The regulation of plasminogen activator inhibitor-1 (PAI-1) and other uPA system members in the gastric epithelium by *Helicobacter pylori*

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy by

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This thesis is dedicated to my loving parents, especially to you mum. You always believed in me and gave me the strength and determination to carry on. I love and miss you both deeply and am so proud to be your daughter. Thank you from the bottom of my heart for everything *xxxx*

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Contents

List of Figures

List of Tables

Abbreviations

The abbreviations used in this thesis are listed below.

α-SMA	α-smooth muscle actin
APC	Activated protein C
ASO	Anti-sense oligonucleotide
ATF	Amino terminal fragment
BrdU	5'-bromo-2'-deoxyuridine
BSA	Bovine serum albumin
C57BL/6	C57Black/6 wild-type mouse strain
CagA	Cytotoxin A
***cag*-PAI**	Cag Pathogenicity Island
CCK2R	Cholecystokinin receptor 2
CGRP	Calcitonin gene-related peptide
COX (-1,-2)	Cyclooxygenase (-1, -2)
CSO	Control scrambled antisense oligonucleotides
DMEM	Dulbecco's Modified Eagles Medium
DTT	Dithiothreitol
EC	Enterochromaffin cell
ECL	Enterochromaffin-like-cell
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGF-R	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial-mesenchymal transition
ERK	Extracellular signal-related kinase
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
FM	Full media
FPRL-1	FPR-like receptor/lipoxin A4 receptor
FVB/N	Background mouse strain for INS-GAS mice
G-17	Amidated heptadecapeptide gastrin
GAS-KO	Gastrin knock-out

GFP	Green fluorescent protein
GI	Gastrointestinal
GMBF	Gastric mucosal blood flow
GPI	Glycosyl-phosphatidyl-inositol moeity
H_2	Histamine
HB-EGF	Heparin-binding epidermal growth factor
HBSS	Hank's balanced salt solution
HCL	Hydrochloric acid
HCO_3^-	Bicarbonate
HDC	Histamine decarboxylase
H. felis	*Helicobacter felis*
H. pylori	*Helicobacter pylori*
HGF	Hepatocyte growth factor
H^+, K^+-ATPase	Hydrogen-Potassium-ATPase
HMW	High molecular weight
HRP	Horseradish peroxidase
IFN-a, -γ	Interferon -alpha, -gamma
IGF-II	Insulin-like growth factor II
IGF-BP5	Insulin-like growth factor-binding protein 5
IHC	Immunohistochemistry
IL	Interleukin
INS-GAS	Transgenic for gastrin driven by insulin promoter
JAK-STAT	Janus kinase/signal transducers activators of transcription
JNK	Jun N-terminal kinase
KGF	Keratinocyte growth factor
LDLR	Low density lipoprotein receptor
LMW	Low molecular weight
LPS	Lipopolysaccharide
LRP	Lipoprotein receptor-related protein
MAPK	Mitogen-activated protein kinase
MI	Myocardial infraction
MMP	Matrix metalloproteinase
MOI	Multiplicity of infection

NaCl	Sodium chloride
NFκB	Nuclear factor kappa-B
NKA	Neurokinin A
NO	Nitric-oxide
NOCs	N-nitroso compounds
NOS	Nitric-oxide synthase
NSAIDs	Non-steroid-anti-inflammatory drugs
oipA	Outer inflammatory protein A
PAI-1	Plasminogen activator inhibitor-1
PAI-2	Plasminogen activator inhibitor-2
PAI-3	Plasminogen activator inhibitor-3
PAI-1 KO	Plasminogen activator inhibitor-1 gene knock-out mice
PBS	Phosphate buffered saline solution
PCI	Protein C inhibitor
PCR	Polymerase chain reaction
PDGF	Platlet derived growth factor
PFA	Paraformaldehyde
PGs	Prostaglandins
PI3K	Phosphoinositol 3-kinase
PMA	Phorbol 12-myristate 13-acetate
PPI	Proton pump inhibitor
RCL	Reactive centre loop
RhoA	Ras homologue gene family member A
ROS	Reactive oxygen species
RT-PCR	Reverse transcription polymerase chain reaction
SCF	Stem cell factor
SFM	Serum free media
SMB	Somatomedin B domain
SP	Substance P
TBST	Tween-Tris-Buffered saline
TFF-1/-2	Trefoil factor family -1/-2
TFFS	Type IV secretory system
TGF-α,- β	Transforming growth factor -alpha, -beta

TIMP	Tissue inhibitor of metalloproteinase
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor -alpha
TRed	Texas red
uPA	Urokinase plasminogen activator
uPAR	Urokinase plasminogen activator receptor
uPAR KO	Urokinase plasminogen activator receptor gene knock-out
VacA	Vacuolating cytotoxin A
VEGF	Vascular endothelial growth factor
VLDL	Very low density lipoprotein
VMAT-2	Vesicular monoamine transporter type-2
VN	Vitronectin
VR1	Vanilliod receptors of type 1
WHO	World Health Organisation
wt	Wild-type

Publications and Presentations

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Abstract

The gastric pathogen *Helicobacter pylori* is linked to gastritis, leading to gastric cancer but the mechanisms are unclear. Gastritis is characterised by remodelling of the gastric mucosa, which requires degradation of the extracellular matrix (ECM). Proteases such as matrix metalloproteinases (MMPs) and urokinase plasminogen activator (uPA), as well as proteases inhibitors such as plasminogen activator inhibitor (PAI)-1 play an important role in this process. Since members of the uPA system (namely PAI-2, uPA and its receptor, uPAR) have been shown to be increased by *H. pylori*, it was hypothesised that PAI-1 expression is also increased in the gastric mucosa during infection, which could play a role in the tissue remodelling observed during the development of gastritis. The aim of this study was to investigate the effect of *H. pylori* on PAI-1 expression in the gastric mucosa, to examine the consequences of this protease inhibitor on human primary gastric epithelial cell proliferation and to determine its initial contribution to gastric mucosal protection during epithelial injury.

Real-time quantitative polymerase chain reaction (Q-PCR) of biopsies from gastric corpus, but not antrum, showed significantly increased PAI-1 expression in *H. pylori* positive patients which was further increased in gastric cancer patients compared to controls. Using immunohistochemistry, it was found that *H. pylori* significantly increased the expression of PAI-1 in primary gastric epithelial cells, particularly in the acid-secreting parietal cells. Expression of PAI-1 in gastric cancer patients was also found in epithelial cells. A transgenic mouse model of gastric remodelling (INS-GAS) infected with *H. felis* showed an increase in PAI-1 expression six months post-infection.

Proliferation assays showed that both *H. pylori* and exogenous uPA stimulated human primary gastric epithelial cell proliferation, which was further increased after PAI-1 knockdown using anti-sense oligonucleotides treatment, consistent with PAI-1 inhibition of endogenous uPA.

Transfection of human primary gastric epithelial cells with uPA, PAI-1 or uPAR promoters, in luciferase reporter constructs, revealed expression of all three in H^+, K^+-ATPase- and VMAT-2-expressing cells; uPA-luciferase was also expressed in pepsinogen-containing cells and uPAR in TFF-1-expressing cells. In each case, expression patterns of the luciferase reporter constructs were similar to that of the endogenously expressed proteins, and all were increased in response to *H. pylori.* However, the virulence factor CagE was required for the stimulation of uPA, but not PAI-1 or uPAR.

The production and use of uPA system transgenic mice in an acute model of gastric mucosal injury revealed that PAI-1 plays a protective role during gastric epithelial damage. Mice in which this gene is deleted displayed a more severe phenotype after challenge compared to wild-type mice, as did uPA over-expressing mice. However, this phenotype was reversed when PAI-1 was over-expressed, consistent with the idea that expression of PAI-1 is vital for the restraint of uPA in order for the initial formation and stabilisation of a haemostatic fibrin plug at the site of epithelial injury to occur.

Together these data indicate that the initial induction of PAI-1 expression in acute *H. pylori* infection is part of a defensive mechanism as it has been demonstrated that PAI-1 plays a pivotal role in the protection of the gastric epithelium after injur y. However, PAI-1 also acts to restrain uPA, therefore

controlling epithelial cell proliferation which is a key characteristic of a preneoplastic, *H. pylori* infected epithelium. During prolonged *H. pylori* infection it can be suggested that sustained increased levels of anti-fibrinolytic PAI-1 contribute to the process of fibrosis, increased proliferation favours the acquisition of mutations which influences tissue remodelling leading to gastric cancer.

Chapter 1

Introduction

1.1 Overview

The Nobel Prize for Physiology or Medicine in 2005 was awarded to Marshall and Warren for their discovery in 1982 of the bacterium *Helicobacter pylori* (*H. pylori*) in patients suffering from inflammation of the gastric mucosa (gastritis) and duodenal ulcer (Marshall & Warren, 1984). It had previously been known that patients suffering from duodenal ulcer displayed an increase in gastric acid secretion (Henning, 1966). Subsequently it became clear that there was relationship between *H. pylori* infection, gastritis and gastric cancer. Gastric cancer is the second most common cause of cancer related death worldwide (Pisani *et al.*, 1999) and the estimated risk of dying from *H. pylori* related disease is 1:30 for men and 1:60 for woman (Axon & Forman, 1997). The mechanisms by which *H. pylori* causes gastritis and gastric cancer remain poorly understood.

This thesis examines the effects of *H. pylori* on several members of the urokinase plasminogen activator (uPA) system within the gastric epithelium. The uPA system consists of uPA itself, its cell surface receptor uPAR and the plasminogen activator inhibitors (PAI)-1, -2. Collectively these proteins play a part in a diverse range of processes including control of fibrinolysis and haemostasis, ovulation, wound healing, fibrosis, cancer cell invasion and metastasis (Andreasen *et al.*, 2000). The uPA system members are expressed in the stomach and have been shown to correlate with a worse prognosis in human gastric cancer (Kaneko *et al.*, 2003). Expression of PAI-2 in the stomach is increased by *H. pylori* (Varro *et al.*, 2004) therefore it is an interesting question to ask if expression of other members of the uPA system are also influenced by *H. pylori,* and if so what are the implications for the gastric mucosa?

