sequence and optimised for real-time PCR. See section 3.2.2.5b for details.
- *Reverse*	5' – CTCACCGCCTCGGCTTGTC – 3'	
IL-8		
- *Forward*	5' – CAATCCTAGTTTGATACTCCC – 3'	From Jenkins *et al.* (2004).
- *Reverse*	5' – AATTACTAATATTGACTGTGGAG – 3'	
IκB		
- *Forward*	5' – ACACTAGAAAACTTCAGATGC – 3'	From Jenkins *et al.* (2004).
- *Reverse*	5' – TGCCTCAGGGCAGCGGAA – 3'	

(**http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nucleotide&val=71051577**) using the corresponding gene ID from the Cancer Pathway Finder array (NM_003376). The criteria used when designing the primers by eye were:-

- Primer length should be approximately 20bp, with a melting temperature of 55 - 65°C to ensure sequence specificity;
- There should be no/ minimal intra-/ inter-sequence complementarity, thereby minimising hairpins, other secondary structures, and primer dimer, as this otherwise reduces PCR specificity and efficiency;
- The primers should not hybridise to other genome sequences. A BLAST search is carried out through the NBCI website (**http://www.ncbi.nlm.nih.gov/BLAST/**) to ensure that this does not occur;
- Ideally, the primer pair should span an intron, thus, if any genomic DNA is amplified it will be of a much larger size than sequences amplified from RNA;
- PCR product should be approximately 100bp in length.

The forward and reverse *VEGF* primer sequences designed on this basis are shown in table 3.2. Once the primers were designed they were analysed using the NetPrimer primer analysis software downloaded from **http://www.premierbiosoft.com/netprimer/netprlaunch/netprlaunch.html**. This software evaluates characteristics of the primers based on the nucleotide sequences, specifically providing important information about their Melting Temperature (T_m), likelihood of hairpins, dimers, palindromes, and the overall rating of the primers. Primers were synthesised by Sigma Genosys (Sigma Genosys, Sigma-Aldrich, Poole, UK) according to the sequences established. The primers were HPLC purified and desalted by the manufacturer. The primers were diluted in filter sterilised dH_2O to a final concentration of 15pmol/µl, and stored at -20°C until required in 50µl working aliquots.

The primer sequences were tested out on sample RNA by first carrying out standard reverse transcription PCR reactions (as described in section 2.4.5), followed by

standard PCR spanning a temperature gradient from 50°C to 65°C in 2°C increments. The PCR products were run on a polyacrylamide gel and visualised by silver staining (section 2.4.5.3).

The resultant gels revealed that the primers generated a clean PCR product of ~100bp in length, with no primer dimer or other non-specific products, at an optimal temperature of 60°C.

3.2.2.5c Statistical Analysis

One-way ANOVA was performed (using SPSS Version 13.0) on the real-time PCR data (after normalising the gene of interest against β-actin values) on each time point separately, in order to identify any significant differences in gene expression between treatments and controls (using Tukey and Duncan Post Hoc tests). Statistical significance was observed when $P < 0.05$ at the 95% confidence interval and 0.05 significance level.

3.2.3 Protein Studies

3.2.3.1 Western Blotting

A series of Western Blots were carried out on protein extracts from control and treated cells as described in section 2.7.3. Proteins of interest in this study were:-

- ERK1/2 (p42/p44), and its phosphorylated, hence active counterpart pERK1/2 in order to assess potential activation of the ERK (p42/p44) MAPK pathway (Yung *et al.,* 1997);
- p38, and its active form, phospho p38, again to assess MAPK pathway activity;

- c-Fos, a downstream gene expression target of MAPK activation;
- Active NFκB in order to assess activation of NFκB signalling;
- IκBα, a downstream gene expression target of NFκB.

All of these primary antibodies were polyclonal antibodies raised in rabbits and were obtained from Abcam (Cambridge, UK) with the exception of pERK (Sigma-Aldrich, Poole, UK) . The primary antibodies were used at a final working dilution of 1:1000. The secondary antibody utilised was Goat Anti-Rabbit Ig-HRP conjugate (Abcam, Cambridge, UK) and was used at a final dilution of 1:3000.

3.2.3.2 Statistical Analysis

One-way ANOVA was performed (followed by Tukey and Duncan post hoc tests) to compare protein levels between control and treated samples at the different time points, at the $P = 0.05$ significance level. Statistically significant differences were those with $P < 0.05$.

3.2.4 NFκB Activity Analysis

3.2.4.1 Harvest of Recombinant NFκB-GFP Fusion Reporter Plasmid

E.coli harbouring the NFκB-GFP fusion plasmid shown in figure 3.5 (kindly donated by Dr Johannes A. Schmid, Center for Biomolecular Medicine and Pharmacology, Vienna, Austria (Schmid *et al.,* 2000)) was grown in 200ml pre-warmed Luria-Bertani (LB) broth (1% $NaCl_2$, 1% Bactotryptone, 0.5% Yeast Extract, 25μg/ml kanamycin) at 37°C in a shaking incubator overnight. Cells were subsequently harvested

by centrifugation using a Beckman J-6M/E centrifuge (Buckinghamshire, UK) at 6000 x g (6000rpm) for 15min at 4°C and total DNA extracted using the QIAgen Maxi-Prep extraction kit (QIAgen, Crawley, West Sussex, UK) as per manufacturers protocol. DNA was quantified by spectrophotometry at A_{260}.

3.2.4.2 Transfection of Human Cell Lines with Reporter Plasmid

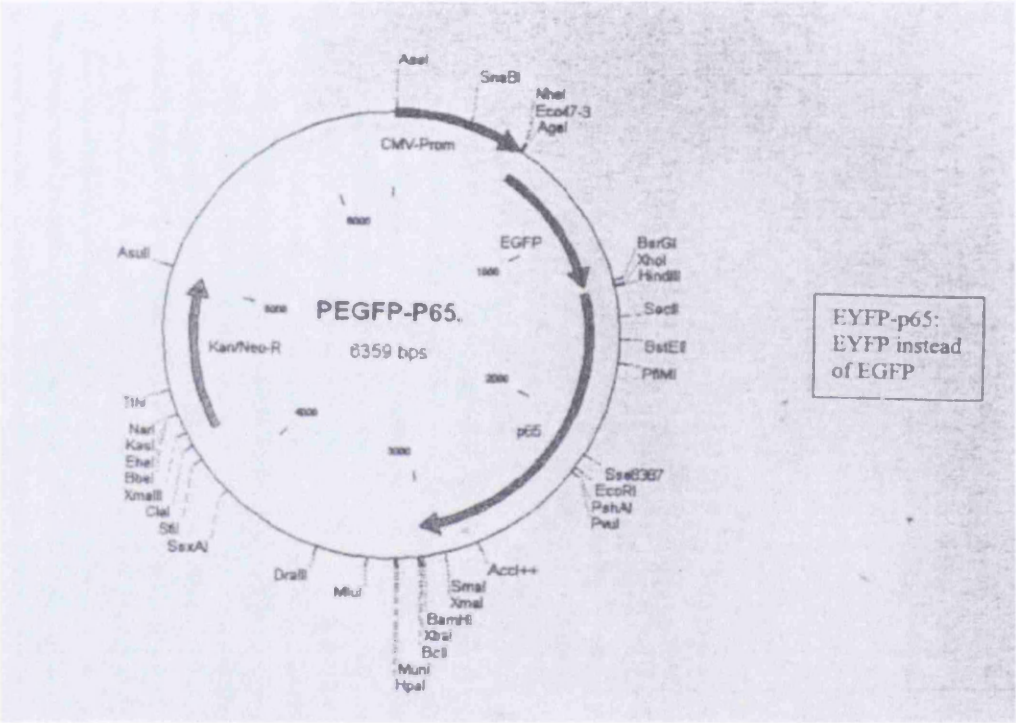
Cell lines (AGS, HGC-27, WILL1) were seeded into chambers of cell culture slides compatible with live cell confocal microscopy (Lab-tek chamber slides with cover, Fisher, Leicestershire, UK) at a density of 1 x 10^5 cells/ml in complete medium (minus antibiotics) and incubated overnight at 37°C in an atmosphere of 5% CO_2. Under these conditions cells attached to the slides generated an even spread. It is essential for transfection that cells are not too confluent or grow in clumps, as evenly spread out cells tend to take up the plasmid with a greater efficiency. The following day the cells were incubated in a transfection cocktail that was prepared as follows for each well (set up as master mixes depending on the number of wells per experiment):-

- 1.6µg plasmid DNA was diluted in 100µl serum free medium (SFM),
- 3µl Lipofectamine (Invitrogen, Paisley, UK) was diluted in 100µl SFM and incubated for 5min at room temperature,
- After incubation, the diluted DNA was added to the diluted Lipofectamine, and the mixture incubated at room temperature for a further 20min,
- To each well, 100µl of the transfection mix was added, together with 400µl complete medium,
- Slides were incubated for 24 – 48hr at 37°C in a humidified atmosphere of CO_2.

Upon a series of trial experiments it was found that the optimal incubation time for AGS and WILL1 cell lines was 24hr, after which the transfection mix was removed from the cells and replaced with medium containing 5% FBS allowing the cells to

recover from the stress of transfection. This reduced background in control and treated samples, since the transfection itself can cause NFκB to become active and translocate to the nucleus.

Figure 3.5 NFκB-GFP (GFP-p65) reporter construct plasmid (kindly donated by Dr J. A. Schmid (Schmid *et al.*, 2000)) used in transfection assays. Successful transfection results in expression of a fluorescent NFκB fusion protein, the cellular localisation of which can be examined using fluorescence microscopy.



The recovery period allows NFκB to shift back to the cytoplasm. For HGC-27 a very poor transfection efficiency was initially observed (< 10%). This was increased to ~ 30% by allowing for transfection to occur over a 48hr period, however, after 24hr had lapsed, 200µl of the transfection mix was removed, and wells were topped up with 300µl complete medium in order to avoid cell death.

3.2.4.3 Dosing cells with H_2O_2

Cells were subsequently treated with H_2O_2 at the following doses and times - control, 30min 250μM H_2O_2, 1hr 250μM H_2O_2, all in triplicate for each cell line.

3.2.4.4 Confocal Microscopy

Following dosing cells were observed live using a Zeiss Confocal laser Scanning Microscope (CLSM) 510 Meta (Herts, UK) using the Argon 488nm excitation laser and the 500nm emission laser to detect green fluorescence from GFP. Cells were observed using the 40x oil objective.

3.3 Results

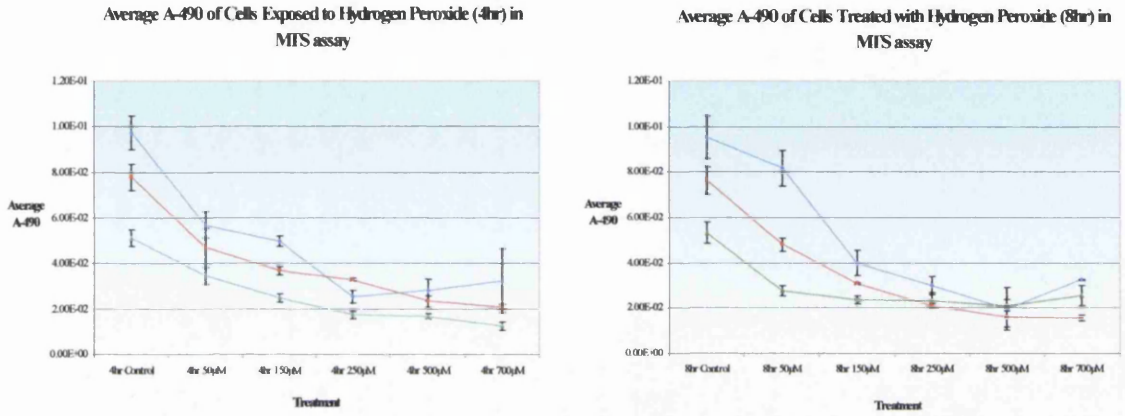
3.3.1 MTS Assay

The MTS assay was carried out on all three cell lines as a preliminary experiment to the H_2O_2 dosing experiments in order to determine cellular toxicity to the model ROS. The assay was carried out over a linear dose range from 50μM – 700μM H_2O_2, as well as across time points – 4, 8, and 24 hours.

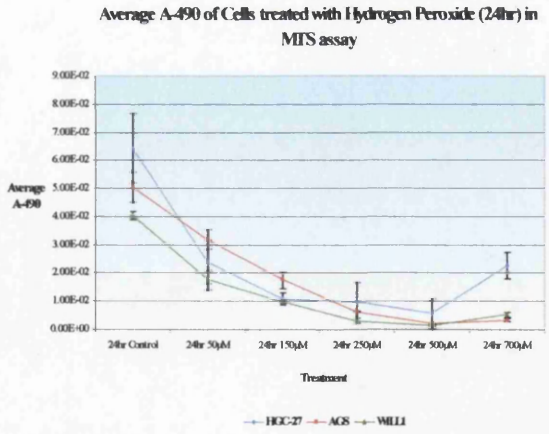
A similar trend of decreasing cell number with increasing dose was observed for all three cell lines, clearly seen in the graphical representation of the data in figure 3.6. The cell lines responded as expected based on their different growth characteristics in cell culture – with HGC-27 displaying the fastest growth kinetics and WILL1 the slowest. The spectrophotometric absorbance at a wavelength of 490nm (A-490/ A_{490}) of the

Figure 3.6 Mean A_{490} (A-490) of HGC-27 treated with dose range of Hydrogen Peroxide (H_2O_2) at different time points – (a) 4hr, (b) 8hr, and (c) 24hr. The A_{490} of the soluble formazan product of the assay corresponds to the number of cells present; an increase would indicate cell proliferation. Here it is clear that there is a decline in the A_{490}, and hence cell number. This indicates that increasing concentration of H_2O_2 leads to cellular toxicity, and this is heightened at the 24hr time point. At the 4 and 8hr time points the cells appear to halve in number at around 150 – 250μM, whereas at 24hr, this occurs at 50μM. Similar trends were seen in all three cell lines tested. N = 3 (i.e. data are averages from 3 experimental repeats).

(a) **(b)**



(c)



soluble formazan product produced during the assay (as a result of cellular metabolism) corresponds to the number of cells present. An increase in absorbance would indicate cell proliferation and increased cell number. Here it is clear that there is actually a decline in

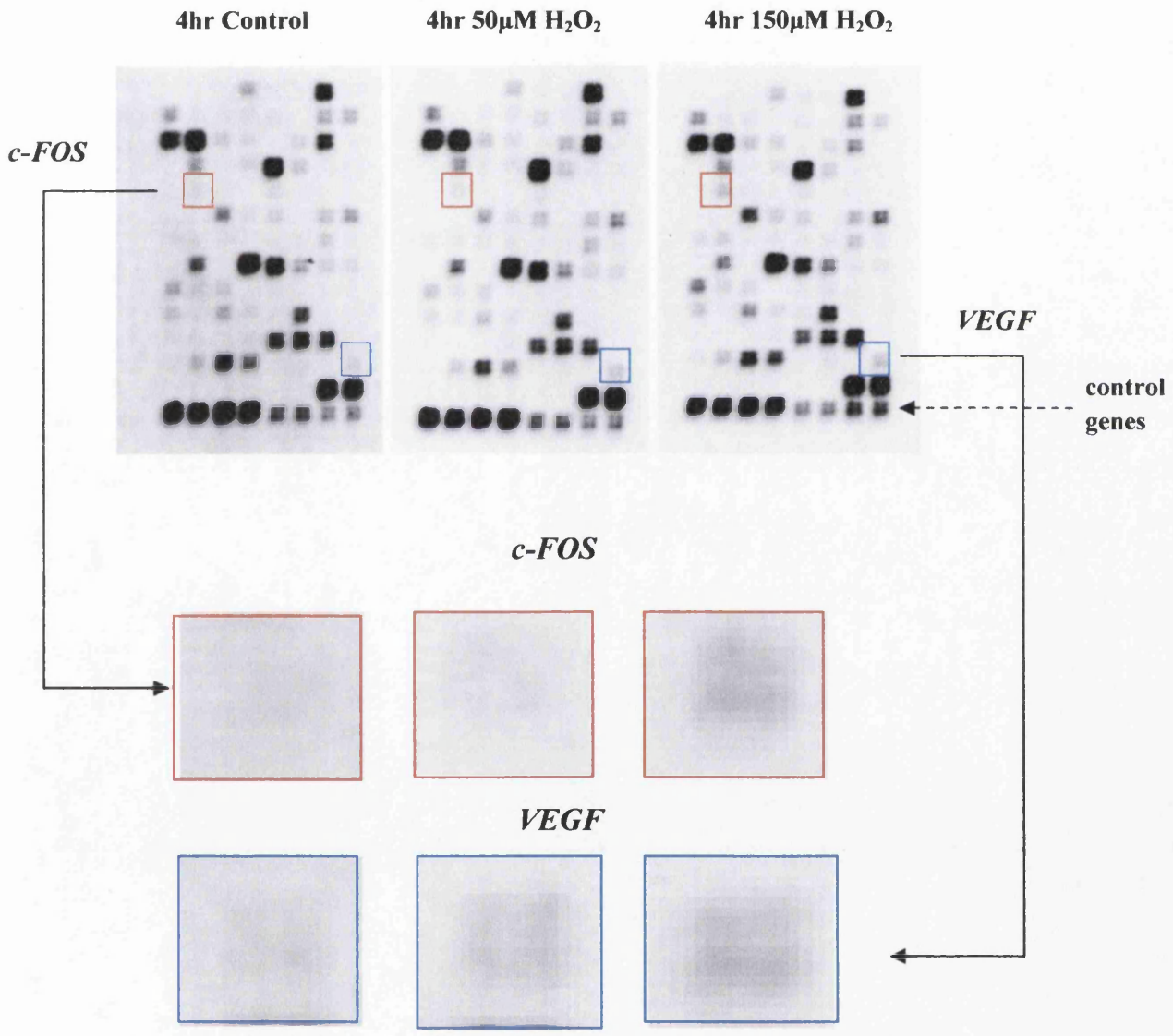
the A-490, and hence cell number. This indicates that increasing concentrations of H_2O_2 lead to cellular toxicity. At the 4 and 8hr time points the cells appear to halve in number (reaching the 50% lethal dose (LD_{50})) at around 150 – 250μM, whereas this appears to be more marked at the 24hr time point, where it occurs at 50μM. From this preliminary study (and similar studies previously carried out in the laboratory) it was decided that concentrations used in the dosing experiments linking H_2O_2 to signalling and gene expression changes would be around the LD_{50}, ranging from 50 – 500μM.

3.3.2 Microarrays

Several microarray experiments were performed on the HGC-27 cell line as a model using a variety of doses of H_2O_2 and time points, in order to study the effect of dose and exposure duration on cellular signal transduction and gene expression dynamics. All experiments were performed in duplicate and excellent reproducibility was observed. The purpose of the microarray experiments was to identify any major signalling changes potentially induced by the model ROS, with a particular emphasis on MAPK and NFκB pathways.

Figure 3.7 shows an example of the results obtained. The control genes (consisting of four human housekeeping genes including β-actin (*ACTB*) and glyceraldehyde-3 phosphate dehydrogenase (*GAPD*), and bacterial plasmid DNA (*PUC18*); refer to appendix AI.1 for details) on all arrays showed excellent hybridisation signals, hence validating RNA quality. At the 4hr time point, the most marked gene expression changes; clearly seen by visual observation of the arrays (highlighted in figure 3.7); were increases in the levels of ***c-FOS*** and ***VEGF*** RNA. Levels of both of these genes can be seen to be low, if detectable at all, in the control cells on inspection of the arrays in figure 3.7. The intensity of the signals on the arrays corresponding to these genes becomes stronger with increasing H_2O_2 concentration. A very similar pattern of gene expression was also observed in cells treated for 8hr, however, the signals appeared to be even more intense

Figure 3.7 Example result of a microarray experiment. It is clear that certain genes are expressed at higher levels (seen as more intense signals on the arrays) in treated versus control cells. The two most obvious changes are highlighted here; being apparent increases in the level of ***c-FOS*** and ***VEGF*** genes. These genes appear to be expressed at very low levels in the control cells, the signal becoming more intense with increasing H_2O_2 concentration. See text for further details. N = 2.



in treated versus control cells in this case. At the 24hr time point,these previously clearly detectable changes in signal intensity (and hence corresponding gene levels) had diminished, and instead what was seen was high levels of expression of both of these genes in control as well as treated cells. It is possible that this is due to the sheer stress of being cultured in the presence of H_2O_2 for this lengthier time point. Another possible explanation for this observation is that over the 24hr duration, the cells are going through a cell cycle, and it is likely that a mitogenic response is taking place (regardless of the presence/ absence of H_2O_2) leading to the observed gene expression profile.

Arrays carried out using RNA from cells treated in acidic (pH5) medium showed the same patterns of gene expression alterations as those observed at neutral pH, and so all subsequent dosing experiments were carried out at neutral pH (for practical ease).

Several other genes present on the arrays appeared to be up- or down-regulated following H_2O_2 treatment, and these gene expression alterations are listed in Table 3.3. Some very interesting gene expression changes as a result of H_2O_2 treatments were observed in the arrays (both Cancer Pathway finder and Nitric Oxide arrays) including genes that impact oxidative stress responses, inflammatory signalling, signal transduction, cell cycle control, DNA damage response, apoptosis and cellular senescence, cellular adhesion, angiogenesis, and invasion and metastasis, all of which are impacted during cancer development. Interestingly, gene expression changes appeared to come about in 'waves' based on timing, the first wave (predominantly 4hr post exposure) brought about up-regulation of DNA damage response genes and cell cycle control genes (e.g. *GADD45A* and *B*, *CDKN1A* (p21)), oxidative stress response genes (e.g. superoxide dismutase (*SOD1*), thioredoxin reductase (*TR1*)), some signal transduction and transcription factor genes (e.g. *MAPK14* (p38), *c-FOS*), and some changes in genes involved in adhesion, angiogenesis (*VEGF*), and invasion and metastasis. Later changes, or a second wave of gene expression alterations (perhaps as a consequence of the signal transduction and transcription factor changes), predominantly included genes involved in apoptosis and inflammation, as well as further genes involved in angiogenesis and invasion and metastasis. This observation is very interesting, and ties in with cellular

Table 3.3 Other H_2O_2 induced gene expression alterations. Genes listed are those whose expression was seen to be consistently altered in duplicate array experiments, and only genes that showed a very clear change in signal intensity compared to controls were considered as expression changes. The genes are categorised according to function. The centre column reflects whether an up- or down-regulation was observed, and the right hand column indicates the time point(s) at which the changes were observed. See text for further details. N = 2.

Gene	↑↓	Time point	Dose(s)
Cell cycle, DNA damage and repair genes			
- *CDKN1A (p21^{Waf1} /p21cip1)*	↑	4hr	250μM
- *GADD45A* and *B*	↑	4, 8, 24hr	250μM
- *OGG1 (8-oxoguanine DNA glycosylase)*	↑	24hr	250μM
- *MDM2*	↓	30min	150μM,
		4hr	150μM
Apoptosis and cell senescence genes			
- *BAD (BCL-2-antagonist of cell death)*	↑	4hr	50, 150μM
- *BCL-2*	↑	24hr	250, 500μM
Signal transduction molecules and transcription factors			
- *C-FOS*	↑↓	↑ 4hr	50, 150,
			250μM
		↑ 8hr	250, 500μM
		↓ 24hr	500μM
- *JUN*	↑	24hr	250μM
- *RAF-1*	↑	4hr	250μM
- *MAPK14 (p38 mitogen activated protein kinase)*	↑	4hr	250μM
- *TNFRSF1A*	↓	24hr	250μM
- *FGF2*	↑	30min	150μM

Table 3.3 continued.

Gene	↑↓	Time point	Dose(s)
Oxidative Stress Response			
- *GCLC* (*Glutamate cysteine ligase*)	↑	4hr	250μM
- *TR1* (*Thioredoxin reductase*)	↑	4hr	150μM
- *SOD1* (*Superoxide dismutase 1 (soluble)*)	↑	8hr	250, 500μM
Inflammation			
- *RANTES*	↑	4hr	150, 250μM
- *IL-6*	↑	24hr	250μM
- *TNF (TNFA)*	↑	4hr	250μM,
		24hr	500μM
Adhesion			
- *ITGAV* (*Integrin aV*)	↑	30min	150μM,
		4hr	250μM,
		24hr	500μM
- *VCAM1*	↑	4, 8hr	250μM
Angiogenesis			
- *VEGF* (Vascular Endothelial Growth Factor)	↑	4hr	150μM,
		8hr	150, 250μM
- *EGFR* (Epidermal Growth Factor Receptor)	↑	24hr	500μM
Invasion and metastasis			
- *MMP-2* (*Gelatinase A*)	↑	4hr	250μM,
		8hr	250, 500μM
- *PAI-1* (*Plasminogen Activator Inhibitor type 1*)	↑	4hr	250μM

responses to oxidative stress, and also relates with several aspects of inflammation and carcinogenesis.

The array images obtained using the Chemi Doc, as detailed in section 3.2.2.4g, were assessed visually, as well as using the array analysis software downloaded from the SuperArray website. From the extensive data analysis carried out it became apparent that often, the changes clearly seen by eye did not match up to the data output from the software. It is plausible that this discrepancy be attributable to varying levels of background signal on the arrays, leading to normalisation functions by the software that may adversely affect the results. Based on this it was decided that the changes detected by visual observation (using the criteria that the gene should be barely detectable, if detectable at all, in the control experiments when looking at up-regulated genes in treated samples, and showing very intense signals in the control samples, which then become barely detectable in the treated samples, when looking at down-regulation), were sufficient on the grounds of these experiments since the gene expression changes noted were to be quantified further using quantitative real-time PCR analysis.

3.3.3 Real-Time PCR – MAPK related Gene Expression

3.3.3.1 *c-FOS* Expression

Real-Time PCR was performed on HGC-27 RNA samples that had been used in the microarray analysis, in order to both validate and quantify the results. Unfortunately due to the toxicity of higher dose treatments at the 24hr time point, some RNA samples were of poor quality and had to be omitted from the final analyses. In addition, RNA extracted from AGS and WILL1 cell lines that had been treated with H_2O_2 as described in section 3.2.2.1, table 3.1, was also analysed by Real-Time PCR for *c-FOS* expression. The doses that AGS and WILL1 were exposed to were based upon the doses that had been seen to cause the most obvious changes in HGC-27. For all three cell lines, the same time points were utilised, and a dose range experiment of H_2O_2 treatments performed at

each time point. Unfortunately, in the final real-time PCR analyses, some doses had to be omitted due to RNA quality being poor, resulting in a lack of reproducibility in the data. The results of the experiments are represented graphically in figure 3.8.

One-way ANOVA (with subsequent Tukey and Duncan post hoc tests) carried out on each time point revealed statistically significant differences in *c-FOS* gene expression levels between control and treated cells for all three cell lines. Significant data (at the 95% confidence interval and 0.05 significance level) are highlighted graphically in figure 3.8.

3.3.3.1a *c-FOS* Expression in AGS

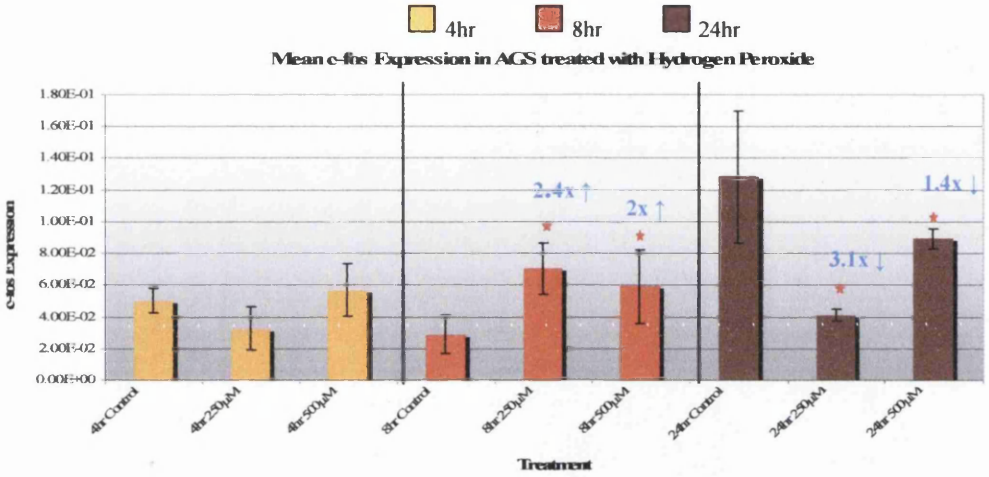
From figure 3.8(a) it can be seen that for AGS, at the 4hr treatment time, there is neither a noticeable trend in *c-FOS* levels, nor any statistically significant difference between control and treated cells.

At 8hr, both H_2O_2 treatments are significantly different to the control, *c-FOS* levels being approximately 2.4-fold, and 2-fold greater than the control level at the 250μM and 500μM H_2O_2 doses respectively.

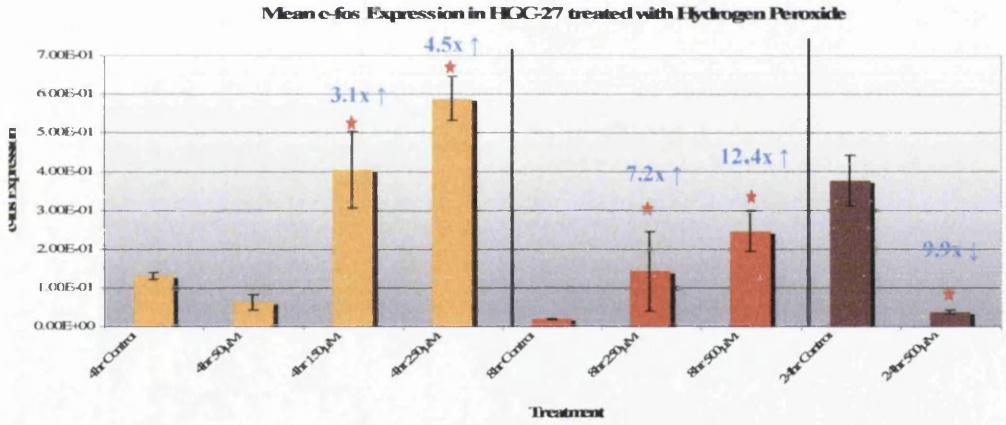
Interestingly, *c-FOS* expression levels show statistically significant decreases in AGS cells 24hr post treatment, the 250μM H_2O_2 treatment resulting in an approximately 3.1-fold decrease, and the 500μM dose leading to an approximately 1.4-fold decrease in *c-FOS* levels.

When contrasting the control levels of *c-FOS* expression at the three different time points in AGS, some clear differences come to light. At 4hr, control AGS cells have a relative *c-FOS* expression level of 5.03E-02, which decreases to 2.89E-02 in the 8hr control (1.7-fold decrease), then dramatically increases to 1.28E-01 in the 24hr control (2.5-fold and 4.4-fold increases compared to 4hr and 8hr controls respectively).

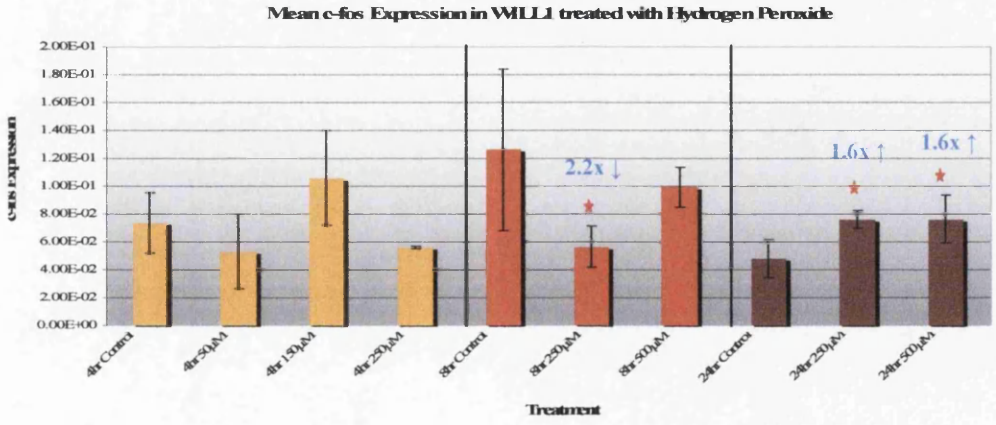
Figure 3.8 Graphs illustrating *c-FOS* gene expression (analysed by real-time PCR relative to β-actin (*ACTB*)) in control and H_2O_2 treated cells over different time points in (a) AGS, (b) HGC-27, and (c) WILL1. Standard error bars shown Statistically significant differences ($P < 0.05$) are highlighted with red stars. See text for further details (based on one way ANOVA). N = 2.



(a)



(b)



(c)

This interesting finding may reflect tissue culture conditions. For example, at 24hr, cells may be stressed due to a longer duration of serum starvation (all treatments are carried out in serum-free culture medium, section 2.2), dosing with H_2O_2 would then add further stress leading to decreases in gene expression and cytotoxicity, the two being intimately linked. At the 4hr and 8hr time points, low levels of *c-FOS* expression in controls may reflect an unstressed cellular environment, dosing then either leads to no significantly detectable changes in gene expression (as at 4hr) or significant increases in *c-FOS* levels (as at 8hr). The cellular stress that may be a factor in the time point differences may be related to the fact that cell culture conditions can impose a state of oxidative stress on the cells (Halliwell, 2003), and that as more time elapses, the oxidative stress becomes more intense, leading to enhanced redox sensitive changes in gene expression, and potentially accounting for the high level of *c-FOS* mRNA in 24hr control cells compared to their 4hr and 8hr counterparts.

A second possible explanation is that cancer cells themselves produce ROS, a phenomenon which has been well documented (Schumacker, 2006; Waris and Ahsan, 2006; Halliwell *et al.*, 1992; Szatrowski and Nathan, 1991), and as such, the ROS levels build up over time leading to the observed time dependent gene expression changes. At 24hr control cells may be saturated by oxidative stress to a point that addition of H_2O_2 leads to cytotoxicity, and so an overall decrease in gene expression.

3.3.3.1b *c-FOS* Expression in HGC-27

Initial review of figure 3.8(b) leads to the observation that there appear to be both dose -, and time - dependent components in the H_2O_2 – induced changes in *c-FOS* levels in the HGC-27 cell line. Dose dependent increases in *c-FOS* levels are apparent at both the 4hr and 8hr exposure times. For example, at the 4hr 150μM and 250μM H_2O_2 doses, *c-FOS* levels increase by approximately 3.1- and 4.5-fold respectively compared to the control, with both increases being statistically significant ($P < 0.05$).

At the 8hr time point, a clear dose dependent response is manifest, *c-FOS* levels increasing by a significant 7.2-fold increase compared to the control at the 250μM treatment and by a significant 12.4-fold increase at the 500μM treatment. Both HGC-27 and AGS display this clear dose dependent response in *c-FOS* expression at the 8hr exposure time point. Perhaps 8hr into culture the cells are accustomed to the cell culture conditions enough that the control cells' expression levels represent that in the unstressed state, exposure to H_2O_2 then elicits a true redox sensitive response in gene expression, reflected in the increased *c-FOS* levels, since *c-FOS* expression can be induced via redox sensitive MAPK signal transduction pathways (Marais *et al.*, 1993; Gille *et al.*, 1992).

Interestingly, HGC-27 cells display the same trend with respect to decreasing *c-FOS* levels at the 24hr time point as AGS. Relative *c-FOS* levels decrease approximately 9.9-fold at the 500μM treatment dose compared to the control in a statistically significant manner. This may again be due to the time enhanced onset/ effect of oxidative stress in the cell culture microenvironment, or due to enhanced generation of ROS from the cancer cells themselves, as discussed in section 3.3.3.1a. The decline in *c-FOS* expression following exposure to H_2O_2 was most probably accountable to cytotoxicity. Indeed, microscopic observation of dosed HGC-27 24hr post exposure revealed an unhealthy cell population in which the integrity of the monolayer was compromised, dominated by rounded floating cells (dead cells), and a lower cell count than the initiated population (may be attributable to necrosis and apoptosis). Several of the treated cell populations yielded very low levels of poor quality RNA, which were hence excluded from the analysis. HGC-27 also displays the same trend in *c-FOS* levels across the 4hr, 8hr, and 24hr control cells as discussed for AGS (low in 4hr control, lower still at 8hr, dramatically increasing at 24hr) in section 3.3.3.1a.

It is worthy of noting that whilst the AGS and HGC-27 cancer cell lines display some marked similarities in terms of their *c-FOS* gene expression dynamics in response to H_2O_2 exposure, there are also some apparent differences. For example, whilst HGC-27 showed clear dose dependent changes in *c-FOS* levels at all three time points, AGS only showed a very clear dose dependent response at the 8hr time point (increase). Based on the data, and the knowledge of the differences in the nature of the cell lines (detailed in

section 3.2.1.1 and 3.2.1.2), one can speculate that due to the more 'primary' characteristics of the AGS cell line, being initiated from a primary gastric tumour that displayed no metastasis (Barranco, 1983), the cells may be more robust to oxidative stress, displaying an adaptive response at the earlier time point, only succumbing to oxidative stress as time progresses. The more advanced HGC-27 cell line derived from a lymph node metastasis of a late gastric tumour, may have enhanced sensitivity to oxidative stress, hence showing more clear cut redox sensitive gene expression responses, even at the 4hr exposure time.

A striking observation comes to light upon inspection of figure 3.8(c) which reveals that *c-FOS* expression dynamics in the primary WILL1 cell line, in response to H_2O_2 treatment, differ completely from those seen in the two cancer cell lines.

3.3.3.1c *c-FOS* Expression in WILL1

WILL1 cells do not show any clear dose dependent expression of *c-FOS* at the 4 and 8hr time points (figure 3.8(c)). The only dose dependency that can be observed is the statistically significant ($P < 0.05$) dose dependent increase in *c-FOS* levels at the 24hr time point. At this time point, *c-FOS* levels increase by approximately 1.6-fold compared to the control at both the 250 and 500μM doses. This pattern of *c-FOS* gene expression in WILL1 differs dramatically from that observed in the two cancer cell lines, which showed some dose dependent increases in *c-FOS* levels at the earlier time points, followed by decreases at the 24hr time point. This is very interesting, since it tends toward the hypothesis that the cancer cells may inherently be more redox sensitive in their cellular responses when compared to a normal cell line. Another observation that supports this theory is that *c-FOS* levels appear to be fairly similar across the controls of the three time points in WILL1 (showing only a slight increase in the 8hr control), suggesting that the normal cell line may either not generate ROS over the course of being in cell culture, or may have better antioxidant systems for returning cells to a state of redox balance.

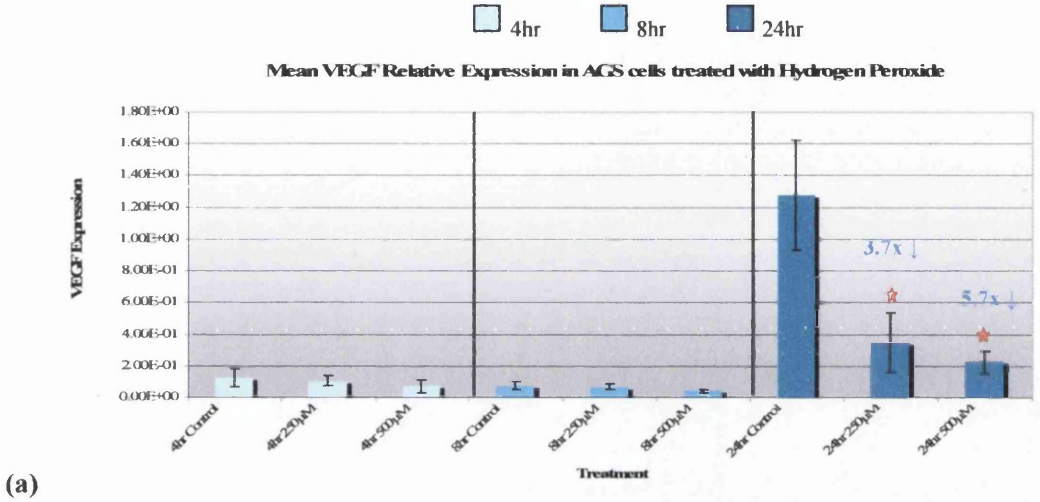
3.3.3.2 *VEGF* Expression

The second predominant gene expression alteration, along with *c-FOS*, that was seen across the array experiments was either up- or down-regulation of the *VEGF* gene encoding vascular endothelial growth factor (*VEGF*). Thus, in a manner similar to that described for *c-FOS* analysis in section 3.3.3.1, samples from HGC-27 (those used in the array experiments), AGS, and WILL1 were analysed for relative *VEGF* expression levels by quantitative real-time PCR analysis. The results are summarised graphically in figure 3.9. Again, some RNA samples were of poor quality and had to be omitted from the final analyses. One-way ANOVA revealed some statistically significant changes in *VEGF* levels ($P < 0.05$), highlighted in figure 3.9.

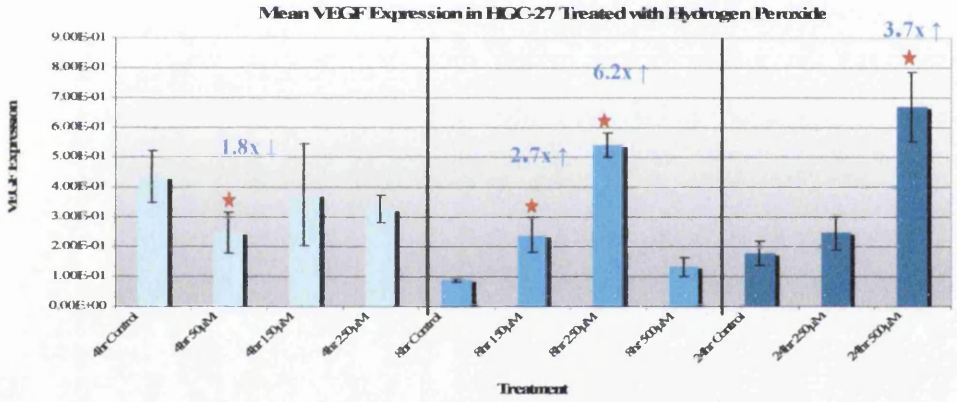
3.3.3.2a *VEGF* Expression in AGS

No noticeable dose dependent responses in terms of *VEGF* gene expression were evident at the 4hr and 8hr time points in figure 3.9(a). Twenty-four hour post exposure, a dose dependent decrease in *VEGF* levels was obvious. Treatment with H_2O_2 doses of 250 and 500μM resulted in statistically significant reduction ($P < 0.05$) in *VEGF* levels, by approximately 3.7-fold and 5.7-fold respectively. A noteworthy observation was that the pattern of gene expression levels in the control cells reflects that seen for *c-FOS* levels in AGS – very low levels at 4hr (1.23E-01), lower still at 8hr (7.57E-02), and then dramatically increasing at 24hr (1.28E+00). Again, this may be a cell culture phenomenon, whereby more time in cell culture leads to increased oxidative stress and hence increased redox sensitive gene expression, the impact of oxidative stress on *VEGF* expression being well documented (Shäfer *et al.*, 2002). The addition of H_2O_2 may then contribute to enhanced oxidative stress which becomes manifest as cytotoxicity and

Figure 3.9 Graphs illustrating *VEGF* gene expression (analysed by real-time PCR relative to *ACTB*) in control and H_2O_2 treated cells over different doses and time points in (a) AGS, (b) HGC-27, and (c) WILL1 cell lines. Standard error bars shown. Statistically significant differences ($P < 0.05$) are highlighted by red stars (based on one way ANOVA). See text for further details. N = 2.



(a)



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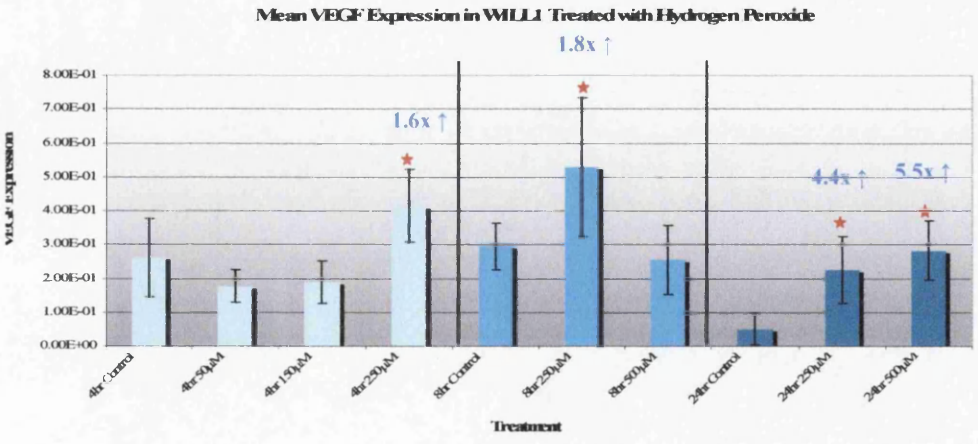


(c)

hence significantly lower *VEGF* levels.

3.3.3.2b *VEGF* Expression in HGC-27

The overall picture of *VEGF* expression levels in HGC-27 (figure 3.9(b)) appears to be dramatically different to that in AGS. At 4hr, exposure to various doses of H_2O_2 appears to have no major effect on *VEGF* levels, other than a slight, yet significant approximately 1.8-fold decrease over the control at the 50μM dose. In contrast, dose dependent increases in *VEGF* levels are evident across the dose range at both the 8hr and the 24hr time point.

Relating the *VEGF* expression pattern with that of *c-FOS* in HGC-27, some differences come to light. While *c-FOS* expression displays very clear and significant dose dependent increases at the 4 and 8hr time points, followed by a dose dependent decrease at 24hr, *VEGF* levels show no trend at 4hr, and then significant dose dependent increases 8 and 24hr exposure times. A possible explanation for this is that *c-FOS* may represent a first wave of redox sensitive gene expression, whilst *VEGF* is representative of a delayed second wave of gene expression response to the oxidative stress signal, and it is quite plausible that the two are linked, since *VEGF* is a target of *c-FOS* at the level of transcription via AP-1 (Fujioka *et al.*, 2004; Ryuto et al, 1996). Indeed, *VEGF* expression is known to be regulated to an extent by upstream redox sensitive MAPK pathways (ERK in particular) (Schäfer *et al.*, 2003; Milanini *et al.*, 1998), as is *c-FOS* further strengthening the link. Interestingly, when comparing gene expression levels in the HGC-27 control cells at the three time points, it appears that there is an opposite trend to that seen in the AGS cell line. Whilst there was a trend toward increasing *VEGF* levels as time elapsed in AGS, the reverse is true for HGC-27, *VEGF* levels being highest in the 4hr control (4.34E-01), decreasing at 8hr (8.78E-02), and 24hr (1.74E-01). It has been reported that in some cell lines, high proliferative rate, as well as serum starvation are associated with low *VEGF* levels (Milanini *et al.*, 1998). It is possible in this instance that both of these factors may explain the decreasing levels of *VEGF* expression in the control

cells over time, since with time, cell division rate is likely to increase (HGC-27 having a greater rate of proliferation compared to AGS), as is the effect of serum starvation.

It appears that in HGC-27, as for *c-FOS*, *VEGF* gene expression is more sensitive/ responsive to H_2O_2 – induced oxidative stress in this experimental system compared to AGS cells. Based on the data available, one can only speculate that these differences may be due to differences in the cell line characteristics previously discussed (section 3.3.3.1b). Perhaps there are biochemical differences in the cell lines that make them either more/ less susceptible to redox regulated gene expression by common pathways, or it is possible that completely different pathways operate in the different cell lines, thereby accounting for the differences in gene expression alterations. Differences in rate of proliferation may also be important, with HGC-27 appearing to have a higher proliferative rate compared to AGS (by observation). Here, the two cancer cell lines show interestingly different responses, and what is even more interesting is that in this case, HGC-27 shows a more similar response to the normal WILL1 cell line.

3.3.3.2c *VEGF* Expression in WILL1

WILL1 (figure 3.9(c)) shows a similar *VEGF* expression pattern to that observed in HGC-27, with no clear trend at the 4hr time point (significant ~ 1.6-fold increase at 250μM dose only), and in this case not such a clear trend 8hr post exposure (significant ~ 1.8-fold increase at 250μM dose only), a clear dose dependent response only being seen at the 24hr time point where *VEGF* levels increase significantly by approximately 4.4-fold at 250μM, and 5.5-fold at 500μM.

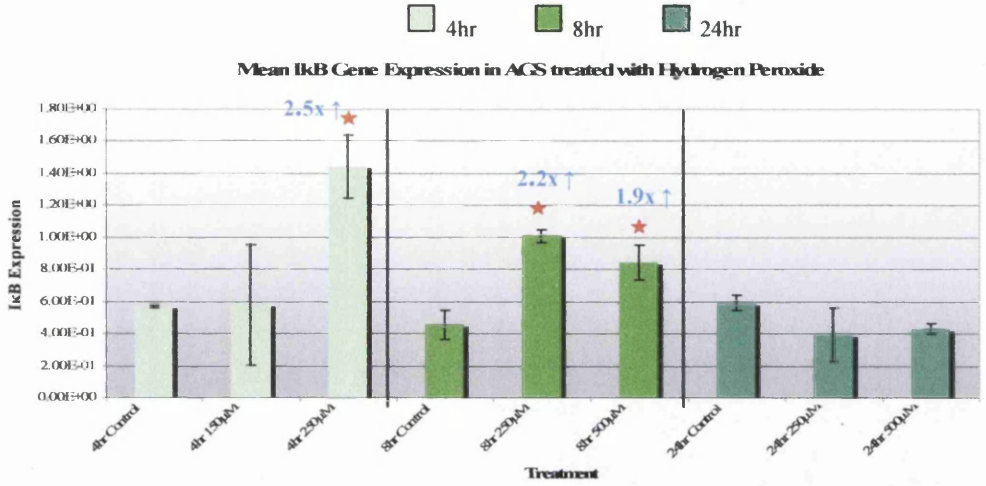
3.3.4 Real-Time PCR – NFκB Related Gene Expression.

As explained in section 3.1.5 *IκB* and *IL-8* expression were used as surrogate markers for NFκB activation, since both have been reported to be transcriptional targets of the transcription factor and have been used in this manner in previously published studies (Jenkins *et al.,* 2004). Interestingly, these genes were not seen to be up-regulated in the microarray experiments, although this cannot be ruled out as their levels may not have been high enough to be picked up by eye (since the arrays require fairly high levels of RNA for detection). Other NFκB regulated genes, e.g. (*VCAM, RANTES, TNFA*) were seen to be up-regulated, and so these two findings, taken together, warranted the study of *IκB* and *IL-8* gene expression by real-time PCR. The results are presented graphically in figures 3.10 and 3.11 and show that *IκB* and *IL-8* follow correlating expression patterns in all three cell lines, and so the trends for the two genes will be described together.

3.3.4a *IκB* and *IL-8* Expression in AGS

Inspection of figure 3.10(a) and figure 3.11(a) reveals that at both the 4 and 8hr time points *IκB* and *IL-8* gene expression show some up-regulation. At 4hr post exposure, both genes are significantly up-regulated at the 250μM H_2O_2 dose, for *IκB,* a fold increase of approximately 2.5 times was evident. For *IL-8,* a significant approximately 2.8-fold increase in gene expression was apparent. Despite these significant increases in gene expression, there is no clear dose dependent increase, since only the highest dose brought about an increase at this time point. Eight hour post exposure, there appears to be more tendency toward a positive dose dependency, being significant for *IκB,* and not significant (and to a somewhat lesser extent) for *IL-8.*

Figure 3.10 Graphical representations of *IκB* gene expression (analysed by real-time PCR relative to *ACTB*) in cells treated with a dose range of H_2O_2 over a range of time points in (a) AGS, (b) HGC-27, and (c) WILL1 cell lines. Standard error bars shown. Statistically significant differences ($P < 0.05$) are highlighted by red stars (based on one way ANOVA). See text for further details. N = 2.