1.2 Gastric architecture

The primary function of the stomach is to temporarily store food and release it slowly into the duodenum. Through the release of acid and pepsin to initiate digestion, the stomach processes the food to a semi-solid chyme which enables better contact with the mucous membrane of the intestine, thereby facilitating absorption of nutrients. The acid environment of the stomach lumen inactivates most micro-organisms although *H. pylori* is a special case since this bacterium is adapted for survival in acid conditions. Several mucosal protective mechanisms exist within the stomach which limit tissue injury, contribute to gastric mucosal protection and inhibit auto-digestion of the stomach (discussed in section 1.2.6).

1.2.1 Organisation of the gastric mucosa

The wall of the stomach is subdivided into two functionally distinct regions. The body of the stomach, also known as the corpus is responsible for acid and pepsinogen secretion and through relaxation of its muscle layer it accommodates most ingested food. The lowermost region is the antrum which produces the hormone gastrin (this stimulates acid production) and grinds the food. In both cases an epithelium is the first/inner layer containing gastric glands; the submucosa is followed by a muscle layer, the serosa and the subserosa (Caletti *et al.*, 1984).

1.2.2 Gastric epithelium

The gastric epithelium is a single layer of cells that lines the interior face of the stomach, providing physical protection from the lumen to the inner layers of the

mucosa. The epithelium consists of a variety of specialised secretory epithelial cells that invaginate to form gastric glands. These are highly organised branching tubular structures that reach deep into the muscularis mucosa. Various differentiated epithelial cell types are found in characteristic locations in the gastric gland and arise from the proliferation and renewal of an undifferentiated, granule-free, multipotent stem cell. This is located in the isthmus or neck region of the gland and forms the precursor cells of the differentiated cell types (Hall, 1989).

The single multipotent stem cell is located in a physiological environment known as a "niche" (Karam & Leblond, 1993a; Dockray, 1999; Brittan & Wright, 2002) and from here cells migrate up or down the gland. Over a period of approximately 3 days, cells that migrate upwards of the neck region differentiate into surface mucous cells on reaching the pit region and secrete mucus to protect the epithelium from the mechanical strain of digestion and the low pH of lumen. At the surface of the gland, pit cells undergo necrosis/apoptosis, are phagocytosed by neighboring cells or are shed into the lumen (Karam & Leblond, 1993b). Cells migrating down the gland give rise to mucous neck cells and acid (HCL) secreting parietal cells forming the neck region of the gland. Histamine producing enterochromaffin-like (ECL) cells and pepsinogen secreting chief cells form the base region of the gland (Karam & Leblond, 1993c; Karam & Leblond, 1993d; Karam *et al.*, 2003). Chief cells are derived entirely from mucous neck cells as they migrate towards the base of the gland.

Gastric glands of the corpus are called oxyntic (from the Greek for acid, *oxys*) glands as they contain the acid secreting parietal cells (Ito, 1987). Figure 1.1

shows a schematic of the main regions of the stomach and a representation of an oxyntic gland which contains parietal, ECL, chief and somatostatin producing D-cells. Other endocrine cells present in the oxyntic glands other than ECL and D-cells are serotonin-releasing enterochromaffin cells (EC) and recently identified ghrelin (X) cells (Bordi *et al.*, 2000a; Rindi *et al.*, 2002). The pyloric glands of the antrum contain gastrin secreting G-cells, D and EC cells (Walsh, 1994; Bordi *et al.*, 2000b). The life span and function of gastric epithelial cells varies from cell to cell with cell types such as surface mucous cells surviving for 2-3 days. Parietal, ECL, G and D-cells are the four cell types crucial for acid secretion (Hou & Schubert, 2006).

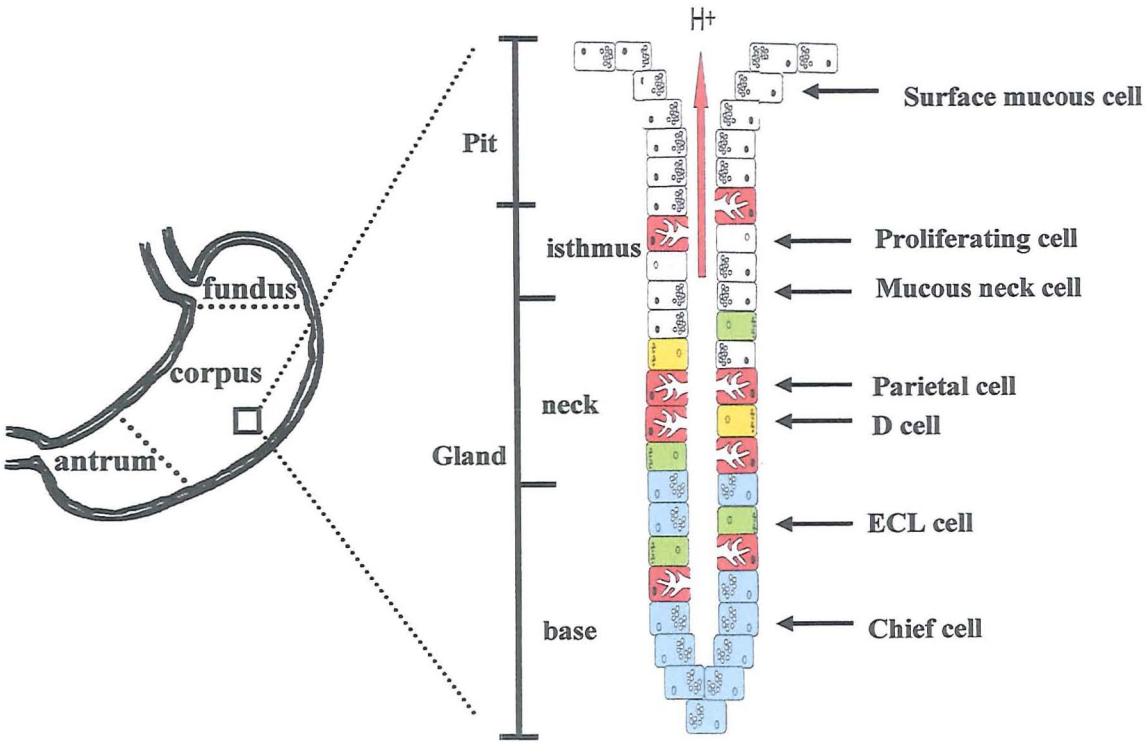

Figure 1.1
Schematic representation of the stomach showing the major anatomical divisions. An enlarged area of the oxyntic mucosa shows a representation of a corpus gastric gland (Adapted from Dimaline & Varro, 2007).

1.2.3 Role of gastric epithelial cells in acid secretion

Formed primarily from the differentiation of pre-parietal cells but also from pre-neck and pre-pit cell precursors (Karam, 1993), the parietal cell was first described by Heidenhain in 1870. It has an average life span in mouse of approximately 20 days (Kirton *et al.*, 2002), is solely found in the corpus mucosa and is responsible for the secretion of 100mM HCL. In addition to this, parietal cells also secrete intrinsic factor required for absorption of vitamin B_{12} (Okuda, 1999).

ECL cells are one of the main endocrine cell types within the stomach and are also confined to the corpus mucosa. They are responsible for the synthesis and secretion of the acid secretagogue histamine and they express the gastrin or cholecystokinin receptor (CCK2R; Schmitz *et al.*, 2001). Since its discovery in 1905 by Edkins, and isolation and purification in 1964, the hormone gastrin which is produced by and released from antral G-cells (Gregory & Tracy, 1964; McGuigan, 1968; Modlin *et al.*, 1997) is now recognised as a major stimulant of acid secretion in the gastric corpus.

Release of gastrin is primarily stimulated by amino acids and peptides in the stomach and acts through the CCK2R. However, although parietal cells express functional CCK2R (Reubi *et al.*, 1997) they play an indirect role in acid secretion (Sachs *et al.*, 1997). The effects of gastrin on parietal cell acid secretion are paracrine as ECL cells are the primary gastrin target which causes the release of histamine (Sandvik *et al.*, 1987).

Histamine released from ECL cells in response to gastrin acts on histamine-H_2 receptors expressed on parietal cells triggering a cascade that initiates the translocation of the proton pump, H^+, K^+-ATPase, from cytoplasmic tubulovesicles to deep invaginations of the apical plasma membrane termed secretory canaliculi. This causes morphological transformation of these cells (Forte & Yao, 1996; Yao & Forte, 2003) and ultimately stimulation of acid secretion (Kitano *et al.*, 2000; Dockray *et al.*, 2001). G-cell release of gastrin is inhibited in a negative feedback mechanism by a decrease in luminal pH caused by acid secretion. This is sensed by antral D-cells which act as chemoreceptors and release somatostatin in response to a pH lower than 3.5 (Brand & Stone, 1988; Dimaline *et al.*, 1991).

1.2.4 Gastric submucosa

The submucosal layers located underneath the epithelium consist of mesenchymal cells (the main type being myofibroblasts), blood vessels, connective tissue, inflammatory cells and nerve fibers. Myofibroblasts were originally described as muscle-like fibroblasts (Gabbiani *et al.*, 1971) and are characterised by the expression of α-smooth muscle actin (α-SMA) and vimentin. Myofibroblasts derive from mesenchymal stem cells (Luria *et al.*, 1971; Friedenstein *et al.*, 1974; Ogawa *et al.*, 2006) through the actions of platelet-derived growth factor (PDGF) and stem cell factor (SCF) and also through transdifferentiation of fibroblasts and smooth muscle cells via PDGF and transforming growth factor (TGF)-β (Powell *et al.*, 1999a; Powell *et al.*, 1999b). Myofibroblasts play an important role in epithelial maintenance including

repairing the epithelium in response to physical damage and bacterial infection through epithelial-mesenchymal interactions (discussed in section 1.4).