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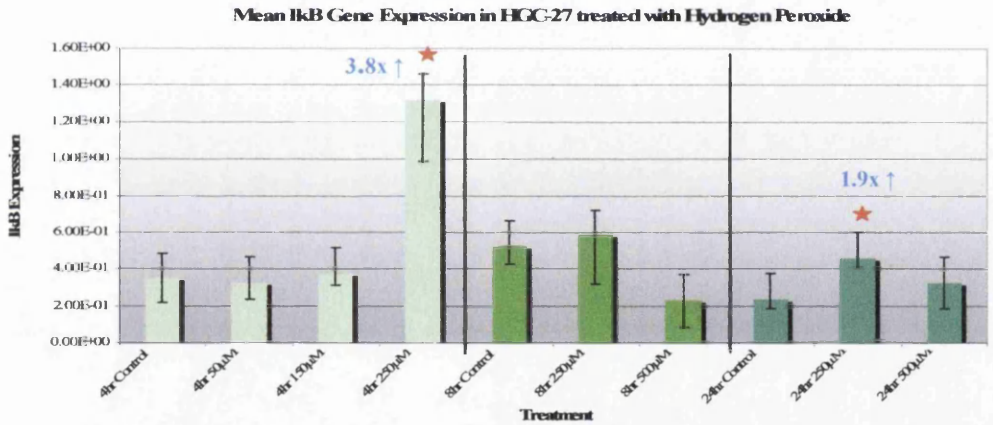

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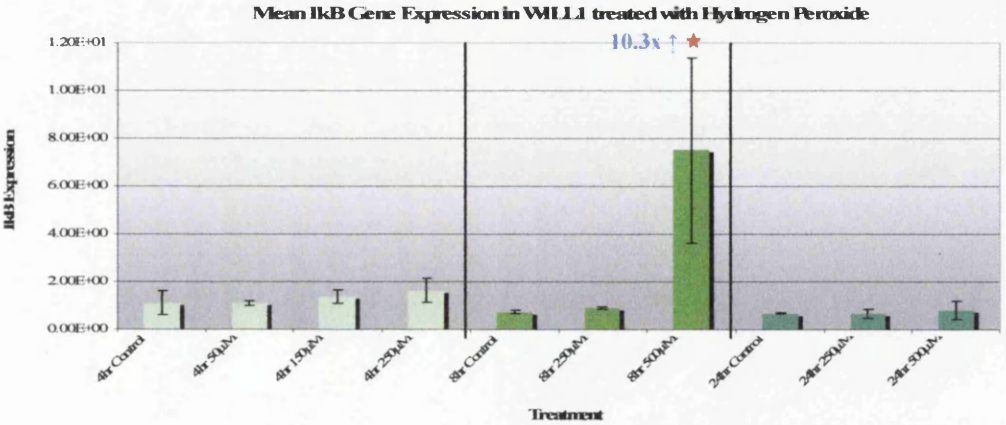
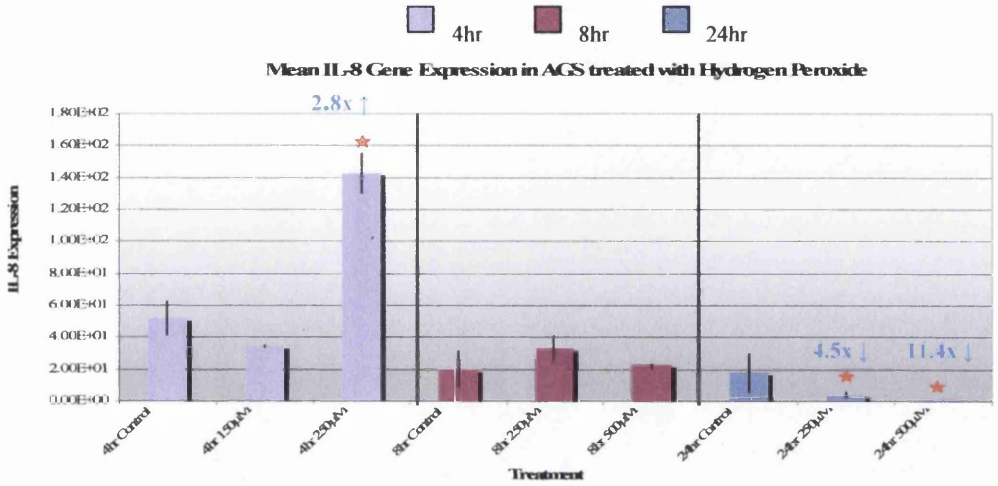
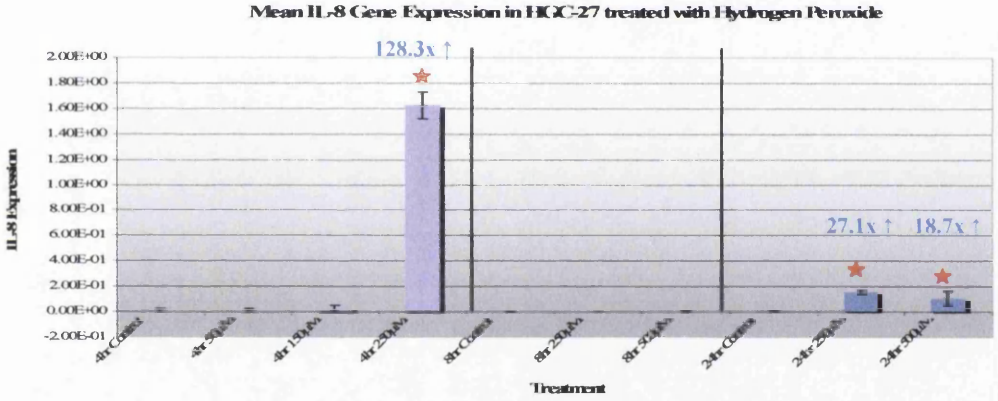


(c)

Figure 3.11 Graphical representations of *IL-8* gene expression (analysed by real-time PCR relative to *ACTB*) in cells treated with a dose range of H_2O_2 over a range of time points in (a) AGS, (b) HGC-27, and (c) WILL1 cell lines. Standard error bars shown, Statistically significant differences ($P < 0.05$) are highlighted by red stars (based on one way ANOVA). See text for further details. N = 2.



(a)



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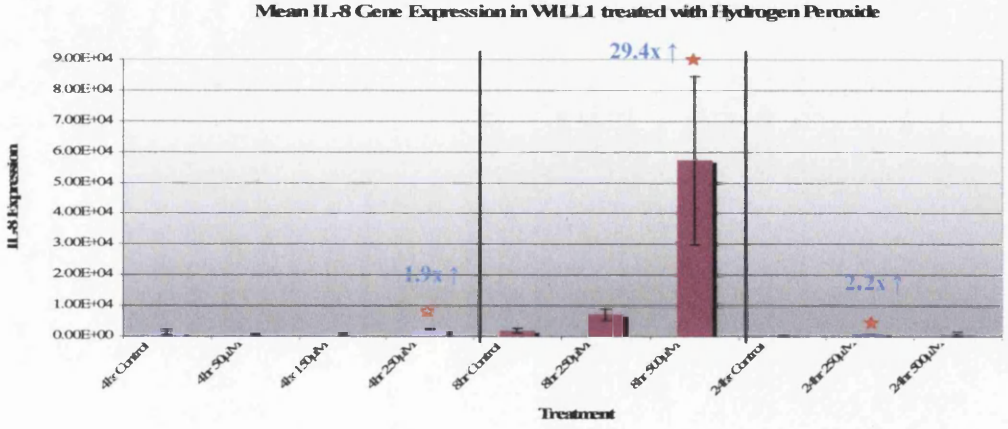


(c)

Twenty-four hour post exposure there is a shift towards a negative dose dependent response, *IκB* expression levels decreasing (albeit not significantly) from control to treated cells. *IL-8* expression shows the same trend, but the decreases are statistically significant, decreasing by approximately 4.5-fold and 11.4-fold in 250μM and 500μM treated cells respectively.

These observations appear to be fairly consistent with findings from other studies which suggest that NFκB – induced *IκB* and *IL-8* expression usually occurs 4 – 8hr post-treatment with activating stimuli (Jenkins *et al.,* 2004). The decreasing expression levels at the 24hr time point are likely due to cytotoxicity as discussed for *c-FOS* and *VEGF.* A point worth noting is that at all time points, control levels of *IκB* and *IL-8* are fairly low and consistent, in contrast to the situation for *c-FOS* and *VEGF,* and that this is the case in all three cell lines examined.

3.3.4b *IκB* and *IL-8* Expression in HGC-27

It is evident from figure 3.10(b) and 3.11(b) that there are no clear-cut trends in *IκB* and *IL-8* gene expression at any of the three time points in HGC-27. At the 4hr time point, both genes show statistically significant increases in gene expression at the 250μM dose, *IκB* level increasing approximately 3.8-fold, and *IL-8* expression levels showing a remarkably high increase of approximately 128-fold compared to control cells, but despite these significant increases, no clear dose dependent increase is observed.

Again, at 8hr there is no clear trend in the results, *IκB* levels shows no significant changes at any of the treatment doses. *IL-8* expression 8hr post exposure also shows no trend, nor any significant results.

IκB expression levels show a very subtle dose dependent increase at the 24hr time point, with one significant approximately 1.9-fold increase in levels at the 250μM dose. There also appears to be a slight increase in levels at the 500μM dose, although this increase is not statistically significant. *IL-8* expression levels show a similar pattern 24hr

post exposure, this time both treatment doses inducing significant increases in *IL-8* RNA levels, with approximately 27.1- and 18.7-fold increases at 250μM and 500μM doses respectively.

Overall, it is difficult to establish a relationship between H_2O_2 exposure and the expression of the two NFκB – regulated genes in HGC-27 since at some doses and time points there are significant increases, and decreases in their RNA levels, with no clear dose dependent patterns.

3.3.4c *IκB* and *IL-8* Expression in WILL1

Again, in this cell line, as for HGC-27, there appears to be no clear trends in the gene expression data (fig. 3.10(c) and fig. 3.11(c)). For *IκB,* at 4hr, H_2O_2 appears to have no effect on expression levels at any treatment dose when compared to the control. *IL-8* expression is only impacted at the 250μM dose at 4hr, significantly increasing approximately 1.9-fold.

At 8hr, both *IκB* and *IL-8* show an initially subtle dose response, with RNA levels increasing slightly (and not significantly) at the 250μM dose, with a subsequent large and significant fold increase at the 500μM dose, increasing by approximately 10.3-fold for *IκB*, and 29.4-fold for *IL-8*.

At the 24hr time point, *IκB* levels do not appear to be affected by treatment with H_2O_2. For *IL-8,* again there is no clear trend in the gene expression alterations with dosing, apart from a significant 2.2-fold increase in gene expression levels at the 250μM dose.

Interestingly, unlike for *c-FOS* and *VEGF,* there doesn't appear to be any clear dose responses in *IκB* and *IL-8* gene expression and no interesting major differences between the cell lines, instead the two genes appear to be up-regulated at very specific doses and time points, and this differs between the three cell lines. Noteworthy

observations are that both AGS and HGC-27 show a maximal induction of both NFκB regulated genes at the 4hr 250μM treatment, and this may be in line with observations in other studies in which gene induction peaked at 4hr in response to other stimuli such as bile acids, which may in fact act through ROS-dependent mechanisms (Jenkins *et al.,* 2007). In WILL1, the maximal gene expression levels are seen following treatment with 500μM H_2O_2 for 8hr. It may be that this normal cell line has better antioxidant defences that curb excessive redox signalling until a certain threshold level is surpassed.

Overall, the most responsiveness (with respect to oxidative stress induced NFκB related gene expression) is seen in the AGS cell line, and this provides support to several published studies that link ROS exposure to the up-regulation of NFκB and subsequently *IL-8* in AGS cells (Seo *et al.,* 2002). In all three cell lines, control levels of both genes are low, and remain so over time, only increasing in response to certain dose treatments. This contrasts with *c-FOS* and *VEGF* control levels, which were in some instances high (section 3.3.3.2a, b, c). The clearest observation from this data is that the two cancer cell lines appear to be much more susceptible to oxidative stress induced *IκB* and *IL-8* gene expression compared to the normal WILL1 cell line, implicating a possible role for aberrant NFκB signalling in carcinogenesis.

3.3.5 MAPK Pathway Studies

Since the central aim of the work in this chapter was to study gene expression changes induced by the model ROS H_2O_2 and the upstream signal transduction pathways, elucidating links between the cellular signalling and the gene expression change endpoints required further experimentation in order to establish if such relationships exist. With respect to the MAPK pathway, this consisted of a MAPK inhibitor study, analysing the effects of MAPK signalling inhibition on a downstream target - *c-FOS*, by real-time PCR, and western blot analysis of active forms of MAPK proteins - phosphorylated ERK (pERK), and phosphorylated p38 (pp38), as well as western blot

analysis of c-FOS protein level to determine if expression changes seen at the RNA level translate to the protein level.

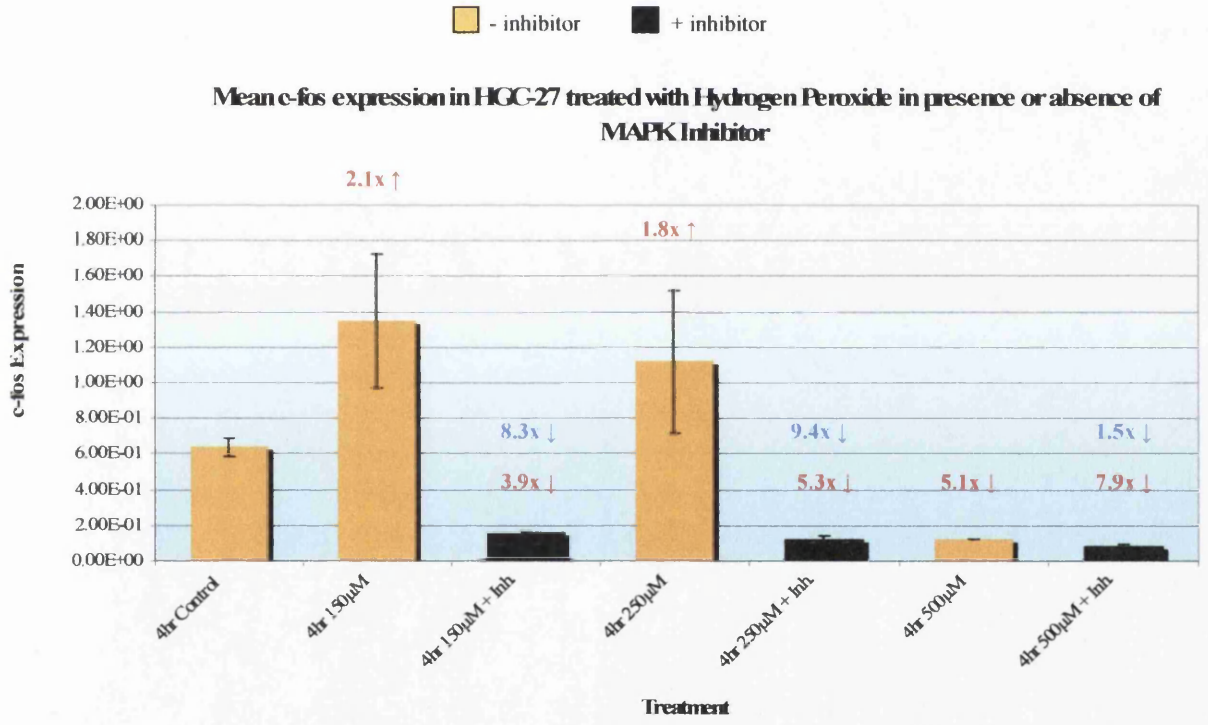
3.3.5.1 MAPK Inhibitor Study

In order to study the potential link between gene expression changes observed, and upstream oxidative stress – induced activation of MAPK pathways a preliminary experiment was conducted using the HGC-27 cell culture system as the model, in which cells were pre-treated with 10μM of the potent MEK/ ERK inhibitor U0126 as described in section 3.2.2.1, and the impact of ERK MAPK inhibition on downstream gene expression targets was assessed by quantitating any changes in *c-FOS* levels using real-time PCR analysis. Prior to experimentation the effectiveness of U0126 to inhibit the ERK MAPK pathway was confirmed by western blots for active pERK (carried out as described in section 3.2.3.1), which showed that pERK levels diminished in inhibitor treated cells compared to a positive control (results not shown). Unfortunately, due to problems with inhibitor induced cytotoxicity (since blocking MAPK pathways has detrimental impacts on cellular biochemistry), the data presented are preliminary, and would need to be repeated further in order to validate the findings and draw strong conclusions. The results are presented graphically in figure 3.12.

It is quite clear that pre-treatment with the MEK/ ERK inhibitor had a downstream effect on *c-FOS* expression levels, since cells treated with H_2O_2 without prior inhibitor treatment resulted in increases in *c-FOS* RNA levels compared to untreated controls, whilst levels in inhibitor treated cells dramatically decreased compared to untreated control cells and cells treated with the same H_2O_2 dose minus inhibitor. Treatment with 150μM H_2O_2 for 4hr resulted in an approximately 2.1-fold increase in gene expression compared with untreated control cells. Pre-treatment with U0126 followed by a 4hr treatment with 150μM H_2O_2 resulted in an approximately 8.3-fold decrease in *c-FOS* levels compared to the 4hr 150μM treated cells without inhibitor, and an approximately 3.9-fold decrease compared to control untreated cells. A similar

effect is seen at the 250μM dose, but such a clear effect is not seen at the 500μM dose possibly due to inhibitor and oxidative stress induced cytotoxicty at this high dose, as well as H_2O_2 induced cytotoxicity in the cells treated in the absence of inhibitor.

Figure 3.12 Graphical representation of average *c-FOS* RNA expression levels in HGC-27 cells treated over a dose range of H_2O_2 either in the absence of MEK/ ERK inhibitor U0126 (yellow bars) or after 30 min pre-treatment with 10μM of the inhibitor (black bars). Approximate fold-changes in gene expression are shown above the bars in the graph. Values in red are fold-changes compared to control values, and those shown in blue represent changes in dosed cells plus inhibitor compared to cells treated at the same dose minus inhibitor. Standard error bars shown. See text for further details. N =2.



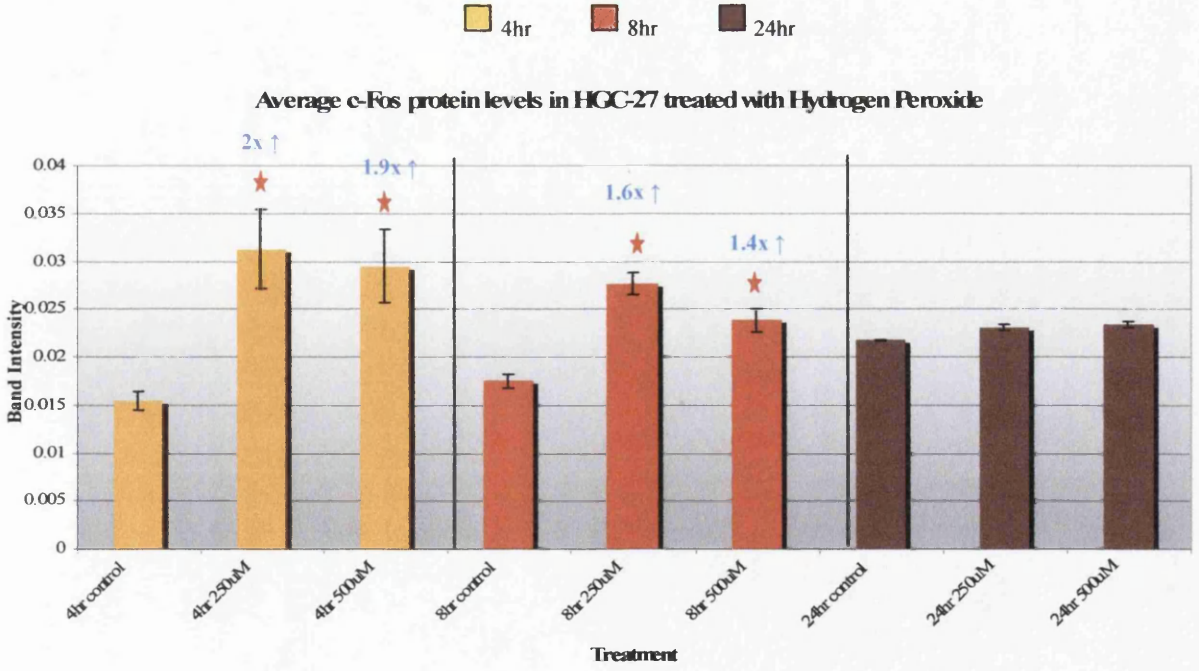
3.3.5.2 Western Blots

Western blots were carried out successfully on protein samples extracted from control and treated cells from AGS, HGC-27, and WILL1 using antibodies against pERK, p38, and fairly successfully using antibody against c-FOS.

3.3.5.2a c-FOS Western Blots

Western blot analysis of c-FOS protein levels in both AGS and WILL1 revealed no significant changes following 4hr, 8hr, and 24hr treatments with 250μM and 500μM H_2O_2 when compared with controls. HGC-27 however, seemed to show a dose dependent increase in c-FOS protein levels at the 4hr time point and to a somewhat lesser extent 8hr post treatment, clear in figure 3.13.

Figure 3.13 c-FOS protein levels in HGC-27 (relative to ACTB) following treatment with either 250 or 500μM H_2O_2 for 4, 8, and 24hr. Protein levels were analysed using western blots for c-FOS, followed by analysis of band intensities using the ImageJ software downloaded from the NIH website **http://rsb.info.nih.gov/ij/download.html**, and normalizing against β-actin protein band intensities (see section 2.7.3 for further details). Standard error bars shown. Fold-changes in treated cells over controls are annotated. Results were analysed using one-way ANOVA and statistically significant results ($P < 0.05$) are highlighted with red stars. N = 2.



Four hour treatment with 250μM H_2O_2 resulted in a 2-fold increase in c-FOS protein levels over control, whilst the 500μM dose induced an approximately 1.9-fold increase in levels. At the 8hr time point, the 250μM dose resulted in an approximately 1.6-fold increase in c-FOS level compared to the level in 8hr control cells, and an approximately 1.4-fold increase in protein levels was seen following treatment with 500μM H_2O_2. One-way ANOVA analysis confirmed that the increases in c-FOS protein levels at the 4 and 8hr 250 and 500μM doses were statistically significant at the 0.05 significance level ($P < 0.05$). No significant changes in protein levels were seen at the 24hr time point. These observations reflect the real-time PCR results for c-FOS RNA levels, and indicate that, at least in the HGC-27 cell line, the gene expression changes seen to be induced by H_2O_2 treatment occur at both the RNA and protein level.

3.3.5.2b pERK Western Blots – Short Exposure Study

For all three cell lines pERK (phospho – p42/p44) protein levels were analysed by western blots relative to total ERK (p42/p44) as described in sections 2.7.3 and 3.2.3.1. The treatments were broken down into two groups – a short exposure study, and a longer exposure study using the same time points as in the gene expression studies.

In the short exposure study, cells were treated with 150μM H_2O_2 (the lowest dose which was seen to cause significant changes in *c-FOS* RNA expression levels) for 5, 10, and 30 minutes. An untreated control and a positive control were included in the experiment. The positive control comprised cells treated with 100ng/ml EGF for 30min, a treatment well documented to cause ERK1/2 (p42/p44) activation (Gao *et al.*, 2005). Interestingly, both cancer cell lines showed very similar changes in phosphorylation levels of ERK following short H_2O_2 exposures. Examples of western blots obtained from the experiments are shown in figure 3.14, and the data presented graphically in figure 3.15. In HGC-27, pERK levels increase approximately 2-fold over the control level as quickly as 5min post treatment, to a similar intensity caused by EGF stimulation (~ 1.9-fold increase). pERK levels then fall back to basal levels by 30min post exposure (fig.

3.15(a)). One-way ANOVA analysis showed that the increase in pERK levels in 5min 150μM H_2O_2 treated and EGF treated cells was significantly different than the control level ($P < 0.05$).

A very similar result was seen in AGS cells, although ERK activation showed a slight delay compared to HGC-27, occurring 10min post treatment compared to 5min, and levels remaining high even at 30min (fig. 3.15(b)). In AGS, 10min post exposure to 150μM H_2O_2, a clear and significant 1.6-fold increase in ERK phosphorylation was seen over the untreated control comparable to the significant 1.7-fold increase induced by EGF in the positive control.

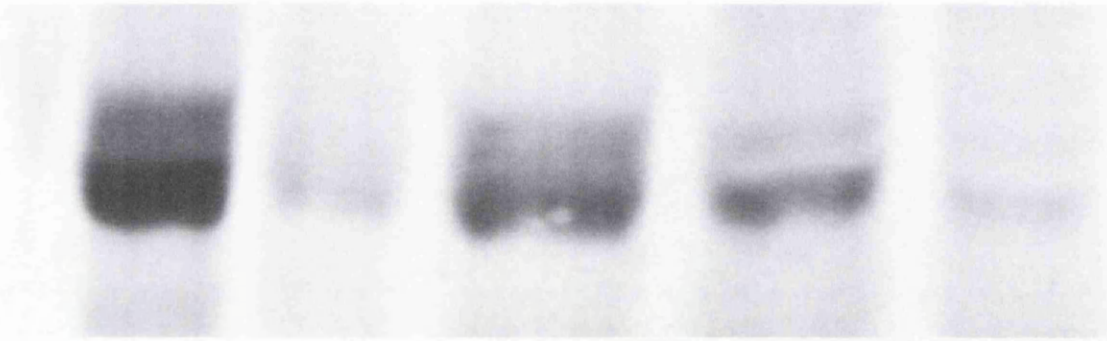
WILL1 showed similar changes in pERK levels to AGS, with a significant 1.6-fold increase in ERK phosphorylation induced 10min post treatment with 150μM H_2O_2 (fig. 3.15(c)).

It is quite clear then, from the results of this short exposure study, that H_2O_2 can induce a very rapid activation of the ERK MAPK pathway in all three cell lines, occurring the quickest in HGC-27 (5min post exposure), and slightly delayed in AGS and WILL1 (10min post exposure). Activation of ERK appears to be sustained longest in AGS, where the level of active pERK remains elevated above control levels 30min post exposure, whilst in HGC-27 and WILL1, levels return to basal control levels by 30min. This may provide an explanation for the difference in timing of gene expression changes between cell lines. For example, *c-FOS* expression is up-regulated 4hr post treatment in HGC-27, whereas up-regulation is not seen in AGS until 8hr post treatment, and it is possible that this may be related to the different dynamics of ERK activation.

In any case, it appears that early changes in ERK signalling induced by H_2O_2 are likely to set in motion the cellular signalling required to lead to the later gene expression changes observed (section 3.3.3).

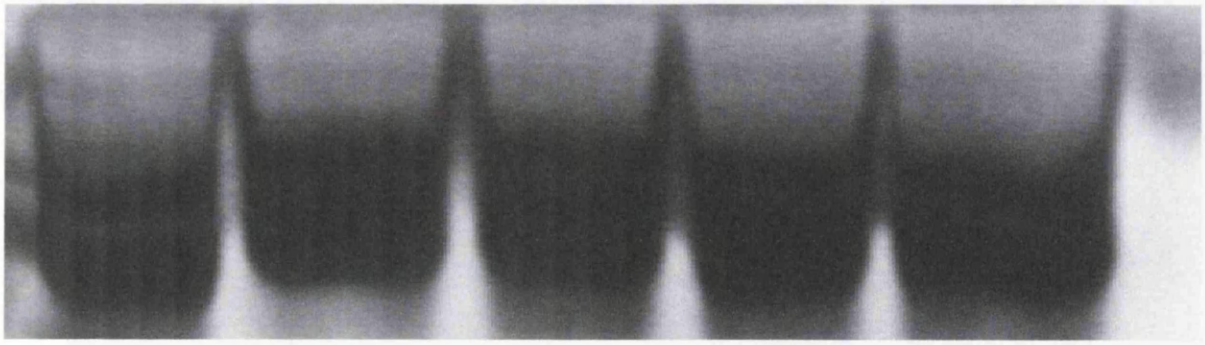
Figure 3.14 An example of a typical western blot result obtained. A blot for pERK (phospho - p42/p44) in HGC-27 cells following short exposures to 150μM H_2O_2, and a corresponding blot for total ERK (p42/p44) for the same samples, so ensuring any changes are true and not due to loading differences. Values shown in blue represent experimental duplicates of the pixel intensity of the bands following imageJ analysis. The values were used in subsequent analyses following normalisation of pERK levels against total ERK. N = 2.

EGF +ve control	-ve Control	5 minute	10 minute	30 minute
121.5	45.9	112.5	85.8	42.8
131.9	89.2	154.0	75.7	29.8



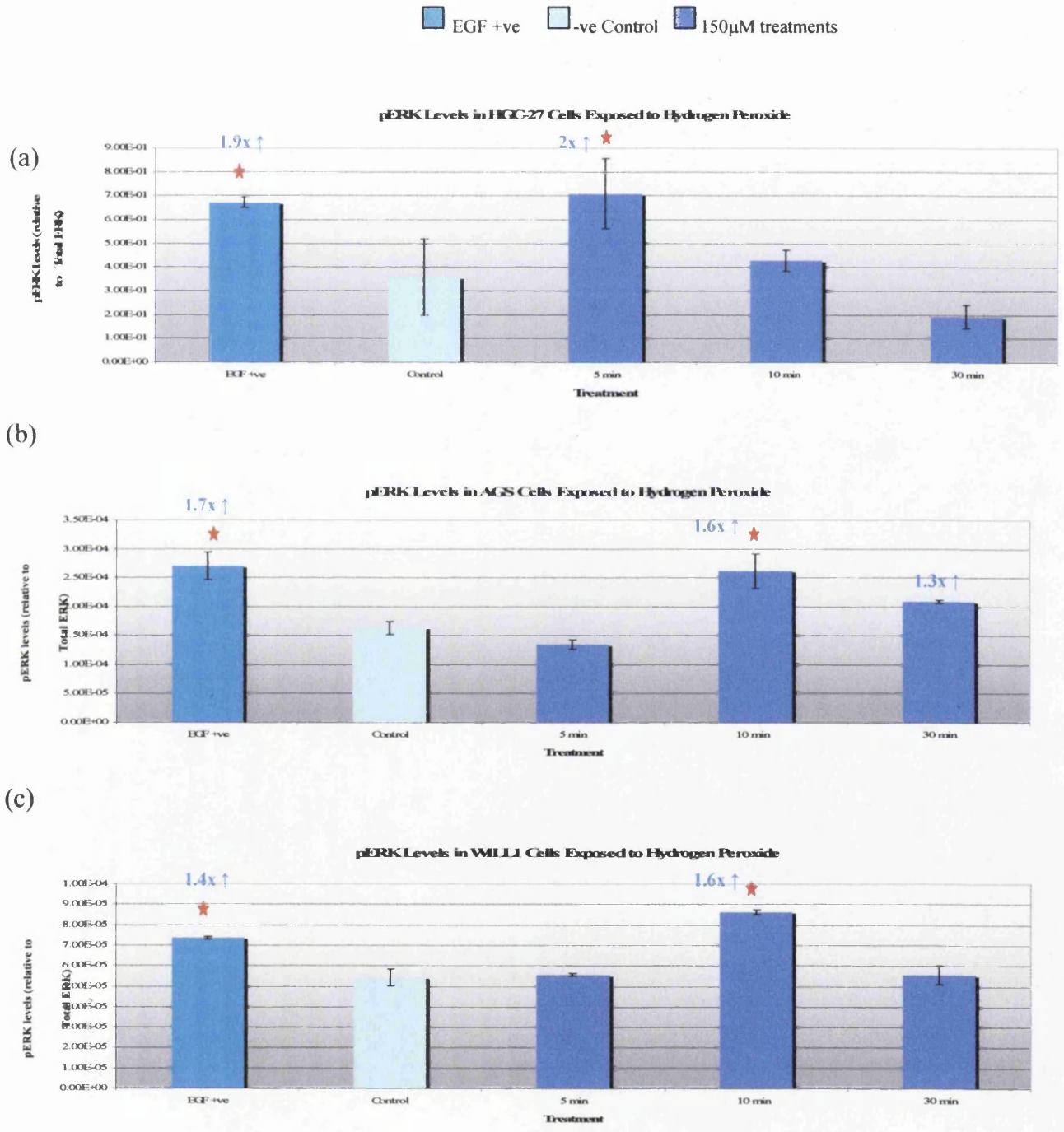
pERK

EGF +ve	-ve Control	5 minute	10 minute	30 minute
185.2	187.0	185.7	186.5	188.5
191.6	189.0	189.7	190.6	188.1



ERK

Figure 3.15 Average pERK (phospho – p42/p44) levels in (a) HGC-27, (b) AGS, and (c) WILL1 analysed by western blots relative to total ERK (p42/p44) following treatments with 150μM H_2O_2 for 5, 10, and 30min. A positive EGF treated control, as well as a negative untreated control were included in the experiments. Standard error bars shown. Fold change differences compared to untreated control levels are annotated (blue) and statistically significant differences ($P < 0.05$) are highlighted with red stars (based on one way ANOVA). See text for further details. N = 2.



3.3.5.2c pERK Western Blots – Long Exposure Study

From figure 3.16 it is clear that long H_2O_2 exposure does not have such a clear impact on ERK activation as does short exposure. In HGC-27 (figure 3.16(a)) there was some increase in pERK levels when cells were treated with 150μM H_2O_2 for both 4 and 8hr, the 8hr exposure resulting in a significant 1.2-fold increase in ERK phosphorylation compared to control levels. No change in pERK levels following 24hr exposure was evident. So from the data it appears that ERK signalling may be rapidly activated by exposure to H_2O_2, but this increased activation subsides over time. It is quite plausible that this would occur, since the initial increase in activation would set in motion a cascade of downstream events leading to changes in gene expression, which can then go on to mediate further changes at the level of the cell independently of ERK activation. Another important consideration to make, however, is the experimental system - although cells are exposed to the ROS for 24hr, it is likely, since H_2O_2 readily diffuses across membranes and exerts its effects intracellularly, that it will be metabolised and broken down to less reactive species as time progresses.

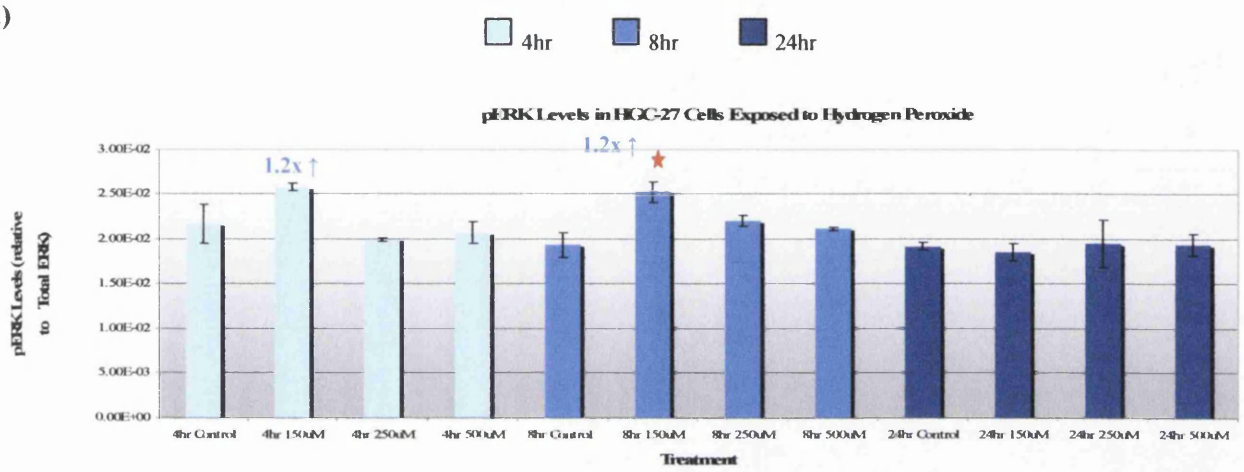
pERK levels in AGS (figure 3.16(b)) show more clear-cut changes following long exposures, increasing 1.4-fold following 150μM treatments at both the 4 and 8hr time points, maximal increase in ERK phosphorylation being evident following a 4hr exposure to 250μM H_2O_2, the changes being statistically significant. Again, no major changes in pERK levels appear to occur at the 24hr time point. These observations tie in with the results for AGS in the short exposure study, in which out of all of the cell lines, AGS appeared to sustain ERK activation the longest, and this may hold true at the longer exposure times.

In WILL1 cells (figure 3.16(c)), H_2O_2 treatment does not appear to cause any changes in ERK phosphorylation levels compared to controls across all time points.

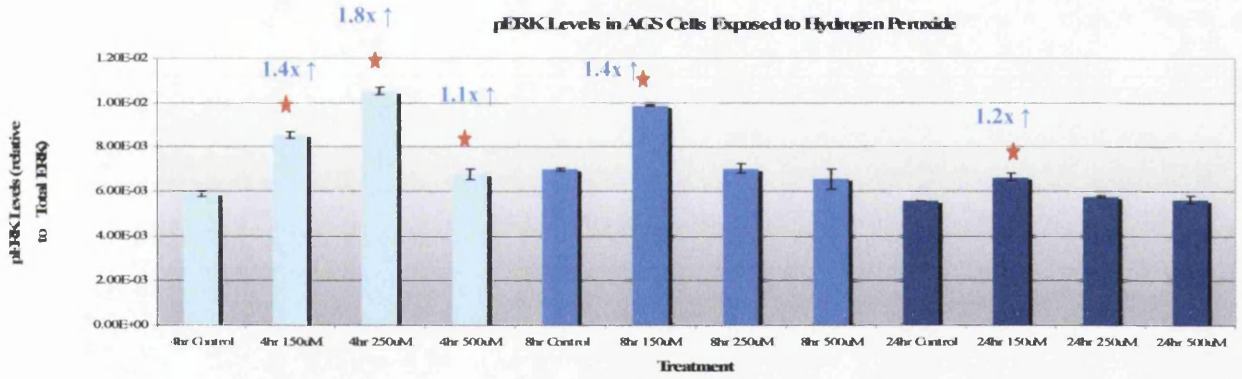
This clear difference between the normal and cancer cell lines can lead to the implication that MAPK signalling may be more sensitive to oxidative stress in the cancer

Figure 3.16 Average pERK levels in (a) HGC-27, (b) AGS, and (c) WILL1 analysed by western blots relative to total ERK following treatments with different doses of H_2O_2 for 4, 8, and 24hr. Standard error bars shown. Fold change differences compared to untreated control levels are annotated (blue) and statistically significant differences ($P < 0.05$) are highlighted with red stars (based on one way ANOVA). See text for further details. N = 2.

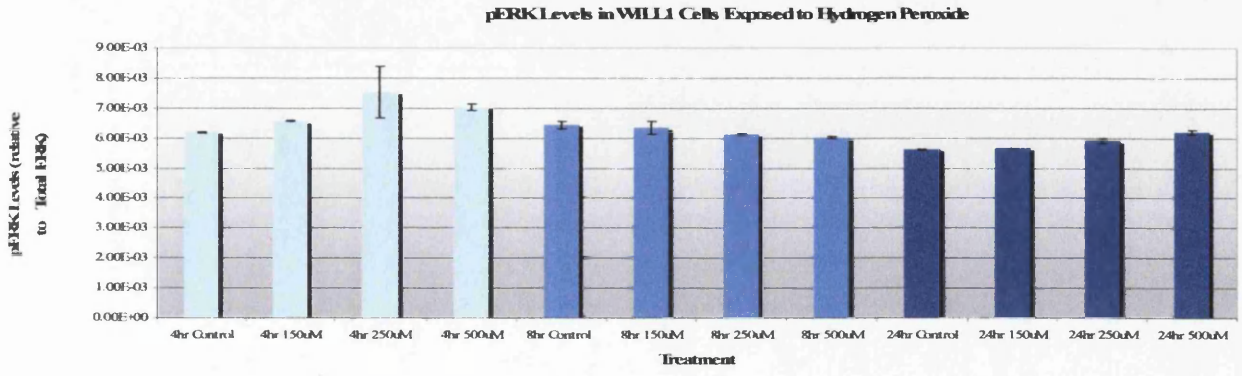
(a)



(b)



(c)



cell lines at longer exposure times, particularly in the less advanced AGS cell line, suggesting that aberrant ERK signalling may be an early change in the development of cancer, and then being a central feature of the progressing cancer phenotype (HGC-27).

3.3.5.2d p38 Western Blots – Short Exposure Study

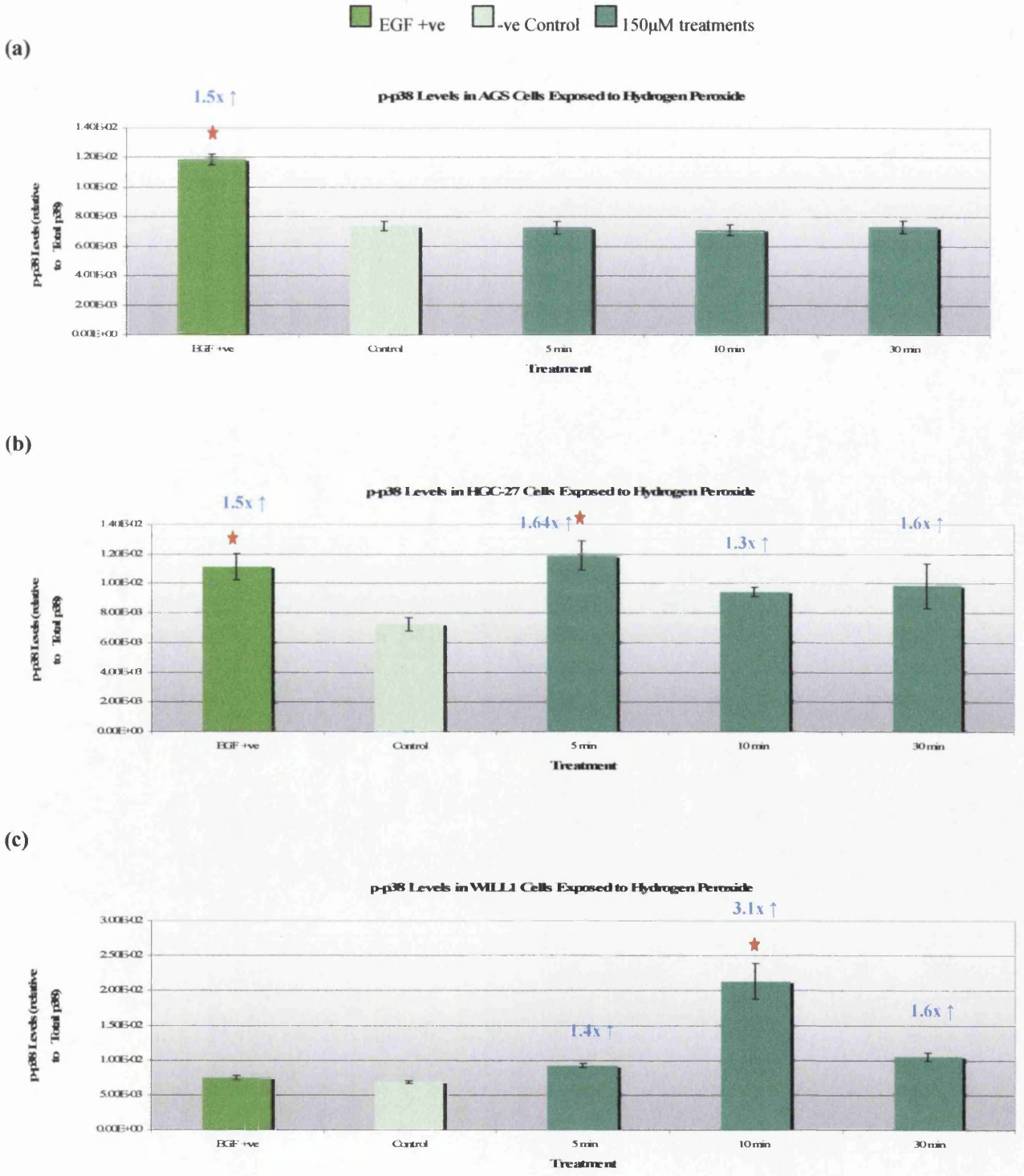
Figure 3.17 illustrates that short exposures to H_2O_2 resulted in a fairly similar p38 activation response as ERK with the exception of AGS. AGS shows a rather contrasting result for p38 compared to ERK (fig. 3.17(a)), whilst 150μM H_2O_2 had induced significant increases in pERK levels at 10min post exposure, remaining high at 30min, no such observation is seen for p38, the ROS seemingly having no effect on p38 activation in this cell line.

Five minute treatment resulted in maximal increase in p38 phosphorylation in HGC-27 (fig. 3.17(b)), levels then slowly returning to basal control levels, as was the case for ERK phosphorylation.

Activation of p38 in WILL1 by H_2O_2 closely resembles the induced activation of ERK in this cell line, levels of active phosphorylated p38 peaking at 10min post exposure with a significant 3.1-fold increase (fig. 3.17(c)).

Western blot for phosphorylated p38 was also carried out on the long exposure samples (as for pERK), however, no major changes in levels of phosphorylated p38 relative to total p38 were evident between control and treated samples at any of the time points in any of the cell lines.

Figure 3.17 Average phosphorylated p38 levels in (a) AGS, (b) HGC-27, and (c) WILL1 analysed by western blots relative to total p38 following treatments with 150μM H_2O_2 for 5, 10, and 30min. A positive EGF treated control, as well as a negative untreated control were included in the experiments. Standard error bars shown. Fold change differences compared to untreated control levels are annotated (blue) and statistically significant differences ($P < 0.05$) are highlighted with red stars (based on one way ANOVA). See text for further details. N = 2.

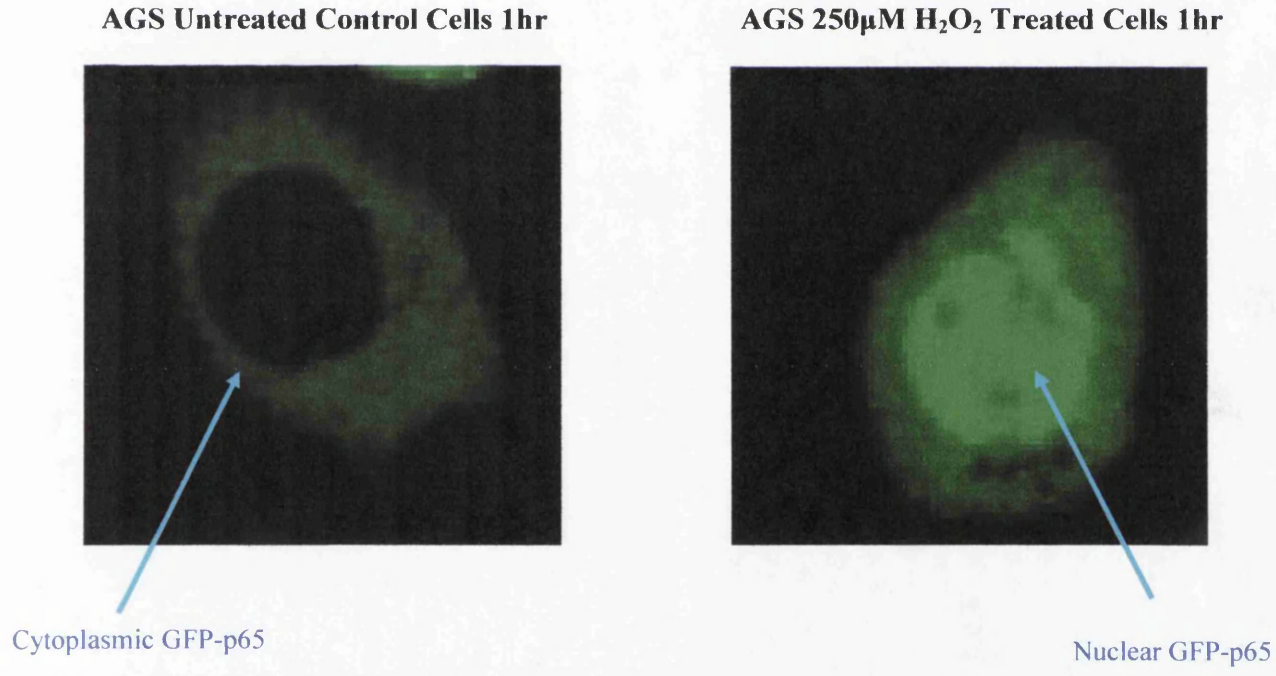


3.3.6 NFκB Pathway Studies

Further studies into the NFκB pathway consisted of western blots for p65 and IκBα proteins and transfection assays using an NFκB-GFP reporter construct, in which activation of NFκB was visualised by translocation of the GFP signal (from the fluorescent GFP-p65 fusion protein) from cytoplasm to nucleus. Very limited success was observed in the western blots, and the results were inconsistent, perhaps owing to the quality of the antibodies. As a result the data was inconclusive and not analysed. In the transfection experiments cells were exposed to 250μM H_2O_2 (since RT-PCR data revealed that this dose most commonly resulted in changes in the expression of NFκB – regulated genes *IκB* and *IL-8*), for 30min and 1hr (since nuclear translocation of the transcription factor should be a fairly rapid event following its activation). Unfortunately, very poor transfection efficiencies were observed using the described protocol (section 3.2.4.2) in all three cell lines, approximately 40%, 30%, and 50% in AGS, HGC-27, and WILL-1 respectively. In addition to this pitfall, a reasonable level of nuclear translocation was observed in control cells, constituting an excessive level of background NFκB activation, possibly induced by the stress of the transfection conditions. Together, these factors made scoring nuclear translocation of NFκB induced by H_2O_2 treatment very challenging. No clear changes in NFκB subcellular localisation were observed in treated HGC-27 compared to control, but this may be due to the very low transfection efficiency observed in this cell line, limiting the number of cells expressing the construct, and the few that did, often showed nuclear localisation of fluorescent p65 in the controls. WILL1 cells showed the best transfection frequency of all three cell lines, indicating the possibility that the cancer cell lines are more sensitive to the stresses of transfection coupled with subsequent oxidant exposure. Despite the reasonable transfection efficiency in WILL1 cells, very little nuclear translocation of NFκB was evident in control versus treated cells. AGS cells also demonstrated a satisfactory transfection efficiency (~40%), and interestingly out of all three cell lines, AGS was the only one to show more marked changes in NFκB subcellular localisation, increased nuclear translocation being observed following H_2O_2 treatment. An example image is shown in figure 3.18. Due to the

experimental difficulties encountered, no quantitative data was obtained, and so the observations simply served as preliminary findings that were indicative of some oxidant exposure induced NFκB activity in AGS cells. The preliminary findings suggest it would be worth pursuing more sophisticated or better optimised methodologies for studying NFκB activation. Due to resource and time restrictions this was not possible in the present study.

Figure 3.18 Confocal laser Scanning Microscopy (CLSM) images of AGS cells obtained using a Zeiss Confocal laser Scanning Microscope 510 Meta (Herts, UK) using the Argon 488nm excitation laser and the 500nm emission laser to detect green fluorescence from GFP. Cells were observed, and images captured, using the 40x oil objective. In the untreated cell population, a predominantly cytoplasmic subcellular localisation of NFκB was observed, whilst in treated cells, an increasing level of nuclear translocation was apparent. See text for further details.



3.4 Discussion

The dire consequences of oxidative stress has been linked to several different types of cancer, affecting diverse tissue types, particularly those in which inflammation is heavily implicated (de Visser and Coussens, 2006; Herrera *et al.,* 2005; Balkwill and Coussens, 2004; Klaunig and Kamendulis, 2004; D'Alessandro *et al.,* 2003; Oshima *et al.,* 2003; Coussens and Werb, 2002; Dröge, 2002; Balkwill and Mantovani, 2001; Conner and Grisham, 1996; Guyton and Kensler, 1993; Cerutti and Trump, 1991). This is exemplified by *H. pylori* – associated gastric carcinogenesis, in which chronic inflammation and accompanying oxidative stress are key players during disease progression (Correa, 2006; Macarthur *et al.,* 2004; Oshima *et al.,* 2003; Shacter and Weitzman, 2002; Obst *et al.,* 2000; Ernst, 1999; Bagchi *et al.,* 1996; Baik *et al.,* 1996; IARC monographs, 1994).

Persistent *H. pylori* infection elicits inflammatory reactions characterised by dense leukocyte infiltration at the infection site with a concomitant release of a plethora of inflammatory mediators and bactericidal free radicals in attempts to ward off infection. Unfortunately *H. pylori* has evolved mechanisms of evading the host inflammatory response, and so, in the absence of intervention therapies, chronic inflammation/ gastritis can develop with often detrimental consequences for the host, since the inflammatory milieu can cause damage to host cells (Correa and Miller, 1998) and can provide a tissue microenvironment which is tumour promoting. The oxidative stress component of the chronic gastric inflammation is believed to be a key factor driving the development of gastric cancer in a subset of individuals. Indeed, one of the mechanisms by which *H. pylori* can cause gastric cancer is via oxidative stress, leading to DNA damage (Obst *et al.,* 2000; Baik *et al.,* 1996), alterations in cell growth by perturbing the proliferation – apoptosis balance (Correa and Miller, 1998; Jones *et al.,* 1997; Peek *et al.,* 1997; Cahill *et al.,* 1996; Moss *et al.,* 1996; Lynch *et al.,* 1995), and changes in signal transduction and gene expression (Tuccillo *et al.,* 2005; Strowski *et al.,* 2004; Caputo *et al.,* 2003; Sepulveda *et al.,* 2002; Chiou *et al.,* 2001; Mitsuno *et al.,* 2001; Peek, 2001; Maeda *et al.,* 2000; Meyer-Ter-Vehn *et al.,* 2000; Keates *et al.,* 1999; Naumann *et al.,* 1999; Sharma *et*

al., 1998; Aihara *et al.*, 1997; Keates *et al.*, 1997). In addition RO/NS associated with infection have been reported to result in decreased antioxidant defence mechanisms, and enhanced expression of inflammatory genes, adhesion molecules, mediators of cellular proliferation and oncogenic transcription factors (Baek *et al.*, 2004; Kim, 2000) all of which can drive cancer development.

The present work set out to further examine the oxidative stress component of gastric disease, with a focus on oxidative stress induced signal transduction and gene expression changes. The MAPK and NFκB signal transduction pathways and their downstream transcription factors targets, AP-1 and NFκB respectively, were essential themes in the studies, since they are heavily implicated in inflammation associated cancers (Basseres and Baldwin, 2006; Karin, 2006; Waris and Ahsan, 2006; Viatour *et al.*, 2005; Karin, 2004; Barnes and Karin, 1997); and are known to have redox sensitive components (Gliore *et al.*, 2006; Manna *et al.*, 1998; Sen and Packer, 1996; Anderson *et al.*, 1994; Schreck and Baeuerle, 1994; Meyer *et al.*, 1993; Schreck *et al.*, 1992; Schreck *et al.*, 1991). Oxidative stress was mimicked using a cell culture model system in which cultured cells were exposed to various doses of H_2O_2 for varying lengths of time (Gille and Joenje, 1992). H_2O_2 was used since it provides a very simple model, is readily available, is easier to handle than several other unstable oxidants, has been used widely in literature (Williams *et al.*, 2005; Jenkins *et al.*, 2001; Ruiz-Laguna and Pueyo, 1999; Wang *et al.*, 1998; Duthie *et al.*, 1997; Kleiman *et al.*, 1990; Moraes *et al.*, 1990), the effects of the reactive species on DNA damage, mutation, and apoptosis are very well documented (Williams *et al.*, 2005; Jenkins *et al.*, 2001; Duthie *et al.*, 1997; Kleiman *et al.*, 1990), and its impacts on cellular signalling and gene expression continue to emerge (Gloire *et al.*, 2006; DeYulia *et al.*, 2005; Guyton *et al.*, 1996) . Important to the signal transduction theme of the studies, H_2O_2 is now generally accepted (together with superoxide) as a carefully regulated metabolite that can relay signals and critical information to the cells nucleus (Veal *et al.*, 2007; Scandalios, 2005). Furthermore, several tumour cells have been shown to constitutively generate H_2O_2 (Xu *et al.*, 2004; Szatrowski and Nathan, 1991) at rates in the range of 100 – 500μM/ 10^4 cells/ hr. Since the physiological levels of ROS generated in inflamed gastric mucosa is not clearly defined (partly due to the lack of sensitive enough assays to detect the transient and

constantly changing levels of reactive metabolites in the stomach, and the added complexity of a diverse array of different RO/NS being present at any one time), a dose range was selected that spanned the LD_{50} of H_2O_2 seen in toxicity tests (50 – 500μM, section 3.3.1), which also covered the range generated in tumour cells (Szatrowski and Nathan, 1991) as well as the dose ranges employed in the literature (Williams *et al.*, 2005; Jenkins *et al.*, 2001; Duthie *et al.*, 1997; Kleiman *et al.*, 1990).

Three different cell lines were used in the model, in order to study potential cell line specific responses to H_2O_2 treatment with respect to magnitude and timing of any induced signalling and gene expression changes. The normal primary cell line WILL1 was used alongside an early gastric cell line AGS derived from a primary gastric tumour (Barranco *et al.*, 1983), and a more advanced gastric cell line HGC-27 derived from a lymph node metastasis (Tetsuo, 1976) so to reveal differences between primary and transformed cells, and cancer cells at different stages of the disease (non-metastatic vs. metastatic).

The initial microarray experiments carried out using RNA obtained from control and dosed HGC-27 cells aimed to identify MAPK and NFκB related signal transduction changes induced by H_2O_2, in relation to dose and exposure time. Several genes were seen to be up- or down- regulated by H_2O_2 exposure, including oxidative stress response, inflammatory signalling, signal transduction, cell cycle control, DNA damage response, apoptosis, adhesion, angiogenesis and, invasion and metastasis related genes, all of which are impacted during cancer development (Hanahan and Weinberg, 2000). Worth noting is the dynamics of gene expression alterations, since they appeared to come in two 'waves' – an early first wave (~ 4hr post exposure) consisting predominantly of up-regulation of DNA damage response and cell cycle genes, oxidative stress response genes, signal transduction and transcription factor genes, and some genes involved in adhesion, angiogenesis, and invasion and metastasis. Later gene expression alterations comprised genes principally involved in apoptosis and inflammatory responses as well as more genes involved in angiogenesis and invasion and metastasis. It maybe that this second wave of gene expression changes is a consequence of the signal transduction and transcription factor changes that were induced at the earlier time points. At the cellular

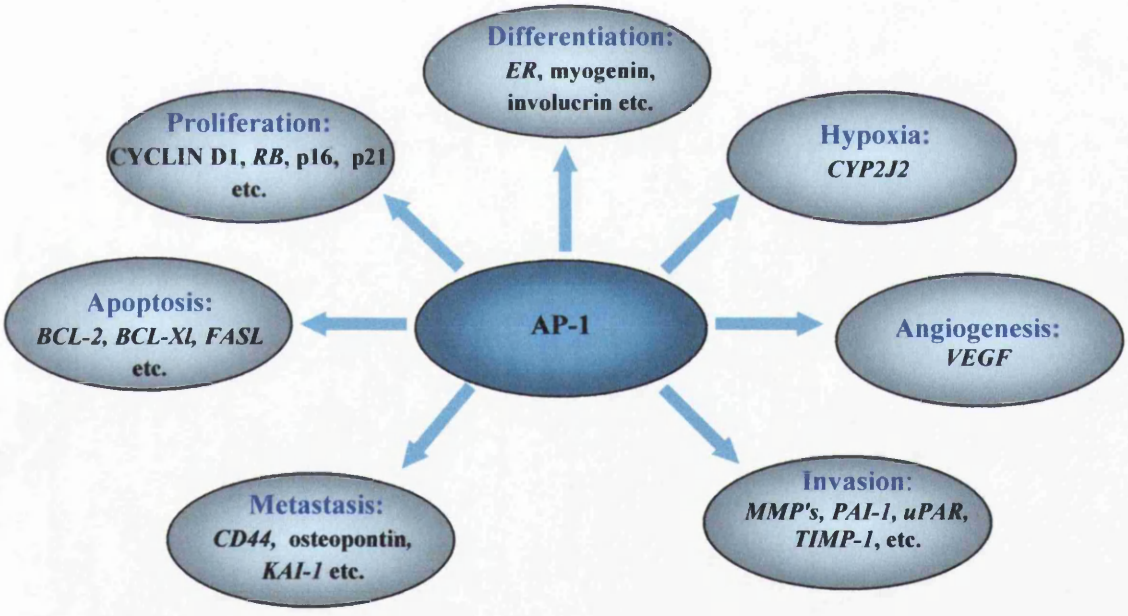
level, the dynamics of the gene expression changes make sense. Indeed, amongst the early signal transduction changes observed were p38 MAPK, well documented to be involved in oxidative stress responses (Dolado *et al.*, 2007; Emerling *et al.*, 2005; Karin, 2004; Freshney *et al.*, 1994), and RAF-1, part of the ERK MAPK pathway, also implicated in oxidative stress responses as well as mitogenesis (Chang and Karin, 2001; Graves *et al.*, 2000; Treinies *et al.*, 1999). It is likely that this induced cellular signalling sets in motion a cascade of cellular events that culminate in the later gene expression changes, notably apoptotic, inflammatory, and angiogenesis genes.

Interestingly, the two most consistently observed changes were up- regulation of *c-FOS* and *VEGF*. c-FOS is a component (together with c-JUN) of the oncogenic AP-1 transcription factor complex implicated in inflammation and carcinogenesis (Wisdom, 1999) (section 3.1.2.1). Induction of AP-1 may indirectly cause inflammation (Abdel Latif *et al.*, 2006) possibly accounting for later up-regulation of the inflammatory response genes. *c-FOS* expression is seen to be up-regulated as part of the early response to H_2O_2 exposure, alongside up-regulation of RAF-1, a MAPKKK in the ERK1/2 (p42/p44) MAPK pathway, a known upstream regulator of *c-FOS* gene expression (Marais *et al.*, 1993; Gille *et al.*, 1992) (sections 3.1.2 and 3.1.2.1, fig. 3.1), providing a link between ROS exposure, signal transduction induction and increase in transcription factor activity. *c-FOS* is known to have oncogenic activity (Miller *et al.*, 1984) and is frequently found to be over expressed in tumour cells (Milde-Langosch, 2005), and the expression of Fos proteins is seen to be crucial for optimal activity of AP-1 regulated genes (Schütte *et al.*, 1989).

Genes involved in invasion and metastasis, proliferation, differentiation and cell survival are seen to be regulated by AP-1 (Shaulian and Karin, 2002; Jochum *et al.*, 2001; Shaulian and Karin, 2001; Van Dam and Castellazzi, 2001; Tulchinsky, 2000). Figure 3.19 summarises the genes known to be downstream targets of AP-1. AP-1 and thus *c-FOS* can influence cell proliferation via cyclin D1 (*CCND1*) (Belguise *et al.*, 2005; Milde-Langosch, 2005) and p21 (*CDKN1A*) (Milde-Langosch *et al.*, 2000), invasion via *MMP9* and *TIMP1*, and *uPAR* (Milde-Langosch *et al.*, 2004; Kim *et al.*, 2004), and angiogenesis via the potent angiogenic factor *VEGF* (Milde-Langosch *et al.*, 2004).

Interestingly some of these genes were seen to be up-regulated in HGC-27 by the H_2O_2 exposure experiments, raising speculations of a link between increased *c-FOS* expression and induction of AP-1 downstream target genes that are heavily implicated in the development of a carcinogenic phenotype.

Figure 3.19 AP-1 responsive genes implicated in cancer development. It is clear that active AP-1 is linked to the underlying pathology of cancer at several levels including apoptosis, proliferation, differentiation, hypoxia, angiogenesis, invasion, and metastasis. See text for further details. Figure adapted from Milde-Langosch, 2005.



Changes in *c-FOS* expression levels, most commonly over-expression, have been reported in several different cancer types including bone (Sunters *et al.*, 2004; Franchi *et al.*, 1998); breast (Milde-Langosch *et al.*, 2004); endometrial (Bamberger *et al.*, 2001; Fujimoto *et al.*, 1995); cervical (Prusty and Das, 2005; Soto *et al.*, 1999); lung (Reddy

and Mossmann, 2002; Volm *et al.*, 1998); skin (Silvers *et al.*, 2003; Sakai, 1990) oesophageal (Wu *et al.*, 2004), hepatocellular (Yuen *et al.*, 2001) pancreatic (Lee and Charalambous, 1994; Wakita *et al.*, 1992), and gastric cancer (Meyer-ter-Vehn *et al.*, 2000) amongst others.

With respect to gastric cancer, *H. pylori* is now clearly a well known aetiological factor. Experimentation by Meyer-ter-Vehn *et al.* (2000) revealed that co-culturing gastric cells with virulent bacterial strains enhanced DNA binding of AP-1, coupled with strong induction of *c-FOS* mRNA expression. In addition *H. pylori* activates the ERK1/2 (p42/p44) MAPK cascade, which ultimately results in increased *c-FOS* expression via Elk-1 (Marais *et al.*, 1993; Gille *et al.*, 1992). Whilst the effect of *H. pylori* on these signalling and gene expression pathways is, to an extent, known, the mechanisms are yet to be elucidated. These observations are interesting on the grounds that several of the gene expression alterations seen in the present study are related to MAPK signalling and AP-1 (by way of *c-FOS*). Since the mechanism by which *H. pylori* acts to induce MAPK signalling and AP-1 activity are not fully defined, I hypothesise here that ROS may play a key role, providing the missing link between infection, inflammation, MAPK signalling, AP-1 activation and related gene expression changes, and hence gastric carcinogenesis.

Real-time PCR analysis provided evidence that oxidant exposure can indeed modulate *c-FOS* gene expression. Both cancer cell lines, particularly HGC-27 showed evidence of dose dependent changes in gene expression following H_2O_2 exposure and the data fit very well with the changes in *c-FOS* levels that were observed in microarray experiments for HGC-27 (section 3.3.2, fig. 3.7). The two cancer cell lines displayed a similar overall trend in *C-FOS* gene expression dynamics in response to oxidant exposure. However, there appeared to be a time delay in significant H_2O_2 induced gene expression changes in AGS compared to HGC-27, H_2O_2 eliciting increases in *c-FOS* levels 4hr post dosing in HGC-27, no such changes being apparent in AGS until the 8hr time point. These differences may reflect inherent differences in the cell biology of the two cancer cell lines. AGS, being derived from a primary gastric tumour (Barranco *et al.*, 1983) may be more resilient to some oxidant induced molecular changes, in this case only longer exposure times resulting in significant oxidant induced *c-FOS* expression

changes. The more advanced HGC-27 cell line is derived from a secondary lymph node metastasis of a gastric adenocarcinoma. It is possible that at the later stages of cancer progression the cells have either become more sensitive to the consequences of oxidative stress, or have accumulated molecular changes that result in aberrant signalling, reflected here in a more rapid response to redox regulated gene expression changes. Indeed, mutations or aberrant expression of components of the RAS-RAF-MEK-ERK1/2 signalling pathway are known to contribute to carcinogenesis (Leicht *et al.*, 2007; Roberts and Der, 2007; Schubbert *et al.*, 2007; Zebisch *et al.*, 2007) affecting tumour initiation, progression and metastasis. In addition it has frequently been noted that differences in cellular biochemistry (e.g. antioxidant enzyme and metabolite levels) as well as differences in metastatic potential are likely to affect how a cell responds to an exposure/ stimulus (Barranco *et al.*, 1983; Nicolson *et al.*, 1978). Differences in proliferation rate could also account for the observed differences, HGC-27 demonstrating a rate greater than that of AGS based on time in cell culture prior to sub-culturing. As such, a higher proliferation rate may account for higher levels of gene expression and/ or enhanced sensitivity.

The primary WILL1 cell line displayed completely different *c-FOS* gene expression dynamics in response to H_2O_2 than the two cancer cell lines, only showing marked changes at the 24hr time point. This is a very interesting finding and leads one to speculate the hypothesis that normal cells may be more resilient to oxidative stress and redox sensitive molecular changes compared to the cancer cells, which may inherently be more redox sensitive (Benhar *et al.*, 2001; Toyokuni *et al.*, 1995). Further support for this comes from the observation that *c-FOS* levels are seen to increase in untreated cancer cells over time, a trend that is absent in WILL1, and is likely to reflect a build up of oxidative stress in the cancer cells over time, owing either to enhanced RO/NS generation in cancer cells themselves (Ding *et al.*, 2007; Obst *et al.*, 2000; Davies *et al.*, 1994a; Davies *et al.*, 1994b; Szatrowski and Nathan, 1991), and/ or less efficient antioxidant systems compared with normal cells (Batcioglu *et al.*, 2006; Navarro *et al.*, 1999). Interestingly *H. pylori* infection has been documented to modulate the activity of cellular ROS scavenging enzymes, such that accumulating ROS in gastric cells fall to sub-lethal doses, and instead result in increased risk of gastric cancer (Smoot *et al.*, 2000).

The second gene seen to be up-regulated in microarray experiments was *VEGF-A*. *VEGF-A*, also known as vascular permeability factor, or *VEGF*, is a prototypic angiogenic factor. It induces endothelial cell differentiation, and is essential during embryogenesis (Risau, 1997; Carmeliet *et al.*, 1996; Ferrara, 1996) and wound repair (Jones *et al.*, 1999). *VEGF* is well documented to play a role in tumour progression via neo-vascularisation, a critical event during malignant transformation which contributes to both local growth of a tumour and its metastatic potential at later stages of the disease (Folkman, 1997; Hanahan and Folkman, 1996). *VEGF* is one of the most potent inducers of tumour angiogenesis (Ferrara, 1995; Kolch *et al.*, 1995). The importance of *VEGF* in tumour angiogenesis and hence development is strengthened by two key observations:-

- Over-expression of *VEGF* and its receptors is seen in several human tumours including brain (Ryuto *et al.*, 1996); hepatocellular (von Marschall *et al.*, 2001); kidney and bladder (Brown *et al.*, 1993a) and gastrointestinal (Brown *et al.*, 1993b) cancers, the elevated levels often correlating with poor prognosis. Over-expression of *VEGF* is also important in other pathologies involving chronic inflammation.
- Inactivation of VEGF protein by neutralizing antibodies inhibits tumour angiogenesis and growth *in vivo* (Kim *et al.*, 1993; Kondo *et al.*, 1993).

VEGF belongs to a family of angiogenic factors, which include *c-FOS* induced growth factor *VEGF*-D (Figf) (Marconcini *et al.*, 1999; Orlandini *et al.*, 1996), and *VEGF* itself may also be regulated by a *c-FOS* mediated mechanism, since the *VEGF* promoter contains an AP-1 binding motif (Ryuto *et al.*, 1996) amongst several Sp1 sites. Of particular relevance to the present findings is that several studies have established a link between ERK MAPK activation and *VEGF* mRNA expression (Strowski *et al.*, 2004; Caputo *et al.*, 2003; Pai *et al.*, 2001; Milanini *et al.*, 1998) most commonly via the Sp1 transcription factor (Strowski *et al.*, 2004; Milanini *et al.*, 1998; Pedram *et al.*, 1997). A variety of cytokines and growth factors including EGF, TGFα and β, IL-8 and IL-6 (Cohen *et al.*, 1996; Detmar *et al.*, 1995; Frank *et al.*, 1995; Brogi *et al.*, 1994) as well as several transforming agents such as RAS and RAF (Grugel *et al.*, 1995) have been shown to induce *VEGF* expression in a variety of cell types often via the ERK MAPK pathway, yet the signalling cascades are yet to be fully elucidated. At the transcriptional level,

regulation of *VEGF* is complex, since its promoter contains binding sites for several transcription factors including AP-2 and Sp1 (Shima *et al.*, 1996; Levy *et al.*, 1995; Hagen *et al.*, 1992; Kadonaga *et al.*, 1987) as well as binding sites for AP-1 (Angel *et al.*, 1987), hypoxia inducible factor (HIF-1) (Ikeda *et al.*, 1995; Levy *et al.*, 1995), and possibly NFκB (Shima *et al.*, 1996). An interesting connection between oxidative stress, MAPK, *VEGF*, and gastric carcinogenesis becomes apparent considering a study by Cho *et al.* (2001) in which H_2O_2 generated by activated neutrophils resulted in MAPK activation, and expression and release of *VEGF* from macrophages. This finding, coupled with several documentations that *H. pylori* can induce *VEGF* expression by way of upstream ERK MAPK activation (Strowski *et al.*, 2004; Caputo *et al.*, 2003), and that *VEGF* is seen to be up-regulated in gastric tumours (Tuccillo *et al.*, 2005; Tian *et al.*, 2001; Yamamoto *et al.*, 1998; Brown *et al.*, 1993b) warranted the further investigation of *VEGF* expression in the present study.

As for *c-FOS*, differences between the three cell lines were also noted in regard to *VEGF* expression dynamics, this time the two cancer cell lines showing very different responses to H_2O_2 exposure associated gene expression changes. Oxidant exposure appeared to have no effect on *VEGF* levels in AGS, aside from decreases in expression 24hr post dosing. In contrast, HGC-27 and WILL1 both displayed some dose dependent increases at the 8hr and 24hr time points. Unlike the induction of increased *c-FOS* expression in HGC-27, which occurred at 4hr post dosing, *VEGF* up-regulation was not observed until 8hr, and also displayed up-regulation at 24hr, whereas *c-FOS* was down-regulated at the later time point. This difference in the timing of gene expression onset may reflect the phases of gene expression changes that were highlighted by the microarray studies which suggested a link between oxidant exposure, ERK MAPK signalling, *c-FOS*, and *VEGF* expression. Based on the available data it appears that compared with AGS, HGC-27 *VEGF* expression is more responsive to H_2O_2 induced oxidative stress. These differences are again likely to be due to cell line specific differences in biochemistry which make them either more or less sensitive to redox regulated gene expression. It is also possible that completely different pathways may operate in the two cell lines, impacting *VEGF* expression in very different ways. Like

HGC-27, WILL1 also shows some dose dependent increases in *VEGF* levels at the 8hr and 24hr time points.

The importance of these findings with respect to gastric carcinogenesis is that since inflammation and its associated generation of ROS are a key element of disease pathogenesis, gene expression changes seen to be induced by ROS may play an important role in the molecular pathways that underlie this. Here it was shown that *c-FOS* and *VEGF* levels can be modified by oxidant exposure, albeit in a time, and cell type dependent manner. Since *H. pylori* as been seen to induce up-regulation of both *c-FOS* and *VEGF* (Tuccillo *et al.*, 2005; Strowski *et al.*, 2004; Caputo *et al.*, 2003; Meyer-Ter-Vehn *et al.*, 2000) by mechanisms which remain to be elucidated, these findings implicate ROS as possible mediators of such bacterium – induced changes.

Changes related to the NFκB pathway were also examined using *IκB* and *IL-8* gene expression levels as markers of NFκB activity. Interestingly, out of the three cell lines examined, AGS appeared to demonstrate the clearest dose dependent changes in *IκB* and *IL-8* expression following H_2O_2 exposure, supporting findings by Seo *et al.* (2002) which linked ROS to enhanced *IL-8* expression and NFκB activation in AGS cells. No dose dependent responses were clear in HGC-27 and WILL1. Instead what was observed was that some doses elicited significant increases in *IκB* and *IL-8* levels at some time points, with no consistent trends. Again, the gene expression patterns were seen to differ somewhat between the cell lines. Some noteworthy observations include that maximal *IκB* and *IL-8* induction was seen following the 4hr 250μM treatment in both AGS and HGC-27. This corresponds to findings by Jenkins *et al.* (2004) which revealed a peak in NFκB – related gene induction at 4hr following exposure to activating stimuli such as bile acids, which may in fact act indirectly via enhancing cellular ROS generation (Jenkins *et al.*, 2007). The 250μM treatment dose also appeared to have the greatest effect on *VEGF* expression at the 4hr and 8hr time points; 250μM and 500μM having the greatest impact on *c-FOS* expression. These doses tend toward the higher side of the LD_{50} observed in MTS assays and are likely to result in cytotoxicity, but also, as evidenced in the present data, significant gene expression changes in the surviving cell population. In WILL1 maximal gene expression induction was seen in response to the 8hr

500μM treatment. The overall picture with respect to *IκB* and *IL-8* gene expression is that the H_2O_2 induced changes differ in all three cell lines, AGS showing the greatest level of responsiveness, and WILL1 showing the least. This may be due to cell line characteristics. The two cancer cell lines appear to be much more susceptible to oxidative stress induced gene expression changes than the normal WILL1 cell line. This may be due to WILL1 possessing more efficient antioxidant defence systems than the cancer cell lines, so helping to maintain a steady redox balance in treated cells, only being perturbed above a threshold level of oxidant exposure. A second possible explanation may be due to aberrant NFκB signalling in the cancer cell lines, making the pathway more redox sensitive than normal. This lends support to the several reports that link aberrant NFκB signalling to carcinogenesis (Karin, 2006; Rayet and Gélinas, 1999).

Based on the data, it is difficult to establish a clear relationship between H_2O_2 exposure and NFκB regulated gene expression. Oxidant exposure does appear to affect *IκB* and *IL-8* gene expression, causing significant up-regulation in some instances, but this occurs in a clearly dose, and cell type specific manner. Several reports in the literature support this observation.

Taken together, the array and real-time PCR results helped to determine that frank cellular exposure to H_2O_2 (and its resultant oxidative stress) results in gene expression changes that appear to be linked to upstream MAPK and NFκB signalling pathways. Further experiments were hence carried out to obtain greater insight into the upstream signalling events. Some support for a link between H_2O_2 exposure and *c-FOS* gene expression to an upstream redox sensitive MAPK pathway was provided by a preliminary study in which the MEK (MAPKK) component of the ERK MAPK pathway was inhibited in HGC-27, and the effects on *c-FOS* expression analysed by real-time PCR. Results showed that *c-FOS* expression could be diminished by inhibitor pre-treatment.

When analysing gene expression changes it is important to look beyond the RNA level to the protein level, since proteins are the ultimate effectors in the cell. As such c-FOS protein levels were examined in the three cell lines. The results revealed that no clear significant changes in c-FOS protein levels in response to H_2O_2 exposure were evident in AGS and WILL1 cell lines, whilst some dose dependent increases in protein

levels were observed in HGC-27 at the 4hr and 8hr time points. The western blot data for HGC-27 was in accordance with the real-time PCR results for *c-FOS* RNA levels indicating that H_2O_2 – induced *c-FOS* gene expression changes occur at both the RNA and protein level, and so is likely to have a knock on effect on cellular physiology and behaviour. In AGS and WILL1 the changes in *c-FOS* RNA levels did not appear to translate to protein level changes. Interestingly in these two cell lines *c-FOS* RNA level changes did not occur to the magnitude seen in HGC-27, leading to the speculation that perhaps only above a certain threshold RNA level do protein level changes occur, possibly owing to factors such as RNA stability, and regulation at either the transcriptional or translational level. Interestingly, upstream signalling pathways such as MAPK cascades can also act to regulate gene expression at the post-translational level, for example, by enhancing mRNA stability and affecting translational control (Chang and Karin, 2001; Kotlyarov *et al.,* 1999). With respect to *c-FOS*, post translational control has been reported by way of phosphorylation by a novel MAPK, FRK (Deng and Karin, 1994), and the involvement of this level of control in the present findings cannot be ruled out.

Upstream p42/p44 ERK activity levels were also examined further at the protein level, using pERK as a marker of ERK activation. In short exposure studies, 150μM H_2O_2 induced significant and rapid activation of ERK signalling. HGC-27 displayed the quickest H_2O_2 induced activation of ERK a mere 5min post exposure. In AGS and WILL1, activation was seen to occur after 10min. The results clearly provide evidence that H_2O_2 can induce very rapid, albeit transient activation of the ERK MAPK pathway in all three cell lines. ERK activation was seen to be sustained the longest in AGS cells, pERK levels remaining elevated above control levels even after 30min whilst it had returned to basal control levels in HGC-27 and WILL1. These differences in dynamics of ERK activation may account for the difference in timing of downstream gene expression changes between the cell lines.

Long exposures to H_2O_2 did not appear to have such a significant impact on ERK activation as the short exposures. Some significant increases in pERK levels were evident in HGC-27 at the 4hr and 8hr 150μM treatments, in AGS at the 4hr 150μM, 250μM,

500μM, 8hr 150μM, and 24hr 150μM treatments, and no significant changes were observed in WILL1. The 150μM dose seemed to most consistently affect pERK levels, this dose also inducing the rapid ERK induction in short exposure experiments, and it was also seen to result in *c-FOS* and *VEGF* up-regulation suggesting that this dose may have the most impact on overall cellular behaviour. This is an interesting finding, since it corresponds to several published studies in which similar H_2O_2 doses resulted in the induction of DNA damage (Duthie *et al.*, 1997; Kleiman *et al.*, 1990), point mutations (Jenkins *et al.*, 2001), and chromosomal aberrations (Williams *et al.*, 2005). Moreover, this dose lies within the range of H_2O_2 levels seen to be generated by several tumour cell lines (Szatrowski and Nathan, 1991).

Of the three cell lines, AGS seemed to be the most responsive with respect to oxidant induced increases in pERK levels at the long exposure times, and this fits in with the observation that ERK activation persisted the longest in AGS cells following the short H_2O_2 exposures. The difference between the cancer cell lines and the WILL1 cell line support the idea that cancer cells are likely to be more sensitive to oxidative stress than the normal cell line. In the cancer cells MAPK signalling may be more sensitive to oxidative stress at longer exposure times, particularly in the less advanced AGS cell line. This suggests that aberrant ERK signalling may be an early change in oxidant linked cancer development driving oncogenic gene expression changes, and is also likely to be a central feature of later disease phenotype (HGC-27). This is of particular relevance since *in vivo* gastric conditions, especially in cases of chronic gastritis (+/ - *H. pylori* infection), are likely to impose persistent oxidant insults on epithelial cells. Cells that may have already undergone an initiating event (e.g. ROS induced mutation) may then be more susceptible to further oxidant driven molecular changes. In addition, ERK is a known survival factor, favouring cell proliferation over apoptosis (Xia *et al.*, 1995) so enhanced activation of ERK signalling in cancer is likely to favour cellular proliferation, and so disease progression.

Short exposures of H_2O_2 were also found to activate the p38 MAPK pathway, in a manner similar to that observed for ERK signalling, at least in HGC-27 and WILL1, 150μM H_2O_2 resulting in an increase in phosphorylated p38 following 5min treatment in

HGC-27 and 10min in WILL1. Such changes were surprisingly not seen in the AGS cell line, the reason behind which is unclear based on the available data but may be due to aberrant p38 signalling in the cell line, or general cell line differences in the oxidant sensitivity of the p38 MAPK pathway. This finding may be of significance since whilst ERK favours cell survival and proliferation, p38 MAPK signalling is linked to the induction of apoptosis (Xia *et al.,* 1995), as such, H_2O_2 - induced activation of ERK, but not p38 in AGS cells may result in a shift in the proliferation – apoptosis balance in favour of proliferation, an event which is central to cancer development (Hanahan and Weinberg, 2000).

With respect to NFκB, some significant changes in the expression levels of *IκB* and *IL-8* were evident in the real-time PCR studies, reflecting a potential oxidant induced activation of NFκB. Reporter gene transfection experiments utilising a GFP-p65 fusion protein construct (Schmid *et al.,* 2000) provided preliminary evidence that H_2O_2 caused nuclear translocation of NFκB in AGS cells. H_2O_2 did not appear to elicit nuclear translocation of NFκB in WILL1. Due to very low transfection efficiency in HGC-27, it was not possible to determine the effects of oxidant exposure on nuclear translocation of NFκB in this cell line, and unfortunately due to difficulties in the experimental protocol, it was not possible to obtain quantitative data for any of the three cell lines. Fine tuning of the protocol and further experimentation would be required to obtain a clearer picture.

In summary, the present work brought to light evidence supporting the importance of oxidative stress in gastric carcinogenesis, demonstrating that in addition to the induction of oxidative DNA damage and mutations, oxidative stress can also act at the level of signal transduction (e.g. MAPK and NFκB) and gene expression (e.g. *c-FOS, VEGF. IκB, IL-8*). Since several of the gene expression changes observed have been reported to be induced by *H. pylori* infection, the findings suggest that infection associated oxidative stress may be at the mechanistic base of the infection induced changes, and add strength to the notion that oxidative stress forms a critical aspect of *H. pylori* – associated gastric cancer.

The most interesting finding was that the oxidant induced changes were often seen to occur in a dose, time, and cell-specific manner, the two cancer cell lines showing

more marked responses than the normal WILL1 cell line. In addition differences were often observed between the two cancer cells, HGC-27 appearing to be more oxidant sensitive with respect to some molecular changes, and AGS being more sensitive to others – likely owing to the different stages of malignancy of the two cell lines – HGC-27 being the more advanced of the two, and as such the differences potentially reflect molecular changes that act at earlier or later stages of disease progression. The *c-FOS* studies, for example, demonstrated that HGC-27 was more responsive to oxidant induced *c-FOS* expression than AGS, and that WILL1 was the least responsive, at both the RNA and protein levels. It is very likely that such cell line and type specific differences are related to differences in cellular biochemistry, such as build up of oxidative stress or less efficient antioxidant systems. Indeed a recent report demonstrated that cancer cells carry greater oxidant loads due to enhanced ROS generation, and that inhibiting antioxidants can kill them preferentially since they will accumulate more severe levels of ROS, thus making them more sensitive to the detrimental effects of ROS than their normal counterparts (Trachootham *et al.,* 2006). This fits nicely with the present findings and suggests that the enhanced oxidant sensitivity of malignant cells can be exploited therapeutically.

Certainly, the further investigation of the biochemical differences between the three cell lines – with a particular focus on how differences in antioxidant systems may be a reflection of the observed differences in oxidant induced signalling and gene expression alterations, is warranted by the present findings. However, since the broader aim of the research was to study the effects of inflammation and ROS on signalling changes in gastric cancer using *in vitro* and *in vivo* models, following chapters focus on these aspects.

Chapter 4

Gastric Epithelial Cell – Inflammatory Cell Co-culture Studies Part I: *Optimisation of HL-60 Immature Neutrophil Cell Line for Respiratory Burst Induction*

4.1 Introduction

The importance of inflammatory processes in the pathogenesis of malignant disease, in particular gastric cancer, is a central theme of the present research and has been detailed in sections 1.4.5 and 1.7. It is now well accepted that chronic inflammation of the gastric mucosa lies at the root of the pathogenesis of gastric cancer (Zavros *et al.*, 2005). This chronic inflammation manifest as chronic gastritis has been linked to *H. pylori* infection (Israel and Peek, 2001; Correa, 1992a; Marshall, 1986) but can also occur in the absence of infection as a result of other insults. Irrespective of the cause, the chronic inflammation is believed to generate a local tissue microenvironment characterised by dense inflammatory cell infiltrates, and an inflammatory milieu of pro-inflammatory cytokines, arachidonic acid derivatives, as well as a plethora of reactive metabolites including RO/NS. Together these factors can instigate and drive carcinogenesis by:-

- Induction of DNA damage, hence increasing the likelihood of initiating mutations (point mutations and chromosomal aberrations). The DNA damage associated with inflammation is often oxidative DNA damage (Farinati *et al.*, 2003; Obst *et al.*, 2000; Shimoda *et al.*, 1994; Weitzman and Gordon, 1990; Lewis and Adams, 1987; Weitzman and Stossel, 1981) caused by the ROS released from activated neutrophils in the locale;
- Shifting the proliferation – apoptosis balance in favour of proliferation. This may be the result of an increase in apoptosis and a compensatory increase in proliferation, or simply as a consequence of decreased apoptosis or increased proliferation (Buttke and Sandstrom, 1994);

- Driving changes in cellular signal transduction and gene expression. This can be the result of shifts in local tissue concentrations of cytokines and growth factors (Yoshimura, 2006; Dobrovolskaia and Kozlov, 2005; Baud and Karin, 2001) or by oxidative stress mediated changes in redox sensitive signal transduction cascades (as demonstrated in chapter 3 for MAPK and NFκB signalling in response to frank H_2O_2 exposures).

Together these changes favour the development of malignant disease at the levels of initiation and promotion, and interestingly can all be attributed to the effects of ROS (Valko *et al.,* 2007; Valko *et al.,* 2006; Klaunig and Kamendulis 2004; Dröge, 2002; Turpaev, 2002; Conner and Grisham, 1996; Dreher and Junod, 1996). The effect of ROS on cellular signal transduction and gene expression was examined in chapter 3.

Since a tissue inflammatory response has both cellular (neutrophils, macrophages, lymphocytes, etc.) and non-cellular components (inflammatory cytokines as well as a diverse array of metabolites including RO/NS) it is important to consider the effects of both components together since this allows a closer modelling of the *in vivo* situation. Indeed it has been suggested that the combinatorial effects of different aspects of the inflammatory response (e.g. ROS and lipid mediators of inflammation) may have a greater impact on cells than single exposures (Lewis and Adams, 1987).

As such, the aims of the present chapter were to optimise a co-culture system in which gastric epithelial cells could be cultured in the presence of inflammatory cells stimulated to undergo an inflammatory response comprising release of RO/NS amongst other pro-inflammatory mediators. This would constitute an *in vitro* model for the effects of components of the inflammatory response on cellular signalling and gene expression that more closely mimics the *in vivo* tissue setting than the H_2O_2 oxidative stress inducing model used in chapter 3, since it takes into account the interaction between different cell types as well as the combinatorial effects of inflammatory mediators and spectrum of RO/NS.

This chapter describes the optimisation of an inflammatory leukocyte cell population induced to undergo an oxidative burst response. The cells could then later be introduced into a co-culture system with gastric epithelial cells so mimicking the tissue

microenvironment characteristic of inflammation (oxidative stress and inflammatory mediators) in order to examine the signalling and gene expression changes that such a setting may instigate. A variety of treatments and techniques were used in the optimisation efforts, all of which are described in the sections that follow.

4.1.1 Importance of a Co-culture System

Since RO/NS are now recognised as key players in the underlying pathogenesis of several diseases including cancer (Valko *et al.*, 2007; Valko *et al.*, 2006; Klaunig and Kamendulis, 2004), great efforts have been put into establishing the cellular effects of the reactive molecular species at several levels from DNA damage and mutation induction, to the effects on cellular lipids and proteins, and changes in cellular signalling and gene expression (Gloire *et al.*, 2006; Williams *et al.*, 2005; Houle *et al.*, 2003; Takada *et al.*, 2003; Lee and Esselman, 2002; Jenkins *et al.*, 2001; Toyokuni, 1999; Meyer *et al.*, 1993; Schreck *et al.*, 1991). Unfortunately the majority of such studies utilised *in vitro* systems in which cells were exposed to chemically generated RO/NS by way of frank exposures. Such experiments do not take into account factors which may be important in the *in vivo* setting such as rate of permeation of the RO/NS into cells, half-life and dose of the RO/NS, as well as the fact that in reality cells are likely to be exposed to mixtures of different RO/NS not just one type. The use of co-culture systems permits the investigation of the cellular effects of biologically generated RO/NS so mimicking the *in vivo* biological microenvironment to a somewhat better extent.

4.1.2 The Inflammatory Model

During tissue inflammation activated inflammatory leukocytes, particularly neutrophils and macrophages, are a major source of RO/NS such as NO, H_2O_2 and $O_2^{\cdot -}$ (Kim *et al.*, 2003; Pedruzzi *et al.*, 2002; Weitzman and Gordon, 1990, Babior, 1984a; Weitzman and Stossel, 1981). ROS are generated by NADPH oxidase which catalyses

the one electron reduction of oxygen to $O_2^{\cdot -}$, the spontaneous dismutation of which leads to H_2O_2 generation (Babior, 1999; Forman *et al.*, 1998), and several other derivative species may be formed as a consequence. This increase on oxygen consumption is termed the 'respiratory/ oxidative burst' (Sandborg and Smolen, 1988; Babior, 1984b) and was first observed in 1933 (Baldridge and Gerard, 1933). This oxidative burst response is normally under stringent regulation such that it only occurs as and when needed thereby maintaining tissue homeostasis with respect to ROS levels so avoiding situations of oxidative stress. If this is not controlled then continued inflammatory leukocyte accumulation and activation with the coupled oxidative stress in tissues can lead to crippling chronic inflammatory conditions. The positive effects of the release of leukocyte generated RO/NS is exemplified during infections where the potent metabolites kill off pathogens. Unfortunately if it is not carefully controlled "collateral damage" in nearby tissues can result (Babior, 1999).

The oxidative burst response in inflammatory leukocytes is dependent upon the activation of NADPH oxidase. NADPH oxidase can be activated by a number of different stimuli, both soluble and particulate in nature (Lundqvist *et al.*, 1996; Lundqvist *et al.*, 1994). Stimuli commonly present at sites of inflammation can lead to the activation of leukocyte NADPH oxidase, the most potent being opsonised microorganisms, C5a complement fragment, N-formyl methionine peptides (e.g. N-Formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP)), platelet activating factor (PAF), leukotriene B_4 (LTB_4), etc. These natural inflammatory stimuli have dual properties as chemotactic factors – instigating the migration of neutrophils and other leukocytes toward sites of inflammation, and as inducers of oxidative burst (Casimir and Teahan, 1994). Synthetic agents such as phorbol myristic acid (PMA) and other phorbol esters, fluoride, and calcium ionophore A23187 are also highly effective at stimulating oxidase activation and oxidative burst.

The metabolic phenomenon of the oxidative burst normally occurs intracellularly in phagocytic inflammatory cells (neutrophils, monocytes, macrophages). Some of the generated ROS may, however, be released extracellularly by way of phagocytosis, or via simple diffusion through the plasma membrane in the case of membrane permeable metabolites such as H_2O_2. In such instances the ROS, unless scavenged by antioxidants

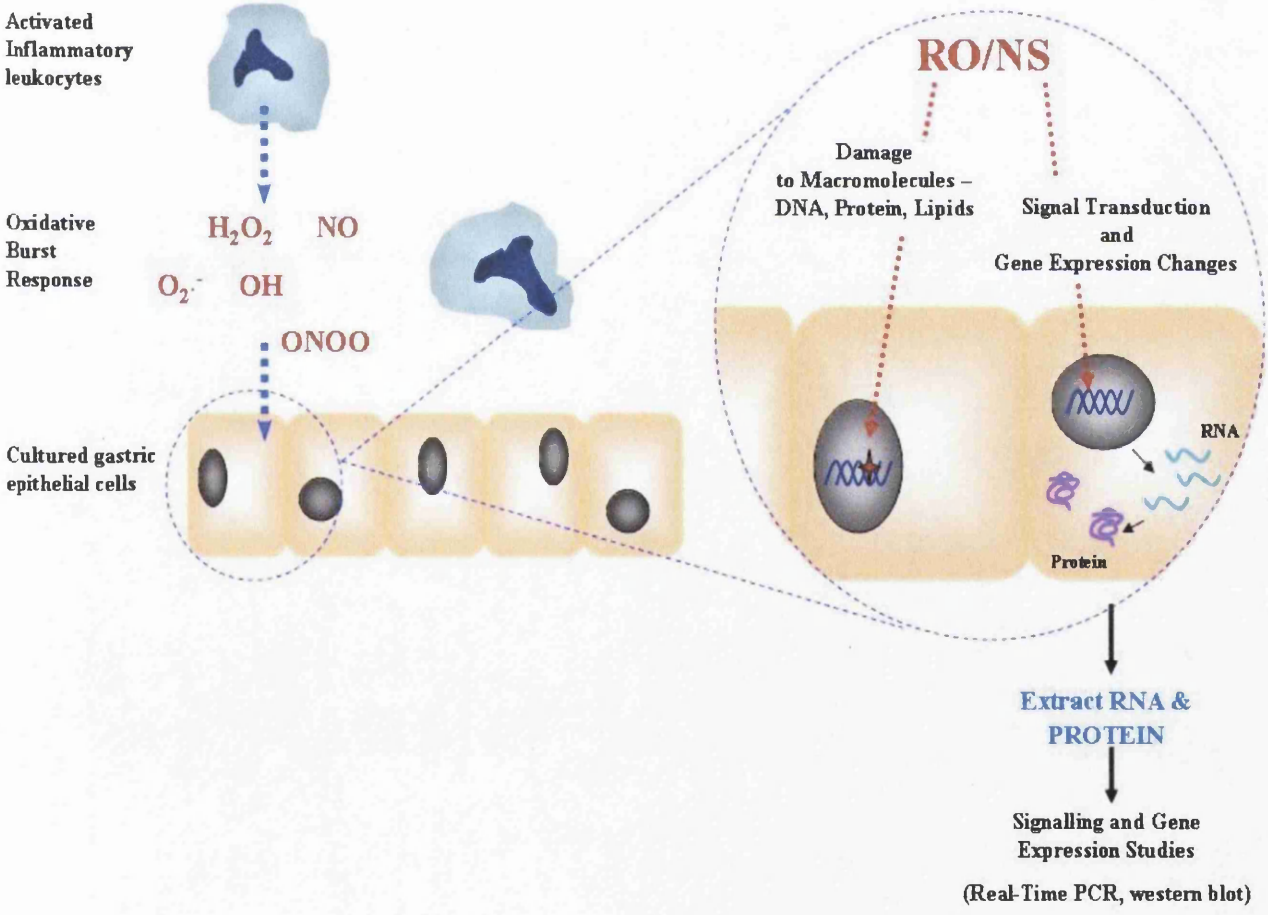
may inflict damage and toxicity on nearby cells, inducing DNA damage and mutation (Farinati *et al.*, 2003; Obst *et al.*, 2000; Shimoda *et al.*, 1994; Weitzman and Gordon, 1990), necrosis and apoptosis (Buttke and Sandstrom, 1994), as well as changes in gene expression and signal transduction (Valko *et al.*, 2007; Valko *et al.*, 2006; Klaunig and Kamendulis 2004).

On these grounds, and going back to the central theme of the present research – to study the effects of oxidative stress and inflammatory processes on signal transduction and gene expression in gastric epithelial cells, an inflammatory model consisting of activated inflammatory leukocytes (stimulated to undergo oxidative burst) co-cultured with gastric epithelial cells was developed. The components of the co-culture system are summarised in figure 4.1 and detailed in the sections that follow.

4.1.3 Use of the HL-60 Cell Line

The use of co-culture experimentation to study cell-to-cell interactions, inflammatory responses, and effects of bacteria such as *H. pylori* on cells has become increasingly well documented in the literature (Stone *et al.*, 2005; Kim *et al.*, 2004; Kim *et al.*, 2003; Yang *et al.*, 2003). Such experimental systems have also proved useful in analysing relationships between activation of inflammatory leukocytes and mutagenesis in near-by cells (Kim *et al.*, 2003) since inflammatory processes are strongly implicated in carcinogenesis and may act by way of RO/NS (Wietzman and Gordon 1990; Weitzman and Stossel, 1981). Studies using inflammatory leukocytes such as neutrophils often utilise cells isolated from fresh blood samples obtained from mice/ rats or consenting individuals (Shen *et al.*, 2002; Watanabe *et al.*, 2001; Knaapen *et al.*, 1999; Ariza *et al.*, 1996), or established cell lines such as the mouse macrophage cell line RAW264 (or its human derivative WBC264-9C) and the human promyelocytic cell line HL-60 (Kim *et al.*, 2003; Lundqvist *et al.*, 1996). The advantage of using cells isolated from fresh blood is that they are more likely to behave normally in terms of an inflammatory response compared to a transformed cell line. However, the use

Figure 4.1 Summary of co-culture system for studying the effects of oxidative stress amongst other components of an inflammatory response on signal transduction and gene expression changes in gastric epithelial cells. In this study the inflammatory component is the HL-60 promyelocytic cell line differentiated to neutrophils and stimulated to undergo an oxidative burst response. RO/NS generated by the intracellular oxidative burst may be released extracellularly by way of phagocytosis or by simple diffusion across the plasma membrane in the case of membrane soluble compounds such as H_2O_2.



of freshly isolated cells does have its drawbacks, for example – their use relies on the availability of fresh blood samples, preferably from the same donor to avoid problems with heterogeneity, and even if the blood is drawn from the same donor there is still a likelihood of obtaining heterogeneous populations of cells that show variations in their response. For this reason the use of an established readily available cell line, which would provide a relatively homogeneous population of cells was preferred in the present work. Although a transformed cell line may respond differently to normal cells, the advantages associated with the cells being readily available as and when needed outweighed the disadvantages.

Since the aim of the work was to mimic an inflammatory response that may be representative of gastritis tissues, in which the inflammatory cell infiltrate is initially predominated by neutrophils (Schmausser *et al.,* 2004; Israel and Peek, 2001; Shimoyama and Crabtree, 1998; Ernst *et al.,* 1997; Kozol *et al.,* 1991) (detailed in section 1.4.5.1), the human promyelocytic cell line HL-60 was utilised since its differentiation into neutrophils can readily be induced using a variety of polar compounds (Martin *et al.,* 1990; Collins *et al.,* 1978).

The HL-60 cell line is a pluripotent leukocyte cell line of the myeloid lineage, which upon maturity can give rise to a variety of cell types including monocytes/ macrophages and granulocytes - neutrophils, eosinophils, and basophils. The cell line was established from a 36-year old Caucasian female with acute promyelocytic leukaemia during leukopheresis (Collins *et al.,* 1977), and has since served an excellent model for the study of myeloid differentiation and function (Vrba *et al.,* 2004; Martin *et al.,* 1990; Birnie, 1988; Collins *et al.,* 1978). The cells are seen to behave as normal leukocytes in respect to their phagocytic capacity and responsiveness to chemotactic stimuli, and production and release of TNFα following stimulation with phorbol myristic acid (PMA) (Gallagher *et al.,* 1979). In regard to their capacity for oxidative burst response, HL-60 have been seen to respond in a similar, if not enhanced, manner to peripheral blood neutrophils upon treatment with stimuli known to induce oxidative burst (Trayner *et al.,* 1995; Sirak *et al.,* 1990). The cell line is pseudodiploid and is positive *for MYC* oncogene expression contributing to its transformed nature.

Under cell culture conditions, 10% of HL-60 cells can spontaneously differentiate into mature myelocytes (e.g. neutrophils, oesinophils) (Collins *et al.*, 1978). This proportion can be enhanced by treatment with polar-planar compounds such as dimethyl sulfoxide (DMSO), butyrate, hypoxanthine, PMA, actinomycin D, retinoic acid, etc. DMSO treatment is seen to cause differentiation of HL-60 cultures such that the cell population becomes a mix of mature myelocytes, metamyelocytes, and banded and segmented neutrophils, with metamyelocytes and banded neutrophils predominating (Collins *et al.*, 1978). It has been observed that functionally, HL-60 grown for 1-3 days in the presence of 1.3% DMSO are capable of $O_2^{\cdot -}$ generation (via oxidative burst), phagocytosis, degranulation, and bacterial killing (Newburger *et al.*, 1979). Longer differentiation periods (up to 9 days) lead to enhanced function, with the maximal performance of each function being 50-100% of that observed in normal neutrophils.

Here, HL-60 were induced to differentiate into neutrophils (or metamyelocytes and banded neutrophils) by incubation with 1.3% v/v DMSO as described by Newburger *et al.* (1979) and Collins *et al.* (1978) for periods of 3, 4, and 5 days in attempts to optimise the treatment regimen for maximal induction of differentiation, whilst at the same time being feasible within the time constraints of the research. Differentiation was subsequently assessed using morphological and functional criteria.

At the morphological level, cells were examined microscopically to check for characteristic features of neutrophils. These include – irregular shape, granulated cytoplasm, and irregular multi-lobate nuclei (in mature neutrophils) or donut-shaped nuclei (in immature banded neutrophils). At the functional level, differentiation status was assessed by measuring oxidative burst capacity of the cells using the Nitro Blue Tetrazolium (NBT) assay (see methodologies section).

4.1.4 fMLP Stimulation

Several published reports on leukocyte oxidative burst have utilised a range of artificial stimuli – such as PMA as well as other phorbol esters, opsonised zymosan, and calcium ionophore A23187 (Muranaka *et al.*, 2005; Kim *et al.*, 2003; Lojek *et al.*, 2002; Lundqvist *et al.*, 1996; Trayner *et al.*, 1995), as well as natural inflammatory stimuli such

as fMLP (Muranaka *et al.,* 2005; Vrba and Modriansky, 2004; Lojek *et al.,* 2002; Shen *et al.,* 2002; Mahomed and Anderson, 2000; DeLeo *et al.,* 1998). The synthetic stimuli tend to act by way of non-receptor mediated mechanisms, whilst the stimuli found naturally at sites of tissue inflammation act via receptor mediated pathways. These receptor mediated pathways are coupled to intracellular signalling pathways by means of heterotrimeric G proteins, leading to both oxidative burst induction and facilitation of migration of leukocytes to the inflamed site (Casimir and Teahan, 1994; Baggiolini *et al.,* 1993). This natural inflammatory response is exemplified by fMLP. Interestingly, the response of leukocytes to fMLP seems to be largely dependent upon the dose of the stimulus. For instance, doses less than 100nM induce neutrophil migration along chemotactic gradients, whilst doses above 100nM induce an oxidative burst response (Casimir and Teahan, 1994).

Since the co-culture model aimed to simulate a typical inflammatory response in the gastric mucosa, commonly caused by *H. pylori* infection, it was decided that a natural inflammatory stimulus would be used to induce an oxidative burst response in HL-60. Unfortunately for safety reasons, the use of *H. pylori* factors was not possible, instead fMLP was chosen as it is widely used in the literature and is seen to be effective at doses between 100nM and 5μM, with 1μM being the most commonly employed dose (Shen *et al.,* 2002; Mahomed and Anderson, 2000; DeLeo *et al.,* 1998). Furthermore, fMLP has been shown to induce H_2O_2 production in HL-60 cells to a similar magnitude to that seen in fMLP stimulated blood neutrophils (Sirak *et al.,* 1990).

Until recently it was believed that undifferentiated HL-60 did not have the capacity to generate RO/NS in response to stimuli, however, Muranaka *et al.* (2005) demonstrated that undifferentiated HL-60 possess NADPH oxidase activity and could generate considerable amounts of ROS in response to stimuli such as fMLP, PMA, and calcium ionophore A23187, and even possibly in the absence of stimuli (Trayner *et al.,* 1995). Differentiation may enhance the oxidative burst potential of HL-60 since it can lead to increased expression of components of the NADPH oxidase (Muranaka *et al.,* 2005). As such, fMLP stimulation of both undifferentiated and differentiated HL-60 was carried out during the optimisation experiments.

4.1.5 Priming with LPS

Whilst various stimuli such as PMA and fMLP are known to induce the oxidative burst in neutrophils, some studies show that pre-treating inflammatory leukocytes (neutrophils, monocytes, etc.) with bacterial lipopolysaccharide (LPS) can lead to a heightened oxidative burst compared to that induced by the stimuli alone (Zughaier *et al.*, 1999a; Zughaier *et al.*, 1999b). LPS has the ability to induce neutrophil activation at very low concentrations and pre-incubation with LPS has been shown to cause a significant "priming" effect for subsequent stimulation using other factors (Nielsen *et al.*, 1994; Aida and Pabst, 1990; Vosbeck *et al.*, 1990; Forehand *et al.*, 1989; Worthen *et al.*, 1988; Haslett *et al.*, 1985; Guthrie *et al.*, 1984). The term "priming" was coined to describe the enhanced leukocyte functions following exposure of neutrophils to bacterial products including LPS (Casimir and Teahan, 1994). Indeed LPS has been seen to cause enhanced generation and release of $O_2^{\bullet -}$ following stimulation with fMLP or PMA (DeLeo *et al.*, 1998; Guthrie *et al.*, 1984). As such, it was decided that in order to optimise the oxidative burst potential in the HL-60 cells, experiments in which the cells were primed with LPS prior to fMLP stimulation would be carried out, and the release of ROS assessed to determine if LPS priming would be a worthwhile component of the final treatment regime of HL-60 prior to co-culture experiments. The dose of LPS used to prime neutrophils was 100ng/ml since this dose has been reported to enhance NADPH oxidase assembly in neutrophils, leading to enhanced oxidative burst response following fMLP stimulation (10-fold increased $O_2^{\bullet -}$ generation) (DeLeo *et al.*, 1998). In order to establish the optimal priming regimen, HL-60 were exposed to LPS for either 1hr or over night periods in both differentiated and undifferentiated cells, and the oxidative burst capacity assessed using a fluorescence based 96-well plate methodology and flow cytometry.