1.2.5 Role of gastrin in gastric epithelium function

In addition to the regulation of acid secretion (discussed in section 1.2.3) the physiological functions of gastrin include roles in the organisation and maintenance of the gastric epithelium (Dockray, 1999; Dockray *et al.*, 2001; Dimaline & Varro, 2007). In the last decade new roles for gastrin have emerged from studies in genetically modified mice in which the gastrin gene is deleted (GAS-KO; Koh *et al.*, 1997; Friis-Hansen *et al.*, 1998), over-expressed (INS-GAS; Wang *et al.*, 1996), or in mice in which the CCK2R gene through which gastrin acts is knocked out (CCK2R -/-; Nagata *et al.*, 1996).

INS-GAS mice develop gastric carcinoma or adenocarcinoma, particularly after *H. pylori* infection. This was demonstrated by *Wang et al* (2000) who showed that chronic hypergastrinaemia in these mice acts in synergy with *Helicobacter* infection, contributing to the rapid onset (approximately 6 months post-infection) of atrophy. These animals show marked hyperplasia, inflammation and had extensive parietal cell loss (67%) with an accelerated progression towards gastric cancer compared to uninfected INS-GAS mice that only showed 40% parietal cell loss with the onset of gastric cancer being much slower (requiring nearly the entire lifetime of the animal,18-20 months). Infection of wild-type animals in the same genetic background not over-expressing gastrin (FVB/N) resulted in moderate foveolar hyperplasia and inflammation, showing only 27% parietal cell loss (Wang *et al.*, 2000).

A marked increase in basal gastric pH and an approximate 10-fold elevation of plasma gastrin concentration is seen in CCK2R -/- mice that also show atrophy of the corpus mucosa associated with a 70% decrease in active parietal cells (Langhans *et al.*, 1997) and a reduced number of D-cells (Nagata et al., 1996). The ECL cells in these animals do not secrete histamine (Chen *et al.*, 2002) and they also have an increased number of gastrin immunoreactive G-cells (Nagata *et al.*, 1996). Hypochlorhydria is seen in GAS-KO mice (Koh *et al.*, 1997) which are a good model for studying tumorigenesis as their phenotype leads to bacterial overgrowth, gastric inflammation, metaplasia and tumour development (Zavros *et al.*, 2002; Friis-Hansen *et al.*, 2006a; Friis-Hansen *et al.*, 2006b; Tu *et al.*, 2007). Taken together, transgenic animal studies have shown that gastrin acting via CCK2R, is not essential for normal epithelial cell proliferation but is important for maintaining the normal cellular composition and function of the gastric mucosa.

Gastrin increases expression of a wide range of genes in gastric epithelial cells which are involved in proteolysis, proliferation, differentiation and migration (Varro *et al.*, 2002a). Among these are members of the uPA system such as PAI-2 and members of the matrix metalloproteinase (MMP) family such as MMP-7 and MMP-9, both of which are involved in tissue remodeling. Gastrin acting via MMP-9 stimulates invasiveness of gastric cancer cells (Wroblewski *et al.*, 2002) and MMP -7 plays an important role in migration of primary human gastric epithelial cells (Wroblewski *et al.*, 2003) and also in epithelial-mesenchymal interactions (discussed in section 1.4; McCaig *et al.*, 2006; Varro *et al.*, 2007). Other gastrin sensitive genes important in epithelial maintenance include trefoil family factor (TFF)- 1 which is required for restitution and repair of the

epithelium and is rapidly up-regulated in response to injury (Sands & Podolsky, 1996; Khan *et al.*, 2003) and the cytoskeleton-membrane linker protein, ezrin which is needed for parietal cell maturation (Pagliocca *et al.*, 2003).

Given its extensive range of effects there is little evidence suggesting that hypergastrinaemia alone predisposes humans to gastric atrophy (characterised by chronic inflammation of the gastric mucosa with decreased numbers of parietal cells and low levels of acid secretion), but it is thought to exert its actions by intensifying the effects of inflammatory conditions such as infection with *Helicobacter* which is also often associated with elevated plasma gastrin concentrations (Harris *et al.*, 2002; Calam *et al.*, 1997) and is discussed above in the context of INS-GAS mice and discussed further in section 1.3.7.1.

1.2.6 Gastric mucosal protection

The stomach is under continuous exposure to potentially hazardous agents. Luminal acid and pepsin constitute a major threat to the gastric mucosa. Reflux of alkaline duodenal contents which contain bile and pancreatic enzymes are additional damaging factors. Infection of the stomach with *Helicobacter* (discussed in section 1.3) has been found to be the main cause of gastritis (inflammation of the gastric mucosa) and gastric ulcers, which is one of the common ailments affecting humans. However, alcohol, cigarette smoking, drugs, reduction in gastric mucosal blood flow (GMBF), stress and a regular intake of non-steroid-anti-inflammatory drugs (NSAIDS), particularly aspirin are examples of exogenous mucosal irritants that can also inflict mucosal injury (Konturek *et al.*, 1981a; Konturek *et al.*, 1981b). The stomach has the ability to defend itself against these noxious agents by having a multitude of structural and

physiological factors in place to reduce tissue damage. These include mucus and bicarbonate secreted by surface epithelial cells, prostaglandins (PGs), modulation of GMBF and a fast acting neural system releasing sensory neuropeptides.

An initial defense barrier is provided by mucus cells which constantly produce a viscid layer of secreted mucus and bicarbonate (Hollander, 1954; Flemstorm, 1977). Gastric mucus consists of a glycoprotein tetramer with overlapping molecules resulting in a gel formation (Allen, 1976; Allen, 1984). Bicarbonate (HCO_3^-) is secreted at a basal rate amounting to 5-10 percent maximal acid output (Flemstorm, 1977) and together these protect the gastric epithelium from luminal acid and pepsin. An unstirred layer where mixing of H^+ and HCO_3^- is limited, gastric diffusion through mucus gel is delayed, allowing HCO_3^- to neutralise the pH at the epithelial surface (Crampton, 1988). Local paracrine agents, especially PGs are potent stimulators of bicarbonate secretion (Garner *et al.*, 1979).

Prostaglandins are the major product of arachidonate metabolism through cyclooxygenase (COX) activity and play a crucial role in protecting the gastric mucosa against injury (Robert *et al.*, 1979; Terano *et al.*, 1987). Two isoforms of COX have been identified (Kargman *et al.*, 1996); COX-1, a constitutive enzyme responsible for the production of PGs that are physiologically important for maintenance of mucosal integrity (Eberhart & Dubois, 1995), and COX-2 which is an inducible isoform (Wallace, 2006). Cyclooxygenase-2 is responsible for the rise in PG levels at sites of inflammation and disease and is induced in inflammatory cells such as fibroblasts and myofibroblasts in gastric ulcers (Takahashi *et al.*, 1998). The stomach can be damaged by NSAIDs principally as

a result of their inhibition of prostaglandin synthesis. This is mediated via abrogation of the secretion of mucus and bicarbonate and by reduction in GMBF (Brzozowski *et al.*, 2005).

If the "muco-bicarbonate layer" is disrupted then GMBF is one way in which the removal of acid is facilitated, preventing its accumulation in tissue. This is influenced by neuronal modulating processes stimulating the release of vasoactive mediators thus increasing GMBF and vascular permeability (Holzer & Sametz, 1986; Holzer *et al.*, 2001), processes crucial for the gastric mucosa to resist the continual onslaught of aggressive agents (Yonei *et al.*, 1990). A reduction in GMBF can cause degeneration of the epithelium by potentiating the effect of mucosal damaging agents, making it vulnerable to injury as it is unable to produce appropriate protective secretions.

The maintenance of gastric mucosal integrity after attack by noxious chemicals is highly dependent on a neural alarm system. Afferent sensory neurons of intrinsic and extrinsic origin (vagal and spinal afferents) are present within the stomach. Extrinsic afferents operate as the main emergency protective system (Wimalawansa *et al.*, 1990), with many of the homeostatic actions of spinal afferents being mediated by the release of neuropeptides from their peripherial nerve endings.

Many primary afferent neurons are sensitive to the excitotoxic action of capsaicin, the pungent ingredient of red pepper/ chillis (Holzer, 1991). This exerts gasoprotective effects and is a unique neuropharmacological tool (Kang *et al.*, 1995; Mozsik *et al.*, 2005). It acts specifically on vanilloid receptors of type 1 (VR1; Caterina *et al.*, 1997), which are expressed on vagal and spinal afferents

(Schicho *et al.*, 2004). Capsaicin is associated with increased GMBF in the gastric mucosa (Holzer, 1990; Holzer *et al.*, 1990) as it stimulates capsaicin–sensitive neurons which are present in the stomach and form a dense plexus around the gastric submucosal vessels (Green & Dockray, 1988).

The effects of capsaicin are mediated through the release of neuropeptide transmitters such as calcitonin gene-related peptide (CGRP) and tachykinins such as substance P (SP) and neurokinin A (NKA) from spinal afferent nerve endings in the gastric mucosa and submucosa (Holzer, 1988; Chen *et al.*, 1992; Yeoh *et al.*, 1995). Calcitonin gene-related peptide is a 37-amino acid, vasoactive neuropeptide which acts on CGRP receptors to enhance the resistance of the gastric mucosa to experimental injury (Holzer *et al.*, 1991). The expression of VR1 and CGRP has been shown to be significantly increased in the epithelial layer of the gastric mucosa in patients with *H. pylori* associated chronic gastritis (Domotor *et al.*, 2007).