4.1.6 Methodologies

4.1.6.1 Assessing Differentiation Status in HL-60: *Microscopic Evaluation and the Nitro Blue Tetrazolium (NBT) Assay*

Differentiated HL-60 appear different at the morphological level compared to their undifferentiated counterparts in that they more closely resemble mature myelocytes. DMSO differentiated HL-60 appear smaller, have a lower nuclear/ cytoplasmic ratio, have less prominent cytoplasmic granules, fewer, if any, nucleoli, and marked indentation, convolution, and segmentation of nuclei compared to immature undifferentiated cells (Collins *et al.,* 1978). On these grounds the success of differentiation induction can readily be determined by microscopic observation of cells.

The Nitro Blue Tetrazolium (NBT) assay has proved to be a very useful assay for the activity and functioning of neutrophils in both normal and diseased states (Belcher and Czarnetzki, 1973; Gordon *et al.,* 1973; Matula and Paterson, 1971; Park, 1971; Grush and Mauer, 1969; Park *et al.,* 1968). The test is routinely carried out in blood samples, although it has been adapted for use in cell culture conditions.

The test works on the basis that mature neutrophils, upon stimulation with a variety of activating stimuli such as bacteria, bacterial extracts or products, PMA, etc. can reduce soluble NBT, by way of oxidants (e.g. $O_2^{\bullet -}$) released during the oxidative burst, resulting in the formation of insoluble formazan deposits which can be detected spectrophotometrically or microscopically. The amount of formazan deposits in stimulated neutrophils is much greater than that seen in unstimulated counterparts (Freeman and King, 1972; Matula and Paterson, 1971; Park *et al.,* 1968), which is dependent on the neutrophils being functionally active (i.e. differentiated to a functionally mature state). Based on these grounds successful induction of neutrophil differentiation in HL-60 should result in enhanced levels of formazan product following stimulation of differentiated HL-60. In this case the N-formyl peptide fMLP was used as the oxidative burst inducing stimulus. Since the ultimate aim of the present work was to optimise oxidative burst induction in leukocytes, a range of fMLP doses (100nM, 1μM,

and 5μM) were used to stimulate HL-60 in order to establish the optimal dose for induction of respiratory burst as well as assess the differentiation status of the cells.

4.1.6.2 Assessing Oxidative Burst Activity in HL-60

The generation and release of H_2O_2 and $O_2^{\bullet -}$ from activated neutrophils during the respiratory/ oxidative burst is recognised as a critical component of both cell-mediated immunity and the underlying pathogenesis of inflammation (Morel *et al.*, 1991). As such a large emphasis has been made on the development of sensitive and reliable methods for detecting oxidative burst activity (Model *et al.*, 1997). In the present work two such methods were employed, a fluorescence based 96-well plate assay and a flow cytometric method. In both cases the fluorescent probe of choice was dihydrodichlorofluorescein diacetate (H_2DCF-DA) since it has been used extensively in the literature (Shen *et al.*, 1999; Trayner *et al.*, 1995; Rao *et al.*, 1992) and is believed to be specific for H_2O_2 (and so indirectly measures $O_2^{\bullet -}$). The probe is very well established for detecting the oxidative burst response in mature myeloid cells and has been seen to be more effective than similar probes (Trayner *et al.*, 1995). To date the detection and measurement of RO/NS in cells and tissues has proven to be very challenging, and whilst the use of fluorescent probes has been criticised on the basis of the potential for false positive results (Rota *et al.*, 1999), they continue to be employed since they provide relatively fast, high-throughput methodologies for the detection of RO/NS. Several citations support the use of H_2DCF-DA since it is a relatively specific probe which allows the real-time monitoring of cellular ROS accumulation (Shen *et al.*, 1999; Trayner *et al.*, 1995). The probe works on the basis that H_2DCF-DA is initially non-fluorescent and membrane permeable but is cleaved by intracellular esterases upon entry to cells yielding a membrane impermeable derivative (DCFH) and so becomes sequestered inside cells (Shen *et al.*, 2002; Robinson *et al.*, 1994; Bass *et al.*, 1983). Oxidation by intracellular ROS then gives rise to fluorescent DCF which also remains inside the cells (Bass *et al.*, 1983), although the leakage of fluorescent DCF from cells has been reported (Ubezio and Civoli, 1994). As such, cells can be 'loaded' with the probe prior to treatment with fMLP

and the oxidative burst response of HL-60 can be detected using fluorescence detecting techniques. The amount of fluorescence detected is proportional to the amount of H_2O_2 (and its reactive derivatives) generated (Robinson *et al.*, 1994).

4.1.6.2a *Fluorescence Based 96-well Plate Assay*

Cellular ROS as well as the leukocyte oxidative burst response can be measured using an automated high throughput 96-well plate based technology described by Trayner *et al.* (1995). The assay works on the basis that the initially non-fluorescent probe H_2DCF-DA can be oxidised, in the presence of H_2O_2 (and its reactive derivatives) released by way of the oxidative burst, to a fluorescent product that can be detected using a fluorometer. This technique permits both the assessment of the differentiation status of cells as well as the oxidative burst response, and the effects of different treatments on cells evaluated simultaneously in a single rapid experiment.

4.1.6.2b *Flow Cytometry*

The analysis of the oxidative burst in inflammatory leukocytes has been very difficult and labour intensive to study, being time consuming and requiring large numbers of cells. Fortunately the development of flow cytometry methodologies has revolutionised research in this area since - owing to the sensitivity of the technique and the single cell analysis approach - significantly fewer cells are required, the techniques are relatively easy and inexpensive to perform, and most importantly they permit the population of cells to be subdivided based on light scatter (dependent on cell size and shape) and fluorescence intensity (dependent on the levels of ROS) (Robinson *et al.*, 1994). Since the principal of the methods is the use of probes that yield fluorescent products (in this case H_2DCF-DA) upon reacting with cellular oxidants, the identification of cell populations with low or high fluorescence intensities allows the identification of distinct populations of cells with low or high oxidative burst potential respectively, as well as the percentage of cells producing ROS following treatment with stimuli.

4.1.6.3 Assessing Inflammatory Response in HL-60: *ELISA for IL-8*

In addition to the generation and release of RO/NS, a typical inflammatory response also involves the release of a plethora of inflammatory mediators such as cytokines/ chemokines. In the case of neutrophils, bacterial infections (e.g. *H. pylori* infection of the gastric mucosa) as well as other inflammatory stimuli can lead to the enhanced generation and release of IL-8 (Altstaedt *et al.,* 1996; Arnold *et al.,* 1994; Cassatella *et al.,* 1992), which, owing to its effect as a potent chemotactic agent for neutrophils, leads to further infiltration of neutrophils to the site of inflammation, and so an enhanced inflammatory response. The enhanced inflammatory response may result as a consequence of the chemokines ability to activate a variety of neutrophil functions including oxidative burst by way of priming an enhanced response to stimuli such as fMLP (Guichard *et al.,* 2005), degranulation (Peveri *et al.,* 1988), and adhesion (Detmers *et al.,* 1990). As such IL-8 can be used as a marker of a neutrophil inflammatory response. Thus, in order to determine if the treatment regimens of HL-60 involving LPS priming and various fMLP treatments caused an inflammatory response beyond the release of ROS, IL-8 protein levels were measured using ELISA.

4.2 Materials and Methods

4.2.1 Cell Culture of HL-60

The human promyeloblastic cell line HL-60 (derived from a 36-yr old Caucasian female with acute promyelocytic leukaemia) was obtained from the American Tissue Culture Collection (ATCC, Teddington, Middlesex, UK) and was cultured as described in section 2.1.1.6. When not in use for experimentation the cells were frozen down in liquid nitrogen following the procedure outlined in section 2.1.1.7. In all experimentation HL-

60 were used between passages 1 – 15, and only cultures with viability ≥ 90% in the trypan blue viability assay (section 2.1.1.8) were used.

4.2.2 Induction of Differentiation in HL-60

Densely populated HL-60 cultures (~ 1 x 10^6 viable cells/ml) were induced to undergo differentiation into neutrophils by the addition of 1.3% v/v dimethyl sulfoxide (DMSO) (Gibco-BRL, Paisley, UK). Cells were exposed to DMSO for periods of 3, 4, and 5 days and subsequently assessed for differentiation status by a variety of means (described in section 4.2.3) in order to confidently determine the optimal regimen for inducing differentiation.

4.2.3 Assessment of Differentiation Status of HL-60

A variety of techniques were employed to assess the differentiation status of HL-60 populations following DMSO treatment so that the success and efficiency of the induction regimens could be determined with a high degree of confidence. These procedures allowed HL-60 differentiation to be evaluated at the levels of cellular morphology and functional biochemistry.

4.2.3.1 *Morphological Evaluation:* Microscopic Examination of Induced HL-60

4.2.3.1a Slide Generation

Following treatment with DMSO cells were harvested by centrifugation at 200 x g (1500rpm) for 5min, and the cells washed to remove all traces of DMSO in sterile PBS (pre-warmed to 37°C) followed by a second 5min centrifugation step. Cells were subsequently re-suspended in 2ml PBS (pH7.4) in clean correspondingly labelled centrifuge tubes and microscope slides of cells generated using the Cytospin4 (ThermoShandon, Cheshire, UK). Glass microscope slides (pre-cleaned with 100% ethanol to remove grease and dirt) were assembled into cytospin clamps together with a

filter card and funnel according to manufacturers instructions, and then placed into the Cytospin. Subsequently 100µl of each cell suspension was introduced into one funnel each and then spun down at 150 x g (1200rpm) for 8min to produce a cytodot on the slides. The cell density on each slide was examined using a light microscope and the respective cell suspension was diluted or concentrated as and if necessary to obtain a single (so scorable) layer of cells on the slides. For each treatment three slides were prepared so that the data would be produced in triplicate. The resultant slides were fixed in 90% methanol at -20°C for 10min and left to air dry prior to storage at -20°C until use.

4.2.3.1b Staining of Slides

Slides were thawed and submerged into a solution of 1.25µg/ml acridine orange in glass coplin jars for 10sec. The slides were then washed to remove excess stain by placing in phosphate buffer (pH6.8) for 10min. Finally the slides were transferred to fresh phosphate buffer and left for 1hr. Slides were air dried in the dark and stored at room temperature away from light.

4.2.3.1c Slide Visualisation and Scoring

Stained slides were viewed under an Olympus BX 50 fluorescence microscope using an UplanF1 100x/1.3 oil objective. Since acridine orange stains nuclei with bright green fluorescence and the cytoplasm orange/red, cells were visualised (after mounting a coverslip with a few drops of PBS (pH7.4)) with a dual-bandpass filter set. Standard FITC and rhodamine filter sets were used to excite and capture green and orange/red fluorescence signals simultaneously. Images were captured with a cooled CCD (charged – coupled device) camera and analysed with the MacProbe version 4.1 software (Applied Imaging, Newcastle Upon Tyne, UK). Undifferentiated HL-60 and differentiated HL-60 cells (referred to as HL-60 and HL-60/N respectively from here on) were identified according to the morphological criteria described by Collins *et al.* (1978) stated below.
Undifferentiated HL-60 cells were identified based on the following morphological features:-

- Round nucleus;
- 2-4 nucleoli visible per nucleus;
- High nuclear/cytoplasmic ratio.

For HL-60/N cells (differentiated):-

- Cells are usually smaller in size;
- Marked indentation, convolution and/or segmentation of the nucleus;
- Nucleoli are usually absent;
- Low nuclear/cytoplasmic ratio.

Three slides were scored for each treatment, with 1000 cells examined per slide, resulting in a total of 3000 cells per treatment. The cells were classified as either normal undifferentiated HL-60 or differentiated HL-60/N based on the criteria listed, and percentages of the cell types were calculated as averages across the three slides scored per treatment.

Statistical analysis was performed using SPSS version 13.0. The data was analysed by way of an unpaired t-test (since the cells were treated in two different and separate manners (control untreated and DMSO treated)) to determine any statistically significant differences in the percentage of differentiated cells between the two treatment groups. Statistical significance was achieved when $P < 0.05$ (confidence levels > 95%).

4.2.3.2 *Biochemical/ Functional Evaluation:* The Nitro Blue Tetrazolium (NBT) Assay

In order to assess the functional differentiation of HL-60 into neutrophils or immature neutrophil-like cells, NBT assay was employed. This assay works on the basis that differentiated neutrophils can be activated to produce and release superoxide, the presence of which leads to the formation of a reduced NBT formazan derivative which can be detected by spectrophotometry. The purpose of the assay was two-fold – firstly to determine the success of DMSO induced differentiation of HL-60, and secondly to serve as

an initial preliminary experiment in the optimisation of neutrophil activation and induction of oxidative burst so establishing the best treatment regimen.

HL-60 that had been cultured in the presence or absence of DMSO for 3, 4, and 5 day intervals were harvested by centrifugation at 200 x g (1500rpm) for 5min. The cells were washed by re-suspending in PBS followed by a second centrifugation step. Next 8 x 10^5 cells were re-suspended in 400µl HEPES-glucose buffer (10mM HEPES; 150mM NaCl; 5mM KOH; 1.2mM $MgCl_2$; 1.3mM $CaCl_2$; 5.5mM D-glucose, pH7.5) in sterile 1.5ml microfuge tubes, labelled according to the treatment regimen, and mixed with 400µl of 0.1% (w/v) NBT (in HEPES-glucose buffer). N-Formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) treatment doses of 100nM, 1µM, and 5µM were set up by adding the appropriate volumes of the inflammatory stimulus to the test set-ups in order to stimulate neutrophil activation, and the reactions incubated for 15min at 37°C. Each reaction was carried out in triplicate. The reactions were stopped by placing the tubes on ice, and subsequently centrifuged for 24sec in a microfuge to pellet down the cells. Supernatant was discarded by gentle suction through a Pasteur pipette, and the pellets washed by re-suspending in 250µl 70% methanol to remove unreduced NBT followed by a second centrifugation step and removal of supernatant. The cells were subsequently lysed by the addition of 500µl 2M potassium hydroxide (KOH), and the reactions incubated overnight in the dark at room temperature. Addition of 600µl DMSO after overnight incubation and subsequent vortexing for 15sec dissolved any blue formazan deposits that may have formed as a result of NBT reduction. The presence of solubilised blue formazan was assessed spectrophotometrically by calculating the A_{620}-A_{450} difference spectrum.

The successful induction of HL-60 differentiation was detected based on the grounds that functionally differentiated cells should generate more reduced NBT than undifferentiated cells. The successful induction of neutrophil activation and oxidative burst was detected on the basis that HL-60/N stimulated with fMLP should result in greater levels of reduced NBT than unstimulated HL-60/N.

4.2.4 Optimisation of Neutrophil Activation and Oxidative Burst Induction: *Combined LPS Priming – fMLP Stimulation Regime*

To determine if LPS priming should be included in the treatment regimen for oxidative burst induction, HL-60 and HL-60/N were primed with LPS (from *Escherichia coli* 0111:B4) for either a 1hr or an overnight incubation period. LPS at a final concentration of 100ng/ml was added to cells that had been cultured for 3 days either in the absence or presence of DMSO, as detailed in section 4.2.2, on the third day, for either 1hr or overnight. Following priming cells were harvested by centrifugation at 200 x g (1500rpm) for 5min, and washed twice in pre-warmed (37°C) PBS followed by another centrifugation step to remove all traces of LPS. The cells were then subject to further treatment with fMLP and oxidative burst capacity assessed using the methods outlined in the sections that follow.

4.2.5 Assessing Oxidative Burst Activity in HL-60

HL-60 and HL-60/N, both unprimed and LPS primed were subject to stimulation with fMLP (see fig. 4.2 and table 4.1) and the oxidative burst response in the cells measured using a fluorescence based 96-well plate assay and flow cytometry. Care was taken to keep fluorescent specimens away from light during the experimental procedures.

4.2.5.1 Fluorescence Based 96-well Plate Assay for Oxidative Burst

A fluorescence based 96-well plate assay for the detection of ROS release from HL-60 following various treatments was carried out according to the method described by Trayner *et al.* (1995) with some modifications. This assay serves dual functionality as both a test for oxidative burst activity, and as a test for myeloid differentiation, hence the results can be used in conjunction with the microscopic evaluation and NBT assay to confirm HL-

60 differentiation status. The assay works on the basis that the initially non-fluorescent probe dihydrodichlorofluorescein diacetate (H_2DCF-DA) can be oxidised, in the presence of ROS released by way of the oxidative burst, to a fluorescent product that can be detected using a fluorometer.

Both HL-60 and HL-60/N that had been grown for 3 days, and HL-60 and HL-60/N that had been grown for 3 days and primed with LPS overnight, were harvested by centrifugation at 200 x g (1500rpm) for 5min and re-suspended in 10ml serum free RPMI. Cells were then seeded into the appropriate wells of 96-well microtitre dishes at 1 x 10^5 cells/ well, the total volume in each well being 100µl. An example of a typical plate set up is shown in figure 4.2. Each treatment was performed in triplicate within a plate, and the plate set up was repeated twice, the final results being the averages of the within and between plate readings. A dilution series of H_2O_2 was included in the plate set up so that a calibration curve of fluorescence to H_2O_2 concentration could be determined, so allowing quantification of the amount of the ROS released from HL-60 cells. Positive controls were also included in which cells were treated with 5µM *tert*-butyl hydroperoxide (TBHP), a known inducer of cellular ROS production, as well as medium blanks. Four separate stock solutions of H_2DCF-DA probe were prepared in Hanks solution (Gibco-BRL, Paisley, UK) either containing just probe, or probe plus the appropriate multiples (depending on the number of wells) of 100nM fMLP, 1µM fMLP, or 5µM TBHP. To all stocks D-glucose was added to a final concentration of 30mM. Next 20µl of the appropriate stock probe solutions were added to corresponding wells in the plate, such that the final concentration of probe was 10µM. The plates were then placed in the Fluostar Galaxy plate reader (BMG Labtech, Ayelsbury, UK) with the in-reader incubator set at 37°C so that the plate could routinely be read whilst the cells were still living, thereby producing real-time results of ROS release over time. The scanning filters were set for fluorescein with an excitation wavelength of 485nm and an emission wavelength of 538nm, and the plates were scanned every 2min for 4hr so producing a kinetic trace of oxidative burst activity.

Figure 4.2 A typical plate set up for the fluorescence based 96-well plate assay for oxidative burst in HL-60. All treatments were carried out in duplicate, and a H_2O_2 dilution series was included to allow calibration of H_2O_2 concentration released from cells. HL-60 = cells grown normally in the absence of DMSO for 3 days; HL-60/N = cells induced to differentiate with DMSO for 3 days; HL-60/ HL-60/N + LPS = cells primed with LPS overnight prior to further treatment. Positive controls were cells treated with 5μM TBHP.

HL-60	HL-60	HL-60	HL-60 + LPS	HL-60 + LPS	HL-60 + LPS		Medium Blank	Medium Blank	Medium Blank		
HL-60 + 100nM fMLP	HL-60 + 100nM fMLP	HL-60 + 100nM fMLP	HL-60 + LPS + 100nM fMLP	HL-60 + LPS + 100nM fMLP	HL-60 + LPS + 100nM fMLP						
HL-60 + 1μM fMLP	HL-60 + 1μM fMLP	HL-60 + 1μM fMLP	HL-60 + LPS + 1μM fMLP	HL-60 + LPS + 1μM fMLP	HL-60 + LPS + 1μM fMLP	**50nM H_2O_2**	**50nM H_2O_2**	**50nM H_2O_2**	**100nM H_2O_2**	**100nM H_2O_2**	**100nM H_2O_2**
HL-60/N	HL-60/N	HL-60/N	HL-60/N + LPS	HL-60/N + LPS	HL-60/N + LPS	**500nM H_2O_2**	**500nM H_2O_2**	**500nM H_2O_2**	**1μM H_2O_2**	**1μM H_2O_2**	**1μM H_2O_2**
HL-60/N + 100nM fMLP	HL-60/N + 100nM fMLP	HL-60/N + 100nM fMLP	HL-60/N + LPS + 100nM fMLP	HL-60/N + LPS + 100nM fMLP	HL-60/N + LPS + 100nM fMLP	**10μM H_2O_2**	**10μM H_2O_2**	**10μM H_2O_2**	**50μM H_2O_2**	**50μM H_2O_2**	**50μM H_2O_2**
HL-60/N + 1μM fMLP	HL-60/N + 1μM fMLP	HL-60/N + 1μM fMLP	HL-60/N + LPS + 1μM fMLP	HL-60/N + LPS + 1μM fMLP	HL-60/N + LPS + 1μM fMLP	**100μM H_2O_2**	**100μM H_2O_2**	**100μM H_2O_2**	**200μM H_2O_2**	**200μM H_2O_2**	**200μM H_2O_2**
HL-60 POSITIVE	HL-60 POSITIVE	HL-60 POSITIVE	HL-60 + LPS POSITIVE	HL-60 + LPS POSITIVE	HL-60 + LPS POSITIVE	**500μM H_2O_2**	**500μM H_2O_2**	**500μM H_2O_2**			
HL-60/N POSITIVE	HL-60/N POSITIVE	HL-60/N POSITIVE	HL-60/N + LPS POSITIVE	HL-60/N + LPS POSITIVE	HL-60/N + LPS POSITIVE						

4.2.5.2 Flow Cytometry

HL-60 and HL-60/N that had been cultured for 3 consecutive days in the absence or presence of 1.3% (v/v) DMSO and HL-60 and HL-60/N that had been cultured in the same manner for 3 days with an additional overnight LPS priming were harvested by centrifugation at 200 x g (1500rpm) for 5min. Cells were then re-suspended in 5ml pre-warmed PBS (37°C) and cell counts obtained (section 2.1.1.8) for each cell suspension. The suspensions were adjusted either by diluting or concentrating so that the final cell count was ~ 1 x 10^6 cells/ml. Appropriate volumes of cell suspension were prepared for each cell type (HL-60, HL-60 + LPS, HL-60/N, and HL-60/N + LPS) to account for the need for 1ml of cell suspension per treatment (summarised in table 4.1). Cells were next 'loaded' with probe by the addition of H_2DCF-DA (Molecular probes, Paisley, UK) to each cell suspension to a final concentration of 20μM followed by a 30min incubation at 37°C in the dark with intermittent agitation. From here on the cell suspensions were protected from the light. Following the incubation period cell suspensions were centrifuged at 200 x g (1500rpm) for 5min and washed in cold (4°C) PBS to terminate probe loading. A second centrifugation step was performed and the cells re-suspended in Krebs Ringer Phosphate (KRP) buffer (118mM NaCl, 5mM KCl, 4mM $MgSO_4$, 1mM $CaCl_2$, 1mM KH_2PO_4, 10mM D-glucose, 16mM $NaHPO_4$ pH7.4) that had been pre-warmed to 37°C at ~ 1 x 10^6 cells/ml. Cells were then equilibrated to 37°C by incubation for 5min.

The cell suspensions were subsequently divided into 1ml aliquots in 5ml polypropylene round bottom flow cytometry tubes (BD Biosciences, Oxford, UK) and fMLP (100nM and 1μM doses) or TBHP (5μM dose) added as necessary to the appropriate tubes such that all treatments in table 4.1 could be analysed by flow cytometry. Four tubes were set up for each treatment corresponding to four different incubation time points – 0min, 10min, 30min, and 45min so that a kinetic trace of ROS detection could be determined. For HL-60 and HL-60/N, cell samples that had not been loaded with probe (i.e. unstained) were also prepared to serve as controls for flow cytometry so allowing setting of voltages and compensating for any spontaneous background fluorescence that the cells may generate.

Table 4.1 Treatment of Cells for Flow Cytometry detection of oxidative burst activity.

Undifferentiated Cells (HL-60)	Differentiated Cells (HL-60/N)
HL-60 Control	HL-60/N Control
HL-60 + LPS	HL-60/N + LPS
HL-60 + 100nM fMLP	HL-60/N + 100nM fMLP
HL-60 + LPS + 100nM fMLP	HL-60 + LPS + 100nM fMLP
HL-60 + 1μM fMLP	HL-60/N + 1μM fMLP
HL-60 + LPS + 1μM fMLP	HL-60/N + LPS + 1μM fMLP
HL-60 +ve Control (+ 5μM TBHP)	HL-60/N +ve Control (+ 5μM TBHP)
HL-60 + LPS +ve Control (TBHP)	HL-60 + LPS +ve Control (TBHP)
HL-60 Control Unstained	HL-60 Control Unstained

Following incubation for the appropriate time periods the samples were placed on ice to stop any further reactions (and prevent cell clumping) and each sample analysed by flow cytometry using the FACSAria (BD Biosciences, NJ, USA) after first running the unstained samples through to take account of any spontaneous fluorescence in the cells. Following termination of the reactions all samples were analysed by flow cytometry within a 30min period, shielding samples from light throughout. The 488nm Argon laser was used for excitation and the cells were identified using forward scatter (FSC) and side scatter (SSC) parameters. The total cell population was detected based on these parameters that detect size (FSC) and granularity (SSC) and a gate was drawn around this cell population (P1) for filter analysis. The P1 population of cells contained a non-fluorescent population of cells, as well as a fluorescent population of cells (P2) – presumably reflecting endogenous ROS activity in the cells. The parameters used for P2 population identification were FSC, SSC, and FITC fluorescence using 502nm long pass filter and 530/30nm band pass filter. The successful induction of oxidative burst (changes in cellular oxidative activity) could be detected as an increase in the percentage of the P2 population, and/ or an increase in the mean FITC fluorescence intensity (MFI).

4.2.6 Assessing Inflammatory Response in HL-60: *ELISA for IL-8*

ELISA for IL-8 was performed on the supernatants of cells that had been treated as summarised in table 4.1 (with the exception of TBHP positive controls). Post-treatment the cells were subject to an additional 4hr incubation at 37°C in serum free RPMI plus 0.5% (v/v) protease inhibitor cocktail (Sigma-Aldrich, Poole, UK) so allowing sufficient time for IL-8 protein to be produced and/ or released into the supernatant. Following treatment and incubation, cells were spun down by centrifugation at 200 x g (1500rpm) for 5min and 1ml supernatant from each treatment aliquotted into correspondingly labelled 1.5ml microfuge tubes. The experiment in its entirety was repeated in duplicate. IL-8 ELISA was then performed on the supernatants using the PeliKine Compact™ human IL-8 ELISA kit (Mast Group Ltd., Merseyside, UK) following the instructions in the manufacturer's protocol with some modifications. The IL-8 capture antibody was first coated onto the provided 96-well plates by adding 55µl to 5.5ml coating buffer (0.5M Carbonate/bicarbonate buffer pH9.6). Capture antibody solution was then added to each well at 50µl/well and the plate incubated at 4°C overnight. The following day excess coating antibody solution was discarded and the plate dried by blotting on tissue. Blocking buffer (1% BSA in PBS) was subsequently added at 150µl/well and the plate incubated for 1hr at room temperature. Blocking buffer was next aspirated and the plate washed 3 X with washing buffer (PBS/ 0.05% Tween). Standards (diluted in PBS with 1% BSA as optimised and prepared as a dilution series from supplied IL-8 standard as 0, 1, 2.5, 6.1, 15.4, 38.4, 96, and 240pg/ml) and samples were subsequently added to the plate at 50µl/well followed by 1hr incubation at room temperature. Samples were then aspirated and the wells washed 4 X with washing buffer. A second incubation with a biotin-conjugated anti-IL-8 antibody was next performed. Biotinylated anti-human IL-8 antibody was diluted in 1X dilution buffer (1:5 dilution in dH_2O of dilution buffer supplied in kit) at 120µl antibody to 12ml buffer per well to be analysed. The antibody solution was then added to the plate at 100µl/ well, the plate covered with an adhesive seal, gently agitated and incubated at room temperature for 1hr. The biotin conjugate was then aspirated and the wells washed 4 X with washing buffer. Next, streptavidin-HRP (provided in the kit) was diluted at 1:10000 in assay buffer (1:5 dilution in dH_2O of assay buffer supplied in kit) and 50µl added per well. The plate

was then incubated for 30min at room temperature after which the solution was aspirated and the plate washed 6 X with washing buffer. HRP substrate solution (prepared by mixing equal volumes of solutions A and B provided in the kit) was subsequently added at 50µl/well followed by a 30min incubation in the dark at room temperature so allowing the coloured (yellow) product to develop. The reactions were stopped by the addition of stop solution (1.8M H_2SO_4) at 50µl/well and the plate read at 450nm using an Anthos HTII plate reader (Labdesign, Sweden). Each sample was analysed in duplicate and the IL-8 concentration in the samples was determined using the average A_{450} reading calibrated against the standard curve.

4.2.7 Statistical Analysis

All statistical analysis was carried out using the SPSS version 13.0 software package. Unless otherwise stated one way ANOVA (followed by Tukey and Duncan post hoc tests) was carried out to compare control and treated cells. Statistical significance was achieved when $P < 0.05$ at the 95% confidence level.

4.3 Results

4.3.1 Microscopic Determination of HL-60 Differentiation

Examination of the slides prepared for the three different periods of differentiation induction revealed that both the 4 and 5 day DMSO treatments were cytotoxic to cells, with dead cells abundant in the examined cell populations. This made scoring of the 4 and 5 day slides very difficult, and only the slides from cells treated for 3 days were scorable. Figure 4.3 summarises the results for the 3 day differentiation treatment as the average percentage of differentiated cells in untreated control HL-60 and DMSO treated HL-60 based on the morphological criteria listed in section 4.2.3.1c, and an example of the cells observed is shown in figure 4.4. It is clear that DMSO treatment induces an increase in the percentage

of HL-60/N cells in the cell population from 11.7% in untreated cells to 39.1% in treated cells. Unpaired t-test revealed that this increase in HL-60 differentiation induced by DMSO after 3 days was statistically significant ($P < 0.05$).

Figure 4.3 Average Percentage of Differentiated HL-60 cells (HL-60/N) observed in untreated control cells and cells that had been treated with 1.3% (v/v) DMSO for 3 days. Standard error bars are shown. Statistically significant results ($P < 0.05$) based on one way ANOVA are highlighted with red stars. See text for further details. N = 3.

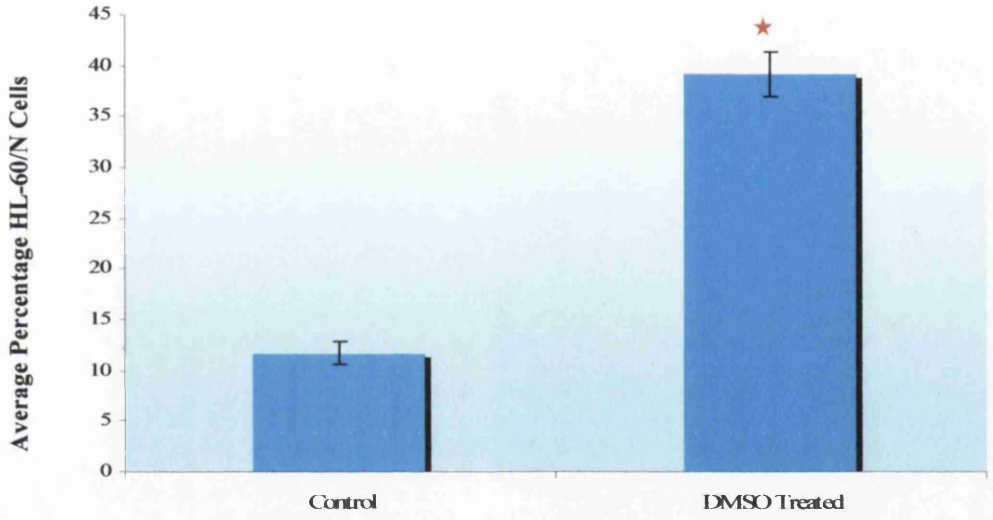
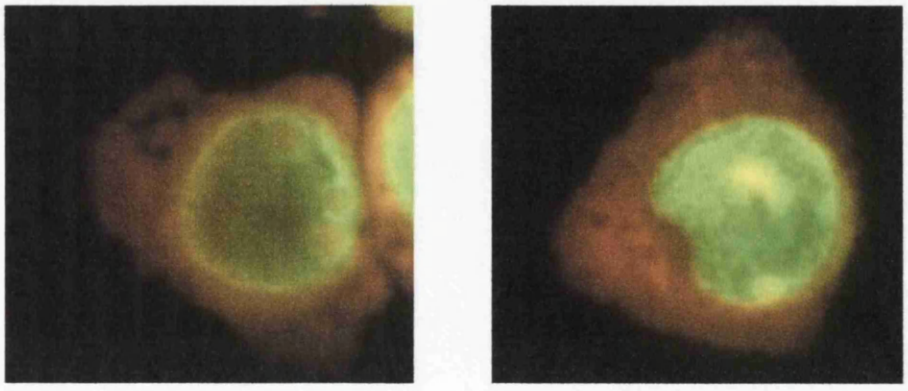


Figure 4.4 Example of HL-60 and HL-60/N cells observed by microscopic examination. Note the round nucleus, presence of nucleoli, and high nuclear/ cytoplasmic ratio in HL-60 compared to the indented nucleus, lack of nucleoli, and low nuclear/ cytoplasmic ratio in HL-60/N.



HL-60 HL-60/N

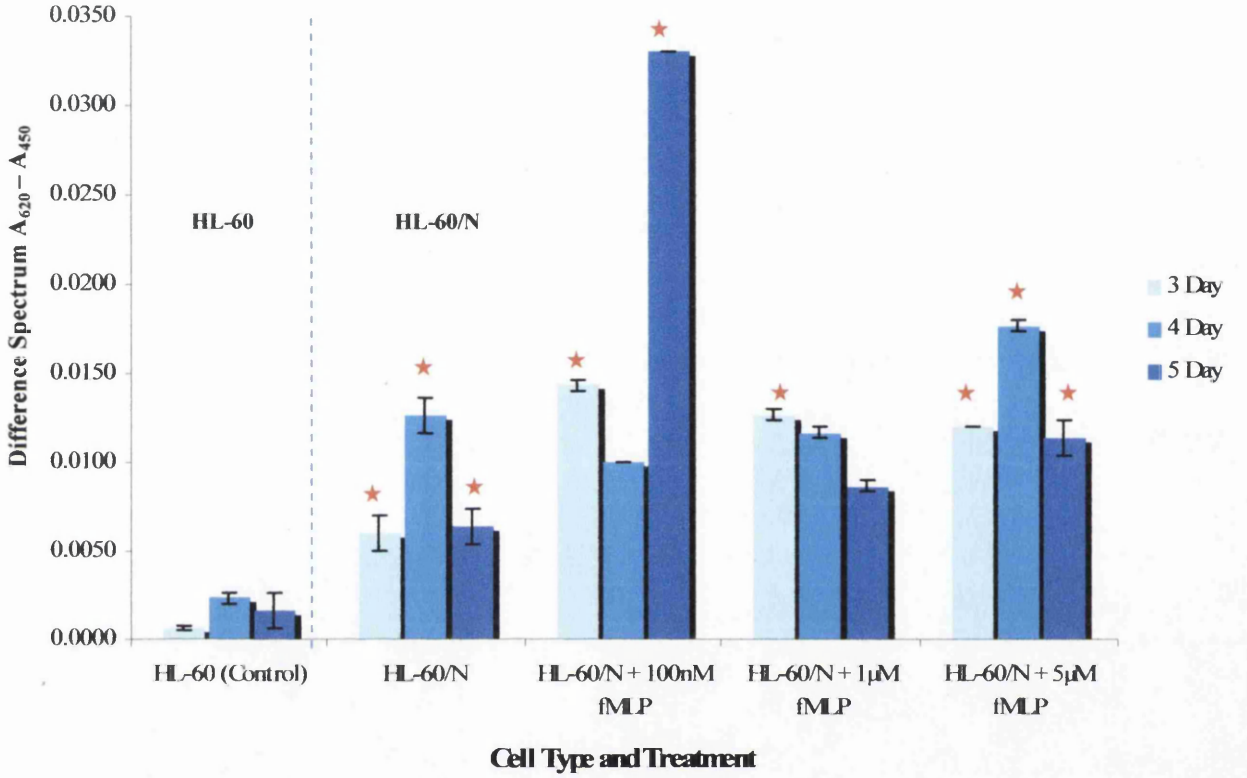
It has been demonstrated that DMSO treatment results in a decrease in HL-60 viability if the cells are grown in the presence of DMSO for more than 3 days (Mollinedo *et al.*, 1998) as well as an increase in the proportion of cells undergoing apoptosis (Mollinedo *et al.*, 1998; Martin *et al.*, 1990). Moreover, HL-60 grown in the presence of DMSO for 1-3 days have been shown to exhibit mature myelocyte functions such as oxidative burst, degranulation, phagocytosis, and bacterial killing (Newburger *et al.*, 1979). Taken together these observations provide support to the current findings that a 3 day DMSO treatment regimen induces significant differentiation of HL-60 whilst retaining adequate cell viability for further experimentation.

4.3.2 NBT Assay

The NBT assay was performed on undifferentiated as well as differentiated HL-60 (HL-60/N) that had been grown in the absence or presence of 1.3% (v/v) DMSO respectively for periods of 3, 4, and 5 days in order to determine the success of differentiation as well as which cell population had a greater inherent capacity for ROS generation and hence NBT reduction. The results were the averages of triplicate treatments and are represented graphically in figure 4.5. It is clear from figure 4.5 that for 3, 4, and 5 day growth periods, HL-60/N resulted in a higher level of NBT reduction than HL-60, confirming functional differentiation and reflecting a higher capacity for ROS generation and release in the differentiated cells. One way ANOVA revealed that in all three cases HL-60/N caused significantly greater reduction of NBT compared to HL-60 ($P < 0.05$).

In addition, the assay was also carried out on HL-60/N that had been treated with 100nM, 1μM, and 5μM doses of the inflammatory response stimulant fMLP for 15min to determine responsiveness of cells. After a 3 day differentiation period, stimulation with fMLP resulted in enhanced NBT reduction in HL-60/N at all three doses used compared to unstimulated counterparts (fig. 4.5), seen to be statistically significant at all three doses ($P < 0.05$) with maximal NBT reduction, and hence ROS generation, seen with the 100nM dose. Interestingly, after a 4 day differentiation period, stimulation of HL-60/N with fMLP

Figure 4.5 Results of NBT Assay carried out in HL-60. The test was carried out on normal untreated HL-60, differentiated HL-60 (HL-60/N) in the absence of fMLP stimulant, and on HL-60/N treated with 100nM, 1μM, and 5μM doses of fMLP stimulant. Standard error bars shown. Statistically significant results ($P < 0.05$) based on one way ANOVA are highlighted with red stars. See text for further details. N = 3.



appeared to have no effect on the level of NBT reduction compared to that detected in unstimulated HL-60/N, with only the 5μM dose causing a significant increase in NBT reduction compared to unstimulated HL-60/N. Following a 5 day differentiation period HL-60/N stimulated with 100nM fMLP gave rise to levels of NBT reduction significantly higher than that seen in unstimulated HL-60/N, 5μM fMLP stimulation also caused a significant, though lesser increase in NBT reduction, whilst the 1μM dose did not appear to have such an effect (fig. 4.5). Thus, 5 day differentiation plus stimulation with 100nM fMLP appeared to cause the maximum induction of respiratory burst in the HL-60/N cells. However, the 3 day differentiation regime plus fMLP stimulation also resulted in significantly enhanced oxidative burst activity, and due to time restraints and practicalities,

it was decided that HL-60/N that had been induced to differentiate by way of 3 day incubation with 1.3% (v/v) DMSO would be used in all subsequent experimentation. In addition, the morphological identification (section 4.3.1) of HL-60/N revealed that 3 day DMSO treatment induced significant HL-60 differentiation whilst 4 day and 5 day treatments may have caused decreased cell viability (possibly by way of necrosis and apoptosis), and this may in fact account for the high levels of reduced NBT following a 5 day DMSO treatment since dying cells tend to have high levels of ROS (Fialkow *et al.*, 2007; Splettstoesser and Schuff-Werner, 2002; Swain *et al.*, 2002). This observation provided further support to the decision to use the 3 day DMSO treatments for further optimisation experiments.

4.3.3 Fluorescence Based 96-well Plate Assay

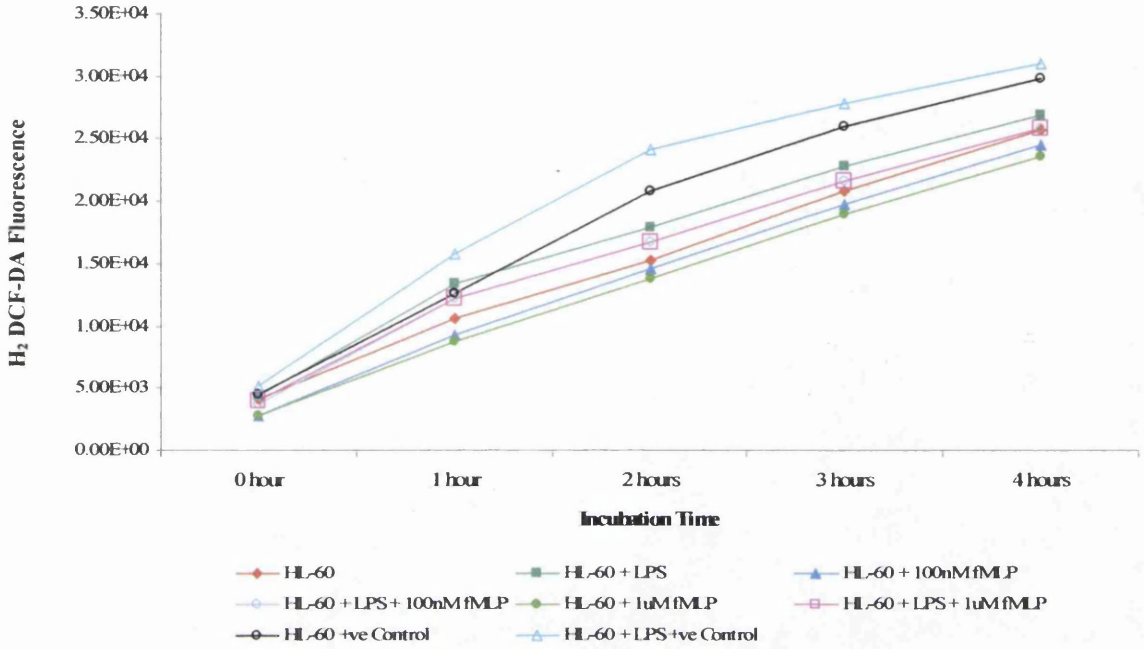
A preliminary experiment in which HL-60 and HL-60/N were primed with LPS for either a 1hr or an overnight incubation period was performed and the capacity of the cells for oxidative burst was assessed using the fluorescence based 96-well plate assay. The results (not shown) indicated that both LPS priming periods resulted in a similar increase in ROS release from cells compared to unprimed counterparts, and as such it was decided that the overnight priming would be used in all subsequent experimentation for practical reasons.

Following on from this, the assay brought to light some interesting observations. Figure 4.6 summarises the time course for the induction of H_2DCF-DA fluorescence in HL-60 (fig. 4.6(a)) and HL-60/N (fig. 4.6(b)) following various optimisation treatments (fig. 4.2) over a 4hr incubation time in the presence of probe. On observation of figure 4.6(a) the following findings become apparent:-

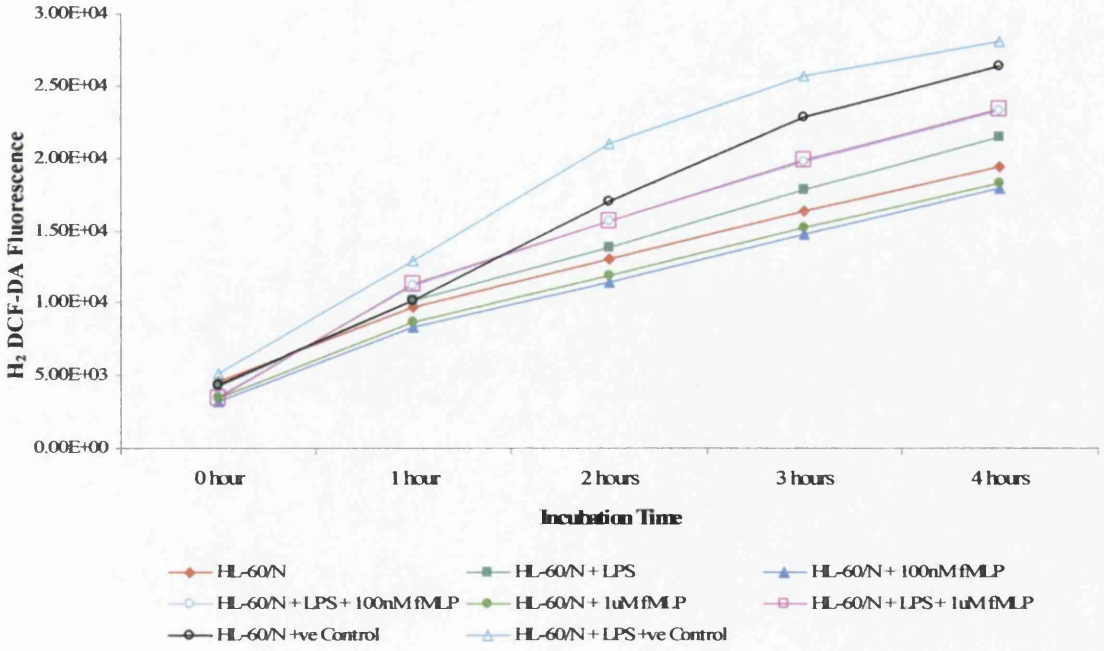
- Overnight priming of cells with LPS results in higher levels of fluorescence and hence ROS generation over the 4hr period compared to unprimed cells;
- Treatment of cells with fMLP alone resulted in a decrease in fluorescence levels compared to untreated HL-60 at both doses used;

Figure 4.6 Time course for the induction of fluorescence in (a) HL-60 and (b) HL-60/N – unstimulated, stimulated with fMLP, primed with LPS, primed with LPS and stimulated with fMLP, or treated with TBHP as a positive control. N = 2.

(a)



(b)



- A combination of overnight priming with LPS plus stimulation of cells with fMLP resulted in enhanced fluorescence induction compared to untreated cells to the same extent for both the 100nM and 1μM doses used, although interestingly the levels of fluorescence were not as intense as that induced by LPS priming alone;
- TBHP caused enhanced fluorescence and hence ROS generation in HL-60, LPS priming followed by TBHP treatment causing the maximal levels of fluorescence out of all treatments;
- The greatest rate of fluorescence induction was observed within the 1hr incubation period.

Figure 4.6(b) brings to light similar findings for HL-60/N:-

- As for HL-60, LPS priming of HL-60/N also resulted in enhanced fluorescence (and hence ROS) levels compared to unprimed cells;
- Both fMLP treatments without prior LPS priming also caused decreased levels of fluorescence in HL-60/N;
- Overnight LPS priming followed by treatment with either 100nM or 1μM fMLP resulted in enhanced levels of fluorescence compared to untreated HL-60/N again to the same extent for both doses, however, in this case, in contrast to HL-60, the coupled priming – stimulation treatments resulted in fluorescence levels greater than the levels induced by LPS priming alone;
- TBHP also caused enhanced fluorescence in HL-60/N, with the LPS priming followed by TBHP treatment again causing the maximal levels of fluorescence out of all treatments;
- As for HL-60, the greatest rate of fluorescence induction in HL-60/N was observed within the 1hr incubation period.

Based on these observations in HL-60 the effect of the treatments on ROS generation could overall be ordered as:-

HL-60 + LPS +ve control > **HL-60 +ve control** > HL-60 + LPS > HL-60 + LPS + 100nM fMLP = HL-60 + LPS + 1μM fMLP > HL-60 > HL-60 + 100nM fMLP > HL-60 + 1μM fMLP with respect to the levels of fluorescence and hence oxidative burst induction.

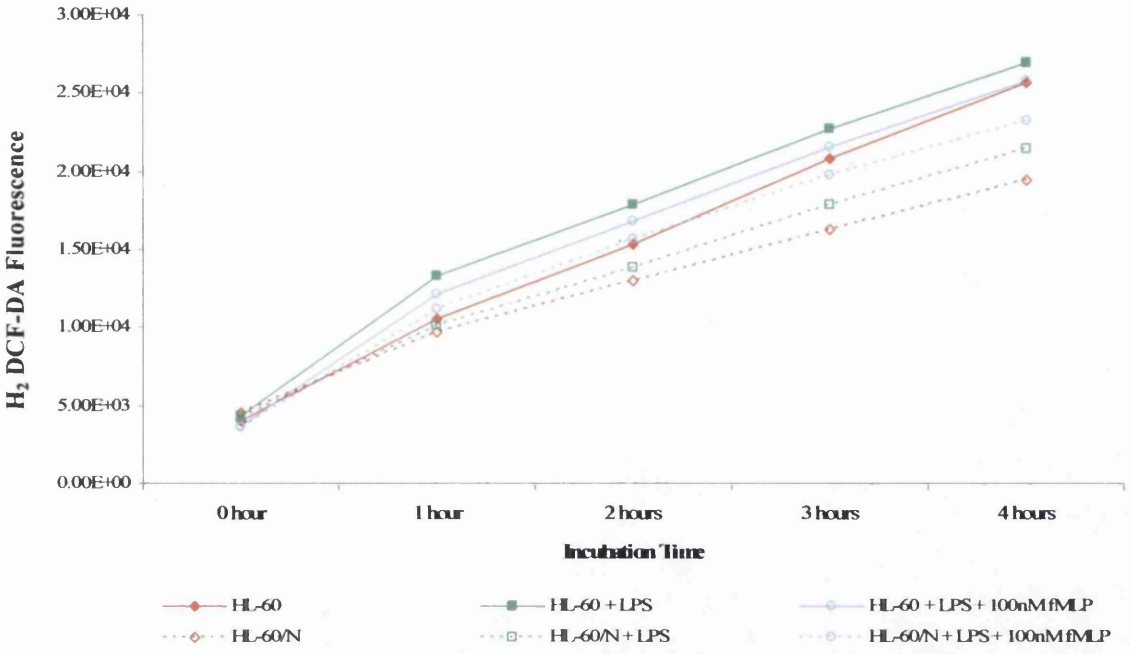
In HL-60/N the overall order of treatments was:-

HL-60/N + LPS +ve control > **HL-60/N +ve control** > HL-60/N + LPS + 100nM fMLP = HL-60/N + LPS + 1μM fMLP > HL-60/N + LPS > HL-60/N > HL-60/N + 100nM fMLP > HL-60/N + 1μM fMLP.

These findings indicate that both HL-60 and HL-60/N can be successfully stimulated to undergo oxidative burst which can be detected using this sensitive 96-well plate based assay, and that the levels of fluorescence, and hence ROS generation, increase over time and persist over the 4hr incubation period. Overnight LPS priming enhances this effect. Interestingly both doses of fMLP induced similar levels of fluorescence. Aside from the positive control treatments, LPS priming alone appeared to have the greatest effect on ROS generation in HL-60, followed by the coupled LPS priming – fMLP stimulation treatments, whilst in HL-60/N the coupled priming – stimulation treatments had the greatest effect. The fact that both the 100nM and 1μM fMLP doses caused very similar levels of fluorescence induction lend support to the results of the NBT assay in which the two doses appeared to have similar effects on the 3 day DMSO differentiated HL-60/N cells, the 100nM dose causing slightly higher induction of NBT reduction compared to the 1μM dose (fig. 4.4). Based on this observation it is apparent that the use of either dose in subsequent experimentation would be sufficient to induce the desired effect of oxidative burst induction.

Interestingly, comparison of the time course of fluorescence induction in HL-60 and HL-60/N revealed that HL-60 may have an overall slightly greater inherent capacity for inflammatory oxidative burst, since untreated HL-60 appeared to cause greater levels of fluorescence induction than HL-60/N and HL-60/N + LPS, and showed very similar results to HL-60/N + LPS + 100nM fMLP (fig. 4.7). This result suggests that both HL-60 and HL-60/N can successfully undergo oxidative burst, and as such either could potentially be used in the final co-culture experiments.

Figure 4.7 Fluorescence induction in HL-60 and HL-60/N controls and following either overnight LPS priming, or a combination of LPS priming and stimulation with 100nM fMLP. N = 2.



An attempt was made to produce a calibration curve of fluorescence against H_2O_2 concentration since H_2DCF-DA can detect H_2O_2 as well as other ROS such as superoxide (Trayner *et al.*, 1995). The doses analysed were 50nM, 100nM, 500nM, 1μM, 10μM, 50μM, 100μM, 200μM, and 500μM. On analysis of the data (not shown) it became evident that the linear detection range for H_2O_2 appeared to be between 200 and 500μM and did not appear to be very accurate or useful for predicting the amount of H_2O_2 released by the cells. In the context of the aims of the present experimentation demonstrating that HL-60/ HL-60/N cells could be induced to undergo oxidative burst was more fundamental than quantification of the amount of ROS released.

4.3.4 Flow Cytometry

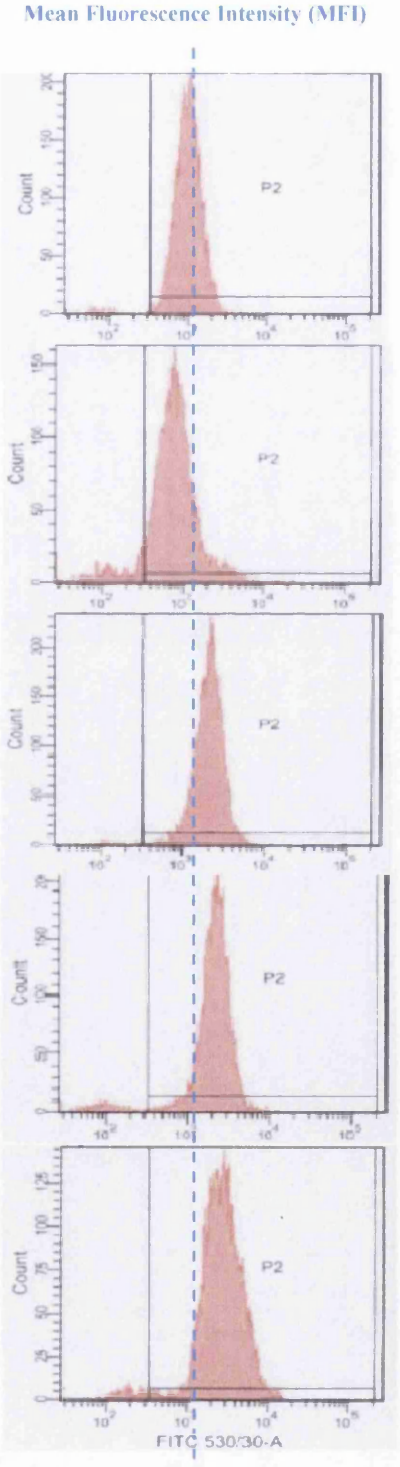
Flow cytometric analysis of HL-60 and HL-60/N cells following a variety of treatments (table 4.1) brought to light a high amount of variation in the oxidative burst

response of the cells. This is likely to reflect a number of cellular and practical reasons. For instance, several reports indicate that the probe can be spontaneously oxidised so yielding fluorescence even in the absence of stimuli (Trayner *et al.,* 1995; Robinson *et al.,* 1994), and that this is commonly due to mitochondrial oxidants, and varies from cell type to cell type (Robinson *et al.,* 1994). Fluorescence may also be detected in seemingly healthy untreated cells due to the induction of apoptosis over time (since apoptosis is linked to increased ROS levels (Macho *et al.,* 1997; Zamzami *et al.,* 1995)), or some other oxidative event. A practical problem that appeared to be encountered was that of quenching/ photo-bleaching, since the amount of fluorescence appeared to decrease over the 45min period, compared to the expected increase on the basis of the other results observed (section 4.3.3). This decrease in fluorescence may also be due to loss of probe from the cells by gradual leakage which has been reported (Ubezio and Civoli, 1994). Another potential problem is one of cell clumping. Activated inflammatory cells have as tendency to clump due to enhanced cell adhesiveness, and the cell clumps can hinder flow cytometric analysis and so bypass detection (Robinson *et al.,* 1994). As such, a large proportion of activated cells may go unmeasured, causing underestimation of oxidative burst activity.

Aside from these practical pitfalls, observation of the data overall revealed some interesting findings, which can be seen from figure 4.8 for HL-60/N cells (undifferentiated HL-60 showed a similar pattern (unless otherwise stated), but to a lesser extent):-

- HL-60/N appeared to be more responsive to fMLP stimulation compared to HL-60;
- fMLP treatment alone caused a decrease in the percentage of fluorescent cells compared to untreated cells, but did not cause marked changes in the mean fluorescence intensity (MFI);
- LPS priming appeared to cause enhanced oxidative burst response in HL-60/N, causing an increase in the MFI, for example, 30min after probe loading, MFI increased approximately 2-fold in LPS primed HL-60/N compared to unprimed counterparts (fig. 4.8);

Figure 4.8 Flow cytometry charts showing DCF fluorescence intensity (FITC 530/30) of HL-60/N cells following 30min incubations with or without stimuli. Positive control cells were treated with 5μM TBHP. See text for further details. Mean Fluorescence Intensities (MFI) significantly different than in control cells ($P < 0.05$) are highlighted (*) based on one way ANOVA. Percentage fluorescent cells significantly different to that in the control are also highlighted (†). N = 2.



Control

% Fluorescent cells: 98.0%

MFI: 1.10

+ 1μM fMLP

% Fluorescent cells: 90.9% †

MFI: 1.12

+ LPS

% Fluorescent cells: 98.1%

MFI: 2.24 *

+ LPS + 1μM fMLP

% Fluorescent cells: 95.0%

MFI: 2.40 *

Positive Control

% Fluorescent cells: 96.5%

MFI: 3.23 *

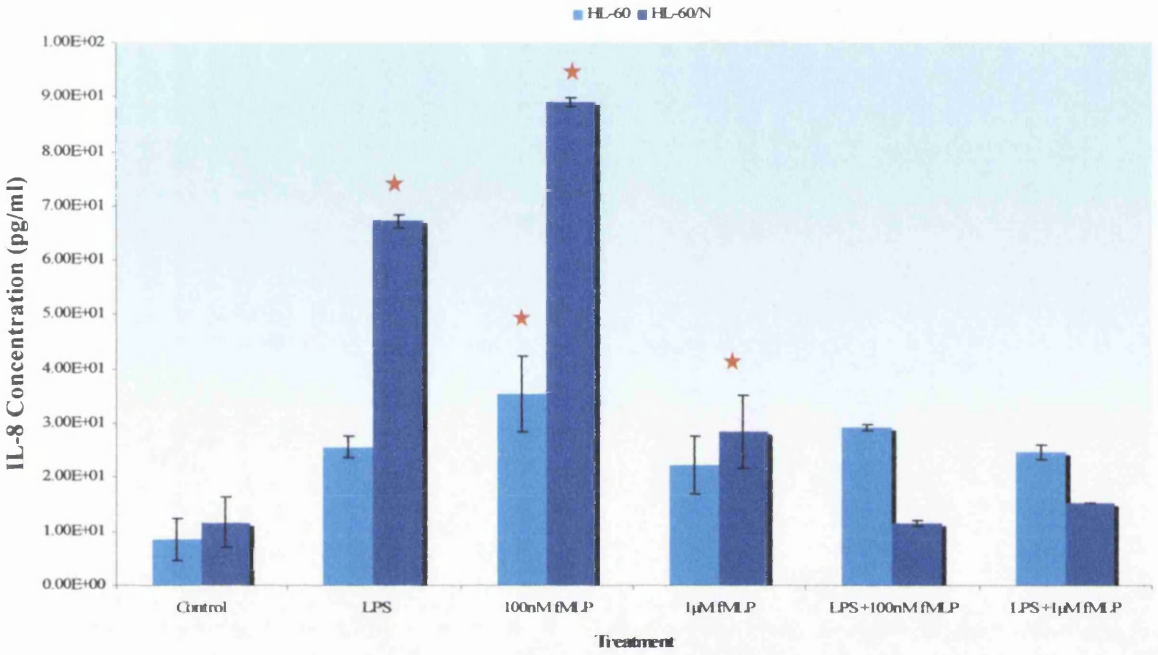
- Coupled LPS priming - fMLP stimulation resulted in enhanced MFI in both HL-60 and HL-60/N, e.g. approximately 2.2-fold increase in LPS primed and 1μM fMLP stimulated HL-60/N (fig. 4.8). The coupled regimen appeared to cause some decrease in the percentage of fluorescent cells, possibly owing to fMLP toxicity, since treatment of cells with fMLP alone also caused a decrease in the percentage of fluorescent cells. It is also possible that the LPS and fMLP together have a combinatorial toxic effect on the cells;
- Fluorescence was detected in cells at time zero at levels comparable to that detected at 10 and 30min. This may be due to the inherent capacity of unstimulated HL-60 and HL-60/N to undergo oxidative burst, or may be due to other cellular oxidative processes (e.g. mitochondrial in origin), or could be due to auto-fluorescence of the probe;
- By 45min the fluorescence intensity appeared to fall in both HL-60 and HL-60/N following all treatments compared to the 0, 10, and 30min time points. It is possible that this is due to photobleaching of the fluorescent signal over time, leakage of the probe from cells (Ubezio and Civoli, 1994), or loss of cells by cell death (necrosis and/ or apoptosis).

One way ANOVA revealed that LPS priming, combined LPS priming – fMLP stimulation, and TBHP treatment (positive control) resulted in significantly enhanced fluorescence intensity, and hence ROS generation compared to controls ($P < 0.05$).

4.3.5 ELISA for IL-8

It is apparent from figure 4.9 that some treatments of HL-60 and HL-60/N resulted in markedly elevated IL-8 protein levels released from cells into the culture medium compared to untreated controls. Interestingly untreated cells also exhibited some

Figure 4.9 ELISA detection of IL-8 levels in HL-60 and HL-60/N following various treatment regimens including LPS priming, fMLP stimulation, and coupled LPS priming – fMLP stimulation. Standard error bars are shown. Treatments that caused significant increases ($P < 0.05$) in IL-8 levels released from cells compared to controls are highlighted with red stars (based on one way ANOVA). N = 2.



IL-8 protein expression and this is in line with several observations that polymorphonuclear (PMN) leukocytes like neutrophils can produce and secrete IL-8 both in the unstimulated and stimulated state (Altstaedt *et al.*, 1996; Arnold *et al.*, 1994; Fujishima *et al.*, 1993; Cassatella *et al.*, 1992; Strieter *et al.*, 1992; Bazzoni *et al.*, 1991). In HL-60 all treatments appeared to induce up-regulation of IL-8 protein expression and release from cells, although one way ANOVA revealed that only the 100nM fMLP treatment caused statistically significant increases in IL-8 levels compared to controls (~ 4.1-fold increase). In HL-60/N all treatments that caused up-regulation of IL-8 levels compared to untreated controls were seen to be statistically significant ($P < 0.05$). Interestingly these were treatments in which cells were treated either with LPS priming alone (~ 5.8-fold increase over control) or fMLP stimulation alone (both doses caused significant IL-8 up-regulation ~ 7.6-fold at 100nM and ~ 2.4-fold at 1μM). Treatments in

which cells were LPS primed and stimulated did not appear to cause notable changes in IL-8 levels, perhaps owing to some antagonistic effects of LPS and the fMLP on *IL-8* gene expression. This may also be related to the increase in ROS in the cells, since coupled priming and stimulation was seen to cause elevated ROS generation in the cells (sections 4.3.3 and 4.3.4). Elevated ROS levels in neutrophils has been linked to apoptosis (Splettstoesser and Schuff-Werner, 2002) and so may account for the low IL-8 levels observed.

Overall HL-60/N appeared to be more sensitive to an inflammatory response at the level of enhanced IL-8 expression and release compared to HL-60.

4.4 Discussion

The aims of the present chapter were to develop an optimised inflammatory cell model consisting of neutrophils which could be stimulated to undergo an oxidative burst, and so could be used in subsequent co-culture experiments. The co-culture experiments would comprise the activated neutrophils co-incubated with gastric epithelial cells so providing a model of inflammatory processes that may be important in early gastric carcinogenesis (starting at gastritis), with a particular emphasis on oxidative stress, by the induction of signal transduction and gene expression changes.

For the inflammatory cell component of the co-culture model the HL-60 cell line was employed. This cell line is a pluripotent immature promyelocytic cell line (Collins *et al.,* 1977) that can be induced to differentiate into morphologically and functionally mature myelocytes. The challenges faced were to optimise a differentiation regimen that could induce both morphological, and more importantly, functional maturity at the level of oxidative burst response in the cells so that a situation of inflammation derived oxidative stress could be introduced in the co-culture system. The experimentation described in this chapter aimed to first optimise the induction of HL-60 differentiation to neutrophils, and then optimise the induction of an inflammatory oxidative burst response, using a variety of techniques to ascertain the success or failure of treatments.

Differentiation of HL-60 was seen to be successfully induced by 3 day incubation in the presence of 1.3% (v/v) DMSO. Whilst a typical differentiation regime may involve longer incubation periods (Collins *et al.,* 1978), several reports suggest that incubation of cells in the presence of 1.25 – 1.3% (v/v) DMSO can lead to marked loss of cell viability and induction of apoptosis after the 3rd or 4th day in culture (Mollinedo *et al.,* 1998; Martin *et al.,* 1990). Furthermore it has been noted that HL-60 grown in the presence of DMSO for 1-3 days exhibited mature neutrophil functions including phagocytosis, oxidative burst, and degranulation (Newburger *et al.,* 1979). Indeed both morphological assessment (by way of microscopy) and functional biochemical assessment (by way of NBT assay) confirmed that a 3 day differentiation regimen induced significant morphological and functional differentiation of cells, giving rise to a population of cells (HL-60/N) that resembled mature myelocytes capable of oxidative burst induction following stimulation with fMLP. The subsequent experimentation aimed to further ascertain and optimise the oxidative burst response in HL-60 cells. Since it has been reported that both undifferentiated and differentiated HL-60 are capable of oxidative burst due to the possession of NADPH oxidase (Muranaka *et al.,* 2005), both HL-60 and HL-60/N were used in the optimisation efforts.

LPS priming, in accordance to several published studies (DeLeo *et al.,* 1998; Nielsen *et al.,* 1994; Aida and Pabst, 1990; Vosbeck *et al.,* 1990; Forehand *et al.,* 1989; Worthen *et al.,* 1988; Haslett *et al.,* 1985; Guthrie *et al.,* 1984), was seen to enhance the oxidative burst capacity of HL-60 and HL-60/N compared to unprimed counterparts when ROS levels in cells were assessed using the fluorescence based 96-well plate assay and flow cytometry. fMLP treatment of cells was seen to cause a decrease in the fluorescence of cells (and hence ROS generation) detected by both the 96-well plate assay and flow cytometry, whilst coupled LPS priming – fMLP stimulation resulted in enhanced fluorescence, with maximal ROS generation detected at 30min using flow cytometry, and persisting over a 4hr period (detected using the 96-well plate based method).

For the most part the 96-well plate assay and the flow cytometry data appeared to correlate, the only discrepancy was that the 96-well plate assay showed that HL-60 may be more sensitive to oxidative burst induction, whilst flow cytometry data suggested that

HL-60/N cells were more responsive. It is likely that this is due to the practical reasons previously discussed (e.g. auto-oxidation of probe, photobleaching, etc.). The advantage of the flow cytometry technique over the 96-well plate assay is that the former allows the detection of the percentage of fluorescent cells and the mean fluorescence intensity (MFI) due to the single cell analysis capabilities of the technique, whilst the 96-well plate assay simply detects overall fluorescence in a well containing ~ 1 x 10^5 cells. A high fluorescence reading in the 96-well plate assay may be the result of a low percentage of highly fluorescent cells (i.e. very high ROS levels in few cells), or a high percentage of dull fluorescent cells (i.e. low ROS levels in many cells), and so the flow cytometry data provides more insight into the population of cells undergoing oxidative burst. Care must also be taken when examining data on ROS detection since ROS can arise from a number of sources aside from oxidative burst, e.g. mitochondrial ROS (Robinson *et al.*, 1994), apoptotic cells (Macho *et al.*, 1997; Zamzami *et al.*, 1995), and are important in the normal physiology and biochemistry of cells (detailed in section 1.6 and chapter 3).

Aside from this discrepancy, overall the two techniques for assaying inflammatory oxidative burst revealed some interesting findings. Noteworthy observations include the apparent oxidative burst capacity of both HL-60 and HL-60/N in the absence of stimuli which has been previously reported (Trayner *et al.*, 1995) (although this may in part be due to spontaneous oxidation and hence fluorescence of the probe (Trayner *et al.*, 1995; Robinson *et al.*, 1994)). As already mentioned, in both cell types LPS priming caused enhanced fluorescence caused by enhanced ROS generation, as did the coupled LPS priming – fMLP stimulation treatment. Interestingly, in HL-60, LPS priming alone appeared to induce a greater oxidative burst response than LPS priming and fMLP stimulation, whilst in HL-60/N, the reverse was true, indicating that HL-60/N may show a response more true to mature neutrophils in which priming is important (Casimir and Teahan, 1994). In both HL-60 and HL-60/N maximal ROS generation was induced by the positive controls (TBHP, LPS priming + TBHP).

In addition to ROS generation, activated neutrophils also release inflammatory mediators such as IL-8 (Altstaedt *et al.*, 1996; Arnold *et al.*, 1994; Fujishima *et al.*, 1993; Cassatella *et al.*, 1992 Strieter *et al.*, 1992; Bazzoni *et al.*, 1991), which may act alone (Kitadai *et al.*, 2000), or in conjunction with ROS (Sasayama *et al.*, 1997; Wozniak *et al.*,

1993) to induce cellular changes involved in gastric carcinogenesis. ELISA data made evident the enhanced cellular release of IL-8 protein following various treatments. LPS priming alone and fMLP stimulation alone resulted in increased IL-8 levels in cell culture medium in both HL-60 and HL-60/N, whilst dual priming – stimulation caused slight yet insignificant increases in IL-8 levels in HL-60, with no such effect seen in HL-60/N. This may be due to an increase in apoptosis (HL-60/N are more sensitive to apoptosis than HL-60 since they are terminally differentiated) which may be linked to the enhanced ROS levels (Macho *et al.*, 1997; Zamzami *et al.*, 1995) induced by these treatments, so hindering IL-8 protein expression. Besides this observation, HL-60/N appeared to be more responsive with respect to IL-8 release, the levels detected reflecting those seen in *H. pylori* infected gastric mucosa (50 – 100pg/ml) (Yamaoka *et al.*, 1998).

On a broader scale, review of all of the data obtained in the optimisation experiments brought to light the following:-

- HL-60/N were overall more responsive than HL-60 with respect to mature inflammatory functions at the level of oxidative burst response and IL-8 release, and so will be used in the final co-culture model (chapter 5);
- Overnight LPS priming significantly enhanced oxidative burst induction and IL-8 release in HL-60/N and so will be used in the final model;
- Combined LPS priming – fMLP stimulation caused more marked generation of ROS in HL-60/N compared to other treatments with both the 100nM and 1μM doses giving rise to similar effects. As such the combined priming – stimulation treatment regimen will be used in co-culture experimentation, with the 100nM fMLP dose being employed to avoid potential problems associated with fMLP toxicity.

Thus, using a variety of optimisation treatments and methodologies to detect their success in inducing neutrophil differentiation and oxidative burst induction, an optimised inflammatory cell model has been developed. Co-incubation of HL-60/N, LPS primed and stimulated with 100nM fMLP for at least 30min (up to 4hr) with gastric epithelial cells should thus lead to a situation of oxidative stress that may be representative of that in gastritis tissues (Shimoyama *et al.*, 2002). Since some of the generated metabolites

such as H_2O_2 can diffuse out of the neutrophils and into the cell culture microenvironment; other ROS also potentially being released by way of phagocytosis, membrane blebbing, and apoptosis; the effects of the ROS on signal transduction and gene expression in gastric epithelial cells can then be evaluated.

Chapter 5

Gastric Epithelial Cell – Inflammatory Cell Co-culture Studies Part II: *Signal Transduction and Gene expression Analysis of Gastric Epithelial Cells in Co-culture*

5.1 Introduction

Gastric cancer is a complex disease in which chronic inflammation of the gastric mucosa (gastritis) is a key early stage in the pathogenesis of the intestinal subtype of the disease (detailed in sections 1.2.1 and 1.3.1). Inflammatory processes are themselves complex – consisting of a highly integrated and controlled network of inflammatory cells, soluble inflammatory mediators, host cells, metabolic by-products, etc. Under normal circumstances this inflammation is tightly controlled and resolved as soon as the infectious agent is removed and/ or the tissue injury healed. Unfortunately in some instances this inflammation may persist, giving rise to chronic inflammatory conditions in which the tissues in the vicinity are constantly exposed to a plethora of inflammatory components which can inflict damage on the host cells. As such chronic inflammation is now recognised as a key driving force in the pathogenesis of several diseases including cancer (section 1.7). In order to further understand the mechanistic nature of the link between chronic inflammation and cancer it is fundamental to gain insights into the complex interactions between inflammatory components and the host tissues.

5.1.1 Tumour Microenvironment

The involvement of host cell – tumour cell interactions and tumour microenvironment in cancer development and progression has had a long standing history, tracing back to 1889 when Stephen Paget wrote:

"When a plant goes to seed, its seeds are carried in all directions, but they can only live and grow if they fall on congenial soil." (Paget, 1889),

hypothesising that the same was true for tumour cells. This theory lay low for almost a century, albeit receiving some attention in a pivotal monograph by Tarin (1972) entitled 'Tissue interactions in carcinogenesis', and saw a greater revival in 1980 when Hart and Fidler demonstrated that tumour metastasis to other organ sites was not random but rather seemed to be targeted to sites with a more favourable microenvironment (Hart and Fidler, 1980). Intense research has followed for over two decades of recognition of the complexity of the disease (Rubin, 1985), and the role of tumour microenvironment has become increasingly well appreciated in recent years (de Visser and Coussens, 2006; Glick and Yuspa, 2005; Rubin, 2003; van Kempen *et al.*, 2003; Ahmad *et al.*, 2002; Cunha and Matrasian, 2002; Cunha *et al.*, 2002; Skobe and Fusenig, 1998). Tissue microenvironment is known to be central to normal development (Anderson *et al.*, 2000; Bissell *et al.*, 1999; Torok-Storb *et al.*, 1999) so it is unsurprising that it plays a key role in determining tumour cell phenotype and cancer progression. The tumour 'stroma' collectively describes all of the components in the local vicinity of the tumour excluding the tumour cells themselves, and consists of a complex array of molecular, mechanical, and cellular components. Both the stromal cells and molecular factors such as cytokines, enzymes, extracellular matrix (ECM) components, etc. play central roles, and complex cross-talk between the cells and molecules is critical in determining the malignant phenotype of the cells in the vicinity (Eshel *et al.*, 2002; Park *et al.*, 2000; Yuan and Glazer, 1999; Witz *et al.*, 1996). The stroma has both temporal and spatial complexity, showing tissue specificity and a high degree of heterogeneity within and between tumours and changes dynamically as a tumour progresses.

The stroma/ tumour microenvironment may influence disease progression by three distinct yet over-lapping mechanisms: -

1. By enhancing genetic instability of tumour cells via mutagenesis (Yuan and Glazer, 1999). For example, chronic inflammation may lead to genetic instability via mutations induced by phagocyte generated ROS (Weitzman and Gordon, 1990) (see section 6.1.2.1);

2. By inducing signalling cascades in tumour cells and thus altering gene expression patterns (Liotta and Kohn, 2001; Bissell *et al.*, 1999; Witz *et al.*, 1996); and
3. By exerting selective pressures on cells – some molecules in the microenvironment may drive cancer development, whilst others may inhibit tumourigenicity (Witz *et al.*, 1996).

The tumour microenvironment (now viewed as the cells and molecules within the tumour itself as well as the host cells in the tissue surrounding the tumour, i.e. the host-tumour interface (Liotta and Kohn, 2001)) can thus act as a double edged sword, since some components may inhibit tumourigenicity and display anti-metastatic properties, whilst others may exert tumourigenic or pro-metastatic effects, the outcome largely depending on the context as a whole and the balance between the factors (Wernert, 1997; Witz *et al.*, 1996). These observations imply that tissue microenvironment is important at all stages of malignant disease, and is likely to have a key role in pre-malignant stages.

Epithelial cancers at different sites in the body frequently develop through a series of pre-malignant stages, and the gradual progression to malignancy is often characterised by increasingly abnormal communications between cells in the tumour microenvironment, i.e. between the pre-malignant epithelial cells and the stromal cells. In some cases the likelihood of initiating mutations in the epithelial cells may be further enhanced by the microenvironment generated by the stromal cells, whilst in other instances changes in the stromal cells (mutations, gene expression changes, etc.) may lead to a reactive stroma which becomes the tumourigenic agent itself, giving rise to an unstable epithelium. This is exemplified by chronic inflammation in gastric tissues driving gastric carcinogenesis which was discussed in some detail in sections 1.3.1, 1.4.5.1, and 1.7, and briefly below.

5.1.2 Chronic Inflammation and Oxidative Stress in Gastric Cancer

The well established link between chronic inflammation and cancer was discussed in detail in section 1.7 as well as in previous chapters. The importance of inflammation in

the pathogenesis of cancer has received such recognition in recent years that it even featured as a cover story in the February 23rd 2004 issue of Time magazine entitled 'The Secret Killer' (**http://www.time.com/time/magazine/article/0,9171,993419,00.html**).

In the context of microenvironment chronic smouldering inflammation generates a hostile stroma upon which the malignant potential of pre-malignant stages of the disease can be enhanced. The inflammatory effector cells found in the microenvironment of a tumour together with soluble mediators of inflammation including cytokines and leukotrienes, and enzymes such as COX-2 and matrix metalloproteinases (MMPs), as well as leukocyte - (and sometimes epithelial -) generated ROS make up an inflammatory milieu that is tumour promoting (Greten *et al.*, 2004; Pikarsky *et al.*, 2004; Wilson and Balkwill, 2002). This can occur via activation of growth factors, as well as signal transduction and gene expression changes. In addition to the tumour promoting effect of chronic inflammation, the inflammatory cells have also been reported to induce initiating mutations via the generation of bactericidal and unfortunately for the host, mutagenic ROS (Weitzman *et al.*, 1990). ROS have been implicated in the pathogenesis of several cancers, oxidative DNA damage induced by ROS being implicated in up to 50% of all human cancers (Beckman and Ames, 1997). In addition, ROS can have a tumour promoting effect by inducing epigenetic changes such as signal transduction and gene expression changes (sections 3.1.1 – 3.1.4). Aberrant cell signalling can come about by way of changes in extracellular growth signals (frequently a consequence of mutations and/ or changes in local tissue microenvironment), changes in the cell surface receptors, and/ or changes in the intracellular signalling cascades that translate the signal to the nucleus (Fedi *et al.*, 1997). Indeed all three of these mechanisms can be induced by excessive ROS, and hence oxidative stress, in the tissue microenvironment (Nathan, 2003; Garcia-Ruiz *et al.*, 1997; Huang *et al.*, 1996; Rao, 1996; Rosette and Karin, 1996; Sen and Packer, 1996; Lo and Cruz, 1995). The effects of ROS on the MAPK and NFκB signalling pathways, two pathways heavily involved in inflammatory signalling and inflammation associated cancers (Baek *et al.*, 2004; Seo *et al.*, 2004; Chu *et al.*, 2003; Chang and Karin, 2001; Li and Karin, 1999), were discussed in detail in chapter 3.

Chronic inflammatory microenvironments can commonly be observed in the gastrointestinal tract, manifest as fairly common conditions such as oesophagitis and

gastritis which can be caused by a variety of factors (MacNaughton, 2006). Gastric cancer of the intestinal subtype has been causally linked to chronic inflammation, progressing through a series of pre-malignant stages (Correa, 1992a) starting with chronic superficial gastritis (chronic inflammation of the gastric mucosa), and progressing through atrophic gastritis, intestinal metaplasia, dysplasia, and ultimately gastric cancer.

The ultimate 'driving force' of gastric carcinogenesis is believed to be chronic gastric inflammation (gastritis) regardless of its cause (Zavros *et al.,* 2005) and is discussed in greater detail in chapter 6. Probably the most common aetiological agent of chronic gastritis is long term infection with *H. pylori* (Israel and Peek, 2001; Correa, 1992a; Marshall, 1986) which may persist throughout an individual's lifetime generating a harsh inflammatory tissue microenvironment with both cellular and humoral components (Matysiak-Budnik and Mégraud, 2006; Graham and Go, 1993), leading to impairment of tissue structure and function. As detailed in sections 1.2.1 and 1.4 – 1.4.5.1, gastric cancer has a complex aetiology in which several factors come into play (age, sex, diet, *H. pylori* infection, etc.), and is further complicated by interactions between factors - both host and bacterial, including host genetic susceptibility, immune response, age at acquisition of infection, and bacterial strain (related to virulence) (section 1.4.5). Such interactions can influence the extent of the chronic inflammation and so the risk of developing gastric cancer, and also provides a possible explanation as to why only a small minority (< 1%) of *H. pylori* infected individuals go on to develop the disease (Correa, 1995).

A possible mechanism through which chronic inflammation may promote gastric carcinogenesis is via oxidative stress, an unavoidable by-product of chronic inflammatory processes, originating from several sources including leukocytes, lymphocytes, and pro-inflammatory cytokines. In *H. pylori* infection inflammatory leukocytes generate and release potent ROS (Zhang *et al.,* 1996; Rautelin *et al.,* 1994) and oxidative stress may also result through the accumulation of bacterial generated superoxide (Nagata *et al.,* 1998), as well as from ROS released by gastric epithelial cells following exposure to bacteria (Ding *et al.*, 2007; Xu *et al.,* 2004; Obst *et al.,* 2000; Bagchi *et al.,* 1996; Davies *et al.,* 1994a; Davies *et al.,* 1994b), and as a consequence of reduced gastric ascorbic acid levels (Hohenberger and Gretschel, 2003; IARC, 1994; Ruiz *et al.,* 1994). Indeed this

chronic infection associated oxidative stress has an impact on cells at the molecular level, being seen to cause marked increases in DNA damage (Farinati *et al.,* 2003; Obst *et al.,* 2000) as well as an increase in the mutation rate (Jenks *et al.,* 2003; Touati *et al.,* 2003); aberrations of cellular signal transduction and gene expression changes (Baek *et al.,* 2004; Seo *et al.,* 2004; Chu *et al.,* 2003; Kim *et al.,* 2001; Lim *et al.,* 2001; Kim *et al.,* 2000; Nardone *et al.,* 1999); and shifts in the proliferation/ apoptosis balance in favour of enhanced cell survival and proliferation (Pritchard and Crabtree, 2006; van Grieken *et al.,* 2003; Correa and Miller, 1998; Piotrowiski *et al.,* 1997; Bechi *et al.,* 1996; Fan *et al.,* 1996; Moss *et al.,* 1996). Together these changes favour the development of neoplastic disease and provide links between chronic inflammation, oxidative stress, and the pathogenesis of gastric cancer.

5.1.3 Aims of the Chapter

Since the overall aim of the present research was to analyse the relationship between inflammation and gastric cancer with an emphasis on signal transduction and gene expression changes; initially using *in vitro* models of inflammatory components and later shifting to an *in vivo* molecular study using inflamed gastric tissue specimens; the present chapter of work set out to investigate the effect of inflammatory leukocytes on signalling and gene expression changes in gastric epithelial cells. In this way the *in vivo* tissue microenvironment in gastritis characterised by dense neutrophil and macrophage infiltrates and a plethora of inflammatory factors including ROS can more closely be mimicked and studied, so providing a steady transition from the initial *in vitro* study with frank H_2O_2 exposures (chapter 3) to the *in vivo* study (chapter 6).

Since cancer has commonly been described as a disease of aberrant signal transduction (Arbiser, 2004; Eshel *et al.,* 2002; Radisky *et al.,* 2001; Fedi *et al.,* 1997; Hunter, 1997; Kohn *et al.,* 1992) it is fundamental to obtain insights into how such changes can be induced. Mis-regulation of the ERK1/2 (p42/p44) MAPK and NFκB signalling cascades, which control the activity of the AP-1 and NFκB transcription

factors respectively, has frequently been reported in *H. pylori* infected gastric tissues and gastric cancer (Maeda *et al.*, 2000; Meyer-Ter-Vehn *et al.*, 2000; Naumann *et al.*, 1999; Aihara *et al.*, 1997; Malinin *et al.*, 1997; Muller *et al.*, 1997). Both of these pathways have been reported to be activated by RO/NS (Genestra, 2007; Gloire *et al.*, 2006; McCubrey *et al.*, 2006; Takada *et al.*, 2003; Jackson *et al.*, 2002; Lee and Esselman, 2002; Zhang *et al.*, 2001; Bowie and O'Neill, 2000) and further support for this was provided in chapter 3. In addition, one mechanism by which *H. pylori* drives gastric carcinogenesis is through oxidative stress (Xu *et al.*, 2004; Farinati *et al.*, 2003; Obst *et al.*, 2000; Asaka *et al.*, 1997; Bagchi *et al.*, 1996; Correa, 1988) derived from bacterial, and host sources (gastric epithelium and inflammatory leukocytes), and so it is quite plausible that inflammation associated oxidative stress may cause such changes in the gastric mucosa, with inflammatory leukocytes likely playing a key role, providing a link between *H. pylori* infection and gastric cancer development.

The effect of inflammatory leukocytes was studied using the co-culture system optimised in the previous chapter. Briefly this involved co-culturing HGC-27 gastric epithelial cells (derived from gastric adenocarcinoma) with the HL-60 promyelocytic cell line that had been induced to differentiate into myelocytes, metamylocytes, and banded neutrophils by culturing cells in the presence of 1.3% (v/v) dimethyl sulfoxide (DMSO) for 3 days prior to co-culture. The resultant population of differentiated cells was deemed HL-60/N for the presence of neutrophils, and was primed overnight with LPS and co-cultured with HGC-27 at different densities. Only the HGC-27 cell line was used in the co-culture studies for practical reasons since both gastric epithelial adenocarcinoma cell lines studied showed similar gene expression and signalling responses in regard to *c-FOS* and *IL-8* expression and ERK1/2 (p42/p44) activation (chapter 3), and of the two cell lines, HGC-27 could be established in steady cell culture more readily and had already been more extensively studied through the array experiments (chapter 3). Since the extent of inflammatory cell infiltrate can vary widely in chronic gastritis, and the extent of inflammation is a recognised risk factor for the development of malignant gastric disease, with more severe inflammation being associated with increased risk (Fox and Wang, 2007; Axon, 2002; El-Omar *et al.*, 2000; Correa, 1995; Gilmour, 1961), HGC-27 was co-cultured with HL-60/N at different ratios in order to reflect different severities of

inflammation. The introduction of 100nM fMLP to the co-culture system stimulated activation of HL-60/N and so the induction of an oxidative burst response. As such the influence of leukocyte derived oxidative stress on HGC-27 at the level of signal transduction and gene expression changes – specifically the ERK1/2 (p42/p44) MAPK pathway (from here-on-in ERK1/2 (p42/p44) will be referred to as ERK) and *c-FOS* and *IL-8* gene expression (in keeping with the previous studies in chapter 3 and further studies in chapter 6) – could be assessed. *c-FOS* expression was assessed as a marker of ERK MAPK signalling since the two were seen to be linked in preliminary experimentation described in chapter 3. In a similar manner *IL-8* was studied as a marker of NFκB activity since *IL-8* expression has been reported to be regulated by NFκB (Jenkins *et al.*, 2004). *VEGF* and *IκB* were not studied in the present work since one marker for each pathway was adequate to gain insights into gene expression and signalling changes induced by co-culture.

5.2 Materials and Methods

5.2.1 Cell Culture of HGC-27

The gastric adenocarcinoma cell line HGC-27 was cultured as described in sections 2.1.1.1 – 2.1.1.4 and 3.2.1. For co-culture experimentation cells between passages 5 – 15 were used, and cells were seeded into 80cm^2 cell culture flasks at a density of 1 x 10^5 cells/ml and incubated at 37°C in an atmosphere of 5% CO_2 overnight prior to the addition of primed HL-60/N. Several flasks of cells were prepared for the co-culture experiments (see section 5.2.3).

5.2.2 Cell Culture, Differentiation, and Priming of HL-60

HL-60 were cultured as described in sections 2.1.1.6 and 4.2.1, induced to differentiate into neutrophils or neutrophil-like cells (mature myelocytes, metamyelocytes, banded neutrophils; population collectively termed HL-60/N), and

primed with LPS following the optimised methods outlined in sections 4.2.2, 4.2.4, and 4.2.5 that were shown to successfully optimise subsequent induction of a measurable oxidative burst response in HL-60/N. Only cells with a viability of $\geq$ 90% were used in subsequent co-culture experimentation. Briefly this involved seeding HL-60 at a density of 1 x 10^6 cells/ml, and allowing cells to grow for 3 days in the presence of 1.3% (v/v) DMSO in order to induce neutrophil differentiation. On the third day in culture LPS was introduced at 100ng/ml and cells incubated at 37°C overnight in order to prime cells for further stimulation. Subsequently cells were harvested by centrifugation at 200 x g (1500rpm) for 5min, and washed twice in pre-warmed (37°C) PBS followed by another centrifugation step to remove all traces of LPS. Six flasks of cells were prepared in the exact same manner in order to achieve the high cell numbers needed for all co-culture experiments. The cells harvested from the six flasks of differentiated and LPS primed HL-60/N were pooled and re-suspended in serum free RPMI (plus 2mM L-glutamate) (Gibco-BRL, Paisley, UK) to a final volume and density of cells that could accommodate for all co-culture experiments (see section 5.2.3 below).

5.2.3 Co-Culture of HGC-27 and HL-60/N

To mimic the gastric tissue inflammation characteristic of gastritis HGC-27 gastric epithelial cells were co-incubated/ co-cultured with primed HL-60/N for 4hr and 8hr periods. Since the density of inflammatory cell infiltrate in gastritis can be highly variable, the extent determining the severity of the inflammation, HL-60/N were introduced to the HGC-27 epithelial cell monolayer (at ~ 75% confluence) at a range of densities in order to emulate different degrees of inflammation. This was achieved by adding HL-60/N cell suspension to HGC-27 at HGC-27 to HL-60/N ratios of 1:1, 2:1, and 4:1. Following addition of HL-60/N to HGC-27, an inflammatory oxidative burst response was stimulated by the addition of 100nM fMLP (demonstrated in chapter 4 to successfully induce a measurable oxidative burst response in HL-60/N) to appropriate flasks. The co-culture experiments performed were:-

1. 4hr control – HGC-27 (1 x 10^5 cells/ml) in 10ml serum free DMEM plus 10ml serum free RPMI
2. 4hr 100nM fMLP control – HGC-27 (in serum free DMEM) plus 10ml serum free RPMI plus 100nM fMLP
3. 4hr 1:1 HGC-27: HL-60/N – HGC-27 (1 x 10^5 cells/ml) plus 10ml HL-60/N cell suspension (1 x 10^5 cells/ml) in serum free RPMI to a final cell/ cell ratio of 1:1
4. 4hr 1:1 HGC-27: HL-60/N + 100nM fMLP
5. 4hr 2:1 HGC-27: HL-60/N (1 x 10^5 cells/ml: 0.5 x 10^5 cells/ml) + 100nM fMLP
6. 4hr 4:1 HGC-27: HL-60/N (1 x 10^5 cells/ml: 0.25 x 10^5 cells/ml) + 100nM fMLP
7. 8hr control
8. 8hr + 100nM fMLP control
9. 8hr 1:1 HGC-27: HL-60/N
10. 8hr 1:1 HGC-27: HL-60/N + 100nM fMLP
11. 8hr 2:1 HGC-27: HL-60/N + 100nM fMLP
12. 8hr 4:1 HGC-27: HL-60/N + 100nM fMLP

Following treatments HL-60/N were removed from HGC-27 by decanting the cell suspension from the flasks and washing the HGC-27 monolayer twice with pre-warmed PBS (37°C) to remove all HL-60/N and any traces of fMLP. The effective removal of all inflammatory leukocyte cells was confirmed by examination of cells under the light microscope. HGC-27 and HL-60/N appear very different morphologically, the former being an attached irregular shaped epithelial cell line much larger in size compared to the rounder HL-60/N cells which grow in suspension, and so the cell types can readily be distinguished and the removal of HL-60/N easily determined. Duplicates for each treatment (1 – 12) were carried out so that RNA could be extracted from HGC-27 following treatment and removal of HL-60/N from one flask, and protein extracted from the other for downstream signal transduction and gene expression analysis.

The experiment in its entirety was performed in duplicate on different days, and the RNA and protein yielded from each treatment pooled and analysed.

5.2.4 RNA Extraction from HGC-27

Following co-culture of HGC-27 and HL-60/N, HL-60/N were removed as described in section 5.2.3 and HGC-27 cells harvested by trypsinisation following the methods detailed in section 2.1.1.4. RNA was subsequently extracted as described in section 2.4. Contaminating DNA was removed from the RNA samples yielded using the DNA*free*™ kit (Ambion, Warrington, UK) according to manufacturer's instructions (section 2.4.3) and RNA quality and quantity assessed following the protocols outlined in sections 2.4.5. RNA samples were aliquotted into 20µl aliquots and stored at -80°C until use to avoid excessive freeze thawing. Samples were used for up to two freeze-thaw cycles and then discarded so avoiding problems associated with RNA stability.

5.2.5 Protein Extraction from HGC-27

Total cellular protein was extracted, processed, and quantified from inflammation exposed and control HGC-27 as described in section 2.6. Protein concentration was quantified using the 2D quant kit (GE Lifesciences, Bucks, UK) as detailed in section 2.6.1 and the samples aliquotted into 20µl aliquots and stored at -80°C until use.

5.2.6 Confirmation of Oxidative Impact of Co-culture on HGC-27: *Analysis of Levels of Oxidised Proteins in HGC-27*

Whilst the successful induction of an inflammatory oxidative burst response in HL-60/N was demonstrated in chapter 4, it was essential to test that under co-culture conditions this oxidative burst generated a setting of oxidative stress that directly impacted HGC-27 at the molecular level. This was achieved by assessing the levels of

oxidised proteins in protein extracts obtained from HGC-27 post co-culture (section 5.2.5) using the OxyBlot™ kit (Chemicon, Hampshire, UK) as per manufacturer's instruction. The assay works on the basis that modification of proteins by ROS introduces carbonyl groups into the protein side chains, which can be modified by treatment with 2,4-Dinitrophenylhydrazine (DNPH) giving rise to dinitrophenyl (DNP) residues which can be readily detected using specific anti-DNP antibodies.

The appropriate volumes of samples constituting 15µg protein were aliquotted in duplicate into 1.5ml microfuge tubes (a positive control provided in the kit consisting of oxidised proteins was also included) followed by the addition of 1 volume 12% (w/v) SDS to denature proteins. Subsequently 1 volume (relative to total volume) of 1X DNPH was added to one of each of the duplicate sample tubes, and 1 volume 1X derivatisation-control solution added to the other as a control to ensure that any changes in antibody binding seen were truly due to ROS mediated protein modifications. The tubes were then incubated at room temperature for 15min. The reactions were subsequently stopped by the addition of 37.5% (v/v) neutralisation solution to each tube. Samples were next spotted onto nitrocellulose membranes by carefully pipetting 1µl of sample at a time, allowing the spot to dry and then reapplying until a total of 10µl was loaded for each sample. The membrane was allowed to dry for 45min prior to a 1hr incubation in blocking buffer (1% BSA in PBS/ 0.05% Tween) at room temperature with gentle agitation to block non-specific sites. The primary antibody solution was prepared as a 1:100 dilution of primary rabbit anti-DNP antibody (supplied in kit) in blocking buffer and the membrane incubated in the solution for 1hr at room temperature with gentle agitation. Next the membrane was rinsed 3 X with PBS/ 0.05% Tween before incubating in secondary antibody solution (1:200 dilution of goat anti-rabbit IgG (HRP-conjugated) (supplied in kit) in blocking buffer) for 1hr at room temperature with gentle agitation. After the incubation period the membrane was rinsed 3 X in PBS/ 0.05% Tween and any excess solution drained prior to chemiluminescence detection using the ECL kit (GE Lifesciences, Bucks, UK) and exposure of the membrane to X-ray film (GE Lifesciences, Bucks, UK) as described in section 2.7.3. The final blot was scanned as a JPEG format image and analysed with respect to signal intensity using the ImageJ software downloaded from the NIH website (**http://rsb.info.nih.gov/ij**, see section 2.7.3). The

assay was performed in duplicate on separate days and results expressed as averages of the data obtained.

5.2.7 Real-Time PCR Analysis of *c-FOS* and *IL-8* Levels

In order to determine if inflammatory conditions and leukocyte-derived ROS in the co-culture system lead to changes in ERK MAPK and NFκB signalling, and as such either increases or decreases in the levels of *c-FOS* and *IL-8* downstream gene expression targets, gene expression levels were analysed by real-time PCR.

Real-time PCR was carried out separately for *c-FOS* and *IL-8* using β-actin (*ACTB)* as an internal standard in both cases (see chapter 3, table 3.2 for primer sequences). For all analyses 500ng sample RNA was used and standard curves were generated using pooled RNA from cell culture and gastric biopsy specimens seen to express *c-FOS* and *IL-8*. The same standard curve RNA was used in each plate of the entire experimental run so minimisation potential sources of variation in the data (see section 2.5.1 for further details).

5.2.8 Western Blot Analysis of pERK Levels

Western blots were performed on post co-culture HGC-27 protein samples for pERK in order to assess ERK MAPK activation in the cells following the methods outlined in sections 2.7.3 and 3.2.3.1.

5.2.9 Statistical Analysis

The SPSS version 13.0 software package was used for statistical analysis. One way ANOVA (followed by Tukey and Duncan post hoc tests) was used to compare data in control and treated samples to determine if co-culture with inflammatory leukocytes

induced significant signal transduction and gene expression changes in HGC-27. Statistical significance was achieved when $P < 0.05$ (confidence levels > 95%).

5.3 Results

5.3.1 Co-culture Induced Protein Oxidation in HGC-27

In order to confirm that co-culture with activated HL-60/N generated an inflammatory setting in which the release of ROS (and hence oxidative stress) could directly impact HGC-27 at the molecular level, the levels of ROS modified proteins in HGC-27 cells post co-culture were measured using the OxyBlot™ oxidised protein detection kit. This assay detects oxidised proteins in whole cell protein lysates using a dot blot type methodology in which oxidised protein side chains are derivatised into moieties that can be specifically and sensitively detected using an antibody when the proteins are spotted onto a membrane. Figure 5.1 shows an example of a typical blot experiment for the 4hr co-culture protein samples from which it is evident that co-culture with HL-60/N either in the presence of absence of 100nM fMLP resulted in increased levels of oxidised proteins compared to untreated or fMLP treated controls. Figure 5.2 summarises the data in graphical format and again illustrates that co-culture of HGC-27 with HL-60/N resulted in enhanced levels of oxidised proteins compared to cells not co-cultured with HL-60/N or treated with 100nM fMLP alone. Using one way ANOVA statistical analysis to compare the results in control and treated cells it was seen that although co-culture with HL-60/N caused a clear increase in protein oxidation in HGC-27, the increases were not statistically significant.

Figure 5.1 Typical result of an OxyBlot™ experiment showing the levels of oxidised protein in 4hr control and treated/ inflammation exposed HGC-27 cells. It is evident that co-culture of cells with HL-60/N, either unstimulated or fMLP stimulated, results in higher levels of oxidised proteins (visible as darker spots) compared to cells not exposed to HL-60/N or treated with 100nM fMLP alone. 1:1 signifies a one to one co-culture ratio of HGC-27 to HL-60/N. Refer to text for further details. N = 2.

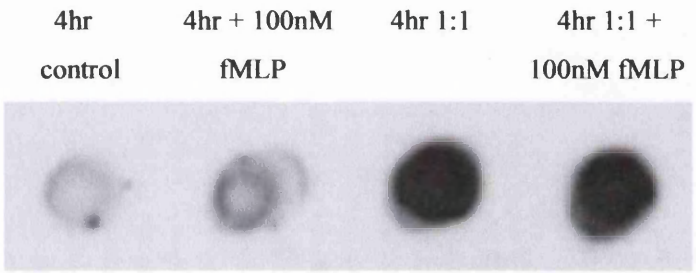
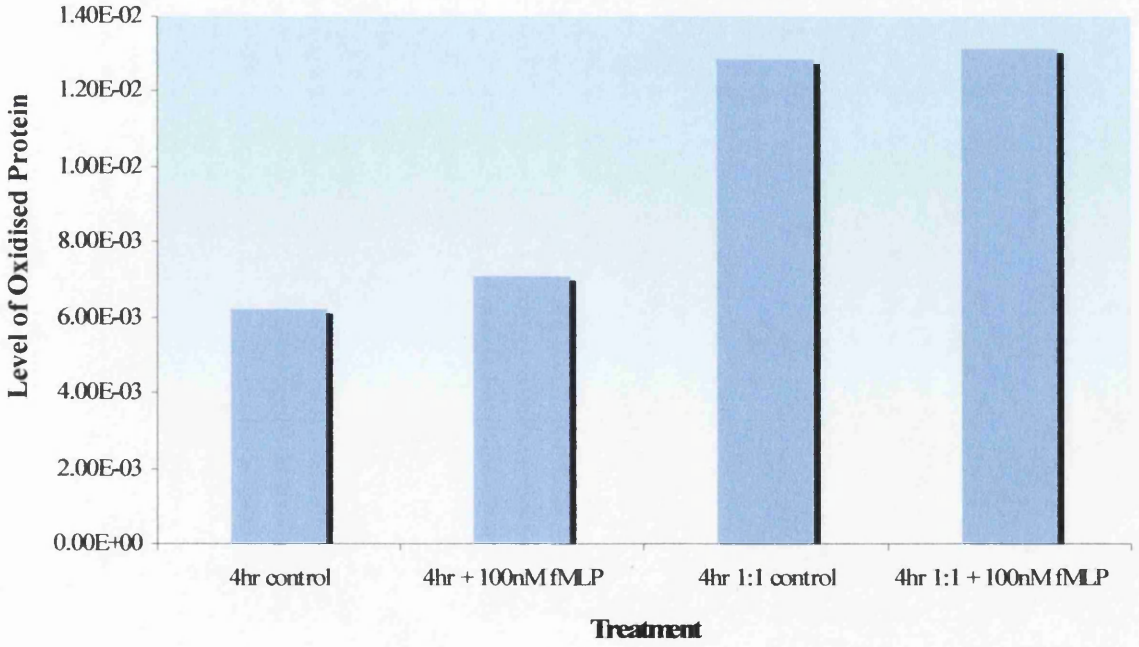


Figure 5.2 Graphical representation of OxyBlot™ assay data. The Blot images obtained were analysed using the ImageJ software (section 5.2.6) and spot intensity was seen to be proportional to the levels of oxidised protein (see fig. 5.1). Standard error too small to be shown as bars. N = 2.



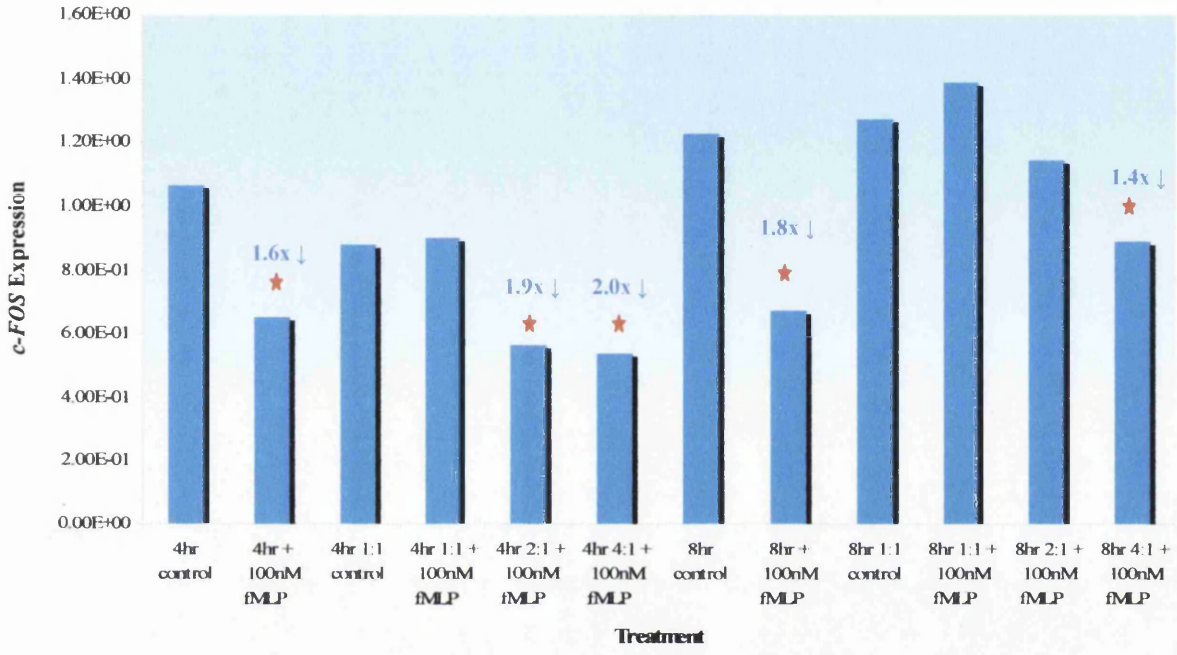
5.3.2 *c-FOS* Gene Expression in HGC-27 Post Co-culture

Figure 5.3 illustrates *c-FOS* expression levels in HGC-27 following various co-culture treatments. Interestingly co-culture with LPS primed HL-60/N in the absence or presence of fMLP stimulation did not cause an expected increase in *c-FOS* expression levels, and interestingly, where statistically significant changes in gene expression levels were observed following co-culture, they tended to be decreases compared to the level in untreated controls ($P < 0.01$). For example, at both 4 and 8hr time points, co-culture of HGC-27 with HL-60/N at a HGC-27 to HL-60/N ratio of 4:1 plus 100nM fMLP resulted in a significant decrease in *c-FOS* level compared to the respective control for each time point (fig. 5.3). A noteworthy observation is that at both time points the level of *c-FOS* expression in control cells appeared to be high and that treatment with 100nM fMLP resulted in significant down-regulation, as did some of the co-culture treatments with fMLP stimulation (2:1 and 4:1 co-cultures + 100nM fMLP at 4hr, and 4:1 co-culture + 100nM fMLP at 8hr). Interestingly, the 1:1 co-culture model, both in the presence and absence of fMLP did not cause significant down-regulation of *c-FOS* gene expression at 4hr and 8hr. This can lead one to speculate that certain conditions of inflammation – in this case high levels of inflammatory cells (1:1) do not cause *c-FOS* down-regulation whilst less marked inflammation (and so less oxidative stress) (2:1 and 4:1), and fMLP treatment alone do. As such, it is likely that in the *in vivo* setting the level of tissue inflammation can influence the outcome of the expression of oncogenic *c-FOS*.