The gastroprotective action of primary transmitters such as CGRP involves secondary messengers such as the powerful vasodilator nitric oxide (NO). Endothelial cells lining the gastric blood vessels release NO, which mediates vascular relaxation induced by vagal stimulation (Palmer *et al.*, 1987) and acts to drive and maintain the gastric microcirculation. The beneficial effects on the gastric mucosa of capsaicin, CGRP and NKA are suppressed by NO synthase inhibitors (Stroff *et al.*, 1996). These inhibitors have been shown to decrease GMBF (Pique *et al.*, 1989; Tepperman & Whittle, 1992) and intragastric administration of NO donors is known to prevent ethanol-induced gastric lesions

(MacNaughton *et al.*, 1989; Lopez-Belmonte *et al.*, 1993). Gastric mucus secretion can also be regulated by NO (Brown *et al.*, 1993).

Excessive ethanol ingestion can result in gastritis characterised by mucosal edema, subepithelial hemorrhages, cellular exfoliation and inflammatory cell infiltration (Guslandi, 1987). Alcohol-induced gastric superficial injury is limited to the epithelium and involves mostly the inter-foveolar epithelium and gastric pits which heals rapidly by epithelial cell migration through a process known as restitution (Lacy *et al.*, 1993). Gastric myofibroblasts can also migrate through the basement membrane and help restitution (Wu *et al.*, 1999). However, the deeper lesions involve intramucosal hemorrhage and vascular engorgement (Guth *et al.*, 1984). Due to damage, microvessels leak inflammatory mediators and vasoconstriction of submucosal arteries results in ischemia which eventually enhances the formation of more severe necrotic mucosal injury. Gastric mucosal damage can be produced by direct application of ethanol and high concentrations elicit injury in parts of the stomach attributed to congestion of the mucosa caused by constrictions of collecting venules (Ohno *et al.*, 1995; Ohno *et al.*, 1999). Ethanol-induced injury due to constriction has been shown to be inhibited by CGRP (Ohno *et al.*, 1995) and by prostaglandin E2 and prostaglandin E4 signaling (Hattori *et al.*, 2008).

1.3 *Helicobacter pylori* infection of the gastric epithelium

After being identified as a separate genus in 1989, the originally named *Campylobacter pyloridis* was renamed *Helicobacter pylori* (*H. pylori*). It is a microaerophilic, S-shaped, gram-negative bacteria known to have colonised the

stomach since ancient times (Linz & Schuster, 2007). Although earlier work identified a spiral bacterium within the stomach, *H. pylori* was only first identified in culture in 1982, and it was shown to be associated with peptic ulcers (Marshall & Warren, 1984). It is now known that this bacterium is strongly associated with gastritis (inflammation of the gastric mucosa) and infected individuals have a susceptibility to either peptic ulcer or gastric cancer.

1.3.1 Epidemiology

Infection with *H. pylori* is usually acquired in early childhood and when left untreated generally persists for the host's entire lifetime (Everhart, 2000). This is in part due to its ability to evade the antimicrobial effects of the immune response (Gobert *et al.*, 2001). People in all geographical locations can carry *H. pylori* but the prevalence of infection rises from about 50% of the population in developed countries, to 90% of the third world (Correa, 1996; Pisani *et al.*, 1999; Ernst, 2000; Everhart, 2000). Over the past decade, progressively fewer children have been shown to carry *H. pylori*, with this decrease being accelerated by the wide-use of medications such as antibiotics and also improved hygiene. Risk factors for acquisition of the bacterium include low socioeconomic status, household crowding, country of origin and ethnicity (Everhart, 2000; Tindberg *et al.*, 2001). The principle reservoir of *H. pylori* is man and infection is probably passed from individual to individual via proposed routes of faecal-oral, oral-oral and gastric-oral. There have also been descriptions of infection spread by means of uncooked vegetables contaminated with sewage (Stone, 1999).

1.3.2 Gastric epithelium remodelling and gastric cancer

H. pylori is recognised as an established causative agent of chronic inflammation of the mucous membranes of the stomach and of peptic ulcer. Individuals colonised with *H. pylori* develop co-existing gastritis, however, most people are asymptomatic. A long-term result of infection leads to the development of chronic gastric inflammation, conferring a significantly increased risk of developing gastric cancer (Forman *et al.*, 1991; Karnes *et al.*, 1991; Parsonnet *et al.*, 1991; Sipponen *et al.*, 1992; Hansson *et al.*, 1993; Correa, 1996; Wu *et al.*, 2003).

Approximately 15% of infected subjects will develop gastric or duodenal ulcer disease and a small proportion (less than 1%) develop neoplasia (Kuipers *et al.*, 1995; Uemura *et al.*, 2001; Go, 2002) thus indicating the involvement of other factors in cancer progression. *H. pylori* associated duodenal ulcer or cancer development are mutually exclusive clinical outcomes and do not develop together. In patients with duodenal ulcer, *H. pylori* colonises the antrum but not the corpus and is associated with increased acid and gastrin secretion (Hansson *et al.*, 1996; McColl *et al.*, 2000a, McColl *et al.*, 2000b; Dockray *et al.*, 2001; Uemura *et al.*, 2001). However, in other patients *H. pylori* can also spread to the corpus of the stomach resulting in inhibition of acid secretion, destruction of parietal and chief cells and progression to gastric cancer (Hansson *et al.*, 1996; Dockray *et al.*, 2001; Uemura *et al.*, 2001).

People infected with *H. pylori* not only have an increased risk of developing gastric cancer but also MALToma and non-Hodgkin's lymphoma of the stomach (Ernst & Gold, 2000; Peek & Blaser, 2002). Although less information is known

regarding the specific factors involved in these two malignancies, the local inflammatory process initiated by *H. pylori* is thought to be of importance (Isaacson & Du, 2005).

Gastric adenocarcinoma is the second leading cause of cancer-related death worldwide (Peek & Blaser, 2002) and approximately 649 000 people die from this malignancy each year (Correa, 2003). Most gastric cancers are sporadic in nature and arise secondary to multiple interacting environmental factors. *H. pylori* is the single most common cause which led to it being classified as a class 1 carcinogen by the World Health Organisation (WHO) and the International Agency for Research on Cancer consensus group (IARC) in 1994 (Houghton *et al.*, 2002; WHO, 1994). These findings have been extended from human patients (Uemura *et al.*, 2001) and several animal models (discussed in section 1.3.7).

The muscularis propria of the stomach is typically invaded by gastric cancer before diagnosis and the 5-year survival rate in the United States is less than 15% (Correa, 2003). Two distinct histological variants of gastric adenocarcinoma have been described each having different epidemiological and pathophysiological features: diffuse-type and intestinal-type gastric cancer.

Intestinal-type gastric adenocarcinoma progresses through a series of distinct histological steps, usually occurs at a late age and predominates in men (Sipponen & Marshall, 2000). These steps are initiated by the transition from normal mucosa to chronic superficial gastritis, leading to atrophic gastritis, intestinal metaplasia and ultimately to dysplasia and adenocarcinoma (Correa, 1998; Peek & Blaser, 2002; Correa, 2004).

Gastritis is characterised by diffuse infiltration of the gastric mucosa by cells representing chronic inflammation such as lymphocytes and macrophages but does not show loss of glands (Dixon *et al.*, 1996), as in atrophic gastritis which is also associated with the introduction of fibrous stromal tissue. At the stage of intestinal metaplasia, original glands and foveolar epithelium are replaced by cells with an intestinal phenotype and mucin-filled goblet cells (Matsukura *et al.*, 1980). Dysplasia is characterized by atypical changes in nuclear morphology and formation of irregular tissue architecture, frequently forming closely packed tubular structures (adenomas) with irregular lumens. All of the atypical cells are confined within the tubular structure but if they go through the basal membrane, they become invasive carcinomas. There is general agreement that the dysplastic epithelium is neoplastic, therefore, dysplasia was also referred to in the past as intraepithelial neoplasia (Rugge *et al.*, 2000). Figure 1.2 depicts the histological stages involved in gastric remodelling (Correa & Houghton, 2007) and other factors thought to be involved in the progression to gastric cancer.

Histologically, intestinal-type gastric cancer consists of gland-like structures that mimic intestinal glands. Diffuse type gastric cancer more commonly affects younger people, affects men and woman equally and consists of individual infiltrating neoplastic cells that do not form glandular structures and are not associated with intestinal metaplasia (Houghton *et al.*, 2002). The differing outcomes of *H. pylori* infection involves specific choreographed interactions between pathogen and host which are dependent on differences between bacterial strains including strain-specific bacterial factors and/or inflammatory responses governed by genetic diversity, environmental influences or variations in the individual host (Blaser & Berg, 2001).

1.3.3 *Helicobacter pylori* bacterial virulence factors

Helicobacter has been postulated to induce growth alterations and malignant transformations of the gastric mucosa through mechanisms involving direct bacterial-host cell contact and/or bacterial secreted products (Mobley, 1997). Since only a relatively small percentage of infected individuals' will progress to neoplasia, it has been suggested that this inconsistency might be in part due to bacterial strain-specific virulence factors (Blaser *et al.*, 1995; Danesh, 1999).

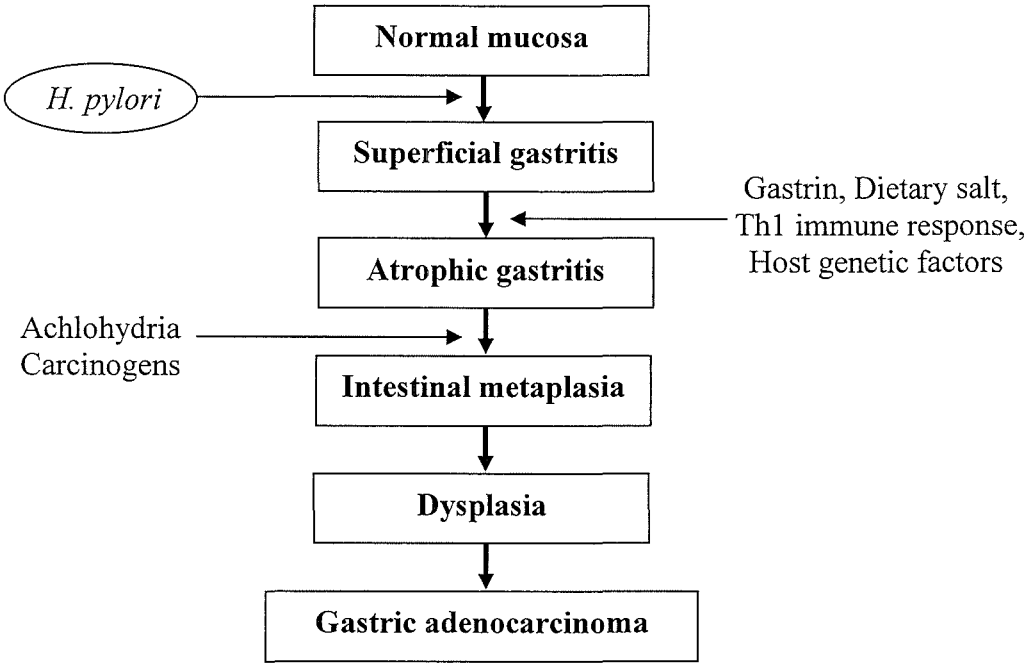

Figure 1.2
Histological steps and factors involved in progression to gastric adenocarcinoma (adapted from Correa, 1992)

Virulence factors are related to the ability of a microbe to induce disease and many are known to be involved in the pathology of *H. pylori* infection. Following colonisation of the gastric epithelium via the involvement of bacterial outer membrane proteins, among the most important virulence factors are the cytotoxin associated antigen pathogenicity island (*cag*-PAI), cytotoxin A (CagA)

and vacuolating cytotoxin A (VacA). On this basis, *H. pylori* strains have been divided into two groups: Type I strains which are *cag*-PAI positive (*cag*+) and *vacA* positive, and Type II which are *cagA*$^-$ and do not produce functional VacA.

H. pylori is able to invade and colonise the harsh environment within the gastric epithelium using several mechanisms including the use of *H. pylori* urease to catalyse the degradation of urea to carbon dioxide and ammonia enabling the survival of *H. pylori* in the low-pH acid environment of the stomach (Mobley *et al.*, 1991). The possession of flagella allows the bacteria to freely move around the gastric epithelium and to colonise the layer between the mucus gel and epithelial cells (Peek, 2005).

1.3.3.1 Outer membrane proteins

Approximately 20% of *H. pylori* in the stomach is found adhered to the surface of mucus epithelial cells (Hessey *et al.*, 1990) involving specialised interactions between host receptors and bacterial adhesins. The bacterium possesses over 30 genes dedicated to the expression of outer membrane proteins, several of which have been classed as adhesins. These have been shown to contribute to the host inflammatory response to *H. pylori* and are likely to be involved in disease progression (Prinz *et al.*, 2001; Mahdavi *et al.*, 2002; Peterson *et al.*, 2006).

One such protein is outer inflammatory protein A (OipA) which can promote interleukin (IL)-8 production and is also associated with bacterial density, gastric inflammation and risk for clinical sequelae such as peptic ulceration (Yamaoka *et al.*, 2002). The adhesin BabA binds to the host fucosylated blood group antigen Lewis-b (Ilver *et al.*, 1998) and SabA adheres to sialylated glycoproteins,

specifically to sialyl- Lewis-X (Mahdavi *et al.*, 2002) causing activation of neutrophils (Unemo *et al.*, 2005; Petersson *et al.*, 2006). It has also been shown that *H. pylori* binds to trefoil factor (TFF)-1 on the epithelial cell surface with the pattern of colonisation mirroring the distribution of TFF-1 (Clyne *et al.*, 2004).

1.3.3.2 *cag* pathogenicity island

The *cag* pathogenicity island (*cag*-PAI) is a well-characterised *H. pylori* virulence factor present in approximately 60% of Western *H. pylori* strains (N.B. PAI in the context of *cag*-PAI bears no relationship to plasminogen activator inhibitor (PAI) described later in this thesis). It is 40-kb encoding a group of approximately 30 genes inserted into the *H. pylori* glutamate racemase gene. Several *cag*-PAI genes encode a type IV secretory system (TFSS) which acts as a molecular syringe, injecting the CagA protein and other bacterial factors into eukaryotic cells (Censini *et al.*, 1996; Tomb *et al.*, 1997; Akopyants *et al.*, 1998; Alm *et al.*, 1999). Its presence is associated with an increased risk of severe gastritis (Peek *et al.*, 1995; Yamaoka *et al.*, 1998), peptic ulcer (Crabtree *et al.*, 1991; Covacci *et al.*, 1993; Cover *et al.*, 1995), atrophic gastritis (Kuipers *et al.*, 1995; Webb *et al.*, 1999) and distal gastric cancer (Blaser *et al.*, 1995; Parsonnet *et al.*, 1997).

A consequence of *cag*-PAI mediated epithelial cell contact is the induction of the neutrophil activating pro-inflammatory chemokine IL-8. Many *cag* island genes including *cagE*, *cagG*, *cag*H and *cag*L but not *cag*A have been found to be necessary for nuclear factor-κB (NF-κB) mediated IL-8 production (Glocker *et al.*, 1998; Crabtree *et al.*, 1999). Although IL-8 expression does not require CagA delivery, it does require a functional TFSS as disruption of the *cagE* gene

abrogates this response (Tummuru *et al.*, 1995; Sharma *et al.*, 1998; Nozawa *et al.*, 2002; Selbach *et al.*, 2002). It has recently been shown that Nod1, an intracytoplasmic pathogen-recognition molecule is responsible for the activation of NF-κB as it senses *H. pylori* peptidoglycan injected into host cells via the TFSS (Viala *et al.*, 2004).

Several studies in gastric epithelial cell lines have demonstrated that *cag*$^+$ *H. pylori* strains are more potent in the stimulation of IL-8 and induce an enhanced inflammatory neutrophilic response in human tissue compared to those strains in which the island is not present (Crabtree *et al.*, 1994; Crabtree *et al.*, 1995; Crabtree & Farmery, 1995; Sharma *et al.*, 1995; Yamaoka *et al.*, 1996; Shimoyama *et al.*, 1998). *cag*$^+$ strains have also been shown to activate mitogen activating protein kinase (MAPK) pathways (Keates *et al.*, 1999; Naumann *et al.*, 1999) activating the proto-oncogenes c-fos and c-jun via activation of the extracellular signal-related kinase (ERK) pathway which in turn activates the transcription factor AP-1 (Meyer-ter-Vehn *et al.*, 2000). The AP-1 family of transcription factors plays a pivotal role in cell proliferation and neoplastic transformation (Angel & Karin, 1991), therefore deregulation of AP-1 proteins or indeed the MAPK pathway may contribute to transformation of gastric epithelial cells.

Both *cag*$^+$ and *cag*$^-$ strains are able to transactivate the epidermal growth factor (EGF) receptor in gastric epithelial cells via activation of heparin binding-EGF (HB-EGF) which is required for Ras-mediated activation of ERK-1/2 and also for the induction of the early growth response gene *Egr-1* which is a regulator of cell growth, differentiation and survival (Keates *et al.*, 2001; Wallasch *et al.*,

2002; Keates *et al.*, 2005; Du *et al.*, 2007). Differences in gene expression induced by wild-type *H. pylori* cag^+ compared with wild-type *H. pylori* cag^- showed many genes to be differentially expressed in the Kato III epithelial cell line with three genes, ADAM 10, amphiregulin and HPYR1 found to be up-regulated *in vivo* in infected human gastric mucosa (Cox *et al.*, 2001).

H. pylori has been shown to up-regulate matrix metalloproteinase-7 (MMP-7) in epithelial cells both *in vivo* and *in vitro* in a Cag dependent manner. *H. pylori* cag^+ strains enhance levels of MMP-7 within the inflamed gastric mucosa which is dependent on activation of ERK1/2 by specific components within the *cag* island (Crawford *et al.*, 2003). Bebb *et al* demonstrated that $cagE^+$ *H. pylori* strains induced higher levels of MMP-7 expression than $cagE^-$ strains (Bebb *et al.*, 2003).

1.3.3.3 CagA

CagA is a 145kDa protein which requires the *cag*-PAI TFSS for it to be translocated into gastric epithelial cells leading to disruption of the epithelial apical-junctional complex and junction-mediated barrier function, processes previously shown to participate in the process of carcinogenesis (Amieva *et al.*, 2003). Infection of gastric epithelial cells (AGS) with $cagA^+$ *H. pylori* induces a 'hummingbird' phenotype characterised by cell spreading, elongated cell growth, lamellipodia and filopodia (Segal *et al.*, 1999). The inflammatory interleukins IL-1β, IL-6, IL-8 and tumour necrosis factor α (TNF-α) have all been detected in patients infected with $cagA^+$ strains of *H. pylori* (Yamaoka *et al.*, 1997).