5.3.3 *IL-8* Gene Expression in HGC-27 Post Co-culture

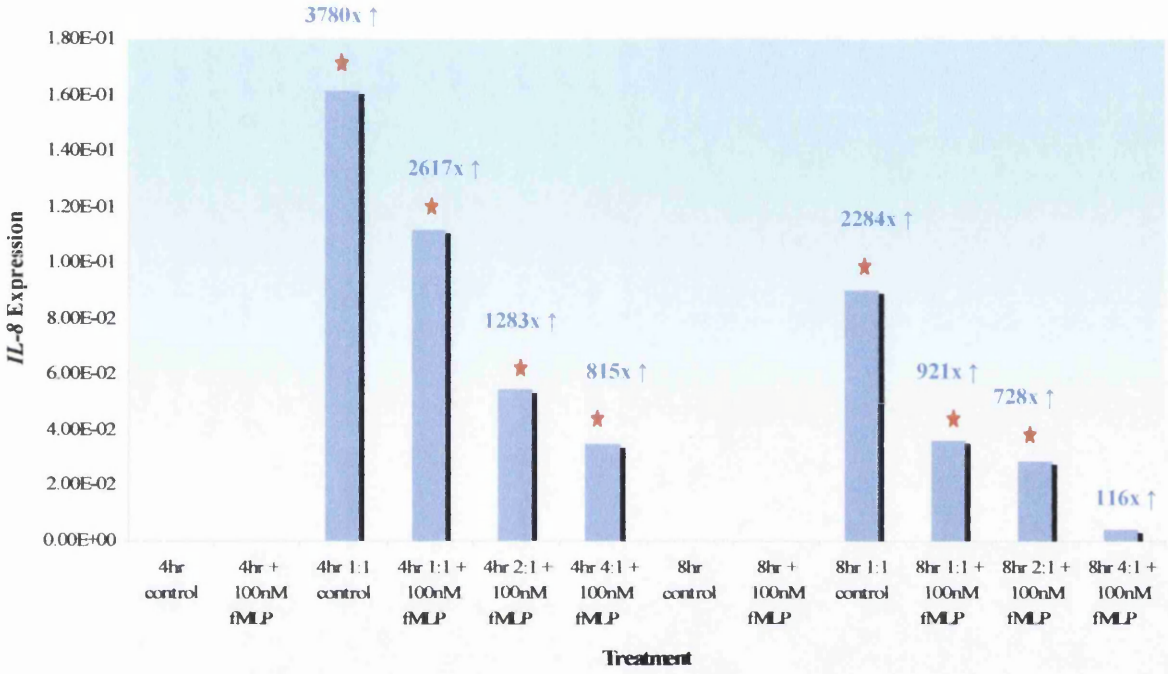
In contrast to the findings for *c-FOS*, *IL-8* gene expression was significantly up-regulated by co-culture of HGC-27 with HL-60/N both in the presence and absence of 100nM fMLP at both 4hr and 8hr exposure times ($P < 0.01$) (fig. 5.4). Interestingly the level of *IL-8* up-regulation appeared to be related to the co-culture ratio, and hence the degree of inflammation and oxidative stress.

Figure 5.3 Graphical representation of average *c-FOS* gene expression (analysed by real-time PCR relative to β-actin) in control, 100nM fMLP treated, and HL-60/N exposed HGC-27 cells (with or without 100nM and with either 1:1, 2:1, or 4:1 HGC-27/ HL-60/N co-culture ratio). Standard error too small to be shown as bars. Statistically significant differences ($P < 0.01$) are highlighted with red stars (based on one way ANOVA). See text for further details. N = 2.



For example, at 4hr post co-culture, a 1:1 co-culture ratio (plus or minus fMLP) resulted in markedly enhanced *IL-8* levels, compared to that in control cells and cells treated with 100nM fMLP alone, than the 2:1 and 4:1 ratios, *IL-8* expression levels declining gradually as the ratio of HGC-27 to HL-60/N increased (fig. 5.4). Another noteworthy observation was that at both the 4 and 8hr exposure times, maximal *IL-8* induction was observed following co-culture of HGC-27 with HL-60 at 1:1 in the absence of fMLP stimulation, and the addition of fMLP appeared to cause a decrease in *IL-8* gene expression. These observations suggest that under conditions of tissue inflammation, up-regulation of *IL-8* gene expression is a likely molecular change. The likelihood of *IL-8* over-expression appears to be positively related to the degree of inflammation, and may be affected negatively by the presence of fMLP in the vicinity.

Figure 5.4 Graph illustrating average *IL-8* gene expression (analysed by real-time PCR relative to β-actin) in control, 100nM fMLP treated, and HL-60/N exposed HGC-27 cells (with or without 100nM and with either 1:1, 2:1, or 4:1 HGC-27/ HL-60/N co-culture ratio). Standard error too small to be shown as bars. Statistically significant differences ($P < 0.01$) are highlighted with red stars (based on one way ANOVA). See text for further details. N = 2.

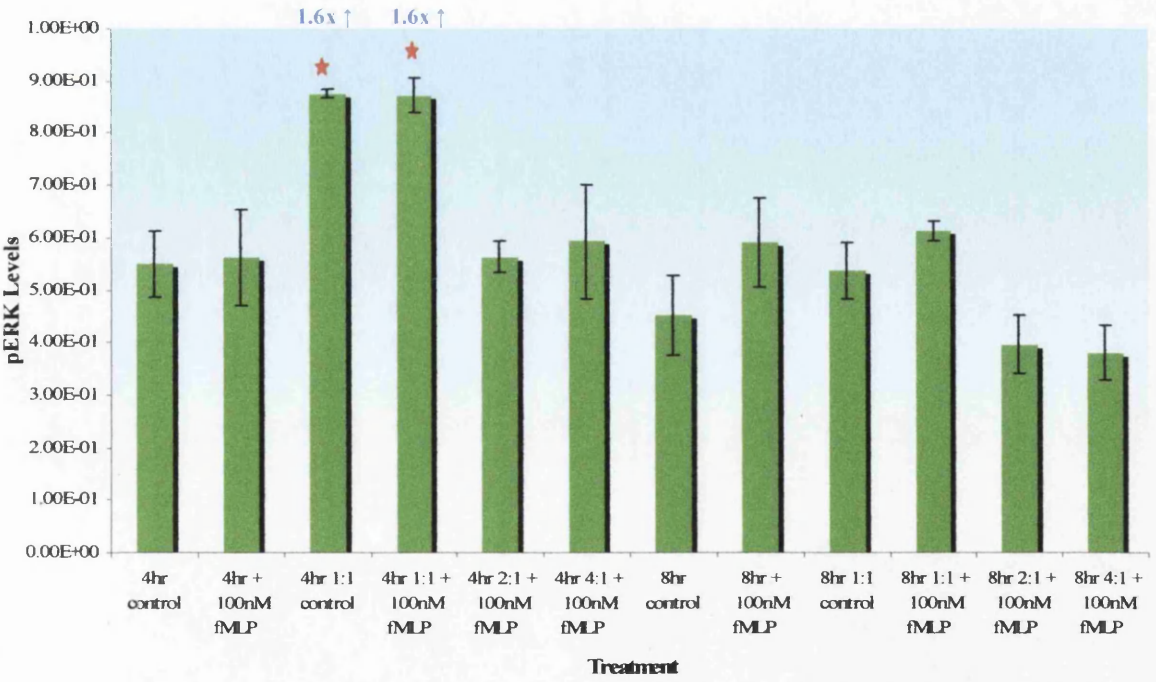


5.3.4 pERK Levels in HGC-27 Post Co-culture

pERK levels in HGC-27 following co-culture experimentation were analysed by western blotting to determine if exposure to inflammatory cells with the associated increase in oxidative stress could cause changes in cellular signal transduction at the level of the ERK MAPK pathway. The results are illustrated graphically in figure 5.5, examination of which reveals that in some instances co-culture of HGC-27 with HL-60/N could induce significant increases in pERK levels in HGC-27 compared to cells not exposed to HL-60/N (seen with one way ANOVA, $P < 0.01$). Specifically, 4hr co-culture with HL-60/N at a 1:1 density with or without fMLP stimulation resulted in significant increases in pERK levels compared to un-exposed control cells and cells exposed to

100nM fMLP alone (~ 1.6-fold increase over un-exposed control cells in both cases). At the 4hr time point, co-culture with HL-60/N at lower ratios of inflammatory cells to gastric epithelial cells did not appear to cause any marked change in pERK levels, suggesting that the induction of ERK signalling is dependent upon the severity of inflammation, significant changes in ERK phosphorylation only being induced in situations of more intense inflammation with greater density of inflammatory cells. At the 8hr time point, co-culture with HL-60/N at any ratio, and in the presence or absence of fMLP stimulation, did not have any significant impact on pERK levels in HGC-27.

Figure 5.5 Average pERK levels in control, 100nM fMLP treated, and HL-60/N exposed HGC-27 cells (with or without 100nM and with either 1:1, 2:1, or 4:1 HGC-27/ HL-60/N co-culture ratio) analysed by western blots relative to total ERK following co-culture experiments. Standard error bars shown. Statistically significant differences ($P < 0.01$) are highlighted with red stars (based on one way ANOVA). N = 2.



5.4 Discussion

The effect of inflammatory leukocytes on *c-FOS* and *IL-8* gene expression, and ERK MAPK signalling in gastric epithelial cells was analysed in the present study using a co-culture system to mimic the inflammatory conditions in gastritis. The relevance of the study stems from the fact that chronic gastric inflammation (gastritis) is heavily implicated in the earliest stages of the pathogenesis of gastric cancer (Zavros *et al.*, 2005; Sipponen *et al.*, 1998; Correa, 1992a). This oncogenic setting of chronic tissue inflammation can be dissected into several components which may act alone, or synergistically to drive the development of malignant gastric disease, and include pro-inflammatory mediators such as cytokines, growth factors, and RO/NS, all of which can be derived from host epithelial cell and leukocyte sources in the vicinity.

The effect of cytokines and RO/NS on cells has been well studied using *in vitro* experimentation whereby cells are directly exposed to various concentrations of the biological molecules or compounds (Genestra, 2007; Gloire *et al.*, 2006; McCubrey *et al.*, 2006; Takada *et al.*, 2003; Klotz, 2002; Lee and Esselman, 2002; Fan *et al.*, 2001; Lin *et al.*, 2001; Suzuki *et al.*, 2001), and indeed chapter 3 described the effects of H_2O_2 exposures on cells with respect to MAPK and NFκB signalling and downstream expression of *c-FOS* and *IL-8*. It is of particular importance, however, to study the combinatorial effects of the compounds that may be present in an *in vivo* inflammatory setting. In response to infection (e.g. *H. pylori*) or injury in the gastric mucosa, inflammatory leukocytes generate and release a diverse array of pro-inflammatory compounds and metabolites. For example, as detailed in section 1.4.5.1, in response to *H. pylori* infection, the gastric epithelium and neutrophils in the vicinity release an array of inflammatory mediators including IL-8 (Crabtree *et al.*, 2004; Crabtree, 1998; Crabtree, 1996a; Crabtree *et al.*, 1994a; Crabtree *et al.*, 1994b) IL-6, IL-1β, TNFα, IFNγ (Smythies *et al.*, 2000; Lindholm *et al.*, 1998; Messa *et al.*, 1996; Mohammadi *et al.*, 1996; Moss *et al.*, 1994; Noach *et al.*, 1994) and a plethora of RO/NS (Mizuki *et al.*, 2000; Rautelin *et al.*, 1993; Mooney *et al.*, 1991). As such, in order to obtain better insights into how pro-inflammatory mediators and RO/NS found persistently at sites of chronic inflammation affect nearby epithelial cells, using an *in vitro* model that more closely mimics the *in vivo*

setting, it is fundamental to expose gastric epithelial cells to the compounds from their natural biological source, i.e. leukocytes. This was achieved using a co-culture system in which the HGC-27 gastric adenocarcinoma cell line was co-incubated with the HL-60/N cell line optimised for oxidative burst induction as described in chapter 4. HGC-27 was co-cultured with LPS primed HL-60/N at HGC-27 to HL-60/N ratios of 1:1, 2:1, and 4:1 in order to mimic different intensities of inflammation since the degree of inflammation may impact an individuals risk of developing gastric cancer, with more severe inflammation being linked to a greater risk of developing the disease (Fox and Wang, 2007; Axon, 2002; El-Omar *et al.*, 2000; Correa, 1995; Correa and Miller, 1995; Gilmour, 1961). Induction of the neutrophil oxidative burst was further enhanced in some instances by the addition of 100nM fMLP. RNA and protein were then extracted from HGC-27 in order to assess any changes in *c-FOS* and *IL-8* RNA levels and ERK MAPK signalling, at the level of ERK phosphorylation, induced by co-culture. OxyBlot™ analysis revealed that the co-culture conditions induced oxidative modifications of proteins in HGC-27, confirming the impact of co-culture derived oxidative stress on the gastric epithelial cells.

With respect to *c-FOS*, exposure of HGC-27 to inflammatory HL-60/N cells, either in the presence or absence of fMLP stimulation, did not cause the expected increase in *c-FOS* expression levels (based on the observations of oxidative stress induced up-regulation of *c-FOS* in chapter 3). Rather, significant down-regulation of the gene was observed following treatment of HGC-27 with 100nM fMLP in the absence of HL-60/N, and following co-culture at 2:1 and 4:1 ratios plus 100nM fMLP (significant decreases in *c-FOS* levels ($P < 0.01$) in all three treatments at 4hr, and 100nM fMLP control and 4:1 co-culture + 100nM fMLP at 8hr). Interestingly no significant changes in *c-FOS* expression levels were apparent following 1:1 co-culture treatments both in the presence and absence of 100nM fMLP at both time points. These findings lead to the speculation that the severity of inflammation may have an impact on *c-FOS* gene expression, since less intense inflammation (lower densities of inflammatory leukocytes) caused down-regulation of *c-FOS* whilst more intense inflammatory conditions (1:1 co-culture ratio) had no apparent effect on *c-FOS* levels. The results are slightly confounded in that *c-FOS* RNA levels were found to be high in un-exposed control HGC-27 for

reasons that are unclear based on the present data. The high levels of *c-FOS* seen in the controls may be due to some aspect of the cell culture system, and as such the observed decreases in *c-FOS* expression may be misleading. In the case that the decreases observed are due to true down-regulation of gene expression, a possible explanation is that *c-FOS* expression levels were saturated in the controls, the introduction of further expression inducing stimulus (e.g. oxidative stress) may then trigger control and negative feedback pathways that either keep *c-FOS* levels constant or result in the observed decreases. A second possibility is that the increase in oxidative stress combined with other aspects of the inflammatory co-culture system may cause a sub-toxic environment in which proliferation of HGC-27 is suppressed so accounting for the decreases in *c-FOS* expression. It is very difficult to account for the findings without further experimentation (e.g. following the proliferation rate of HGC-27 post co-culture), but it is clear that the severity of inflammation is likely to determine the outcome of *c-FOS* gene expression, and this is likely to be the case in the *in vivo* gastric setting.

The data on *IL-8* gene expression revealed some very interesting findings. At both 4hr and 8hr time points a similar trend was observed whereby the 1:1 co-culture ratio in the absence of fMLP caused maximal *IL-8* induction which then gradually declined when fMLP was added to the system and as the density of HL-60/N in the co-culture system decreased (2:1 and 4:1) (fig. 5.4). At the 4hr time point all four co-culture treatments caused significant increases in *IL-8* levels compared to un-exposed controls, whilst at the 8hr time point the 1:1 co-culture, 1:1 co-culture plus fMLP, and 2:1 co-culture plus fMLP resulted in significant up-regulation ($P < 0.01$). These results contrast with the results for *c-FOS* since co-culture treatments caused significant up-regulation of *IL-8* in HGC-27 with a clear trend. A noteworthy finding is that, as was the case for *c-FOS*, the level of *IL-8* gene expression appeared to be related to the co-culture ratio and hence the degree of inflammation. In this case, *IL-8* gene expression was seen to be maximal in HGC-27 exposed to the highest density of HL-60/N (1:1). Interestingly the addition of fMLP to the co-culture system appeared to have a negative impact on *IL-8* gene expression levels at both time points. This can be seen clearly when comparing the level of *IL-8* RNA in HGC-27 exposed to HL-60/N at a ratio of 1:1 in the absence of 100nM fMLP compared to the 1:1 co-culture plus 100nM fMLP. This could be due to an inhibitory effect caused

fMLP, or the combination of LPS priming and fMLP. This speculation fits the IL-8 ELISA data in chapter 4 where it was seen that LPS priming of HL-60/N alone and treatment with 100nM fMLP alone caused significant increases in IL-8 protein levels in HL-60/N, whilst combined LPS priming – fMLP stimulation resulted in no significant change in IL-8 levels compared to untreated HL-60/N controls. This finding is very interesting since it conflicts with several reports in the literature that both LPS and fMLP are potent inducers of *IL-8* gene expression (Han *et al.,* 2007, Huang *et al.,*,2001; Innocenti *et al.,* 2001; Cassatella *et al.,* 1992) with LPS acting by way of induction of ROS generation and concomitant activation of redox sensitive NFκB (Han *et al.,* 2007). Interestingly however, review of the literature brought to light that the effects of LPS and fMLP on *IL-8* gene expression varies greatly in different cell lines, likely owing to the different gene expression control pathways that predominate in different cell types (Carlson *et al.,* 2007; Liboni *et al.,* 2005; Cassatella *et al.,* 1992). In this case LPS is not likely to directly impact gene expression in HGC-27 since it is not present in the co-culture system. However, LPS priming of HL-60/N may indirectly effect expression changes in HGC-27 by altering the way HL-60/N cells respond to fMLP, so influencing the release of pro-inflammatory mediators and RO/NS which may impact cell signalling and gene expression in HGC-27. With respect to fMLP, Carlson *et al.* (2007) demonstrated that at physiological concentrations (10 – 100nM), fMLP can inhibit TNFα induced activation of NFκB, providing a possible explanation for the observed decrease in *IL-8* gene expression since NFκB is a key regulator of *IL-8* expression (Jenkins *et al.,* 2004). Based on the present data the cause of the apparent decrease in *IL-8* levels following addition of 100nM fMLP is unclear and would require further experimentation to obtain greater insights.

As observed for both *c-FOS* and *IL-8* gene expression, the induction of ERK phosphorylation (and hence MAPK signalling) also appeared to be related to the density of HL-60/N in co-culture, and hence to the extent of inflammation. Specifically the levels of pERK in HGC-27 increased significantly ($P < 0.01$) when the cells were co-incubated for a 4hr period with HL-60/N at the highest density, i.e. 1:1 co-culture, both in the absence and presence of 100nM fMLP (~ 1.6-fold increase in pERK levels over un-exposed control cells in both cases). At the 4hr time point both the 2:1 and 4:1 co-culture

treatments (both plus 100nM fMLP) had no significant impact on pERK levels in HGC-27. At the 8hr time point none of the co-culture treatments caused significant changes in ERK phosphorylation, leading to the conclusion that only the 1:1 co-culture treatments, and hence the most intense inflammatory cell culture microenvironement can induce ERK activation, and that ERK phosphorylation appears to occur within a 4hr period, with the increase in phosphorylation being transient since it is not seen at 8hr. Relating the findings to *in vivo* circumstances, these observations suggest that in some instances of gastric inflammation, most likely severe inflammation with dense leukocyte infiltrates, the tissue microenvironment can induce ERK MAPK signalling by way of ERK phosphorylation, and that this is likely to occur in a transient manner, possibly responding to dynamic/ fluctuating changes in the inflammatory conditions, e.g. waves of increased oxidative stress, which could lead to a more consistent overall ERK MAPK signalling. In addition, an initial fairly rapid activation of ERK signalling may set in motion a series of downstream signalling and gene expression events that lead to changes in cell behaviour. In some instances such epigenetic changes may drive the development of an oncogenic phenotype. Interestingly, whilst pERK levels appeared to correlate with *c-FOS* expression following H_2O_2 treatment of HGC-27 in chapter 3, no such association was seen here. It may be that some other aspect of the co-culture system impacted *c-FOS* expression, overshadowing any possible effects of ERK MAPK signalling. It is also possible that, in the co-culture system, factors that both induce and inhibit *c-FOS* expression may be present, and so antagonism between factors may lead to a delayed induction of ERK regulated *c-FOS* expression beyond the time scale of the present experimentation. It must also be noted that the possible discrepancy in the *c-FOS* data (mis-leadingly high levels in controls) may account for the observed differences in pERK and *c-FOS* levels. Further experimentation would be required to ascertain the underlying mechanisms for the observations.

In summary the present work demonstrated that co-culture of HGC-27 gastric epithelial adenocarcinoma cells with HL-60/N inflammatory leukocytes could cause activation of ERK MAPK signalling by way of increased ERK phosphorylation, and increased expression of *IL-8* (implying possible involvement of NFκB signalling), but not significant increases in *c-FOS* gene expression. It is quite likely that these changes are

caused by the oxidative stress conditions generated by HL-60/N in the co-culture setting (confirmed in the OxyBlot studies for detection of oxidative protein modifications), and as such the data for pERK and *IL-8* fit nicely with the data in chapter 3 which demonstrated that frank exposure of HGC-27 to various doses of H_2O_2 could cause activation of pERK and over-expression of *IL-8*. The data for *c-FOS* gene expression following co-culture do not fit the H_2O_2 exposure data in chapter 3, and so it is likely that other pathways for controlling *c-FOS* expression come into play in the co-culture setting. Indeed, whilst it is quite plausible that oxidative stress in the co-culture system causes the observed signalling and gene expression changes, so tying the present observations to those in chapter 3, other factors must not be ruled out. For example, the combinatorial effects of several different RO/NS may also be coupled to synergistic/ antagonistic effects of other pro-inflammatory mediators that may be released from HL-60/N such as cytokines (e.g. IL-8 – see chapter 4), leukotrienes, growth factors, etc. In addition direct cell – to – cell contact between HGC-27 and HL-60/N may influence signal transduction and gene expression. Based on the present data and experimental design the mechanistic nature of the signalling and gene expression changes cannot clearly be determined and further experimentation would be required, for example, repeating the experiments with the addition of antioxidant enzymes or compounds would ascertain any involvement of RO/NS in the observed changes.

What is clear is that in both *in vitro* models studied (chapter 3 and present), components of an inflammatory response; be it RO/NS, cytokines, cell – to – cell contact, etc.; appear to induce significant signal transduction and gene expression changes in gastric epithelial cells. The data provides potential mechanistic links between chronic inflammation and gastric carcinogenesis. It is important, however, to bear in mind that *in vitro* observations may not always translate to the *in vivo* tissue setting, and as such it is fundamental to study these changes in gastric biopsy specimens.

Chapter 6

ERK1/2 MAPK Activity and *c-FOS* gene expression in pre-malignant gastric tissues.

6.1 Introduction

A common feature of all cancers is the genetic instability that they come to exhibit with advancement of disease (Hanahan and Weinberg, 2000; Loeb and Loeb, 2000; Cahill *et al.,* 1999; Lengauer *et al.,* 1998). An initiating mutation followed by a series of further mutational hits can result in the evolution of a tumour mass via complex, and yet to be fully understood mechanisms involving natural selection at the cellular level (Merlo *et al.,* 2006; Aranda-Anzaldo, 2001, Rubin, 2001; Cahill *et al.,* 1999; Vogelstein and Kinzler, 1993; Nowell, 1976). A single mutation in a cell may drive waves of cellular multiplication and clonal expansion, further cellular complexity being accumulated in the process resulting in tumour growth, disorganization, and enhanced malignant potential. This process is dependent upon the generation of cells with different proliferative capacities in a group of growing cells. The cells that exhibit a growth advantage are selected for during the waves of clonal expansion resulting in tumour formation. This growth advantage may arise by several distinct yet interrelated mechanisms: -

1. Mutation and/ or down-regulation of tumour suppressor genes by genetic or epigenetic mechanisms,
2. Mutation and/ or up-regulation of oncogenes via genetic or epigenetic mechanisms,
3. Aberrations in signal transduction (often a consequence of 1. and 2.).

Accumulating genomic instability is at the root of these changes (Oda *et al.,* 2005; Zheng *et al.,* 2004). The ultimate outcome of such molecular changes is that normal cells give

rise to highly malignant derivatives that display physiological and molecular characteristics that confer a selective growth advantage (Nowell, 1976; Foulds, 1954).

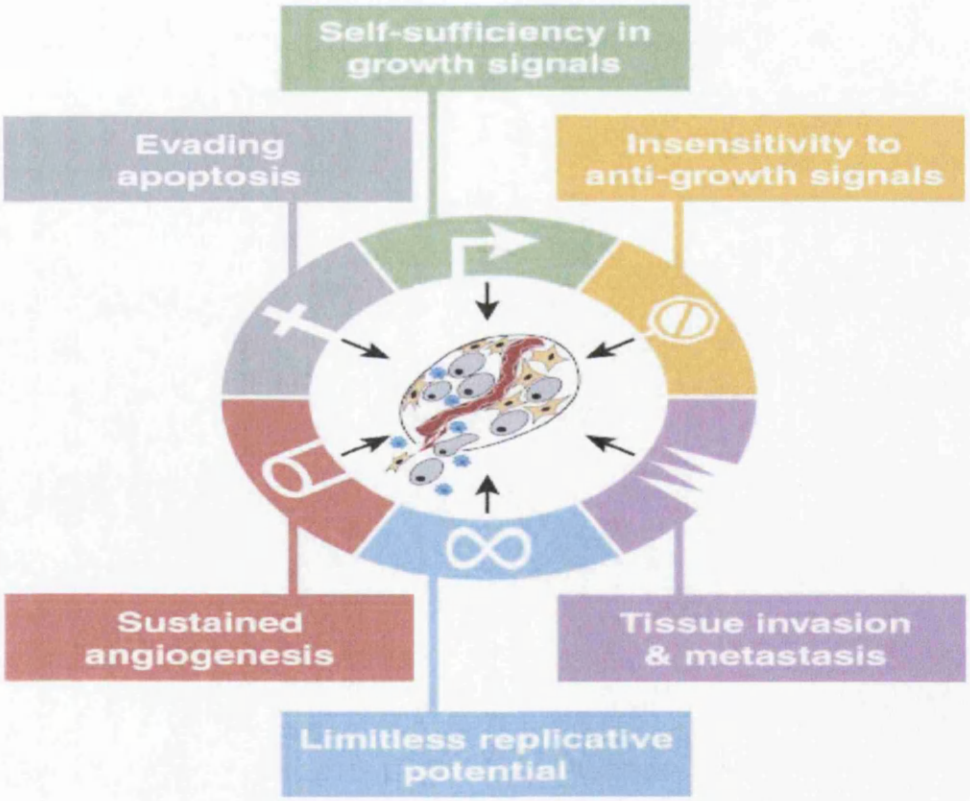
A tumour resulting from the accumulation of molecular changes is essentially a heterogeneous mass of cells, with distinct groups of cells originating from different rounds of clonal expansion, all harbouring one type of growth advantage or another. The multiple layers of complexity of cancers are evident in that over 200 types of the disease are known, affecting different sites in the body, different tumour subtypes being found in different organs, and the tumours themselves showing heterogeneity in their cellular and molecular make-up. Despite this, in recent decades some common features of cancer cells have come to light, summarised in figure 6.1. Of particular interest in the present investigations is the fact that all of these functional characteristics can result from changes in cellular signal transduction, and that this can be linked to our ever increasing understanding of the role of reactive oxygen species (ROS) in both the initiation and promotion of malignant disease (detailed in chapters 1 and 3) with a focus here on gastric cancer.

6.1.1 Signal Transduction and the Cancer Phenotype

Cell behaviour is largely orchestrated by complex networks of inter- and intra-cellular signal transduction pathways. Research over the past two decades or so has put a huge emphasis on unravelling the complexities of signal transduction pathways at the level of pathway components, individual signal transduction modules, and interactions between pathways. As such the 'cellular circuitry', likened to integrated electronic circuits in regard to their intricacy, has been delineated to an extent (section 1.8, fig. 1.10), key features being inducing signals/ ligands, cell surface receptors, and the flow of molecular information from the cell surface to the nucleus involving kinases and phosphatases. More importantly, the mechanisms by which such signalling goes awry in disease states have come to be better understood (Finkel and Gutkind, 2003), including atherosclerosis, asthma, diabetes, rheumatoid arthritis, neurodegenerative diseases, and cancer.

Cancer, in fact, has often been described as a disease of mis-regulated signal transduction (Arbiser, 2004; Eshel *et al.*, 2002; Radisky *et al.*, 2001; Fedi *et al.*, 1997; Hunter, 1997; Kohn *et al.*, 1992) since cancer cells proliferate and grow when they should not, and do not undergo programmed cell death (i.e. apoptosis) when they should, resulting in a shift in cellular homeostasis to excessive proliferation, a situation which can provide the background upon which further molecular anomalies can lead to neoplastic progression.

Figure 6.1 Common features of cancer cells. It is hypothesised that most if not all human cancers acquire this set of functional capabilities, although the means by which they are acquired during disease development can vary greatly. Taken from Hanahan and Weinberg, 2000.



The aberrant signal transduction is often a causal effect of mutations in oncogenes and tumour suppressor genes which are frequently components of the signal transduction pathways themselves.

Proliferation and growth in normal cells requires the processing of mitogenic signals, often from growth factors, extracellular matrix components or cell – to – cell contact. In contrast, tumour cells can proliferate in the absence of such stimuli, and often generate many of their own growth signals (autonomous signalling), resulting in a 'self-sufficiency in growth signalling' phenotype (Hanahan and Weinberg, 2000). Defective signalling can arise as a result of – changes in extracellular growth signals (frequently a consequence of mutations and/ or changes in local tissue microenvironment), changes in the cell surface receptors, and/ or changes in the intracellular signalling cascades that translate the signal to the nucleus (Fedi *et al.*, 1997). The signal transduction alterations in cancer cells favours their increased proliferation, and can result from over-activity of cellular survival and mitogenic signalling, the decreased activity of apoptotic signalling, or both resulting in an imbalance in cellular homeostasis. In some cases excessive apoptotic signalling can lead to enhanced mitogenic signalling in attempts to compensate for the disproportionate cell death, again tilting the balance toward increased proliferation. The balance between mitogenic and apoptotic signalling is exemplified by the fine balance between the ERK1/2 (p42/p44) and JNK/ p38 MAPK pathways; the former favouring proliferation, and the latter two driving apoptosis; disruption of which is manifest in many cancers (Dolado *et al.*, 2007; Dhillon *et al.*, 2007; Kennedy *et al.*, 2007). The NFκB pathway is also seen to play a pivotal role, mis-regulation again influencing cell survival (Bubici *et al.*, 2006) (section 1.8.2 and 3.1.3).

In previous decades, cancer research focussed on a somewhat reductionist view of tumours, where tumours were viewed simply as the cancer cells and the genes within them. This view changed dramatically when it was realised that tumours are often complex and could function as independent organs, capable of regulating their own signalling, gene expression, and hence behaviour irrespective of their host. They consist of a heterogeneous population of cells – both malignant in nature, and normal stromal cells such as fibroblasts and inflammatory cells, intertwined with endothelial cells making up capillaries so providing tumours with their own blood supply (van Kempen *et*

al., 2003; Radisky, 2001; Hanahan and Weinberg, 2000; Bissell *et al.*, 1999) as well as non-cellular matrix components. In addition to the autonomous growth signalling in cancer cells themselves, their growth can also be influenced by changes in the paracrine signalling from normal cells in the vicinity (Hanahan and Weinberg, 2000). In this way the normal host cells may ultimately support neoplastic progression, since they can provide the appropriate microenvironment that favours neoplasia.

The importance of tissue microenvironment at all stages of neoplastic disease, in particular gastric cancer, have been outlined in sections 5.1.1 and 5.1.2 with a particular emphasis on chronic inflammation and oxidative stress, since these conditions appear to provide a context upon which gastric carcinogenesis can occur (Obst *et al.*, 2000; Stadtländer and Waterbor, 1999; Correa and Shiao, 1994).

6.1.2 Gastric Cancer – Link with Chronic Inflammation and Oxidative Stress

Whilst the incidence of gastric cancer has seen a steady decline in recent years, it sustains its reputation as the second largest cause of cancer related death worldwide (Parkin *et al.*, 2001), largely due to late diagnosis and a dismal prognosis. At the histological level, the intestinal subtype is deemed the 'epidemic' type, and develops through a well defined series of pre-malignant steps (also known as the Correa model (Correa, 1992a; Correa, 1988; Correa, 1975)) outlined in figure 1.3 and section 1.2.1. Briefly, the sequence of events begins with chronic superficial gastritis (chronic inflammation of the gastric mucosa), subsequently progressing through atrophic gastritis, intestinal metaplasia (IM), dysplasia, and ultimately gastric cancer.

Chronic inflammation has long been viewed as the crucial primary stage of gastric carcinogenesis, and as such is believed to be the ultimate 'driving force' of disease pathogenesis regardless of its cause (Zavros *et al.*, 2005). Several factors may cause chronic gastric mucosal inflammation/ gastritis, including physical stress, dietary factors, medications, etc. However, chronic infection with the bacterium *H. pylori* is the most common aetiological agent (Israel and Peek, 2001; Correa, 1992a; Marshall, 1986). Colonisation of the gastric epithelium with the bacterium induces a chronic host inflammatory reaction consisting of cellular and humoral components that may persist

throughout an individual's lifetime (Matysiak-Budnik and Mégraud, 2006; Graham, 1993). The extent of chronic inflammation depends on several interacting factors – both host and bacterial, including host genetic susceptibility, immune response, age at acquisition of infection, and bacterial strain (related to virulence) (section 1.4.5). This multi-factorial nature of gastric disease may provide and explanation as to why only a small minority (< 1%) of *H. pylori* infected individuals go on to develop gastric cancer (Correa, 1995).

Although the link between chronic inflammation and gastric cancer is now clearly recognised, the mechanisms remain to be fully elucidated. Chronic inflammation may promote gastric carcinogenesis by the generation of a hostile tissue microenvironment characterised by oxidative stress. Oxidative stress is an unavoidable by-product of chronic inflammation, originating from several sources including leukocytes, lymphocytes, and pro-inflammatory cytokines. In response to *H. pylori* infection inflammatory leukocytes generate and release potent ROS (Zhang *et al.*, 1996; Rautelin *et al.*, 1994). In addition to ROS, leukocytes also release a diverse array of cytokines into the local microenvironment which can, in turn, further exacerbate the oxidative stress by recruiting more leukocytes (e.g. IL-8 is a potent neutrophil recruiter) and enhancing generation of ROS (e.g. TNFα (Garcia-Ruiz *et al.*, 1997; Lo and Cruz, 1995)). Oxidative stress may also arise from an accumulation of bacterial generated superoxide (Nagata *et al.*, 1998), from bacterial induction of ROS generation and release from epithelial cells (Ding *et al.*, 2007; Obst *et al.*, 2000; Teshima *et al.*, 1998; Bagchi *et al.*, 1996; Davies *et al.*, 1994a; Davies *et al.*, 1994b), and from reduced gastric ascorbic acid levels (Capurso *et al.*, 2003). Chronic inflammation associated oxidative stress linked to *H. pylori* infection has been reported to cause elevated levels of oxidative DNA damage in the gastric epithelium (Farinati *et al.*, 2003; Obst *et al.*, 2000) as well as an increase in mutation rate (Jenks *et al.*, 2003; Touati *et al.*, 2003); perturbation of cellular signal transduction leading to gene expression changes (Baek *et al.*, 2004; Ding *et al.*, 2004; Seo *et al.*, 2004; Chu *et al.*, 2003; Kim *et al.*, 2001; Lim *et al.*, 2001; Kim *et al.*, 2000; Nardone *et al.*, 1999; Muller *et al.*, 1997); and shifts in the homeostatic balance between proliferation and apoptosis in the favour of enhanced cell survival and proliferation (Pritchard and Crabtree, 2006; van Grieken *et al.*, 2003; Piotrowiski *et al.*, 1997; Bechi *et*

al., 1996; Fan *et al.*, 1996; Moss *et al.*, 1996). All of these changes can together initiate and drive neoplastic disease, and it is quite plausible that a mechanistic molecular link exists between chronic inflammation, accompanying generation of ROS and the pathogenesis of gastric cancer, changes in signal transduction and gene expression being the amongst the likely candidates that connect the molecular dots.

6.1.2.1 Signal Transduction and Gene Expression Changes in Gastric Cancer

The importance of aberrant signal transduction in the pathogenesis of cancer cannot be overlooked, and has been discussed in the context of *H. pylori* – associated gastric carcinogenesis in previous chapters (sections 1.8.1, 1.8.2, 3.1.4, and fig. 3.3). Central here are the MAPK and NFκB families of signalling pathways that lead to the activation of AP-1 and NFκB transcription factors amongst others (Maeda *et al.*, 2000; Meyer-Ter-Vehn *et al.*, 2000; Naumann *et al.*, 1999; Aihara *et al.*, 1997; Malinin *et al.*, 1997; Muller *et al.*, 1997). *H. pylori* has been shown to be capable of activating both families of signal transduction pathway *in vitro,* leading to downstream gene expression changes – up-regulation of *IL-8* being the best characterised (Peek, 2001; Maeda *et al.*, 2000; Meyer-Ter-Vehn *et al.*, 2000). These molecular changes appear to be critical in *H. pylori* – associated gastric carcinogenesis leading to changes in cellular proliferation, angiogenesis, invasion and metastasis. Other molecular changes that have been observed which may also be attributable to changes in cellular signal transduction include up-regulation of cyclin D1 (*CCND1*)– only seen in gastric tumours and may be caused by excessive MAPK signalling (Belguise *et al.*, 2005; Milde-Langosch, 2005); *COX-2* over-expression – reported in *H. pylori* positive gastritis, precancerous lesions (atrophic gastritis and intestinal metaplasia), as well as in gastric cancer (Nardone *et al.*, 2004; Tatsuguchi *et al.*, 2004), and may be caused by aberrant activity of NFκB; and *c-FOS* – seen to be over-expressed in *H. pylori* – infected gastric mucosa and precancerous lesions (Yang *et al.*, 2003b) as well as in gastric cancer (Meyer-ter-Vehn *et al.*, 2000). *c-FOS* is a key component of the AP-1 transcription factor detailed in sections 1.8.1 and 3.1.2.1 which controls the expression of genes related to cell cycle control such as cyclin D (*CCND1*) (Belguise *et al.*, 2005; Milde-Langosch, 2005) amongst others.

In gastric tumour samples signalling changes that have been reported in the literature include up-regulation of the *EGF-R/ ERB-B* and *HER2/ NEU* cell surface receptors (Moon *et al.*, 2005; Barnard *et al.*, 1995; Yarden and Ullrich, 1988); increased activity of transforming growth factor – beta1 (*TGF-B1*) (Hawinkels *et al.*, 2007); and down-regulation of EGFR related protein (*EGFRP*) (Moon *et al.*, 2005), a negative regulator of EGF-R. In addition aberrant ERK1/2 (p42/p44) MAPK signalling has been detected in gastric cancer (Liang *et al.*, 2005; Wang *et al.*, 2003).

So it is clear that changes in cellular signal transduction are critical in the pathogenesis of gastric cancer, driving progression at all stages of the disease. Unfortunately the data on signal transduction changes in pre-malignant stages of the disease are lacking with respect to the pathways affected, and the mechanistic nature of the induction of changes. As such, this is a fertile area of research, which promises, with improved knowledge and understanding, the hope of identifying early molecular targets for therapeutic intervention.

6.1.3 Aims of the Chapter

With regard to signal transduction changes, *H. pylori* has been seen to rapidly activate ERK1/2 (p42/p44), p38, and JNK MAPKs, and NFκB signalling in cell culture systems leading to the activation of AP-1 and NFκB transcription factors and the expression of downstream genes (Meyer-Ter-Vehn, 2000; Keates *et al.*, 1999; Naumann *et al.*, 1999). Such changes have also been seen to be induced by ROS (Genestra, 2007; Gloire *et al.*, 2006; McCubrey *et al.*, 2006; Takada *et al.*, 2003; Jackson *et al.*, 2002; Zhang *et al.*, 2001; Sen and Packer, 1996) (refer to sections 3.1.2 and 3.1.3 for further details), and as in previous chapters, I speculate here that inflammation associated ROS and oxidative stress may provide a mechanistic link between *H. pylori* infection and gastric disease on the basis of two key observations:-

1. Infection is known to cause chronic gastric inflammation with accompanying release of ROS into the gastric tissue microenvironment and is recognised as one

mechanism by which *H. pylori* is believed to act (Correa, 2006; Farinati *et al.*, 2003; Obst *et al.*, 2000; Baik *et al.*, 1996), and

2. ROS induced formation of DNA damage and changes in cellular proliferation are important factors in the development of gastric cancer (Farinati *et al.*, 2003; Stadtländer and Waterbor, 1999; Baik *et al.*, 1996; Correa and Shiao, 1994).

Again, the focus of the work is cellular signal transduction and gene expression changes at the levels of the ERK MAPK pathway (specifically the ERK1/2 (p42/p44) pathway; further references to ERK thus concern ERK1/2 (p42/p44)) and a downstream gene expression target *c-FOS*. This chapter describes studies that were carried out to assess ERK MAPK activity and *c-FOS* gene expression in pre-malignant gastric biopsies, so linking *in vitro* work that was carried out in previous chapters showing that ROS could lead to ERK activation and *c-FOS* up-regulation, to an *in vivo* setting, following the same theme of signal transduction changes and downstream consequences on gene expression. ERK MAPK signalling was examined as a measure of general ROS induced signal transduction *in vivo*. Alterations in NFκB signalling were assessed in the pre-malignant biopsies at the level of *IL-8* RNA levels by a colleague in the laboratory (Mrs. Jane Mikhail).

The hypothesis being tested is that redox sensitive signal transduction changes such as ERK MAPK signalling and downstream gene expression changes may be important in pre-malignant stages of Correa's pathway to gastric cancer. This hypothesis is formed on the grounds that gastritis and its preceding chronic inflammation are characterised by dense leukocyte infiltrates and high levels of ROS (Morgan *et al.*, 2003; Marnett, 2000) and hence oxidative stress, which have been demonstrated to cause *P53* mutations (Morgan *et al.*, 2003) and chromosomal aberrations (Williams *et al.*, 2005), and may drive early changes in signal transduction and gene expression that could play a role in progression of pre-malignant disease toward gastric cancer.

The ERK MAPK pathway has been implicated in carcinogenesis and is known to be activated by *H. pylori* infection (Chen *et al.*, 2006; Meyer-Ter-Vehn *et al.*, 2000), although the mechanism remains obscure. By assessing ERK activity in pre-malignant gastric biopsies from patients with or without *H. pylori* infection, an association between

the two can be further established. Also, increased levels of ERK activity have been reported in gastric cancer (Liang *et al.*, 2005), warranting the investigation of changes in ERK MAPK signalling in pre-malignant stages of the disease. In addition, by linking findings to the inflammatory state of the tissues it may be possible to determine whether or not chronic inflammatory processes lead to ERK activation in pre-malignant gastric disease, so providing a mechanistic link that may relate to *in vitro* findings in previous chapters, where oxidative stress was seen to activate ERK signalling and downstream *c-FOS* expression. *c-FOS* has been reported to be up-regulated early on in pre-malignant gastric lesions in a Mongolian gerbil model (Yang *et al.*, 2003b), and hence may play a pivotal role in tumour progression. The present study serves to provide *in vivo* human data on the expression status of *c-FOS* in pre-malignant gastric tissues, as well as assessing the potential role of *in vivo* chronic inflammation in the up-regulation of the gene, again in order to relate findings to the *in vitro* findings in previous chapters so providing a more complete picture.

6.2 Materials and Methods

6.2.1 Enrolment of Patients to the Study

Prior to the *in vivo* studies, ethical approval was obtained from the local board of ethics (Iechyd Morgannwg Health Local Research Ethics Committee) for all aspects of the investigation that required the handling of fresh human tissues.

Patients over the age of 18 (and thus able to give consent) attending upper GI endoscopy clinic at Singleton and Morriston District General Hospitals (Swansea, UK) were invited to participate in the study. Patients were briefed about the study and were provided with written information. Those then wishing to participate in the study gave verbal and written consent by signing a consent form (see appendix II). Exclusions to the study were patients taking proton pump inhibitors or nonsteroidal anti-inflammatory drugs (NSAIDs), those who had had previous upper GI surgery, had undergone recent *H. pylori* eradication, or could not adequately give consent. At the same time as patients were asked to give consent, a questionnaire was also carried out (see appendix II) to

obtain information about sex, age, ethnicity, family history of GI tract disease, diet, smoking, alcohol and drug intake, and previous *H. pylori* infection.

Biopsy samples were taken from consenting patients during endoscopy using a standard gastric biopsy forceps. Three adjacent small biopsy samples were taken from what appeared to be diseased sites in the gastric mucosa (fundus, body, or antrum), and three samples from an adjacent unaffected 'normal' area as an internal control. All biopsies were rinsed with water to remove blood prior to placing in appropriate storage buffers. Of the three biopsies at each site, one was placed in formaldehyde and sent for histological examination and assessment of *H. pylori* status (by GI pathologist Dr. Paul Griffiths), one was placed in RNA*later*™ (Ambion, Warrington, UK), and one was placed in 100mM Tris-base pH10 + 0.01% protease inhibitor cocktail (Sigma-Aldrich, Poole, UK). The RNA*later*™ rapidly permeates tissues and inactivates RNases, thereby stabilising RNA by preventing its degradation. The Tris-base buffer with protease inhibitors stabilises cellular proteins. The latter two biopsy samples were placed on ice and transported back to the laboratory for RNA and protein extraction respectively.

Twenty-five patients were sampled in total. The male: female ratio of the patients was approximately 1.3: 1 and their median age at sampling was 68.5 with a range of 28 – 85 years.

6.2.2 RNA Extraction from Biopsies

Total RNA was isolated from tissue samples by the TRIspin method (Reno *et al*, 1997). Biopsies were placed, using sterile forceps, into microfuge tubes and the samples kept on ice throughout. Biopsy tissue was homogenised in 500µl Trizol reagent (Invitrogen, Paisley, UK) using an Ultra-Turrax T8 homogeniser (IKA- Werke GMBH & Co, Staufen, Germany). If several RNA extractions were to be performed at a time, the homogeniser head was washed in distilled water followed by 100% ethanol and a final rinse in fresh distilled water between samples so avoiding cross contamination.

Subsequent to homogenisation, the tissues were incubated at room temperature for 5min prior to addition of 100µl chloroform (Sigma-Aldrich, Poole, UK) to the resultant tissue lysate. The tubes were next vigorously agitated for 15sec and then

allowed to stand for another 5min at room temperature. The samples were centrifuged for 15min at 4°C and 11,300 x g (11,000rpm) resulting in phase separation to a lower red phenol/ chloroform phase, a white interphase and an upper colourless aqueous phase containing the RNA. The aqueous phase of each sample was then transferred to a fresh 1.5ml microfuge tube and one volume of 70% ethanol added and immediately mixed in by pipetting. All subsequent steps performed to purify the extracted RNA utilised the RNeasy Mini kit (QIAgen, Crawley, West Sussex, UK) following the manufacturer's recommendations.

Contaminating DNA was removed from samples using the DNA*free*™ kit (Ambion Ltd, Cambridgeshire, UK) and RNA yields quantified by spectophotometry, as outlined in the methods sections 2.4.3 and 2.4.4, to determine both concentration and the 260/280 ratio. Only samples with a ratio within 1.7 – 2.2 were considered free of DNA contamination, and so used for subsequent analysis. RNA quality was assessed as outlined in section 2.4.5. Each resultant RNA sample was then divided into 20µl working aliquots, stored at - 80°C until use.

6.2.3 Protein Extraction from Biopsies

Biopsies were placed into microfuge tubes containing 500µl ice cold modified RIPA buffer (50mM Tris-HCl pH7.4, 1% IGEPAL, 0.25% Sodium Deoxycholate, 1mM EDTA, 1mM Sodium orthovanadate, 1mM Sodium fluoride, plus 1mM AEBSF and 1µg/ ml Leupeptin) using sterile forceps. The samples were homogenised on ice using an Ultra-Turrax T8 homogeniser (IKA- Werke GMBH & Co, Staufen, Germany) at high speed for 3 X 1 min intervals until tissue was evenly dispersed into solution. 1 volume of 2X western blot loading (Lamelli) buffer (250mM Tris pH6.8, 4% (w/v) SDS, 10% (v/v) glycerol, 0.006% (w/v) bromophenol blue, 2% (v/v) β-mercaptoethanol) was subsequently added to samples, the samples gently mixed by inverting and boiled at 100°C in a heating block to denature proteins. Protein concentration was determined using a 2D Quant Kit (GE Lifesciences, Bucks, UK) as detailed in section 2.6.1. Samples were then divided into 20µl working aliquots and stored at - 80°C until use.

6.2.4 Real-time PCR for *c-FOS* Gene Expression

In order to determine whether increases or decreases in *c-FOS* expression were selected for at particular pre-malignant histological grades, *c-FOS* levels in biopsy specimens were assessed by real-time PCR as described in section 2.5 – 2.5.1.3 and 3.2.2.5a. The PCR primers used in the real-time analysis were *c-FOS* and β-actin (*ACTB*) (refer to chapter 3 table 3.2 for primer sequences). For the analysis 500ng sample RNA was used and standard curves were generated from pooled RNA from cell culture and gastric biopsy specimens seen to express *c-FOS*. The same standard curve RNA was used in each plate of an experimental run for all replicates so ruling out a potential source of variation in the quantitative data (refer to section 2.5.1.1 for further details).

6.2.5 Western Blotting

Western blots were performed on biopsy protein samples for pERK in order to assess ERK MAPK activation in pre-malignant gastric tissues according to the methods outlined in sections 2.7 – 2.7.3 and 3.2.3.1.

6.2.6 Correlation Between *c-FOS* Gene Expression and pERK Levels

The existence of any possible relationship between *c-FOS* RNA and pERK protein levels in the biopsy samples were assessed in order to investigate links between ERK MAPK signalling and *c-FOS* expression. This was achieved by Pearson's test to examine correlation between the two variables. Statistical significance was observed when $P < 0.05$.

6.3 Results

6.3.1 Patients

A total of 25 patients were successfully enrolled into the pre-malignant gastric study, and biopsy samples collected. The details are summarised in table 6.1. The male: female ratio of the patients was approximately 1.3: 1 and their median age at sampling was 68.5 with a range of 28 – 85 years.

At the time of sampling biopsies taken from areas of the gastric mucosa that the endoscopist deemed normal were labelled 'N', and biopsies taken from what appeared to be diseased sites were labelled 'D'. The results of the histo-pathological diagnoses are presented in table 6.1. No information was available regarding the inflammatory scores in the specimens based on the Sydney system (Dixon *et al.,* 1994) since this is not a routine part of pathological reporting in the Swansea NHS trust.

It can be seen from table 6.1 that the endoscopic diagnosis was not always consistent with the true histo-pathological diagnosis. This sampling problem was difficult to avoid, since the majority of the patient pool was over the age of 60, and in view of the fact that gastritis has a tendency to spread throughout the gastric mucosa with age (Siurala *et al.,* 1985; Morson *et al.,* 1980), it was often challenging to identify a truly normal region of the stomach. Gastritis and inflammation are diagnosed by endoscopy simply by looking for red irritated regions of the stomach mucosa (not clearly defined lesions), but it must not be overlooked that other factors may cause such irritation, for instance alcohol, excessive salt intake, medications, etc. and as such, biopsies that were believed to be of diseased stomach were often found to be normal. The few matched normal and diseased specimens were generally obtained from younger patients (late forties to early fifties), and in attempts to obtain greater numbers of matched specimens slightly younger patients were targeted. However, the majority of patients attending outpatient endoscopy clinic presenting with upper GI complaints were of an older age pool. A consequence of this is that very few (if any after exclusion criteria were applied)

Table 6.1 Summary of patients successfully recruited for pre-malignant gastric biopsy study. At the time of collection, biopsies taken from what appeared to be 'normal' sites in the gastric mucosa were labelled 'N', and those taken from what appeared to be diseased sites labelled 'D'. Histological diagnosis often revealed that the endoscopic diagnosis did not match up to the true histology due to difficulty in clearly defining diseased sites within the stomach, especially when inflammation was present. See text for further details. Clo tests were carried out only when patients presented with symptoms suspect of *H. pylori* infection, otherwise diagnosis of infection could be determined from histology. Some patients were omitted from the final analysis for either clinical reasons (*) as described in section 6.2.1, or due to poor RNA yield and/ or quality from the biopsy specimens (†).

Patient	Sex	Age at collection	Clo test taken	Clo test result	Histological Diagnosis	Additional Notes
1	M	85	Y	-	N = Mild chronic inflammation with Intestinal Metaplasia (IM) D = Reflux/ chemical-type gastritis	
2	F	28	Y	-	N = Normal body mucosa D = Normal body mucosa	
3	F	78	Y	+	N = Mild superficial chronic inflammation with scanty *H. pylori* D = Chronic inflammation with IM and *H. pylori*	
4*	F	78	N		N = Normal body mucosa D = Antral mucosa, mild non-specific chronic inflammation	Treated for a previous *H. pylori* infection
5†	F	75	N		N = Ulcer debris, antral mucosa reflux/ chemical type gastritis D = Normal body mucosa	Clo test negative on previous OGD Previous pre-pyloric gastric ulcer june 2003

continued.....

Table 6.1 continued.

Patient	Sex	Age at collection	Clo test taken	Clo test result	Histological Diagnosis	Additional Notes
6	M	46	N		N = Normal body mucosa D = Normal body mucosa	
7	F	61	Y	-	N = Normal body mucosa D = Normal body mucosa	
8	M	71	Y	-	N = Severe proximal chronic inflammation with atrophy and IM D = Mild distal gastritis (†)	No atrophy in specimen D raises suspicion of autoimmune gastritis, and possible reflux/ chemical gastritis distally.
9	M	53	N		N = Normal body mucosa D = Normal body mucosa	
10	M	71	Y	+	N = Gastritis with *H. pylori* D = Gastritis with *H. pylori*	
11	M	77	N		N = Inflamed with IM D = Inflamed with IM	
12	F	78	N		N = Moderate chronic superficial gastritis of body with abundant *H. pylori* D = Moderate chronic pan-mucosal gastritis with IM and abundant *H. pylori*	Has small pre-pyloric ulcer

continued.....

Table 6.1 continued.

Patient	Sex	Age at collection	Clo test taken	Clo test result	Histological Diagnosis	Additional Notes
13	F	68	N		N = N/A D = Mild active chronic inflammation	No normal collected as Patient was unable to tolerate procedure further
14*	M	43	Y	-	N = N/A D = Antral mucosa, mild non-specific chronic inflammation	No normal collected for research purposes due to time restraints patient on proton pump inhibitors
15	M	65	N		N = Normal body mucosa D = Normal body mucosa	Previously had mild flat erosive gastritis in pre-pyloric region, and currently has Barret's oesophagus with no dysplasia
16	M	75	N		N = Mild active chronic superficial gastritis and abundant *H. pylori* D = Antral mucosa showing mild chronic inflammation. No *H. pylori*	
17*	F	55	Y	-	N = Normal body mucosa D = Mild pan-mucosal non-specific chronic inflammation. No atrophy, IM or *H. pylori*	Previous *H. pylori* infection, treated
18	M	47	Y	-	N = Normal body mucosa D = Mild, focal, non-specific chronic inflammation (†)	

continued.....

Table 6.1 continued.

Patient	Sex	Age at collection	Clo test taken	Clo test result	Histological Diagnosis	Additional Notes
19	F	71	Y	+	N = Mild, superficial chronic inflammation, and scanty foci of acute inflammation. Scanty *H. pylori* (†) D = Mild chronic inflammation, early IM and moderate numbers of *H. pylori*	
20	M	69	Y	-	N = Normal body mucosa D = Normal body-type mucosa with a few antral type glands	
21	F	73	Y	-	N = Normal body mucosa D = Normal antral mucosa (†)	
22	F	44	Y	-	N = Normal body mucosa D = Normal body mucosa	
23	M	65	Y	+	N = Mild, superficial chronic gastritis with scanty *H. pylori* D = Mild glandular atrophy and a focus of IM are present	
24	M	36	Y	-	N = Normal body mucosa D = Normal antral mucosa	
25	M	69	Y	+	N = Proximal body mucosa, severe, active, chronic superficial gastritis with abundant *H. pylori* D = Antral mucosa, moderate, active, chronic superficial gastritis with abundant *H. pylori*	

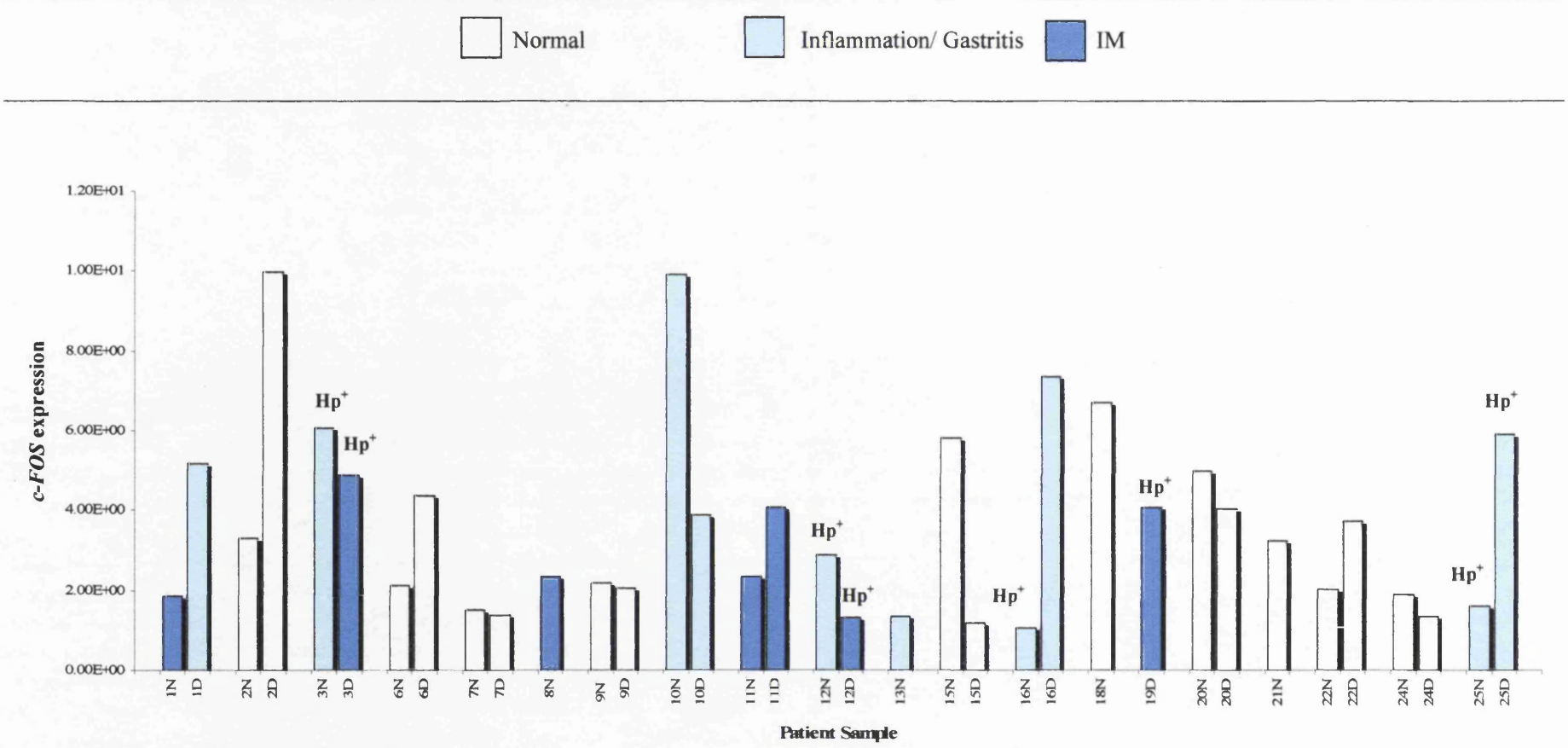
matched specimens were obtained, and so in the RNA and protein expression analyses, the biopsies were classified according to histo-pathology and each patient assessed individually. These findings really highlight the difficulties that can be encountered in such clinical studies.

6.3.2 *c-FOS* Expression

Real time PCR analysis indicated that expression levels of *c-FOS* were highly variable in the pre-malignant gastric biopsy samples. Figure 6.2 highlights the variability in *c-FOS* expression levels in the sample cohort studied. For each histo-pathological subgroup a lot of variation was seen, with no clear trend as to whether up- or down-regulation of the gene was selected for with disease progression, or at particular pre-malignant histological stages. Overall the variability in *c-FOS* expression levels was manifest at both the inter- and intra-patient levels.

. A noteworthy observation is that in cases where the 'N' and 'D' biopsies from a patient were of the same pre-malignant classification, the *c-FOS* levels in the two samples was markedly different in some instances. For example, in the two normal biopsies (2N and 2D) that were obtained from patient 2 from distinct yet adjacent sites in the stomach one exhibited a relative *c-FOS* level of 3.29, while an approximately 3-times higher level of 9.96 was detected in the second normal biopsy. This variation was not limited to normal samples (patients 2, 6, 15, and 22), and can also be seen for inflammation/ gastritis (patient 10, 16, and 25), and IM samples (patient 11). This is an interesting observation since it suggests that *c-FOS* expression tends to vary spatially within the gastric mucosa of some individuals whereas it is fairly consistent in others (e.g. patients 7, 9, 20, 24). It may be that the spatial differences in *c-FOS* expression are related to localised differences in the gastric microenvironment, for instance, small segments of irritated cells exposed to cellular stresses such as oxidative stress may lay adjacent to relatively unstressed cells; alternatively, some regions of the stomach may exhibit a greater intensity of inflammation/ inflammatory milieu,

Figure 6.2 The average *c-FOS* expression levels (relative to β-actin (*ACTB*)) across all pre-malignant gastric biopsy specimens. Samples from patients with *H. pylori* infection are highlighted **Hp$^+$**. IM = intestinal metaplasia.



characterised by more severe inflammatory leukocyte infiltration and a plethora of pro-inflammatory factors, compared to segments in near proximity. These factors could potentially impact the level of *c-FOS* expression at both the levels of upstream signalling pathways (in particular the ERK MAPK pathway discussed in previous chapters) and more directly at the level of transcription, and so may account for the observed variability. In addition the differences in expression levels may be related to the fact that, due to the problems encountered with obtaining diseased and matched normal samples, biopsies were often from different anatomical regions of the stomach. Differences in the cell types and the local microenvironment at the different sites may then account for the observed results.

Another interesting observation was that in a subset of patients in which an inflammation/ gastritis sample and an IM sample were obtained, the level of *c-FOS* expression was higher in the inflammation/ gastritis biopsies compared to the IM biopsies. This is evident in patients 1, 3, and 12 (fig. 6.2) and suggests that some component of tissue inflammation may favour enhanced *c-FOS* gene expression which is absent in IM samples. It is quite plausible that this may be related to the level of inflammation and ROS in the tissue samples of different histo-pathology, since a study by Morgan *et al.* (2003) demonstrated high levels of ROS in gastritis samples compared to normal stomach tissue as well as IM and gastric cancer. This would lead to the speculation that enhanced ROS in inflammation/ gastritis samples could trigger oxidative stress signalling and *c-FOS* expression.

Whilst no clear trends are apparent, figure 6.2 indicates there is an overall tendency towards higher *c-FOS* expression in normal and inflammation/ gastritis samples compared to the IM samples, and this again ties in with the study of Morgan *et al.* (2003). No trend was observed with respect to samples obtained from *H. pylori* infected individuals (patients 3, 12, 16, 19, and 25), with varying *c-FOS* levels in all samples – some exhibiting higher expression (e.g. patient 3) compared to others (e.g. 12). Intra-patient variation is also evident, with *c-FOS* levels being quite distinct between the patient biopsy pairs (e.g. patient 25).

Overall, based on the relatively small size of the pre-malignant biopsy sample cohort, and the difficulties encountered during endoscopic sampling, it is very difficult to

draw strong conclusions as to the involvement of *c-FOS* in pre-malignant gastric disease. Ideally a larger sample cohort, preferably consisting of within patient matched normal and diseased gastric tissue (in the present study no matched normal and diseased samples were studied in the RNA analysis), all taken from the same anatomical region of the stomach; e.g. antrum; in order to rule out differences in gene expression at different sites, would be studied in order to obtain a clearer picture of any trends that may exist.

The variability in the *c-FOS* expression data does not rule out possible signalling changes in the pre-malignant tissues, and so ERK MAPK activity was assessed at the level of western blots for pERK.

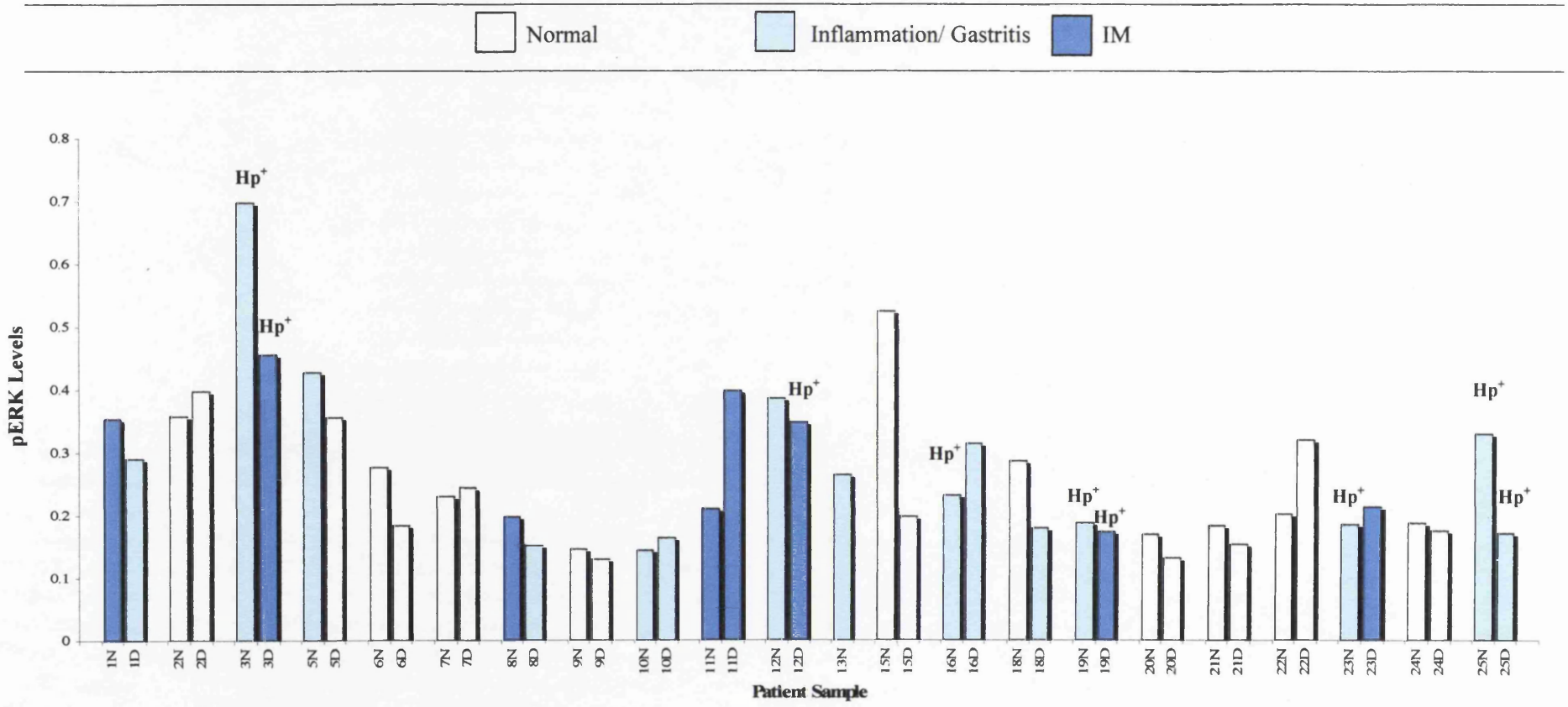
6.3.3 Patients for Protein Studies

Of the 25 biopsy sample pairs collected, protein was successfully extracted with good yields from all samples. As a result only patients that were excluded for clinical reasons (table 6.1) were omitted from the western blot analyses.

6.3.4 ERK MAPK Activity in Pre-malignant Gastric Biopsies

Observation of figure 6.3 reveals that pERK levels in pre-malignant gastric tissues display a somewhat similar pattern to *c-FOS* gene expression (fig. 6.2) particularly with respect to the high degree of variability between patients. Figure 6.3 highlights the amount of variation in pERK levels in all samples analysed. Interestingly in the two matched normal and diseased samples that were obtained (patients 5 and 18, both matched normal and inflammation/ gastritis), patient 5 displayed a subtle increase in pERK levels in the inflammation/ gastritis specimen compared its matched normal counterpart (~ 1.2-fold increase), whilst patient 18 showed a decrease in pERK level in the inflammation/ gastritis biopsy compared to the matched normal sample (~ 1.6-fold decrease). As such the high level of variation in pERK levels in the small sample cohort made it difficult to determine if any relationship between ERK signalling and disease truly existed.

Figure 6.3 The average pERK levels (relative to total ERK) across all pre-malignant gastric biopsy specimens. Samples from patients with *H. pylori* infection are highlighted **Hp$^+$**. IM = intestinal metaplasia.



As for *c-FOS* expression (fig. 6.2), one of the most interesting findings with respect to pERK levels was that marked intra-patient differences were evident in cases where the 'N' and 'D' biopsies from a patient were of the same pre-malignant classification (fig. 6.4). For example, in the two normal biopsies that were obtained from patient 6 (6N and 6D), from distinct yet adjacent sites in the stomach, one displayed a relative pERK level of 2.76E-01, whilst the level detected in the second normal biopsy was 1.84E-01. A similar trend was seen in normal patients 15 and 22, the most marked differences seen in patient 15 where one biopsy had a relative pERK level of 5.23E-01 compared to an approximately 2.6-fold lesser value of 1.99E-01 in the second sample.

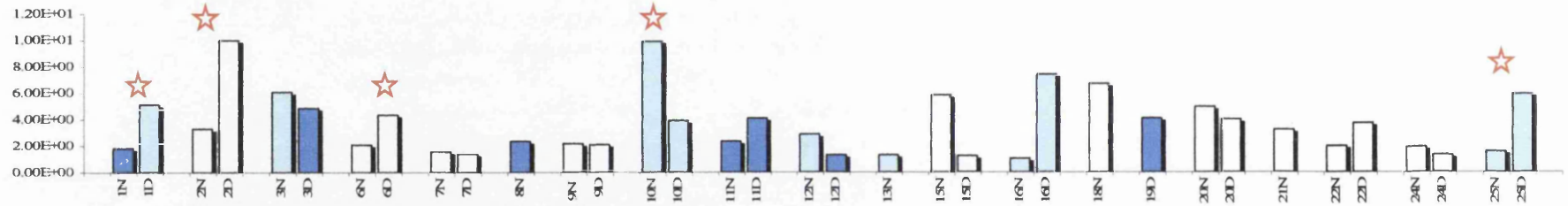
Overall there appear to be no clear trends relating pERK levels and histo-pathology, the levels being highly variable across normal, inflammation/ gastritis, and IM samples, as well as samples from *H. pylori* positive patients.

Again, as for *c-FOS* RNA levels, this variability was not restricted to patients with two normal biopsies, but was also evident for inflammation/ gastritis (patient 25), and intestinal metaplasia (patient 11). In some patients however, as for *c-FOS*, the pERK levels were fairly consistent where the two biopsies were of the same histo-pathology, for example, patients 2, 7, 9, 10, 20, and 24. Again it is possible that this intra-patient variation in ERK activity may be a consequence of spatial differences in gastric microenvironment, and may be related to samples being obtained from different anatomical regions of the stomach as detailed in section 6.3.2.

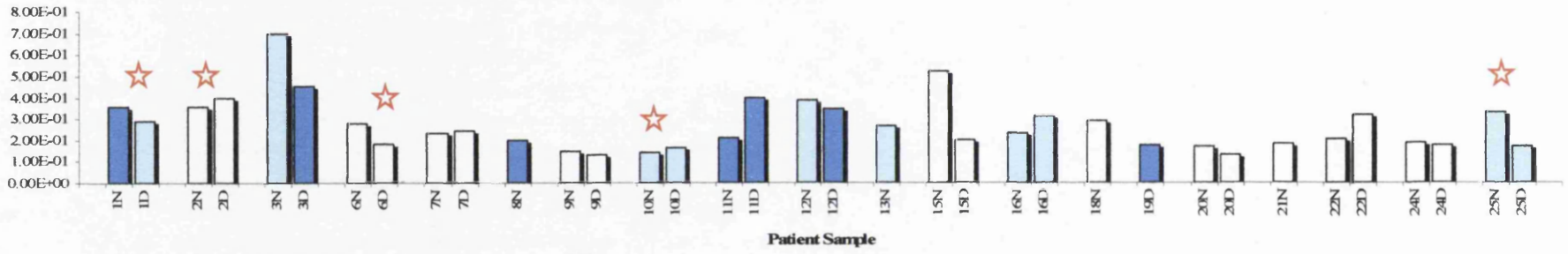
When comparing *c-FOS* expression data with the pERK western blot data, it became apparent that there was some correlation between *c-FOS* and pERK levels, going back to the link between ERK MAPK activity and *c-FOS* gene expression studied in chapter 3. This is highlighted in figure 6.4 where it is clear that upon comparison of only the patients that yielded two biopsy samples, in most cases the *c-FOS* expression patterns were reflective of the pERK levels (in ~ 67% of the patients). In patients 1, 6, and 25, opposite patterns were observed, for example, in patient 1 biopsy 1N showed a low level of *c-FOS* expression, the level being much higher in biopsy 1D, whilst biopsy 1N exhibited a higher level of pERK which was seen to be diminished in biopsy 1D. In the remainder of the patients (2 and 10) *c-FOS* expression levels differed between the 'N'

Figure 6.4 c-fos and pERK levels in pre-malignant gastric biopsy patients which were assessed for both RNA and protein changes. Overall there appears to be some correlation in c-fos RNA expression levels and pERK levels in the samples, however, in some patients (highlighted with red stars) quite distinct trends were observed, for example, in patient 6, biopsy 6N displayed a lower level of c-fos compared to 6D, whilst 6N displayed a higher level of pERK when compared to 6D. Refer to text for further details.

c-fos



pERK



and 'D' biopsies, whilst pERK levels were fairly consistent, for example, in patient 2 biopsy 2N displayed a low level of *c-FOS* RNA, the level being much higher in biopsy 2D, whereas pERK levels were consistent in both biopsy samples.

Interestingly, using Pearson's test for association between *c-FOS* and pERK levels in samples, it was found that there was no statistically significant correlation between the two variables ($P > 0.05$) due to the 33% of cases where *c-FOS* gene expression and pERK levels were not reflective of one another. This is an interesting observation since it suggests that there may be an association between ERK MAPK activity and *c-FOS* gene expression at the level of transcription in some cases, as was seen in chapter 3. However, the fact that ERK activity and *c-FOS* expression are not always reflective of each other, in some cases being opposite, lend to the notion that other mechanisms for controlling *c-FOS* expression are in place, and that some negative feedback may exist between the upstream signal transduction pathway and its downstream gene expression target, further strengthening the findings in chapter 3.

This may vary from individual to individual, and may be related to other factors such as diet, family history, smoking habits, etc. Attempts were made to identify such factors which may contribute to gene expression and signal transduction differences by way of a clinical questionnaire (see appendix II). Unfortunately no clear associations were apparent due to the large amount of inter- and intra-patient differences in the data, and the sample cohort not being large enough to allow sufficient groupings according to dietary factors, cigarette smoking, alcohol habits, and family history.

6.4 Discussion

ERK MAPK signalling changes and *c-FOS* RNA expression changes in pre-malignant gastric tissues were analysed in the present study to determine if such changes are involved in early stage disease pathogenesis in Correa's pathway to gastric cancer, and to assess if any changes could be related to chronic tissue inflammation.

Twenty-five patients were successfully recruited into the studies yielding tissue samples from gastritis and intestinal metaplasia (IM) pre-malignant stages. Chronic

inflammation and gastritis samples were grouped together in one histological grouping (inflammation/ gastritis).

c-FOS RNA expression levels were seen to be highly variable at both the inter- and intra-patient level and no clear trends were apparent, apart from a slight tendency toward higher *c-FOS* levels in normal and inflammation/ gastritis samples compared to IM, and *H. pylori* status did not appear to affect gene expression. These subtle differences may be related to the ROS levels in the tissues since a previous study by Morgan *et al.* (2003) demonstrated that ROS levels were highest in gastritis samples, followed by normal tissues, then IM, and cancer, the present observations tying in with these findings. Based on these results and the pitfalls in the experimental design (regarding the small sample cohort and difficulties in obtaining matched normal and gastritis tissues), the involvement of *c-FOS* in gastric carcinogenesis could not be clearly determined. It is unsurprising that *c-FOS* RNA expression showed such marked variation since gastric tumours, like other GI tract tumours are often seen to be highly unstable and genetically heterogeneous (Owonikoko *et al.*, 2002; Lindforss *et al.*, 2000) (often showing marked differences in gene expression patterns in different segments of a solid neoplasm), and so the genetic profile of the pre-malignant stages is likely to be highly variable too (Wang *et al.*, 2007). It is important to note that whilst *c-FOS* RNA levels showed marked variability, this may not translate to the protein level, and so the involvement of c-FOS in gastric carcinogenesis can not be ruled out based on these findings. As such, it would be important to assess c-FOS protein levels in the biopsy samples.

Other factors that may account for the variability in gene expression include a variety of environmental and host factors. For example, some individuals may harbour genetic polymorphisms in key inflammatory genes that lead to heightened inflammatory responses. Genetic polymorphisms of *IL-1β* amongst other pro-inflammatory cytokines have been associated with increased gastric cancer risk (Rad *et al.*, 2004; El Omar *et al.*, 2003; Rad *et al.*, 2003; Figueiredo *et al.*, 2002; Machado *et al.*, 2001; El Omar *et al.*, 2000). These polymorphisms can give individuals harbouring them an enhanced genetic susceptibility to developing gastric cancer since they are likely to develop more severe inflammatory responses (with more dense leukocyte infiltrate, cytokine release and ROS generation) to gastric injury or infection, and a positive association exists between the

intensity of chronic inflammation and gastric cancer. Dietary factors and smoking habits have also been linked to gastric cancer risk (Lunet *et al.,* 2005; Tsugane *et al.,* 2004; Bergin *et al.,* 2003; Fox *et al.,* 2003; Fox *et al.,* 1999; Tredaniel *et al.,* 1997) and may influence pre-malignant changes, however, no such association was evident in the present study. This was determined based on the answers provided in patient questionnaires. However, one must be wary of the accuracy of such information since patients often do not provide full details about diet and often underestimate their smoking habits.

The gastric tissue microenvironment may also account for the variability in *c-FOS* gene expression since it is subject to dynamic changes due to exposures to a variety of compounds such as food allergens, alcohol, medications, bile acids etc. as well as temporal-spatial variations in oxidative stress (the gastric mucosa is constantly exposed to waves of luminal oxidative stress arising from digested food, bacteria, shed mucosal cells, etc. (Smith *et al.,* 1996; Fukumura *et al.,* 1995; Hiraishi *et al.,* 1994)), gastric acidity, and ascorbic acid content of gastric juice, all of which may influence signal transduction and gene expression changes. A recent study by Jenkins *et al.* (2007) showed that bile acids can affect cellular signal transduction (specifically NFκB signalling) through ROS mediated mechanisms, and so the fluctuating levels of bile acids in gastric juices bathing cells may lead to fluctuations in local oxidative stress. These factors can vary in the different anatomical regions of the stomach due to the differences in the cell types and secretions found at the different sites. This was a pitfall of the present studies since biopsies were often taken from different regions of the stomach, and is likely to contribute to the variation in the expression data. Ideally the experiments would be repeated with all samples taken from the same stomach region, e.g. the antrum, so removing a potential source of variation. The variability in the expression data may also stem from the differing proportions of diseased: normal cells in the biopsy specimens. In instances where the majority of cells in a diseased biopsy are normal stromal cells, their expression levels may mask any abnormal patterns in the diseased cells.

As for *c-FOS*, there was an overall marked amount of variation in the pERK data. Interestingly, in some patients pERK and *c-FOS* levels were seen to be related to an extent, supporting findings in chapter 3 which demonstrated that ERK activation was

linked to downstream *c-FOS* gene expression. The observation that ERK activity and *c-FOS* levels were not always associated suggests that other mechanisms of controlling *c-FOS* expression are likely to be important. It is possible that a mechanism distinct from ERK MAPK signalling was regulating *c-FOS* expression in cases where the two were not reflective, and perhaps negative feedback between the two could account for cases where pERK levels were seen to be increased and *c-FOS* levels appeared to be decreased.

In summary, the present studies have demonstrated that *c-FOS* expression and ERK activity are highly variable in gastric biopsy samples and that no clear involvement of such molecular changes in pre-malignant gastric tissues could be established based on the experimental design. The gene expression and signalling changes observed may be the end points of several mechanisms. For instance, since chronic inflammation and gastritis are accompanied by tissue injury and cell loss mediated by ROS (Ding *et al.*, 2007; Naito and Yoshikawa, 2001), any increases in ERK signalling and *c-FOS* expression in such samples are unsurprising since they provide the mitogenic signalling and gene expression changes required to evoke proliferation, so replacing lost cells (Chang and Karin, 2001; Treinies *et al.*, 1999). If such persistent oxidative stress signalling changes occur in a subset of individuals it is possible that these early changes set in motion a cascade of cellular events that may drive gastric carcinogenesis.

The key findings from the studies were:-

- pERK activity and *c-FOS* RNA expression were seen to be highly variable in the sample cohort study, with no clear trends regarding the pre-malignant stages and the onset of the changes, hence the involvement of the signalling and gene expression changes in gastric carcinogenesis remains unclear;
- *H. pylori* status did not appear to impact *c-FOS* expression levels or ERK activity;
- Some association between pERK and *c-FOS* was apparent, strengthening findings in chapter 3 that implicated ERK MAPK signalling as an upstream control of ROS induced *c-FOS* gene expression;
- There was a high degree of variability in the data, possibly reflecting inter-patient differences in diet, genetic susceptibility, etc., and intra-patient variation, likely due to spatial differences in gastric tissue microenvironment. Normal gastric

mucosa has previously been shown to be more genetically unstable than matched oesophageal tissue (Williams *et al.*, 2005), and so it is unsurprising that genetic heterogeneity be present in pre-malignant lesions. The variation may also be a consequences of samples being taken from different anatomical regions of the stomach, and so it would be worthwhile to repeat the experiments with all samples being obtained from the same region, e.g. antrum;

- There were pitfalls in the experimental design that made it difficult to establish the significance of the molecular changes with respect to pre-malignant histology. The root of the problems lay in the difficulty in clearly distinguishing gastritis and normal tissues endoscopically. This resulted in a patient pool with very limited matched normal and diseased samples.

Further studies with a larger sample cohort (to remove the effects of variation) and better success with obtaining matched normal and diseased samples (all from the same region of the stomach) would ascertain whether changes in ERK signalling and *c-FOS* expression are important pre-malignant molecular changes on the path to gastric cancer.

Chapter 7

Final Discussion

The potential involvement of RO/NS in disease and ageing has been an area of interest since 1956, when Harman first proposed the free radical theory of ageing, in which it was speculated that cumulative cellular damage induced by radicals may contribute to the ageing process (Harman, 1956). Since then, a boom in the field of free radical research has provided insight and strength to the hypothesis that RO/NS play crucial roles in biology and medicine (Scandalios, 2002; Beckman and Ames, 1998; Guyton and Kensler, 1993; McCord and Fridovich, 1969). A particular area of interest included the now very well documented involvement of reactive metabolites in cancer development (Valko *et al.*, 2007; Fukuruma *et al.,* 2006; Ristow, 2006; Waris and Ahsan, 2006; Klaunig and Kamendulis, 2004; Oshima *et al.,* 2003; Klaunig *et al.,* 1998; Poulsen *et al.,* 1998; Wink *et al.,* 1998; Knight, 1995; Toyokuni *et al.,* 1995; Feig *et al.,* 1994; Guyton and Kensler, 1993; Trush and Kensler, 1991; Breimer, 1990; Vuillaume, 1987; Ames, 1983).

Oxidative stress and carcinogenesis are intimately intertwined in a manner which can both initiate disease, and drive its further progression, since oxidants are complete carcinogens - impacting the initiation and promotion stages via the induction of DNA damage and mutations, and enhanced cellular proliferation respectively (Valko *et al.*, 2006; Behrend *et al.,* 2003; Olinski *et al.,* 1998; Poulsen *et al.,* 1998; Dreher and Junod, 1996; Cerutti and Trump, 1991). Excessive oxidative stress can instigate oncogenic changes in cells, and cells that go on to develop a transformed oncogenic phenotype often produce elevated levels of reactive species, which can go on to drive further disease progression. Indeed, it is often observed that cancer cells/ tumour samples contain greater levels of RO/NS and hence oxidative and nitrosative stress than their normal disease free counterparts (Obst *et al.,* 2000; Davies *et al.,* 1994a; Davies *et al.,* 1994b; Halliwell *et al.,* 1992; Szatrowski and Nathan, 1991). Experimentation by Szatrowski and Nathan (1991) revealed that several different tumour cell lines constitutively generate H_2O_2 at rates between 100 – 500μM/ 10^4 cells/ hr over several hours at a constant rate. Thus a vicious

cycle exists, resulting in cells being continuously bombarded with oxidants and their damaging effects.

The present work set out to further evaluate the involvement of components of an inflammatory response, in particular ROS and resultant oxidative stress, in gastric carcinogenesis. Gastric cancer of the intestinal sub-type is well documented as developing through a defined series of pre-malignant stages starting with chronic gastritis and gradually progressing through atrophic gastritis, intestinal metaplasia, dysplasia, and ultimately invasive gastric cancer (Correa and Shiao, 1994; Correa, 1992a; Correa, 1988; Correa *et al.,* 1975) with chronic inflammation being central in both the initiation and promotion of the disease (Zavros *et al.,* 2005; Israel and Peek, 2001; Ernst, 1999; Ernst *et al.*, 1997; Correa, 1992a). With the realisation of the global burden of the disease, constituting the 2nd largest cause of cancer related deaths worldwide, large research efforts have brought to light key findings with regard to the pathogenesis and underlying molecular biology of the disease (detailed in chapter 1).

Chronic inflammation of the gastric mucosa/ gastritis, regardless of its cause, is now generally accepted as an early driving force in gastric carcinogenesis (Zavros *et al.,* 2005). In the case of *H. pylori* – associated gastritis, the infection elicits a marked inflammatory response, characterised by infiltration of leukocytes which release a plethora of pro-inflammatory mediators and bactericidal free radicals in attempts to ward off infection (Peek and Crabtree, 2006; Peek and Blaser, 2002; Ernst *et al.*, 1997). Unfortunately for the host, the bacterium has evolved mechanisms of evading the inflammatory response, and as such, in the absence of antibiotic intervention therapy, persistent infection may develop, leading to chronic inflammation and a hostile gastric tissue microenvironment in which leukocyte - (and gastric epithelium -) generated reactive metabolites can inflict harm on the host cells (Correa and Miller, 1998) (detailed in section 1.4.5.1). Both bacterial factors and host inflammatory response contribute to disease pathogenesis by conferring an increase in oxidative stress. Based on these grounds, it is speculated that the oxidative stress component of *H. pylori* infection maybe a key driving force in disease development and progression, since the reactive species can account for almost all of the molecular changes seen to be induced by the bacterium, either acting independently or synergistically. *H. pylori* infection is known to cause

oxidative DNA damage (Obst *et al.,* 2000; Baik *et al.,* 1996), alterations in cell growth by perturbing the proliferation – apoptosis balance (Correa *et al.,* 2004; Moss *et al.,* 2001; Correa and Miller, 1998; Peek *et al.,* 1997; Cahill *et al.,* 1996; Moss *et al.,* 1996), and changes in signal transduction and gene expression (Tuccillo *et al.,* 2005; Strowski *et al.,* 2004; Caputo *et al.,* 2003; Sepulveda *et al.,* 2002; Chiou *et al.,* 2001; Mitsuno *et al.,* 2001; Peek, 2001; Maeda *et al.,* 2000; Meyer-Ter-Vehn *et al.,* 2000; Keates *et al.,* 1999; Naumann *et al.,* 1999; Sharma *et al.,* 1998; Aihara *et al.,* 1997; Keates *et al.,* 1997). These cellular effects can also be induced by oxidative stress (Dreher and Junod, 1996; Guyton and Kensler, 1993; Cerutti and Trump, 1991), and links between *H. pylori* infection, oxidative stress, and such changes is well established (Baek *et al.,* 2004; Seo *et al.,* 2004; Xu *et al.,* 2004; Chu *et al.,* 2003; Kim *et al.,* 2000; Bagchi *et al.,* 1996) further strengthening the notion that *H. pylori* – associated gastric carcinogenesis can be mediated by oxidative stress. Indeed the bacterium is known to cause inflammation and ROS generation and release in gastric epithelial cells (Xu *et al.,* 2004; Obst *et al.,* 2000; Bagchi *et al.,* 1996; Farinati *et al.,* 1996). Two recent studies into protein changes induced by *H. pylori* in gastric epithelial cells speculate that ROS are fundamental to the changes – resulting in decreased antioxidant defence mechanisms, and enhanced expression of inflammatory genes, adhesion molecules, mediators of cellular proliferation and oncogenic transcription factors (Kim, 2005; Baek *et al.,* 2004).

Despite these advances the mechanistic nature of disease development with respect to the effects of inflammation remains to be clearly defined. The overall aims of the present research were to obtain further insights into the link between inflammation and gastric cancer at the molecular level (with an emphasis on signal transduction and gene expression changes) using a combination of *in vitro* and *in vivo* models. The hypothesis being tested was that: -

Chronic gastric inflammation & accompanying generation of Reactive Oxygen Species (ROS) plays a significant role in gastric carcinogenesis by the induction of molecular changes (signal transduction, and gene and protein expression changes).

In vitro tissue culture models were first employed – one in which cells (two gastric cancer cell lines (AGS and HGC-27) and one normal fibroblast cell line (WILL1)) were exposed to various doses of a model ROS, hydrogen peroxide (H_2O_2), for varying lengths of time in order to mimic the oxidative stress component of a gastric inflammatory response (chapter 3); and one in which gastric epithelial adenocarcinoma cells (HGC-27) were co-cultured with inflammatory leukocytes so more realistically mimicking an *in vivo* inflammatory response (chapter 5). The *in vitro* studies were then translated to an *in vivo* study in which signalling and gene expression changes consistently observed in the cell culture studies were assessed in pre-malignant gastric biopsies in order to determine whether such changes may be truly important in early stages of gastric carcinogenesis (chapter 6).

The data presented in chapter 3 revealed that H_2O_2 treatment of cells in culture (with sub-toxic doses) can cause some significant changes in cellular gene expression, impacting DNA damage response, cell cycle control, apoptosis, signal transduction, inflammation, angiogenesis, invasion and metastasis, all of which are known to be affected in neoplastic development (Hanahan and Weinberg, 2000). This finding strengthens the notion that oxidative stress is a key driving force in cancer development. Microarray experiments on HGC-27 RNA from control and treated cells shed some light on the impact of oxidative stress on MAPK signalling, reflected in up-regulation of p38 MAPK (*MAPK14*), *RAF1* (MAP3K), and *c-FOS*, and the potential involvement of the NFκB pathway, since several NFκB regulated genes were seen to be up-regulated in response to H_2O_2 exposure (e.g. *VCAM*, *RANTES*, *TNFA*). The most consistent gene expression alterations detected in the array experiments were up-regulation of *c-FOS* and *VEGF*, the levels of which were subsequently quantified by real-time PCR. The real-time PCR gene expression studies were carried out on RNA from three cell lines – AGS, HGC-27 and WILL1 (treated in the same manner), and confirmed that frank oxidant exposure could indeed induce significant changes in *c-FOS* and *VEGF* levels, showing dose dependency in some instances. Interestingly the outcome seemed to differ between the cell lines, reflecting possible differences in the redox sensitivity of the different cells, different antioxidant profiles, or differences in the signalling pathways. Most often it was seen that the cancer cells (ASG and HGC-27) showed clearer dose responses at earlier

time points than the normal WILL1 cell line. This may, in addition to the aforementioned reasons, be due to aberrant signal transduction in the cancer cell lines, possibly brought about by mutation. Aberrant signalling, such as ability to trigger excessive signalling in response to stimuli that would not normally elicit such a marked response, or self-sufficiency in signalling, is in fact one of the hallmarks of cancer (Hanahan and Weinberg, 2000). Interestingly, the importance of altered responsiveness to stimuli or a so-called 'response modification' has been proposed to be central to carcinogenic development by oxidants for over twenty years (Cerutti, 1989, Cerutti, 1985). The involvement of MAPK signalling in the response to H_2O_2 was validated by western blotting, which revealed rapid activation of ERK1/2 (p42/ p44) MARK signalling in all three cell lines, and p38 signalling in HGC-27 and WILL1. ERK signalling was most persistent in AGS cells, and the apparent absence of p38 signalling in this cell line is suggestive of a shift toward enhanced proliferation since ERK signalling favours cell survival and proliferation whilst the p38 pathway favours apoptosis (Halliwell and Gutteridge, 2007).

Some evidence of a link between ERK signalling and downstream *c-FOS* expression was provided by a preliminary inhibitor study, however, further experimentation would be required to validate this. It is also important not to overlook the complexity of MAPK signalling when trying to establish links, since several layers of control exist, often as autocrine loops. For example, MAPKs can indirectly regulate the expression of both their activating ligands and their inhibitors, so introducing inherent control in MAPK signalling (Chang and Karin, 2001), which is often lost in cancer cells. Interestingly MAPK signalling can regulate the expression of *VEGF* (seen to be up-regulated in the present studies), VEGF protein, in turn, upon release from cells, can act in an autocrine manner to activate further MAPK signalling (Yashima *et al.,* 2001; Doanes *et al.,* 1999; Takahashi *et al.,* 1999). In addition, some reports suggest that VEGF can modulate cellular redox status (Abid *et al.,* 2001) such that it can impact NFκB signalling (Wang *et al.,* 2001). This is important in the context of gastric carcinogenesis, and suggests a possible role for VEGF not only in the later angiogenic stages of disease, but also early on in the progression sequence at the stage of gastritis. Gastritis associated tissue injury and inflammation results in enhanced ROS levels in affected tissues

compared to controls (Morgan *et al.,* 2003). Based on these findings ROS exposure has the potential to induce *VEGF* over-expression. This may then go on to result in further production of ROS, enhanced MAPK and NFκB signalling, enhanced inflammatory cytokine expression, and an overall hostile tissue microenvironment. It is possible that VEGF, may, in addition to H_2O_2 exposure, account for some of the NFκB related gene expression changes observed. The potential link between *c-FOS* and *VEGF* expression is also interesting, since if a true link can be validated by further experimentation, it would suggest that *VEGF* may act as *c-FOS* effector in malignancy, providing a link between the oncogene and angiogenesis (Marconcini *et al.,* 1999).

Looking at the data as a whole, the overall picture is that oxidant exposure can elicit enhanced MAPK signalling, and up-regulation of the nuclear oncogene *c-FOS*, a component of the AP-1 transcription factor, and potentially enhanced NFκB signalling, albeit in a dose, time, and cell line specific manner. This can favour neoplasia since it can shift the proliferation – apoptosis balance toward enhanced proliferation and enhanced cell survival, and can enhance inflammation through the induction of inflammatory response genes such as *IL-8* and *TNFA* (Karin, 1995). This can then further drive enhanced inflammatory responses, proliferation, cell survival, and angiogenesis (by IL-8). IL-8 also drives the generation of ROS (Naito and Yoshikawa, 2002) leading to further oxidative stress resulting in a vicious cycle in which a hostile microenvironment of oxidative stress is favoured. Taken together these findings strongly support the hypothesis that oxidative stress is a key driving force in carcinogenesis, not only by the induction of mutations, but also via signal transduction and gene expression changes. The H_2O_2 doses that caused the most significant changes correspond to doses that have been reported to cause point mutations and chromosome damage (Williams *et al.,* 2005; Jenkins *et al.,* 2001; Duthie *et al.,* 1997; Kleiman *et al.,* 1990), and also lie in the range of ROS levels reported to be generated by cancer cells (Szatrowski and Nathan, 1991), making the findings of clinical significance. As such, dose and timing of exposure seems to be important, low doses causing no significant changes, and excessively high doses causing cytotoxicity and may also inactivate signal transduction (e.g. high levels of oxidative stress inhibit MAPK signalling via the activation of phosphatases (Jackson *et al.,* 2002)). Matysiak-Budnika and Mégraud (2006) reported that the intensity of gastric

inflammation (as a consequence of gastritis (+/ - *H. pylori* infection)) determines an individuals risk to gastric cancer, and it may be that this is related to the levels of ROS in the tissues, and the lengths of time for which cells are exposed. A very recent study by Ding *et al.* (2007) demonstrated that both *H. pylori* infection and exposure to H_2O_2 caused dose dependent increases in ROS generation by gastric epithelial cells, and that this was enhanced by pre-treatment with inflammatory cytokines. This finding provides yet further weight to the hypothesis that ROS are central to gastric carcinogenesis, but also implies added complexity since inflammatory mediators are likely to have an impact. This warranted the further research that was carried out in subsequent chapters which took a further interest in the inflammatory component of disease pathogenesis.

Chapters 4 and 5 set out to take the *in vitro* studies to a more biologically/ clinically relevant level by mimicking an inflammatory response as it is likely to occur *in vivo* with RO/NS originating from a biological source (inflammatory leukocytes) in conjunction with other inflammatory mediators. Specifically chapter 4 involved optimising an inflammatory response in the promyelocytic HL-60 cell line (giving rise to a mature myelocyte cell population (consisting of myelocytes, metamylocytes, and banded neutrophils with the capacity for oxidative burst) in preparation for subsequent co-culture experiments in which this inflammatory cell population was co-incubated with gastric epithelial adenocarcinoma cells (HGC-27) and any consequent signalling and gene expression changes in HGC-27 analysed. After optimisation efforts to induce neutrophil differentiation (since neutrophils are one of the most common inflammatory cell types present at sites of infection and inflammation (Yoshikawa and Naito, 2001; Crabtree, 1996a; Kozol, 1990)), priming, and induction of a detectable oxidative burst as well as release of inflammatory cytokines, a treatment regimen was established. Briefly this involved culturing HL-60 in the presence of 1.3% (v/v) dimethyl sulfoxide (DMSO) for 3 days prior to co-culture. The resultant population of differentiated cells was deemed HL-60/N for the presence of neutrophils, and was primed overnight with LPS and co-cultured with HGC-27 at different densities. The degree of inflammatory cell infiltrate can fluctuate widely in chronic gastritis, and the extent of inflammation is recognised as a risk factor for the development of malignant gastric disease, the more severe the inflammation the greater the risk (Fox and Wang, 2007; Axon, 2002; El-Omar *et al.*,

2000; Correa, 1995; Gilmour, 1961). As such, HGC-27 was co-cultured with HL-60/N at different ratios (HGC-27: HL-60/N at 1:1, 2:1, and 4:1) in order to reflect different severities of inflammation. Activation of HL-60/N was then stimulated via the introduction of 100nM fMLP to the co-culture system resulting in an oxidative burst response.

Utilising this co-culture model in chapter 5, the influence of leukocyte derived oxidative stress on HGC-27 at the level of signal transduction and gene expression changes – specifically the ERK1/2 (p42/ p44) MAPK pathway and *c-FOS* and *IL-8* gene expression (in keeping with the previous studies in chapter 3 and further studies in chapter 6) was analysed. *c-FOS* expression was assessed as a marker of ERK MAPK signalling since the two were seen to be linked in preliminary experimentation described in chapter 3, in addition to assessing ERK activation at the level of ERK phosphorylation. In a similar manner *IL-8* was studied as a marker of NFκB activity since *IL-8* expression has been reported to be regulated by NFκB (Jenkins *et al.*, 2004). Following co-culture, RNA and protein were extracted from cells and the levels of oxidised proteins measured to determine the impact of the co-culture system with respect to oxidative stress. The results confirmed that the system generated an inflammatory setting in which release of ROS (and hence oxidative stress) could directly impact HGC-27 at the molecular level since co-culture with HL-60/N in the absence and presence of fMLP was seen to cause an increase in the levels of oxidised proteins in the gastric epithelial cells. Interestingly co-culture did not cause the expected increase in *c-FOS* expression as was observed as a consequence of chemical induced oxidative stress in chapter 3. Instead where significant changes in *c-FOS* levels were seen, they tended to be significant decreases. The most interesting observation was that *c-FOS* expression level appeared to be related to the level of 'tissue inflammation' in the co-culture system, with no down-regulation seen in the 1:1 co-culture both in the absence or presence of fMLP stimulation, whilst significant decreases in *c-FOS* expression were evident at both the 2:1 and 4:1 co-culture systems. This raised the speculation that the severity of inflammation may have an impact on *c-FOS* gene expression, since less intense inflammation (lower densities of inflammatory leukocytes – 2:1 and 4:1 co-culture) caused down-regulation of *c-FOS* whilst more intense inflammatory conditions (1:1 co-culture) had no significant effect on *c-FOS*

levels. High levels of *c-FOS* were seen in the un-exposed controls, and as such the decreases in *c-FOS* expression observed may be misleading, and may in fact be related to some aspect of the cell culture system. Possible explanations for the observed decreases in *c-FOS* levels, in the case that the decreases were due to true down-regulation of gene expression, include negative feedback control; whereby *c-FOS* expression is saturated in un-exposed controls, the introduction of gene expression inducing stimuli in the co-culture system may then instigate down-regulation of expression; and/ or a sub-toxic effect of excessive oxidative stress (amongst other aspects of the co-culture system) leading to decreased proliferation in HGC-27 and so an overall reduction in gene expression. Without further experimentation (e.g. assessing the proliferation rate of HGC-27 post co-culture) it is very difficult to establish reasoning behind the observations.

Unlike *c-FOS*, co-culture caused significant up-regulation of *IL-8* gene expression. An interesting finding that came to light however, was that as for *c-FOS*, *IL-8* expression also appeared to be related to the co-culture ratio and hence to the degree of inflammation and oxidative stress. For example, at both the 4hr and 8hr time points the 1:1 co-culture system in the absence of fMLP caused maximal induction of *IL-8* compared to the 2:1 and 4:1 co-culture systems in which HGC-27 cells were exposed to lower densities of HL-60/N and thus less severe inflammatory conditions. Since *IL-8* expression was seen to be consistently up-regulated in the co-culture experiments, albeit being related to the degree of inflammation, one can speculate that *IL-8* is a likely molecular change under conditions of tissue inflammation which has previously been reported (Naito and Yoshikawa, 2002; Maeda *et al.*, 2001; Shimada and Terano, 1998; Crabtree and Lindley, 1994), with the likelihood of up-regulation being positively related to the degree of inflammation.

As for *c-FOS* and *IL-8*, ERK phosphorylation was seen to be related to the co-culture ratio and so the severity of inflammation, with significant increases in pERK levels being evident following exposure of HGC-27 to HL-60/N at a ratio of 1:1 both in the absence and presence of fMLP stimulation, with the lower HL-60/N densities (2:1 and 4:1) having no effect. Interestingly there appeared to be some discrepancy between *c-FOS* expression and ERK activation, since the expected positive association between the

two (based on the findings in chapter 3) was not apparent following co-culture. It may be that some other aspect of the co-culture system impacted *c-FOS* expression so overshadowing any possible effects of ERK MAPK signalling, and further experimentation would be required to ascertain the underlying mechanisms for the observations.

Based on these findings one thing that became very clear was that the severity of inflammation (likened to a 'dose' dependency) is likely to determine the outcome of *c-FOS* and *IL-8* gene expression as well as ERK MAPK signalling, and that this is likely to be the case in the *in vivo* gastric setting. It is then likely that only in a small number of individuals in which the degree of inflammation is severe enough, but at the same time sub-toxic/ sub-lethal to the cells (i.e. within a 'dose' window, or above a threshold 'dose'), do such changes which may drive gastric carcinogenesis occur, and may help to account for the frequent observation that only a subset of individuals with chronic gastritis (+/ - *H. pylori* infection) go on to develop malignant gastric disease (Peek and Crabtree, 2006; Farthing, 1998).

Together the two *in vitro* models of components of inflammatory gastritis brought to light that MAPK and NFκB signalling changes together with downstream gene expression targets can be altered quite clearly in situations of oxidative stress (chapter 3) and to a reasonable (although less clear) extent as a consequence of exposure to inflammatory leukocytes as a biological source of RO/NS and inflammatory mediators. In order to determine if such alterations were of clinical significance they were also examined in gastric biopsy specimens in an *in vivo* study.

In chapter 6 ERK MAPK signalling and *c-FOS* gene expression were assessed as markers of oxidative stress induced signalling and gene expression changes *in vivo.* Since the *in vitro* studies demonstrated that oxidative stress could induce such changes, it was important to determine if the findings translated to the *in vivo* tissue setting, specifically looking at pre-malignant gastric tissues. The hypothesis being tested was that redox sensitive signal transduction changes such as ERK MAPK signalling and downstream gene expression changes may be important in pre-malignant stages of Correa's pathway to gastric cancer so driving disease pathogenesis. Twenty-five patients were successfully recruited into the studies yielding tissue samples from inflammation/ gastritis and

intestinal metaplasia pre-malignant stages as well as samples from *H. pylori* infected individuals. Results indicated that whilst *c-FOS* expression levels appeared to be overall high in normal and chronic inflammation/ gastritis tissues compared to IM samples, there was a great amount of variation in expression levels (both at the inter- and intra-patient levels), which seem to reflect the levels of ROS detected in pre-malignant gastric tissues by Morgan *et al.* (2003) suggesting that ROS may have an impact on the gene expression changes. Overall, it was not possible to determine the involvement of *c-FOS* expression in gastric carcinogenesis and its relation to pre-malignant lesions (in particular inflammation/ gastritis). pERK levels also showed a lot of variation across the samples with no clear trends with respect to ERK activity and pre-malignant disease progression nor *H. pylori* status. It was seen that there was some association between pERK and *c-FOS* in the samples providing further strength to findings in chapter 3 that implicated ERK MAPK signalling as an upstream control of ROS induced *c-FOS* gene expression, as well as indicating that the changes observed in the *in vitro* studies are likely to be important at the clinical level. The *in vivo* study suffered pitfalls in the experimental design which largely made the data inconclusive. A fairly small sample cohort was obtained with very few matched normal and diseased samples, biopsies often taken from different anatomical regions of the stomach, due to difficulties in diagnosing pre-malignant lesions at the endoscopic level. These problems resulted in data that could not be analysed in a statistically sound manner and so any relation between *c-FOS* expression, pERK levels, and tissue inflammation (and hence oxidative stress) could not be clearly established. The findings highlight the difficulties that can be encountered in clinical studies.