Once inside the cell, CagA is subsequently phosphorylated by members of the Src family of kinases (Asahi *et al.*, 2000; Odenbreit *et al.*, 2000; Stein *et al.*, 2000) resulting in activation of C-terminal Src kinase, inhibiting the activity of Src leading to a decrease in the levels of phospho-CagA in a negative feedback loop (Selbach *et al.*, 2003; Tsutsumi *et al.*, 2003). Phospho-CagA interacts with several host signalling molecules, activating protein kinase cascades (Segal *et al.*, 1999). It activates both ERK which is a member of the MAPK family and a eukaryotic phosphatase (SHP-2) leading to morphological changes within the host cell due to rearrangement of the actin cytoskeleton (Higashi *et al.*, 2002; Higashi *et al.*, 2004).

The three prime repeat regions of *cagA* contain EPIYA motifs (consisting of glutamine-proline-isolucine-tyrosine-alanine), the number of which determines the level of CagA phosphorylation (Argent *et al.*, 2004). The EPIYA motif has a dual function in both membrane association and tyrosine phosphorylation which are both critically involved in the activity of CagA to deregulate intracellular signalling.

In gastric epithelial cells non-phosphorylated CagA intracellularly interacts with *c*-Met inducing a motogenic response (Churin *et al.*, 2003) and it also induces inactivation of Crc kinase, dephosphorylation of cortactin, and adapter molecule Grb-2 (Mimuro *et al.*, 2002; Selbach *et al.*, 2003). In AGS cells, *H. pylori* that co-express CagA with another virulence factor, HspB, (Dunn *et al.*, 1992; Macchia *et al.*, 1993) a protein involved in gastric carcinogenesis (Iaquinto *et al.*, 2000; De Luca *et al.*, 2003), are able to influence cell growth by inducing an increase in cyclin D3 and phosphorylated Retinoblastoma (Rb) gene product (De

Luca *et al.*, 2003; De Luca *et al.*, 2004). Interestingly, expression of both CagA and HspB are required to induce these effects in AGS cells, suggesting that co-operation among different *H. pylori* proteins is necessary to induce cell cycle alterations in infected cells.

1.3.3.4 VacA

An independent *H. pylori* locus associated with gastric adenocarcinoma development is *vacA*. This encodes a secreted protein that induces large cytoplasmic vacuole formation in eukaryotic cells (Cover & Blaser, 1992a; Cover & Blaser, 1992b; Phadnis *et al.*, 1994). The toxin inserts itself into the epithelial cell membrane allowing bicarbonate and organic anion release, facilitating the formation of transmembrane pores which permeabilise the gastric epithelium to urea (Papini *et al.*, 1998; Szabo *et al.*, 1999; Tombola *et al.*, 1999; Jungblut *et al.*, 2000).

All *H. pylori* strains possess the *vacA* gene, yet only approximately 50% of isolates express a toxin which has vacuolating activity (Cover *et al.*, 1994; Atherton *et al.*, 1995). This is due to two major sequence variations within *vacA* located to both the *vacA* secretion signal sequence which are known as allele types s1a, s1b, s1c or s2 and to the midregion known as allele types m1 or m2 (Atherton *et al.*, 1995; van Doorn *et al.*, 1998; van Doorn *et al.*, 1999a). Enhanced gastric cell injury is associated with strains possessing the m1 allele (Ghiara *et al.*, 1995; Atherton *et al.*, 1997) and increased risk for distal gastric cancer compared to *vacA* m2 strains (Gerhard *et al.*, 1999; Miehlke *et al.*, 2000; Figueiredo *et al.*, 2002).

vacA induces apoptosis in gastric epithelial cells which is dependent upon strain variations in the structure of VacA (Kuck *et al.*, 2001; Cover *et al.*, 2003). It has also been shown that increased concentration of VacA are found in the gastric juice of patients with duodenal ulcers compared to those without, thus suggesting the quantity of VacA could also be important in determining clinical outcome of disease (Papini *et al.*, 1998). VacA binds to a unique receptor-type protein tyrosine phosphatase (PTPξ) which is a member of a family of receptor like enzymes that are involved in regulation of proliferation, differentiation and adhesion (Fujikawa *et al.*, 2003).

Recent observations describe that VacA contributes to evasion of *H. pylori* to the adaptive immune response by interfering with T cell proliferation and activation via multiple mechanisms. VacA actively suppresses proliferation and activation of transformed Jurkat T cells *in vitro* via inhibition of nuclear factor of activated T cell (NFAT)-mediated IL-2 signalling (Gebert *et al.*, 2003). As well as this, VacA can also block activation of primary human T cells which is independent of NFAT (Sundrud *et al.*, 2004).

1.3.3.5 *Helicobacter felis*

H. pylori can only colonize a limited number of hosts including non-human primates, germ-free or barrier raised piglets, germ-free dogs and recently, laboratory raised cats. Mice experimentally infected with *H. pylori* develop gastritis although this is less severe than that seen in human *H. pylori* infection (Ferrero *et al.*, 1994; van Doorn *et al.*, 1999b). However, a mouse-adapted strain of *H. pylori,* SS1, has proven useful in modelling gastric disease in mice (Lee *et al.*, 1997; Sutton *et al.*, 2000).

Infection of mice with the related gastric species *Helicobacter felis* (*H. felis*) results in severe gastritis (Sakagami *et al.*, 1996) and infection of C57BL/6 mice with *H. felis* mimics many of the pathogenic changes commonly found in humans infected with *H. pylori* (Lee *et al.*, 1990; Wang *et al.*, 1998). Although both *H. felis* and *H. pylori* are closely related (Dewhirst *et al.*, 1994), *H. felis* is deficient in production of the *H. pylori* CagA and the cytotoxin VacA (Xiang *et al.*, 1995).

1.3.4 Environmental factors

Many environmental factors, particularly relating to dietary components are thought to affect the pathogenesis of gastric adenocarcinoma. The correlation between high salt diets, *H. pylori* infection and gastric cancer has been reported which appeared to be most prevalent in Japanese people (Go, 2002). These findings are consistent with a recent Japanese study on a large group of humans which also suggested that salt is an important environmental cofactor in patients infected with *H. pylori* and with atrophic gastritis (Shikata *et al.*, 2006). Salt is a dietary factor reported to promote *H. pylori* induced progression towards gastric atrophy and gastric cancer by increasing *H. pylori* colonisation and cellular proliferation in the antrum and corpus of C57BL/6 mice infected with the SS1 strain of *H. pylori* (Fox *et al.*, 1999).

H. pylori infected subjects on proton pump inhibitor (PPI) therapy to decrease acid secretion have been shown to have a modified gastric chemistry (Mowat *et al.*, 2000). At a neutral pH (Labenz *et al.*, 1996), ingested nitrite (derived from dietary nitrate) is not reduced to nitric oxide by ascorbic acid but accumulates in the stomach (Lundberg *et al.*, 2008). Proton pump inhibitor therapy also lowers the intragastric concentration of ascorbic acid, probably because of the relative

instability of the vitamin at a higher pH (Mowat & McColl, 2001). It is recognized that an elevated nitrite-to-ascorbic acid ratio predisposes to the formation of potentially carcinogenic *N* - nitroso compounds (NOCs) and *H. pylori* infection in animals has been shown to increase their susceptibility to NOC induced cancers (Sugiyama & Asaka, 1998).

1.3.5 *Helicobacter* and gastrin

Infection with *H. pylori* is accompanied by marked changes in gastric physiology and a role for gastrin in the progression of mucosal damage and development of cancer in response to this bacterium has emerged (reviewed Rozengurt & Walsh, 2001). Basal plasma gastrin concentrations are increased approximately two fold in *H. pylori* infected subjects and maybe elevated six fold in response to food (Dockray *et al.*, 2001). Hypergastrinaemia occurs early in the course of human *H. pylori* infection (Levi *et al.*, 1989) and the mechanism by which this occurs involves IL-1β and TNFα stimulated release of gastrin from antral G-cells (Weigert *et al.*, 1996).

Gastrin is negatively regulated by the release of somatostatin from D-cells which is inhibited by the pro-inflammatory cytokines TNF-α and interferon-gamma (IFN-γ; Beales *et al.*, 1997; Zavros *et al.*, 2003). *H. pylori* infection also reduces somatostatin with decreased levels reported in the antrum of infected patients (Kaneko *et al.*, 1992). Following *H. pylori* eradication in patients with duodenal ulcer disease there is an increase in the amount of somatostatin mRNA suggesting that the number of D-cells are reduced by infection (Moss *et al.*, 1992). The trophic actions of gastrin in the gastric mucosa and its interaction

with *Helicobacter* have been further studied in mice over-expressing amidated gastrin (INS-GAS; discussed in section 1.3.7.1).

1.3.6 Host response to *H. pylori* infection

Several other factors are now thought to influence the susceptibility of infected *H. pylori* subjects to developing gastric carcinoma such as the nature of the immune response elicited towards *H. pylori.* This involves both humoral and cellular immune responses and has long been considered an important determinant of the pathology induced in the particular host (reviewed in Wilson & Crabtree, 2007). The gastric mucosa of infected patients has increased concentrations of pro-inflammatory cytokines such as IL-6, IL-8 and TNF-α (Crabtree *et al.*, 1991; Crabtree *et al.*, 1993; Peek *et al.*, 1995; Crabtree, 1996), which are likely to modulate exocrine and endocrine cell function, affecting local acid secretion, the colonisation pattern of *H. pylori* and thus the distribution of gastritis.

Pro-inflammatory cytokines are also responsible for leukocyte recruitment and are crucial for inflammation since IL-8 secretion is directly linked to severity of gastritis (Peek *et al.*, 1995). In addition to cytokine-mediated activation of leukocytes, *H. pylori* also directly activates monocytes and macrophages via toll-like receptor (TLR)-2 which are receptors involved in the innate immune response, resulting in the induction of lymphocyte T-helper (Th)-1 (Mandell *et al.*, 2004). T lymphocyte deficient mice were found to be largely protected from gastric mucosal changes due to *H. pylori* infection, demonstrating a direct involvement of T lymphocytes in gastric cancer progression (Eaton *et al.*, 2001). The pattern of cytokine expression in both human and the Mongolian gerbil

gastric mucosa infected with *H. pylori* is T-helper type 1 (Th1) polarised (Crabtree *et al.*, 2004; Yamaoka *et al.*, 2005) and probably accounts for the increased levels of plasma gastrin and reduced amounts of somatostatin in infected subjects (as discussed in section 1.3.5).

Host gene polymorphisms in the proinflammatory IL-1 gene cluster (IL-1B encoding IL-1β and IL-1RN encoding its receptor) increase the risk of gastric cancer and its precursors in the presence of *H. pylori* (el-Omar *et al.*, 2000). In defined patient cohorts, cytokine gene polymorphisms in the progression to gastric cancer have also been extensively studied and clearly play a role in the risk of *H. pylori* induced gastric adenocarcinoma. Potentially important polymorphisms have been found in IL-2, IL-6, IL-8, IL-10 and IL-12 (Perez-Perez *et al.*, 2005; Lobo *et al.*, 2005; Lu *et al.*, 2005b; Navaglia *et al.*, 2005; Ohyauchi *et al.*, 2005; Togawa *et al.*, 2005; Zabaleta *et al.*, 2006).

Several human cancers are initiated by chronic inflammation for example colon cancer by inflammatory bowel disease and oesophageal adenocarcinoma by gastroesophageal reflux disease (Houghton & Wang, 2005). A relationship between chronic inflammation and cancer was established as early as 1863 (Virchow, 1863). Chronic inflammation in the gastric mucosa as a result of *H. pylori* infection could promote gastric cancer development by promoting processes such as DNA damage, apoptosis and hyperproliferation of gastric epithelial cells. *H. pylori* infection increases the expression of cyclooxygenase (COX)-2 (Franco *et al.*, 1999; McCarthy *et al.*, 1999), which has been shown to promote carcinogenesis by inhibiting apoptosis (Kim *et al.*, 2000; Wambura *et*

al., 2002), increasing invasion, angiogenesis (Chang *et al.*, 2005; Wu *et al.*, 2005) and proliferation (Wambura *et al.*, 2002).

The involvement of oxidative stress in gastrointestinal carcinogenesis is also well established (Seril *et al.*, 2003). *H. pylori* infection leads to an increase in expression of an important polyamine catabolic enzyme, spermine oxidase, SMO(PAOh1) which oxidises spermine to produce the DNA damaging reactive oxygen species (ROS), H_2O_2 (Xu *et al.*, 2004). Inflammation results in nitric oxide (NO) and ROS production by leukocytes, macrophages and gastric epithelial cells (Bagchi *et al.*, 1996; Mannick *et al.*, 1996; Wilson *et al.*, 1996), resulting in DNA damage and mutations (Jaiswal *et al.*, 2001). Apoptosis of epithelial cells is also promoted by chronic inflammation (Moss *et al.*, 1996; Piotrowski *et al.*, 1996). *H. pylori* infection can elicit a strong host response and an accumulation of mutations arising from hyperproliferation of epithelial cells and DNA damage caused by NO and ROS could lead to the formation of malignant transformations.

1.3.7 Animal models of *Helicobacter*-induced gastric carcinoma

Many *in vivo* studies have employed experimental animal models, especially infection of inbred C57BL/6 mice, hypergastrinaemic INS-GAS mice or Mongolian gerbils infected with *Helicobacter* (reviewed in Pritchard & Przemeck 2004; Peek & Crabtree, 2006) to provide an insight into the host, bacterial and environmental factors involved in gastric carcinogenesis.

Conventional human gastric carcinoma cells lines such as AGS, MKN28, MKN45 and KATO III have been useful for the *in vitro* investigation into the effects *H. pylori* has on cell signalling, gene expression, apoptosis and

proliferation (Chang *et al.*, 1993; Jones *et al.*, 1999; Peek *et al.*, 1999; Chiou *et al.*, 2001; Iwamoto *et al.*, 2005). However, limited information regarding the roles of these factors in gastric carcinogenesis can be obtained since they are all derived from established tumours. The development of mouse, and more recently human (Wroblewski *et al.*, 2003) primary epithelial cell cultures might prove to be more useful in providing an insight into the progression to gastric carcinoma.

1.3.7.1 INS-GAS mice

INS-GAS mice express human gastrin under the control of the 0.4kb of the insulin promoter resulting in the production of amidated gastrin in pancreatic β cells and a 2 fold elevation (~150 pM) in plasma amidated gastrin levels at 1-3 months (Wang *et al.*, 1996). They show gastric mucosa hyperplasia which is associated with increased levels of TGF-α and HB-EGF. Initial hyperacidity is followed by hypoacidity, and at the age of 5 month onwards there is a steady decline in parietal cell number and invasive carcinomas are seen by the age of 20 months (Wang *et al.*, 2000).

INS-GAS mice are an attractive model of gastric cancer as they display many of the distinct histological and epidemiological steps as humans in the progression to intestinal-type adenocarcinoma. Both *H. felis* (Wang *et al.*, 2000; Fox *et al.*, 2003b) and *H. pylori* (Fox *et al.*, 2003a; Fox *et al.*, 2003b) have been shown to act synergistically with chronic hypergastrinaemia to accelerate the progression of gastric cancer with histopathological changes already present at 6-7 months in infected animals. Infection of INS-GAS mice induces gastric adenocarcinoma but is restricted to males which respond more aggressively and rapidly to infection, consistent with the greater incidence of gastric carcinoma in men (Fox

et al., 2003b). Removal of *cagE* temporally slows down but does not abolish pathological progression (Fox *et al.*, 2003a), in agreement with the finding that CagE is important for the assembly of the TFSS that plays a role in the transfer of CagA from *Helicobacter* into the epithelial cell (Guillemin *et al.*, 2002).

Prior to the development of disease there is an increase in mucosal IL-1β levels. However, this is not gender specific (Fox *et al.*, 2003a). The only major drawback of this useful model is that hypergastinaemia preceded rather than results from *Helicobacter* infection and the site where gastric adenocarcinomas occurs is the only major difference between humans and the INS-GAS model of gastric cancer. Gastric adenocarcionmas tend to develop in the antrum or at the junction of the antrum and corpus (Correa, 1996; Peek & Blaser, 2002) whereas gastric cancer in INS-GAS mice develops more frequently in the corpus (Fox *et al.*, 2003b).

1.3.7.2 *Helicobacter* vaccination

Even though current antimicrobial treatment regimens efficiently eradicate *H. pylori*, antibiotic resistance and reinfection remains a serious problem (Graham, 1998; Soto *et al.*, 2003; Koletzko *et al.*, 2006). Many strategies using animal models of infection have been attempted in order to try to develop either protective prophylactic or therapeutic vaccines against *H. pylori* which is an exciting prospect. Mice (Corthesy-Theulaz *et al.*, 1995) and ferrets (Cuenca *et al.*, 1996) in addition to beagle dogs (Rossi *et al.*, 2004) and Rhesus monkeys (Dubois *et al.*, 1998) have all shown that therapeutic immunisation reduces *H. pylori* colonisation and gastritis. A recently developed vaccine model in the

Mongolian gerbil showed no postimmunisation gastritis following *H. pylori* challenge in infected animals (Jeremy *et al.*, 2006). To date there have been very few clinical studies testing the immunogenicity and safety of potential *H. pylori* vaccines (Michetti *et al.*, 1999; Kotloff *et al.*, 2001; Banerjee *et al.*, 2002) and the ability to translate experimental model studies to human remains an elusive goal.

1.4 Epithelial-mesenchymal interactions and signalling

Two-way interactions between epithelial and underlying mesenchymal cells (such as myofibroblasts) are crucial for normal mucosal organisation and this occurs during development (Goke *et al.*, 1998), wound healing (McKaig *et al.*, 1999; Watanabe *et al.*, 2000) and progression to cancer. These interactions were first demonstrated in 1967 when the separation of embryonic epithelium of kidney, lung, salivary gland or pancreas from native mesenchymal cells resulted in the failure of these tissues to undergo branching morphogenesis but resumed to normal when these cell types were reintroduced (Grobstein, 1967).

The mechanism involved in epithelial-mesenchymal signalling often includes expression of specific growth factors by mesenchymal cells, which act in a paracrine fashion to stimulate epithelial cells. Intestinal mesenchymal cells do not express the hepatocyte growth factor (HGF)-receptor (c-Met) yet release HGF which acts via c-Met expressed on intestinal epithelial cell to stimulate proliferation (Goke *et al.*, 1998; Prat *et al.*, 1991; Fukamachi *et al.*, 1994). Keratinocyte growth factor (KGF) provides another example of this interaction as it originates solely from mesenchymal cells (Brauchle *et al.*, 1994; Boismenu & Havran, 1994) and acts exclusively on epithelial cells (Rubin *et al.*, 1995)

where it plays a role in wound healing after injury (Werner *et al.*, 1992; Staiano-Coico *et al.*, 1993). Breast cancer epithelial cells show an up-regulation of PDGF yet do not expresses the PDGF-receptor. However, this is expressed by myofibroblasts (Ichiki *et al.*, 1995; Bostrom *et al.*, 1996) which show increased proliferation and migration when treated with PDGF (Sommer *et al.*, 2002; Tangkijvanich *et al.*, 2002).

Epithelial and mesenchymal cells are present in the specialised microenvironment of the gastric stem cell "niche" and are important for stem cell regulation and development via mesenchymal-epithelial cross-talk (Brittan & Wright, 2002; Brittan & Wright, 2004). Dysfunction of this system is likely to contribute to the process of epithelial tumour development arising on a background of chronic tissue injury or inflammation, an example of which is provided by the development of gastric cancer in patients infected with *H. pylori*.

The mechanisms of gastric cancer progression remain largely unknown but it does involve remodelling of the epithelium and alterations in cell type/ number as well as altered expression of growth factors, extra-cellular matrix (ECM) proteases and protease inhibitors (Dimaline & Varro, 2007). *H. pylori* increases the release of MMP-7 (a member of a family of proteolytic enzymes important in maintenance and remodelling of interactions between epithelial cells and basement membranes; McCawley, 2001) from the gastric epithelium (Wroblewski *et al.*, 2003). This stimulates gastric myofibroblasts to produce both insulin-like growth factor II (IGF-II) and IGF-binding protein (IGFBP)-5 (Hemers *et al.*, 2005; McCaig *et al.*, 2006). In turn, MMP-7 cleaves IGFBP-5 and the free bioavailable IGF-II not only acts on myofibroblasts to stimulate

migration, proliferation and adhesion of but also stimulates proliferation of gastric epithelial cells (McCaig *et al.*, 2006). These mechanisms might play an important role in altering the niche that favours premalignant changes in the gastric epithelium.

1.5 Urokinase plasminogen activator (uPA) system

The uPA system is an enzymatic system central to a spectrum of physiological processes with its primary function being the control of fibrinolysis. Binding of the protease uPA to its receptor, uPAR initiates a proteolytic cascade resulting in the conversion of the zymogen plasminogen into plasmin. Plasmin has a wide range of functions and can degrade fibrin, extracellular basement membrane components such as fibrinogen and fibronectin (Liotta *et al.*, 1981; Mochan & Keler 1984; Goldfarb *et al.*, 1986), whilst activating others such as MMPs (Keski-Oja *et al.*, 1992; Baramova *et al.*, 1997; Kazes *et al.*, 1998). The serpin inhibitor α2-antiplasmin is the primary inhibitor of plasmin (Wiman & Collen, 1978). The production of plasmin by the uPA system is tightly regulated by the activity of plasminogen activator inhibitors (PAIs) of which there are three: PAI-1, -2 and -3 (Figure 1.3). The activity of the uPA system also activates certain growth factors such as HGF (Naldini *et al.*, 1992; Mars *et al.*, 1993), TGFβ (Yee *et al.*, 1993) and IGF (Remacle-Bonnet *et al.*, 1997).

Tissue remodelling during normal and pathological processes such as embryogenesis (Werb *et al.*, 1999), wound healing (Lund *et al.*, 1999), angiogenesis (Pepper, 2001) and cancer invasion (Johnsen *et al.*, 1998) are regulated by the presence of ECM degrading proteases and their corresponding

inhibitors. As well as its role in fibrinolysis, numerous studies (often utilising uPA system transgenic mice) have highlighted an involvement of this system in most of these processes (Dano *et al.*, 1985; Liotta *et al.*, 1991; Andreasen *et al.*, 2000).

1.5.1 Urokinase plasminogen activator

The conversion of plasminogen to plasmin is activated by the highly specific protease uPA. The 2.5kb uPA mRNA (Verde *et al.*, 1984) gives rise to a 411 amino acid, 53-kDa catalytically inactive single-chain polypeptide known as pro-uPA or sc-uPA (Skriver et al., 1982; Wun *et al.*, 1982; Andreasen *et al.*, 1997). Pro-uPA is released from many cell types and lacks plasminogen-activating activity. It is cleaved at lysine 158-159 to yield its two-chain, active form, also known as high molecular weight uPA (HMW-uPA) or tc-UPA.

Plasmin is an important activator of uPA (Dano *et al.*, 1985; Andreasen *et al.*, 1997) and a positive feedback mechanism exists in which plasmin generates uPA activity which in turn leads to the formation of new active plasmin from plasminogen in a highly potent amplification mechanism (Petersen *et al.*, 1988). Several other proteases have been shown to catalyse activation of uPA including cathepsin B and cathepsin G (Kobayashi *et al.*, 1991), plasma kallikrein (Ichinose *et al.*, 1986), mast cell tryptase (Stack & Johnson, 1994) and nerve growth factor gamma (Wolf *et al.*, 1993). Certain proteins such as the angiogenic growth factors vascular endothelial growth factor (VEGF) can also activate uPA (Prager *et al.*, 2004).

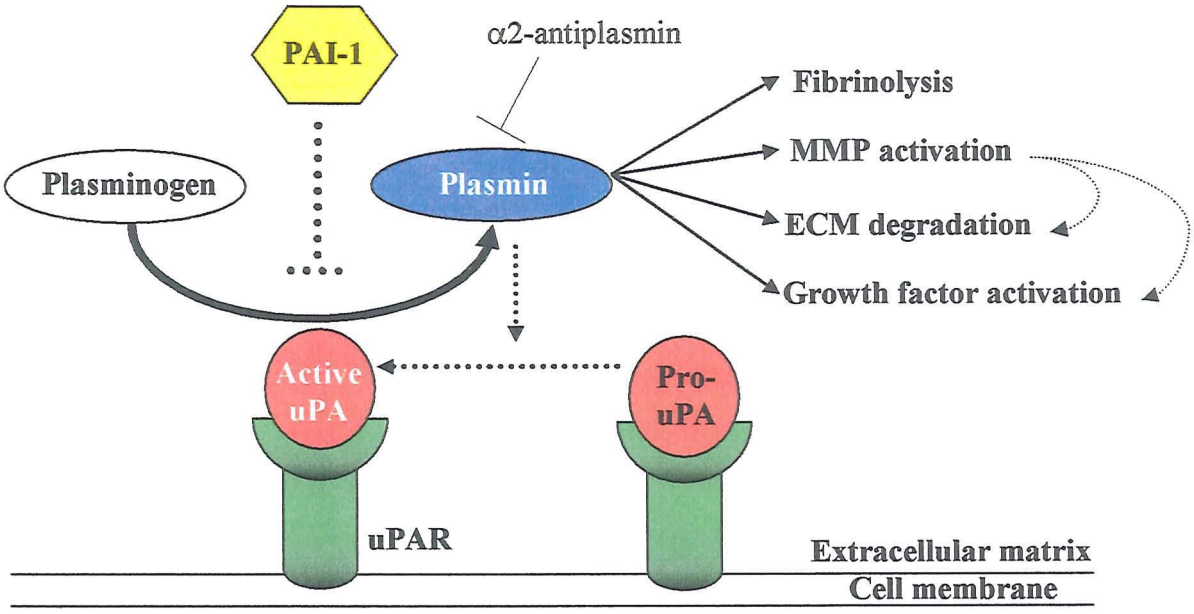

Figure 1.3
Schematic outline of the urokinase plasminogen activator (uPA) system
After binding to uPAR, uPA in its active form converts plasminogen to plasmin which is able to control fibrinolysis, breakdown the extracellular matrix (ECM) and activate growth factors and matrix metalloproteinases (MMPs), enhancing extracellular proteolysis. The plasminogen activator inhibitor PAI-1 (and PAI-2) control plasmin production by inhibiting uPA. α2-antiplasmin is also a specific inhibitor of plasmin.

High molecular weight-uPA is a two-chain protein (A and B chain), linked by a single sulphide bond. The tertiary structure of uPA is divided into three main domains. The N-terminal A-chain (light chain) growth factor domain (residues 1-49) contains the binding site for uPAR at residues 21-32 (Appella *et al.*, 1987; Saksela & Rifkin, 1988; Blasi & Carmeliet, 2002). This is followed by a kringle domain (residues 50-131) which together make up the amino-terminal fragment (ATF; Stoppelli *et al.*, 1985). The carboxyl-terminal region (B-chain/ heavy chain) contains the catalytic serine protease domain (residues 159-411).

Further cleavage of active uPA in the linker region between the kringle and serine protease domain (at lysine135-lysine136) splits uPA into two fragments:

the ATF fragment and B-chain (also designated low molecular weight uPA, LMW-uPA; Kasai *et al.*, 1985a; Kasai *et al.*, 1985b). LMW-uPA is soluble and maintains its proteolytic activity but does not bind to the uPAR receptor (Irigoyen *et al.*, 1999; Alfano *et al.*, 2005). Both pro-uPA and HMW-uPA bind to uPAR with high affinity (0.1-1nM) but the conversion of pro- to HMW-uPA occurs more efficiently when the zymogen is bound to uPAR (Irigoyen *et al.*, 1999). Binding of uPA to its cell surface receptor significantly increases the rate of its catalytic activity towards plasminogen at least 20-fold (Ellis *et al.*, 1989; Quax *et al.*, 1991).

The human gene coding for uPA is located on chromosome 10 and consists of 11 exons and 10 introns (Ibanez-Tallon *et al.*, 1999). The 5' flanking sequence contains several elements that indicate a tight transcriptional regulation (Ibanez-Tallon *et al.*, 1999; Ibanez-Tallon *et al.*, 2002). Upstream of the TATA box lies a CG rich region containing several binding sites for the transcription factor Sp1. This has a prominent role in uPA gene expression in PC3 cells and is targeted by several signal transduction pathways (Benasciutti *et al.*, 2004). Approximately 2 kb upstream of the transcription start site is a well-characterised enhancer containing an upstream Ets (PEA3) binding site and two AP-1 binding sites. There is a cooperativity mediator region containing the binding sites for upstream enhancer factor (UEF) transcription factors 1-4 (Ibanez-Tallon *et al.*, 1999; Ibanez-Tallon *et al.*, 2002). Two more elements have been identified in the regulatory region of the human uPA gene: an NK-κB binding site which mediates the transcriptional induction of gene expression by phorbol esters in the absence of the enhancer region (Hansen *et al.*, 1992) and a cell type-specific silencer (Cannio *et al.*, 1991).