Looking at the research presented as a whole some interesting themes emerged. In relation to the overall aims of the studies, much of the data presented lent support to the hypothesis that oxidative stress (amongst other aspects of the tissue inflammation characteristic of gastritis) may drive early stages of the pathogenesis of gastric cancer by way of signal transduction and associated gene expression changes. In chapter 3 it was seen that chemically induced oxidative stress could clearly induce MAPK signalling and downstream gene expression changes, in particular *c-FOS*. NFκB – related gene expression changes as a consequence of this oxidative stress were also evident to an

extent. When the ERK – related signalling and gene expression changes were then analysed in the second *in vitro* model, which more closely mimicked the biological conditions of tissue inflammation, it was seen that whilst inflammatory conditions could induce ERK signalling, *c-FOS* over-expression was not induced, suggesting that under such conditions, in which oxidative stress as well as other components of the inflammatory response may simultaneously impact different aspects of cellular biochemistry and signalling, any association between ERK signalling and *c-FOS* expression may be overshadowed. *IL-8* was seen to be up-regulated in these studies, bringing to light potential involvement of the NFκB pathway.

Based on the *in vitro* findings it became fundamental to determine if such changes in *c-FOS* gene expression and ERK signalling could also be detected in pre-malignant gastric biopsies. The finding that *c-FOS* expression was overall higher (across individual patient samples) in normal and inflammation/ gastritis tissues compared to IM, together with the apparent relation between *c-FOS* expression levels and the ROS levels previously detected in the different histo-pathological stages (Morgan *et al.*, 2003) were the most interesting findings from the *in vivo* study, since together they support the hypothesis that oxidative stress is a key player in the induction of potentially oncogenic signalling and gene expression changes in pre-malignant gastric tissues. With respect to ERK signalling, the data also revealed a high degree of variability, with no clear trends being observed across the pre-malignant stages. Overall, due to the discussed problems encountered with the clinical study, the data obtained did not permit any solid conclusions to be drawn regarding *c-FOS* expression and pre-malignant gastric disease progression. Based on the findings the involvement of *c-FOS* expression changes and ERK signalling in gastric carcinogenesis are unclear and cannot be ruled out without further experimentation (ideally with a larger sample cohort consisting of matched control and diseased samples (all obtained from the same anatomical region of the stomach)). NFκB – related gene expression was also assessed in the pre-malignant biopsies by a colleague in the laboratory (Mrs. Jane Mikhail) by looking at *IL-8* as a marker of NFκB activity, and preliminary data revealed that *IL-8* was up-regulated in gastritis samples as well as in samples from *H. pylori* infected patients, leading to speculations that aberrant NFκB signalling is a likely event in gastric carcinogenesis.

Overall the data from the *in vitro* and *in vivo* studies provides evidence that oxidative stress amongst other components of inflammation can cause signalling and gene expression changes that may be important in driving the development of gastric cancer at the level of MAPK and NFκB signalling and downstream gene expression changes.

In chapter 3, aside from the effects of chemically induced oxidative stress on signal transduction and gene expression, the notable differences in responses to oxidant induced changes between the three cell lines emerged as a fundamental finding from the studies. Oxidant induced changes were often seen to occur in a dose, time, and cell line specific manner, with the two cancer cell lines (AGS and HGC-27) showing more marked responses than the normal WILL1 cell line. Additionally, differences were often seen between the two cancer cells, with HGC-27 appearing to be more oxidant sensitive with respect to some molecular changes, and AGS being more sensitive to others. This is likely due to the different stages of malignancy of the two cell lines – HGC-27 being the more advanced of the two, and so the differences may reflect molecular changes that come into play at earlier or later stages of disease progression. In the *c-FOS* expression data, for example, up-regulation was induced more rapidly in HGC-27 compared to AGS. It is very likely that such differences reflect inherent differences in the cell biology of the two cancer cell lines. Since AGS is established from a primary gastric tumour (Barranco *et al.,* 1983) this cell line may be more resilient to some oxidant induced molecular changes, with the more advanced HGC-27 cell line (derived from a secondary lymph node metastasis of a gastric adenocarcinoma) being more sensitive to some of the consequences of oxidative stress. Alternatively, being more advanced, HGC-27 is more likely to have accumulated molecular changes that result in aberrant signalling (e.g. mutations or aberrant expression of components of the RAS-RAF-MEK-ERK signalling pathway which are known to contribute to carcinogenesis (Leicht *et al.,* 2007; Roberts and Der, 2007; Schubbert *et al.,* 2007, McCubrey *et al.,* 2006)). Interestingly, it has frequently been noted that differences in cellular biochemistry (e.g. antioxidant enzyme and metabolite levels) as well as differences in metastatic potential are likely to affect how a cell responds to an exposure/ stimulus (Aune and Pogue, 1989; Barranco *et al.,* 1983; Nicolson *et al.,* 1978), and such differences may help to explain the cell line

specific responses. Another plausible explanation lies in differences in proliferation rate. HGC-27 demonstrated a higher rate of proliferation than that of AGS (observed simply based on cell culture properties), and so higher proliferation rate may account for higher levels of gene expression and/ or enhanced sensitivity in HGC-27.

The primary WILL1 cell line was the least responsive of the three cell lines and this raised the hypothesis that normal cells may be more resilient to oxidative stress and redox sensitive molecular changes compared to the cancer cells, which may inherently be more redox sensitive (Benhar *et al.*, 2001; Toyokuni *et al.*, 1995). In addition, the observation that *c-FOS* levels were seen to increase in untreated cancer cells over time but not in WILL1, led to the speculation that oxidative stress can build up in the cancer cells over time which may make them more redox sensitive. This build up of oxidative stress is likely to be due either to enhanced RO/NS generation in cancer cells themselves (Ding et al., 2007; Schumacker, 2006; Benhar *et al.*, 2001; Halliwell *et al.*, 1992; Szatrowski and Nathan, 1991; Aune and Pogue, 1989), and/ or less efficient antioxidant systems compared with normal cells (Batcioglu *et al.*, 2006; Navarro *et al.*, 1999). The balance between oxidants and antioxidants is fundamental here. In cancer cells lower levels of cellular antioxidants can lead to a build up of oxidants. If the balance is such that oxidant levels are maintained at sub-lethal levels an individual may be at a greater risk of gastric carcinogenesis. Interestingly *H. pylori* infection has been reported to impact the activity of cellular ROS scavenging enzymes, such that accumulating ROS in gastric cells fall to sub-lethal doses, resulting in an increased risk of gastric cancer (Smoot *et al.*, 2000).

Differences between the cell lines were also noted for *VEGF*, *IκB*, and *IL-8* expression, as well as in ERK and p38 signalling, and were detailed in section 3.4.

Since the overall aim of the present research was to assess the involvement of ROS and inflammation in cancer associated signal transduction and gene expression changes initially using *in vitro* models (chapters 3, 4, and 5) and then shifting to an *in vivo* study (chapter 6), time did not permit the further study of the signal transduction pathways in detail. None-the-less, the findings have opened up the doors to continued research. Further studies in continuation of the findings in chapter 3 that would be of particular interest would be to study in greater depth the differences observed between

the three cell lines. The differences in cell line responsiveness to H_2O_2 exposure are likely to reflect inherent biochemical differences in the cells such as variations in antioxidant defence systems. In recent years there has been an increase in evidence that H_2O_2 is an important signalling molecule and that antioxidant enzymes play key roles as sensors and regulators of H_2O_2 – induced signal transduction (Veal *et al.*, 2007). Based on this it would certainly be worth examining differences in the antioxidant profiles of the cell lines in relation to their response to oxidant exposures so to gain insights into the observed differences. One way in which this may be achieved would be to examine the levels of a few antioxidant enzyme markers such as superoxide dismutase and catalase in the cells by way of western blotting. Such studies may also be important in a clinical context since inter-individual differences in antioxidant enzyme levels may account for differences in susceptibility to disease. Additionally, it has recently been reported that cancer cells carry greater oxidant loads due to enhanced ROS generation, and that by inhibiting antioxidants it may be possible to achieve targeted killing of the cancer cells since they will accumulate more severe (often lethal) levels of ROS rendering them more sensitive to the detrimental effects of ROS than their normal counterparts (Trachootham *et al.*, 2006). As such, further insights into the oxidant and antioxidant profiles of normal cells versus cancer cells, as well as cancer cells at different stages of the disease, would be a fruitful area of further research that could lead to findings which would add further weight to such reports. The enhanced oxidant sensitivity of malignant cells may then be exploited therapeutically.

The cell line differences may also be due to differences in proliferation rates and induction of apoptosis, higher proliferations could account for overall higher levels of gene expression in one cell type versus another, as could high induction of apoptosis (or death via necrosis) account for lower gene expression. As such, it would be of interest to study in greater detail the proliferation rates and induction of cell death. A variety of experimental techniques are available that could be employed for studying proliferation and cell death (apoptosis and necrosis) including cell counting of live cells routinely during cell culture, scoring of apoptotic and necrotic cells in Geimsa stained slide preparations (Fenech *et al.*, 1999), as well as more elaborate techniques such as ELISAs for caspase enzymes to more sensitively assay the induction of apoptosis, and methods

that employ fluorescent antibodies for cell surface markers of proliferation and apoptosis followed by fluorescent microscopy and/ or flow cytometry based visualisation.

It would also be worthwhile to conduct more inhibitor studies to validate links between MAPK signalling and *c-FOS* expression, and analysing a potential link between *c-FOS* and *VEGF* gene expression by inhibiting *c-FOS* and analysing the impact on *VEGF* (possibly using siRNA for *c-FOS*). To further pursue the NFκB studies it would be worth optimising the transfection experiments using the GFP-p65 reporter plasmid to gain further insights into the involvement of NFκB activation. Ideally this would be examined in real-time using live cells, directly following the cytoplasmic to nuclear shuttling of the transcription factor under the confocal microscope. Alternatively the cellular localisation of NFκB could be examined via immunohistochemistry using a fluorescent antibody coupled with visualisation by standard fluorescence microscopy.

Since the induced signal transduction and gene expression changes observed are likely to favour proliferation and cell survival, it would be worth studying the effects of oxidant exposure on cellular proliferation and apoptosis (using the methods previously discussed) in order to establish if indeed a shift in the proliferation – apoptosis balance toward enhanced proliferation exists. This would be of particular interest to gain further understanding into the observation that of all three cell lines AGS appeared to sustain ERK activation the longest and did not show activation of the p38 MAPK pathway based on the data presented. Such signalling dynamics would likely favour enhanced proliferation since ERK is a survival factor and drives enhanced proliferation, whilst p38 generally induces apoptosis (Halliwell and Gutteridge, 2007). Proliferation and apoptosis studies could help to determine if such changes impact the cells at the physiological level causing shifts in the proliferation – apoptosis balance, such that the changes may drive gastric carcinogenesis.

Stemming from the co-culture studies, given more time and resources it would be worthwhile to further optimise the inflammatory model such that the cause(s) of the observed changes could be more clearly identified. Since gene expression and signalling changes observed may be due to any one or a combination of the effects of oxidative stress, inflammatory mediators, and cell – to – cell contact between the gastric epithelial cells (HGC-27) and the inflammatory leukocytes (HL-60/N), further experiments in

which each factor could be eliminated and signalling and gene expression changes analysed would help obtain further insights. For example by co-culturing HGC-27 and HL-60/N in trans-well plates whereby the two cell lines would be separated by the trans-well filters, through which only soluble mediators and RO/NS of certain molecular dimensions could pass, would rule out any effects of cell – to – cell contact. The impact of oxidative stress could be determined by the introduction of potent broad spectrum antioxidants into the system, if the previously observed changes were no longer apparent following antioxidant treatment this would confirm the involvement of RO/NS in inducing the changes. In order to study any possible effects of inflammatory mediators released by HL-60/N a combination of the trans-well co-culture system and sequestering RO/NS by the introduction of antioxidants could be employed. Alternatively the effects of inflammatory mediators, such as IL-8, on signal transduction and gene expression in HGC-27 could be more directly studied by exposing HGC-27 to physiologically/ clinically relevant doses of the mediators and analysing gene expression and signalling changes that may be induced.

Since chapter 3 highlighted that induced ERK MAPK signalling is highly likely to be an upstream event in inducing *c-FOS* gene expression, it would be of interest to study in greater detail the apparent discrepancy between ERK signalling and *c-FOS* expression in the co-culture experiments where no apparent association was seen between the two. It is plausible that other aspects of the co-culture system may be influencing *c-FOS* expression, for example the presence of factors that both positively and negatively effect *c-FOS* gene expression may be present leading to delayed ERK regulated *c-FOS* gene expression that could not be detected within the time scale of the experimentation. As such, it would be worth repeating the experiments for longer periods of time such as 12hr and 24hr to see if any *c-FOS* over-expression came to light. The further experiments described in which the two cell types could be separated in trans-well plates also removes the cell – to – cell contact factor that could potentially affect ERK associated *c-FOS* gene expression, and so conducting the co-culture experiments in this manner may also make clearer any signalling and gene expression changes. It would be important, also, to carry out ERK inhibitor studies in this context in order to see if inhibiting ERK signalling has any knock on effects of *c-FOS* and/ IL-8 gene expression. NFκB studies would also be

interesting to follow up, looking directly for activation of the transcription factor by assessing its subcellular localisation following co-culture. As detailed in section 5.4 the discrepancy in the *c-FOS* data (in that *c-FOS* levels were high in controls and falling following some of the co-culture treatments) may be due to a sub-toxic effect of the oxidative stress and/ inflammation in the system leading to decreased proliferation rate and hence an overall reduction in *c-FOS* gene expression. In order to establish if the decreased *c-FOS* levels were due to reduced proliferation rate, simple experiments to assess any changes in proliferation rate of HGC-27 post co-culture could be performed. To take the co-culture studies to the next level, making them more clinically relevant to *H. pylori* associated gastric carcinogenesis it would also be fascinating to include the bacterium itself, or bacterial extracts in the model. This could involve direct co-culture of *H. pylori* with gastric epithelial cells, the use of trans-well plates, and a combination of gastric epithelial cells, inflammatory leukocytes, and *H. pylori* such that the bacterium would elicit an inflammatory response more representative of the true *in vivo* situation. Finally, following the cell line differences observed in chapter 3, it would be interesting to repeat the co-culture studies with AGS and WILL1, so identifying if the different cell lines also respond differently in an inflammatory setting.

Given more time it would also be valuable to expand the *in vivo* study so as to include a larger sample cohort with more matched normal and diseased samples, with the samples being obtained from the same anatomical region of the stomach (e.g. antrum) in order to eliminate the problems encountered with the studies that hindered data analysis. This may even out the observed high degree of variability in the data which likely reflected inter-patient differences in diet, genetic susceptibility, etc. as well as intra-patient variation due to spatial differences in gastric tissue microenvironment (possibly a consequence of sampling from different regions). As such, this would permit a more confident analysis of the importance of ERK activation and *c-FOS* over-expression in the molecular pathogenesis of gastric cancer. To determine if changes detected in the biopsies are associated with/ attributable to oxidative stress, it would be worthwhile to assess the levels of markers of oxidative stress in the tissue samples such as levels of nitrotyrosine and inducible nitric oxide synthase (iNOS) by way of immunohistochemistry in a similar manner to Mannick *et al.* (1996). In addition, to

elaborate on the apparent association between the degree of inflammation and gene expression changes observed in the co-culture studies, a key area of future research would be to study *c-FOS* gene expression and ERK signalling in gastric biopsies in relation to grades of inflammation (inflammatory scores assessed by a pathologist based on the updated Sydney system (Dixon *et al.,* 1994)), in order to determine if the level of *c-FOS* expression and ERK activation are related to the severity of inflammation in the *in vivo* setting. To further the study it would also be interesting to collect gastric juice and blood samples from the patients at the time of biopsy collection, and analyse the levels of oxidants and antioxidants (in a manner similar to You *et al.* (2000) and Ruiz *et al.* (1994)) so determining if individuals with a greater oxidant and/ or lower antioxidant load may be more susceptible to the observed signalling and gene expression changes. Finally pERK and *c-FOS* levels could be assessed in dysplastic tissues as well as in gastric tumour tissue to establish if the changes are important in later stages of the disease as well as the earlier inflammatory stages.

In summary the extensive research conducted, and the data presented, implicate an important role for oxidative stress amongst other components of tissue inflammation in gastric carcinogenesis via the induction of signal transduction and gene expression changes, specifically seen here to impact MAPK and NFκB signalling and downstream gene expression changes. The findings certainly add strength to the notion that inflammation and oxidative stress are key players in gastric carcinogenesis, likely impacting disease pathogenesis as early as gastritis. In addition, the gene expression changes seen to be induced by oxidative stress have been reported to be induced by *H. pylori*, and as such the findings implicate oxidative stress as a potential mechanism through which the bacterium may exert its effects. On these grounds it is clear that therapeutic approaches that block the generation of, and/ or sequester excessive RO/NS in pre-malignant gastric tissues, such as antioxidant supplementation, and/ or administration of anti-inflammatory drugs (Nardone and Rocco, 2004; Correa, 2004; Jones-Blackett *et al.,* 1999), are likely to be very useful in the prevention and management of intestinal type gastric cancer. In addition, more specific therapies targeting the signal transduction pathways affected, may prove useful (Kaminska, 2005; Karin, 2004).

Whilst the data helped to establish a potential role of ROS and inflammation in pre-malignant gastric disease the picture remains somewhat incomplete, having opened up the doors to more fruitful investigations. Further research in this arena will surely add to our understanding of the underlying molecular pathogenesis of gastric malignancy and may aid in the development of novel therapies at the level of antioxidant and anti-inflammatory based regimens so helping to reduce the global burden of this devastating disease.

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Appendix I – Array Gene Tables

AI.1 Human Cancer Pathway Finder Array Gene Table.

Array Layout Table with Gene Symbol and Position Information

HS-006

AKT1	ANGPT1	ANGPT2	APAF1	ATM	BAD	BAX	BCL2
1	2	3	4	5	6	7	8
BCL2L1	BIRC5	BRCA1	BRCA2	CASP8	CASP9	CCND1	CCNE1
9	10	11	12	13	14	15	16
CD44	CDC25A	CDH1	CDK4	CDKN1A	CDKN1B	CDKN2A	CFLAR
17	18	19	20	21	22	23	24
COL18A1	CTNNB1	EGF	EGFR	ERBB2	ETS2	FGF2	FGFR2
25	26	27	28	29	30	31	32
FLT1	FOS	HGF	ICAM1	IFNA1	IFNB1	IGF1	IL8
33	34	35	36	37	38	39	40
ITGA1	ITGA2	ITGA3	ITGA4	ITGA5	ITGA6	ITGAV	ITGB1
41	42	43	44	45	46	47	48
ITGB3	ITGB5	JUN	KAI1	KISS1	MAP2K1	MAPK14	MDM2
49	50	51	52	53	54	55	56
MMP1	MMP2	MMP9	MTA1	MICA (MUC18L)	MYC	NCAM1	NFKB1
57	58	59	60	61	62	63	64
NFKBIA	NME4	PDGFA	PDGFB	PIK3CB	PIK3R1	PLAU	PLAUR
65	66	67	68	69	70	71	72
PRKDC	CHEK2 (RAD53)	RAF1	RASA1	RB1	S100A4	SERPINB2	SERPINB5
73	74	75	76	77	78	79	80
SERPINE1	SRC	TEK	TERT	TGFB1	TGFBR1	THBS1	THBS2
81	82	83	84	85	86	87	88
TIMP1	TNF	TNFRSF10B	TNFRSF12	TNFRSF1A	TNFRSF6	TP53	VEGF
89	90	91	92	93	94	95	96
PUC18	PUC18	PUC18	Blank	Blank	Blank	GAPD	GAPD
97	98	99	100	101	102	103	104
PPIA	PPIA	PPIA	PPIA	RPL13A	RPL13A	ACTB	ACTB
105	106	107	108	109	110	111	112

HS-006 gene table

Position	*UniGene*	Genebank	Symbol	Description	Gene name
1	Hs.71816	NM_005163	AKT1	v-akt murine thymoma viral oncogene homolog 1	Akt-1
2	Hs.2463	NM_001146	ANGPT1	Human angiopoietin-1 mRNA	Angiopoietin 1
3	Hs.115181	NM_001147	ANGPT2	Homo sapiens angiopoietin-2 mRNA	Angiopoietin-2
4	Hs.77579	NM_001160	APAF1	Apoptotic protease activating factor	Apaf-1
5	Hs.194382	NM_000051	ATM	Ataxia telangiectasia mutated (includes complementation groups A, C and D)	ATM
6	Hs.76366	U66879	BAD	BCL2-antagonist of cell death	Bad
7	Hs.159428	L22474	BAX	BCL2-associated X protein	Bax
8	Hs.79241	M14745	BCL2	B-cell CLL/lymphoma 2	Bcl-2
9	Hs.305890	Z23115	BCL2L1	BCL2-like 1	Bcl-x
10	Hs.1578	U75285	BIRC5	Apoptosis inhibitor 4 (survivin)	Survivin (API4)
11	Hs.194143	U68041	BRCA1	Breast cancer 1, early onset	BRCA1
12	Hs.34012	X95177	BRCA2	Breast cancer 2, early onset	BRCA2
13	Hs.19949	NM_001228	CASP8	Caspase 8, apoptosis-related cysteine protease	FLICE
14	Hs.100641	U60521	CASP9	Caspase 9, apoptosis-related cysteine protease	Mch6
15	Hs.82932	M64349	CCND1	Cyclin D1 (PRAD1: parathyroid adenomatosis 1)	Cyclin D1
16	Hs.9700	M73812	CCNE1	Cyclin E1	Cyclin E1
17	Hs.169610	M59040	CD44	CD44 antigen (homing function and Indian blood group system)	CD44
18	Hs.1634	NM_001789	CDC25A	Cell division cycle 25A	CDC25a, phosphotase
19	Hs.194657	Z13009	CDH1	Cadherin 1, type 1, E-cadherin (epithelial)	E-cadherin
20	Hs.95577	M14505	CDK4	Cyclin-dependent kinase 4	Cdk4
21	Hs.179665	L47233	CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	$p21^{Wafl}$ /p21Cip1
22	Hs.238990	U10906	CDKN1B	Cyclin-dependent kinase inhibitor 1B (p27, Kip1)	p27Kip1
23	Hs.1174	U26727	CDKN2A	Cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)	p16ink4
24	Hs.195175	AF010127	CFLAR	CASP8 and FADD-like apoptosis regulator	CASPER
25	Hs.78409	AF018081	COL18A1	Collagen, type XVIII, alpha 1	Endostatin
26	Hs.171271	NM_001904	CTNNB1	Catenin (cadherin-associated protein), beta 1 (88kD)	B catenin
27	Hs.2230	X04571	EGF	Epidermal growth factor (beta-urogastrone)	EGF
28	Hs.77432	X00588	EGFR	Epidermal growth factor receptor	EGFR
29	Hs.323910	M11730	ERBB2	v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 2	Erb-2
30	Hs.85146	J04102	ETS2	Human erythroblastosis virus oncogene homolog 2 (ets-2) mRNA	c-Ets2
31	Hs.284244	NM_002006	FGF2	Fibroblast growth factor 2 (basic)	FGF2
32	Hs.278581	M55614	FGFR2	Fibroblast growth factor receptor 2	FGFR2
33	Hs.138671	NM_002019	FLT1	Homo sapiens vascular endothelial growth factor receptor (FLT1) mRNA (Fms-related tyrosine kinase 1)	FLT1
34	Hs.25647	V01512	FOS	Human cellular oncogene c-fos	c-Fos
35	Hs.809	X57574	HGF	Hepatocyte growth factor (hepapoietin A; scatter factor)	HGF
36	Hs.168383	NM_000201	ICAM1	Intercellular adhesion molecule 1 (CD54), human rhinovirus receptor	ICAM-1
37	Hs.37026	NM_024013	IFNA1	Homo sapiens interferon, alpha 1	IFNA1
38	Hs.93177	M28622	IFNB1	Interferon, beta 1, fibroblast	IFN-b1
39	Hs.85112	M27544	IGF1	Insulin-like growth factor 1 (somatomedin C)	IGF-1
40	Hs.624	M17017	IL8	Interleukin 8	IL-8
41	Hs.116774	X68742	ITGA1	Integrin, alpha 1	Integrin a1
42	Hs.271986	X17033	ITGA2	Integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)	Integrin a2/ LFA1b

Position	*UniGene*	Genebank	Symbol	Description	Gene name
43	Hs.265829	M59911	ITGA3	Integrin, alpha 3 (antigen CD49C, alpha 3 subunit of VLA-3 receptor)	Integrin a3
44	Hs.40034	L12002	ITGA4	Integrin, alpha 4 (antigen CD49D, alpha 4 subunit of VLA-4 receptor)	Integrin a4
45	Hs.149609	X06256	ITGA5	Integrin, alpha 5 (fibronectin receptor, alpha polypeptide)	Integrin a5
46	Hs.227730	X53586	ITGA6	Integrin, alpha 6 subunit	Integrin a6
47	Hs.295726	NM_002210	ITGAV	Integrin, alpha V (vitronectin receptor, alpha polypeptide, antigen CD51)	Integrin aV
48	Hs.287797	NM_002211	ITGB1	Integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)	Integrin b1
49	Hs.87149	J02703	ITGB3	Integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)	Integrin b3 (CD61)
50	Hs.149846	J05633	ITGB5	Integrin, beta 5	Integrin b5
51	Hs.78465	J04111	JUN	v-jun avian sarcoma virus 17 oncogene homolog	c-jun
52	Hs.323949	NM_002231	KAI1	Kangai 1 (suppression of tumorigenicity 6, prostate...)	KAI1
53	Hs.95008	NM_002256	KISS1	KiSS-1 metastasis-suppressor	KISS1
54	Hs.3446	NM_002755	MAP2K1	Protein kinase, mitogen-activated, kinase 1 (MAP kinase kinase 1)	MEK1
55	Hs.79107	NM_001315	MAPK14	p38 mitogen activated protein (MAP) kinase	p38 MAPK
56	Hs.170027	Z12020	MDM2	Mouse double minute 2, human homolog of; p53-binding protein	Mdm2
57	Hs.83169	X05231	MMP1	Matrix metalloproteinase 1 (interstitial collagenase)	Collagenase-1 (MMP-1)
58	Hs.111301	J03210	MMP2	Matrix metalloproteinase 2 (gelatinase A, 72kD gelatinase, 72kD type IV collagenase)	Gelatinase A (MMP-2)
59	Hs.151738	J05070	MMP9	Matrix metalloproteinase 9 (gelatinase B, 92kD gelatinase, 92kD type IV collagenase)	Gelatinase B (MMP-9)
60	Hs.101448	U35113	MTA1	Metastasis associated 1	Mta-1
61	Hs.90598	NM_000247	MICA	Homo sapiens MICA gene, allele MUC-18	MUC-18
62	Hs.79070	X00364	MYC	v-myc avian myelocytomatosis viral oncogene homolog	c-myc
63	Hs.167988	U63041	NCAM1	Neural cell adhesion molecule 1	NCAM1
64	Hs.83428	M58603	NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105)	NFkB
65	Hs.81328	M69043	NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	IkBa (mad3)
66	Hs.9235	NM_005009	NME4	Homo sapiens non-metastatic cells 4, protein expressed in (NME4), mRNA	Nm23-E4
67	Hs.37040	X06374	PDGFA	Platelet-derived growth factor alpha polypeptide	PDGF a
68	Hs.1976	NM_002608	PDGFB	Platelet-derived growth factor beta polypeptide (simian sarcoma viral (v-sis) oncogene homolog)	PDGF2/SIS
69	Hs.239818	NM_006219	PIK3CB	Phosphoinositide-3-kinase, catalytic, beta polypeptide	PI3K p110b
70	Hs.6241	M61906	PIK3R1	Phosphoinositide-3-kinase, regulatory subunit, polypeptide 1 (p85 alpha)	PI3K p85 alpha
71	Hs.77274	D00244	PLAU	Plasminogen activator, urokinase	uPA
72	Hs.179657	NM_002659	PLAUR	Human urokinase-type plasminogen activator receptor	uPAR
73	Hs.155637	NM_006904	PRKDC	Homo sapiens protein kinase, DNA-activated, catalytic polypeptide	DNA-PK
74	Hs.146329	NM_007194	CHEK2	Protein kinase Chk2	Chk2/Rad53
75	Hs.85181	X03484	RAF1	v-raf-1 murine leukemia viral oncogene homolog 1	c-raf-1
76	Hs.758	NM_002890	RASA1	RAS p21 protein activator (GTPase activating protein) 1	GAP
77	Hs.75770	M15400	RB1	Retinoblastoma 1 (including osteosarcoma)	Rb
78	Hs.81256	NM_002961	S100A4	Homo sapiens S100 calcium-binding protein A4 (calcium protein, calvasculin, metastasin, murine place	Mts1
79	Hs.75716	J02685	SERPINB2	Human plasminogen activator inhibitor	PAI-2

Position	*UniGene*	Genebank	Symbol	Description	Gene name
80	Hs.55279	NM_002639	SERPINB5	Serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 5	Maspin
81	Hs.82085	M16006	SERPINE1	Plasminogen activator inhibitor, type I	PAI-1
82	Hs.198298	K03218	SRC	Human c-src-1 proto-oncogene	c-Src
83	Hs.89640	NM_000459	TEK	TEK tyrosine kinase, endothelial (venous malformations, multiple cutaneous and mucosal)	Tie-2
84	Hs.115256	AF015950	TERT	Telomerase reverse transcriptase	Telomerase
85	Hs.1103	X02812	TGFB1	Transforming growth factor, beta 1	TGFb1
86	Hs.220	L11695	TGFBR1	Transforming growth factor, beta receptor I (activin A receptor type II-like kinase, 53kD)	ALK-5
87	Hs.87409	NM_003246	THBS1	Homo sapiens thrombospondin 1 (THBS1)	Thrombospondin1
88	Hs.108623	L12350	THBS2	Human thrombospondin 2 (THBS2) mRNA	Thrombospondin2
89	Hs.5831	NM_003254	TIMP1	Tissue inhibitor of metalloproteinase 1 (erythroid potentiating activity, collagenase inhibitor)	TIMP1
90	Hs.241570	X01394	TNF	Tumor necrosis factor (TNF superfamily, member 2)	TNFa
91	Hs.51233	AF016266	TNFRSF10B	Homo sapiens TRAIL receptor 2/DR5	Trail Receptor (DR5)
92	Hs.180338	U74611	TNFRSF12	TRAMP/Apo-3/DDR3	DR3 (Apo3)
93	Hs.159	M33294	TNFRSF1A	Tumor necrosis factor receptor superfamily, member 1A	TNFR1
94	Hs.82359	X63717	TNFRSF6	Tumor necrosis factor receptor superfamily, member 6	Fas (Apo-1) (CD95)
95	Hs.1846	M14694	TP53	Tumor protein p53 (Li-Fraumeni syndrome)	p53
96	Hs.73793	M32977	VEGF	Vascular endothelial growth factor	VEGF
97	N/A	L08752	PUC18	PUC18 Plasmid DNA	pUC18
98	N/A	L08752	PUC18	PUC18 Plasmid DNA	pUC18
99	N/A	L08752	PUC18	PUC18 Plasmid DNA	pUC18
100	Blank	Blank	Blank	Blank	0
101	Blank	Blank	Blank	Blank	0
102	Blank	Blank	Blank	Blank	0
103	Hs.169476	M33197	GAPD	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH
104	Hs.169476	M33197	GAPD	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH
105	Hs.342389	NM_021130	PPIA	Homo sapiens peptidylprolyl isomerase A	Cyclophilin A
106	Hs.342389	NM_021130	PPIA	Homo sapiens peptidylprolyl isomerase A	Cyclophilin A
107	Hs.342389	NM_021130	PPIA	Homo sapiens peptidylprolyl isomerase A	Cyclophilin A
108	Hs.342389	NM_021130	PPIA	Homo sapiens peptidylprolyl isomerase A	Cyclophilin A
109	Hs.119122	NM_012423	RPL13A	Ribosomal protein L13a (23 Kda highly basic protein)	RPL13A
110	Hs.119122	NM_012423	RPL13A	Ribosomal protein L13a (23 Kda highly basic protein)	RPL13A
111	Hs.288061	X00351	ACTB	Beta Actin	b-actin
112	Hs.288061	X00351	ACTB	Beta Actin	b-actin

AI.2 Human Nitric Oxide Array Gene Table.

Array Layout Table with Gene Symbol and Position Information

HS-034

ABCC1 1	ADM 2	AGTR1 3	ALAS2 4	BCL2 5	BNIP3 6	CCL2 7	CCL3 8
CCL5 9	CDKN1A 10	CFTR 11	COL1A1 12	COL2A1 13	COL3A1 14	CSF1 15	CXCL2 16
CYP19 17	CYP2D6 18	CYP3A4 19	DDIT3 20	EDN1 21	EDNRB 22	EGR1 23	EPO 24
FLJ37174 25	FLT1 26	FMR1 27	FN1 28	FTL 29	GADD45A 30	GADD45B 31	GCLC 32
GCLM 33	HBG1 34	HLA-DRA 35	HMOX1 36	HPRT1 37	HRMT1L1 38	HRMT1L2 39	HSPA4 40
HSPCA 41	ICAM1 42	IGFBP1 43	IL10 44	IL1B 45	IL2 46	IL4 47	IL6 48
IL8 49	ILK 50	JUNB 51	LAMC1 52	MMP1 53	MMP2 54	MMP7 55	MMP9 56
MRP1-ESTs 57	MT1G 58	MYB 59	MYC 60	NCAM1 61	NFKBIA 62	NOS2A 63	OGG1 64
OPRM1 65	PDGFA 66	PDGFB 67	PLA2G2D 68	PLAT 69	POLYDOM 70	PPP1R15A 71	PRKDC 72
PRKG1 73	PRMT3 74	PTGS2 75	S100A10 76	SERPINE1 77	SFTPA1 78	SHC1 79	SLC6A6 80
SLC7A1 81	SLC7A2 82	SOD1 83	SOD2 84	SPARC 85	TFRC 86	TGFB1 87	TIMP1 88
TNF 89	TNFRSF1A 90	TNFRSF6 91	TXNRD1 92	TYR 93	VCAM1 94	VEGF 95	ZNF2 96
PUC18 97	PUC18 98	PUC18 99	Blank 100	Blank 101	Blank 102	GAPD 103	GAPD 104
PPIA 105	PPIA 106	PPIA 107	PPIA 108	RPL13A 109	RPL13A 110	ACTB 111	ACTB 112

HS-034 gene table

Position	UniGene	Genebank	Symbol	Description	Gene name
1	Hs.89433	NM_004996	ABCC1	ATP-binding cassette, sub-family C (CFTR/ MRP), member 1 (multiple drug resistance protein 1)	MRP1
2	Hs.394	NM_001124	ADM	Adrenomedullin	Adrenomedullin
3	Hs.89472	NM_031850	AGTR1	ANGIOTENSIN RECEPTOR 1	AT1
4	Hs.323383	NM_000032	ALAS2	Aminolevulinate, delta-, synthase 2 (sideroblastic/hypochromic anemia)	ANH1
5	Hs.79241	M14745	BCL2	B-cell CLL/lymphoma 2	Bcl-2
6	Hs.79428	AF002697	BNIP3	BCL2/adenovirus E1B 19kDa interacting protein 3	Nip3
7	Hs.303649	X14768	CCL2	Chemokine (C-C motif) ligand 2, small inducible cytokine A2 precursor (Monocyte chemotactic protein 1)	MCP1/SCYA2
8	Hs.73817	M23452	CCL3	Chemokine (C-C motif) ligand 3 (Macrophage inflammatory protein 1-alpha)	MIP-1a/SCYA3
9	Hs.241392	NM_002985	CCL5	Chemokine (C-C motif) ligand 5; small inducible cytokine A5	SCYA5/RANTES
10	Hs.179665	L47233	CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	P21/Waf1/CIP1
11	Hs.663	M28668	CFTR	Cystic fibrosis mRNA, encoding a presumed transmembrane conductance	CFTR
12	Hs.172928	NM_000088	COL1A1	Collagen, type I, alpha 1	COL1A1
13	Hs.81343	NM_001844	COL2A1	Collagen, type II, alpha 1 (primary osteoarthritis, spondyloepiphyseal dysplasia, congenital)	COL2A1
14	Hs.119571	NM_000090	COL3A1	Collagen, type III, alpha 1 (Ehlers-Danlos syndrome type IV, autosomal dominant)	COL3A1
15	Hs.173894	M37435	CSF1	Colony stimulating factor 1 (macrophage)	MCSF
16	Hs.75765	NM_002089	CXCL2	Chemokine (C-X-C motif) ligand 2, Macrophage inflammatory protein-2-alpha precursor (MIP2-alpha)	MIP-2a/GROb
17	Hs.79946	Y07508	CYP19	Cytochrome P450, subfamily XIX (aromatization of androgens)	ARO1
18	Hs.333497	NM_000106	CYP2D6	Cytochrome P450, subfamily IID (debrisoquine, sparteine, etc., -metabolizing), polypeptide 6	CYP2D6
19	Hs.178738	NM_017460	CYP3A4	Cytochrome P450, subfamily IIIA (niphedipine oxidase), polypeptide 4	CYP3A4
20	Hs.337761	S40706	DDIT3	DNA-damage-inducible transcript 3	GADD153/CHOP
21	Hs.2271	Y00749	EDN1	Endothelin 1	Endothelin 1
22	Hs.82002	D90402	EDNRB	Endothelin receptor type B	ETRB/ETB
23	Hs.326035	X52541	EGR1	Early growth response 1	Krox-24
24	Hs.2303	NM_000799	EPO	Homo sapiens erythropoietin	EPO
25	Hs.322469	AK091014	FLJ37174	Homo sapiens cDNA FLJ37174 fis, clone BRACE2028406, mRNA sequence	FLJ37174
26	Hs.381093	NM_002019	FLT1	Fms-related tyrosine kinase 1 (vascular endothelial growth factor/vascular permeability factor receptor)	VEGFR1
27	Hs.89764	NM_002024	FMR1	Fragile X mental retardation 1	FRAXA
28	Hs.287820	X02761	FN1	Fibronectin 1	Fibronectin-1
29	Hs.128899	NM_000146	FTL	Ferritin, light polypeptide	FTL
30	Hs.80409	M60974	GADD45A	Growth arrest and DNA-damage-inducible, alpha	GADD45
31	Hs.110571	AF078077	GADD45B	Growth arrest and DNA-damage inducible, beta	GADD45 b
32	Hs.151393	AK094940	GCLC	Glutamate-cysteine ligase, catalytic subunit	GCS
33	Hs.89709	NM_002061	GCLM	Glutamate-cysteine ligase, modifier subunit	GCLM
34	Hs.283108	NM_000559	HBG1	Hemoglobin, gamma A	HBG1
35	Hs.76807	K01171	HLA-DRA	Major histocompatibility complex, class II, DR alpha	HLA-DRA1
36	Hs.202833	X06985	HMOX1	Heme oxygenase (decycling) 1	HEME1
37	Hs.82314	M31642	HPRT1	Hypoxanthine-guanine phosphoribosyltransferase	HPRT1
38	Hs.235887	NM_001535	HRMT1L1	HMT1 hnRNP methyltransferase-like 1 (S. cerevisiae)	PRMT2
39	Hs.20521	NM_001536	HRMT1L2	HMT1 hnRNP methyltransferase-like 2 (S. cerevisiae)	PRMT1
40	Hs.90093	L12723	HSPA4	Human heat shock protein 70	Hsp70
41	Hs.289088	X15183	HSPCA	Heat shock 90kDa protein 1, alpha	HSP90A/HSP86

Position	UniGene	Genebank	Symbol	Description	Gene name
42	Hs.168383	NM_000201	ICAM1	Intercellular adhesion molecule 1 (CD54), human rhinovirus receptor	ICAM-1
43	Hs.102122	NM_000596	IGFBP1	Insulin-like growth factor binding protein 1	IGFBP1
44	Hs.193717	M57627	IL10	Interleukin 10	IL-10
45	Hs.126256	M15330	IL1B	Interleukin 1, beta	IL-1b
46	Hs.89679	U25676	IL2	Interleukin 2	IL-2
47	Hs.73917	M13982	IL4	Interleukin 4	IL-4
48	Hs.93913	M14584	IL6	Interleukin 6 (interferon, beta 2)	IL-6
49	Hs.624	M17017	IL8	Interleukin 8	IL-8
50	Hs.6196	NM_004517	ILK	Integrin-linked kinase	P59
51	Hs.198951	X51345	JUNB	Jun B proto-oncogene	Jun-B
52	Hs.214982	J03202	LAMC1	Laminin, gamma 1 (formerly LAMB2)	LAMB2
53	Hs.83169	X05231	MMP1	Matrix metalloproteinase 1 (interstitial collagenase)	Collagenase-1
54	Hs.111301	J03210	MMP2	Matrix metalloproteinase 2 (gelatinase A, 72kD gelatinase, 72kD type IV collagenase)	MMP-2
55	Hs.2256	X07819	MMP7	Matrix metalloproteinase 7 (matrilysin, uterine)	MMP-7
56	Hs.151738	J05070	MMP9	Matrix metalloproteinase 9 (gelatinase B, 92kD gelatinase, 92kD type IV collagenase)	MMP-9
57	Hs.13188	N59493	MRP1-ESTs	Human HepG2 partial cDNA, clone hmd5d04m5, mRNA sequence	MRP1-ESTs
58	Hs.433391	NM_005950	MT1G	Metallothionein 1G	MT1G
59	Hs.1334	M15024	MYB	V-myb avian myeloblastosis viral oncogene homolog	c-Myb
60	Hs.79070	NM_002467	MYC	V-myc avian myelocytomatosis viral oncogene homolog	c-Myc
61	Hs.167988	U63041	NCAM1	Neural cell adhesion molecule 1	NCAM
62	Hs.81328	M69043	NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	IKBA/MAD-3
63	Hs.193788	L09210	NOS2A	Nitric oxide synthase 2A (inducible, hepatocytes)	NOS
64	Hs.96398	NM_002542	OGG1	8-oxoguanine DNA glycosylase	OGH1
65	Hs.2353	L25119	OPRM1	Opioid receptor mu 1	MOR1
66	Hs.37040	X06374	PDGFA	Platelet-derived growth factor alpha polypeptide	PDGF a
67	Hs.1976	NM_002608	PDGFB	Platelet-derived growth factor beta polypeptide (simian sarcoma viral (v-sis) oncogene homolog)	PDGF2/SIS
68	Hs.189507	NM_012400	PLA2G2D	Phospholipase A2, group IID	SPLASH
69	Hs.274404	NM_000930	PLAT	Homo sapiens plasminogen activator, tissue (PLATa)	T-PA
70	Hs.271350	NM_024500	POLYDOM	Likely ortholog of mouse polydom	FLJ13529
71	Hs.76556	NM_014330	PPP1R15A	Homo sapiens protein phosphatase 1, regulatory (inhibitor) subunit 15A	GADD34
72	Hs.155637	NM_006904	PRKDC	Homo sapiens protein kinase, DNA-activated, catalytic polypeptide	DNA-PK
73	Hs.2689	NM_006258	PRKG1	Protein kinase, cGMP-dependent, type I	PRKG1
74	Hs.152337	BC019339	PRMT3	Protein arginine N-methyltransferase 3 (hnRNP methyltransferase S. cerevisiae)-like 3	PRMT3
75	Hs.196384	NM_000963	PTGS2	Homo sapiens prostaglandin-endoperoxide synthase 2 (prostaglandin G/Hsynthase and cyclooxygenase)	Cox-2
76	Hs.119301	NM_002966	S100A10	S100 calcium binding protein A10 (annexin II ligand, calpactin I, light polypeptide (p11))	ANX2L
77	Hs.82085	M16006	SERPINE1	Serine (or cysteine) proteinase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	PAI-1
78	Hs.301254	NM_005411	SFTPA1	Surfactant, pulmonary-associated protein A1	SFTPA1
79	Hs.81972	U73377	SHC1	SHC (Src homology 2 domain-containing) transforming protein 1	SHC
80	Hs.1194	Z18956	SLC6A6	Solute carrier family 6 (neurotransmitter transporter, taurine), member 6	TAUT
81	Hs.2928	NM_003045	SLC7A1	Solute carrier family 7 (cationic amino acid transporter, y+ system), member 1	CAT-1
82	Hs.153985	AL832016	SLC7A2	Solute carrier family 7 (cationic amino acid transporter, y+ system), member 2	CAT-2

Position	UniGene	Genebank	Symbol	Description	Gene name
83	Hs.75428	X02317	SOD1	Superoxide dismutase 1, soluble (amyotrophic lateral sclerosis 1 (adult)) [Cu-Zn]	Cu/ZnSOD
84	Hs.372783	NM_000636	SOD2	Superoxide dismutase 2, mitochondrial	IPO-B/MNSOD
85	Hs.111779	NM_003118	SPARC	Homo sapiens secreted protein, acidic, cysteine-rich (osteonectin)	ON
86	Hs.77356	M11507	TFRC	Transferrin receptor (p90, CD71)	TFRC
87	Hs.1103	X02812	TGFB1	Transforming growth factor, beta 1 (Camurati-Engelmann disease)	TGFb1
88	Hs.5831	NM_003254	TIMP1	Tissue inhibitor of metalloproteinase 1 (erythroid potentiating activity, collagenase inhibitor)	TIMP1
89	Hs.241570	X01394	TNF	Tumor necrosis factor (TNF superfamily, member 2)	TNFA
90	Hs.159	M33294	TNFRSF1A	Tumor necrosis factor receptor superfamily, member 1A	TNFR1
91	Hs.82359	X63717	TNFRSF6	Tumor necrosis factor receptor superfamily, member 6	Fas/Apo-1/CD95
92	Hs.13046	NM_003330	TXNRD1	Thioredoxin reductase 1	TR1
93	Hs.2053	NM_000372	TYR	Homo sapiens tyrosinase (oculocutaneous albinism IA)	Tyrosinase
94	Hs.109225	M30257	VCAM1	Vascular cell adhesion molecule 1	VCAM-1
95	Hs.73793	NM_003376	VEGF	Vascular endothelial growth factor	VEGF
96	Hs.192285	NM_021088	ZNF2	Zinc finger protein 2 (A1-5)	A1-5
97	N/A	L08752	PUC18	PUC18 Plasmid DNA	pUC18
98	N/A	L08752	PUC18	PUC18 Plasmid DNA	pUC18
99	N/A	L08752	PUC18	PUC18 Plasmid DNA	pUC18
100	Blank	Blank	Blank	Blank	0
101	Blank	Blank	Blank	Blank	0
102	Blank	Blank	Blank	Blank	0
103	Hs.169476	M33197	GAPD	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH
104	Hs.169476	M33197	GAPD	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH
105	Hs.342389	NM_021130	PPIA	Homo sapiens peptidylprolyl isomerase A	Cyclophilin A
106	Hs.342389	NM_021130	PPIA	Homo sapiens peptidylprolyl isomerase A	Cyclophilin A
107	Hs.342389	NM_021130	PPIA	Homo sapiens peptidylprolyl isomerase A	Cyclophilin A
108	Hs.342389	NM_021130	PPIA	Homo sapiens peptidylprolyl isomerase A	Cyclophilin A
109	Hs.119122	NM_012423	RPL13A	Ribosomal protein L13a (23 Kda highly basic protein)	RPL13A
110	Hs.119122	NM_012423	RPL13A	Ribosomal protein L13a (23 Kda highly basic protein)	RPL13A
111	Hs.288061	X00351	ACTB	Beta Actin	b-actin
112	Hs.288061	X00351	ACTB	Beta Actin	b-actin

Appendix II – Patient Documents for *in vivo* Study

AII.1 Patient Information Leaflet.

TITLE:
Research Project looking at the cause of gastric disease.
[Gene expression analysis of *Helicobacter pylori* induced gastric disease.]

Professor JN Baxter, Endoscopy Unit, Morriston Hospital, Dr L Thomas, Endoscopy unit, Singleton Hospital.

Patient information leaflet for endoscopy patients
Version number: 3
26th January 2005

When you come to the endoscopy unit for the endoscopy that has been arranged for you, you will be invited to take part in a research study. Before you agree to this, please read this information leaflet and discuss it with friends or family. If anything is unclear and you need to ask any questions please contact us before (see end of leaflet for contact information), or when you attend for the appointment. Please take time to decide if you wish to participate.

Why are we doing this research?
A germ is often found in the stomach, that can cause different diseases in the stomach. In our study, we want to look for this germ and then, very closely at the cells lining the stomach. Hopefully this will help explain what triggers disease, and improve our ability to diagnose and treat gastric diseases.

Why have I been chosen for this study?
This study intends to enrol patients attending the endoscopy clinics at Singleton /Morriston Hospital, you have been chosen as you are one of these patients.

Do I have to take part?
It is up to you to decide whether or not you wish to take part in the study. If you do not wish to take part, the endoscopy that has been arranged for you will take place as planned, and your future care will not be affected. If you agree to take part you may change your mind at any time.

What will happen if I take part?
If you do agree then you will be asked questions, by a doctor when you arrive in the hospital, and then asked to sign a consent form and participate. If you are taking certain tablets or have had surgery on your stomach, then we may not ask you to participate. If you agree to take part, then it will not involve any additional hospital visits.
What do I have to do?

When you come to the endoscopy unit we will need to collect certain details from you. Your age, sex, family history, eating and drinking habits, smoking habits and medication history. All the information we collect will remain confidential. This will take no longer than ten minutes.

What is the procedure being tested?
We plan to analyse (in detail) the active genes present in stomach tissue, this is to be performed in the molecular biology lab at Swansea University. By analysing the active genes of diseased tissue versus normal tissue, we hope to better understand the basis of the disease. In addition the level of certain factors, such as dietary antioxidants will be assessed in blood and gastric juice samples.

What are the alternatives for diagnosis or treatment?
There is currently no alternative to endoscopy for looking for diseases of the stomach.

What are the possible side-effects of taking part?
During the endoscopy we routinely need to take 2-3 small samples of tissue to help us make a diagnosis. This we do with a biopsy, whereby we take very small bites of tissue, we also sometimes collect some cells for analysis with a small brush. For this study we will need 2-3 extra biopsies and 1-2 brushings, which we can look at closely in the laboratory. Both procedures take a minute or two and you will be unable to feel them. A Blood sample and gastric juice sample will also be collected.

What are the possible disadvantages and risks of taking part?
Taking any biopsies carries a very small risk, hence taking extra biopsies for this study theoretically extends this risk. However, the doctors involved in this study are very experienced at collecting biopsies during endoscopy and have had no previous problems with taking biopsies from the stomach of patients.

What are the possible benefits of taking part?
From our studies of your (and other patient's) stomach tissue, blood, and gastric juice, we may be able to understand the mechanism whereby certain stomach diseases occur. This knowledge may therefore aid in our efforts to diagnose and treat patients with gastric disease.

What if something goes wrong?
There are no special compensation arrangements to anyone taking part in this research project. You are a patient of Swansea NHS Trust and as such the NHS complaints mechanisms are available to you if needed.

Will my taking part be kept confidential?
Yes, your tissue, blood and gastric juice samples will be analysed at Swansea University, but your name will be removed first, hence you will not be identified.

What will happen to the results of this study?

When the research study stops all the information will be analysed and the results used to improve our knowledge of gastric disease. They may be published in the scientific literature, but you will not be identified.
After the tissue samples have been analysed for this research project they will be incinerated, following the guidelines at the University.

Who is organising and funding this study?
The research study is under the supervision of Professor J Baxter, Dr G Jenkins, Professor J Parry. Professor Baxter is a consultant surgeon at Morriston hospital and supervises endoscopy lists at Morriston hospital. He, or one of his registrars, under his supervision, will take the biopsies, blood sample, and gastric juice sample at Morriston Hospital. Dr Thomas or one of her colleagues will take the biopsies, blood, and gastric juice samples at Singleton Hospital. Dr Jenkins and Professor Parry are molecular biologists in University of Wales Swansea and are responsible for the supervision of the laboratory work, in accordance with the University guidelines. The study is being funded by a charity called Tenovus.

Who has reviewed this study?
This research project has been approved by the Local Research Ethics Committee.

If you need any further information please contact Professor Baxter and his team at Morriston Hospital (01792 703573)

Thank you for taking the time to read this leaflet.

Professor Baxter.
Dr. L. Thomas.

AII.2 Patient Consent Form.

Gene expression analysis of *Helicobacter pylori* induced gastric disease.

Professor Baxter, Consultant Surgeon, Morriston Hospital.
Dr Thomas, Consultant Gastroenterologist, Singleton Hospital.

Patient consent form
Version 3

I have read the patient information leaflet and received a verbal explanation of the proposed research project.

I consent to the taking of additional biopsies and brushings during the endoscopic procedure for research purposes.

I agree for information about me to be held but I understand that the information I give will not be communicated to anyone outside the research team.

Patient name:

Patient Signature: Date:

I have supplied the written information on this research project, given a verbal explanation and answered any questions posed.

Investigator name:

Investigator signature: Date:

Gene expression analysis of *Helicobacter pylori* induced gastric cancer.

Professor Baxter, Consultant Surgeon, Morriston Hospital.
Dr Thomas, Consultant Gastroenterologist, Singleton Hospital.

Patient profile

Name:

Date of birth: Sex: Race:

Family history of GI cancer:

Family history peptic ulcer disease:

Smoking history:

Alcohol history:

Dietary habits (salt, fruit, vegetables, meat):

Drug history:

Employment history:

Endoscopic diagnosis (+ location):

Histological diagnosis:

Helicobacter pylori status: [1] histological [2] PCR

Helicobacter pylori strain: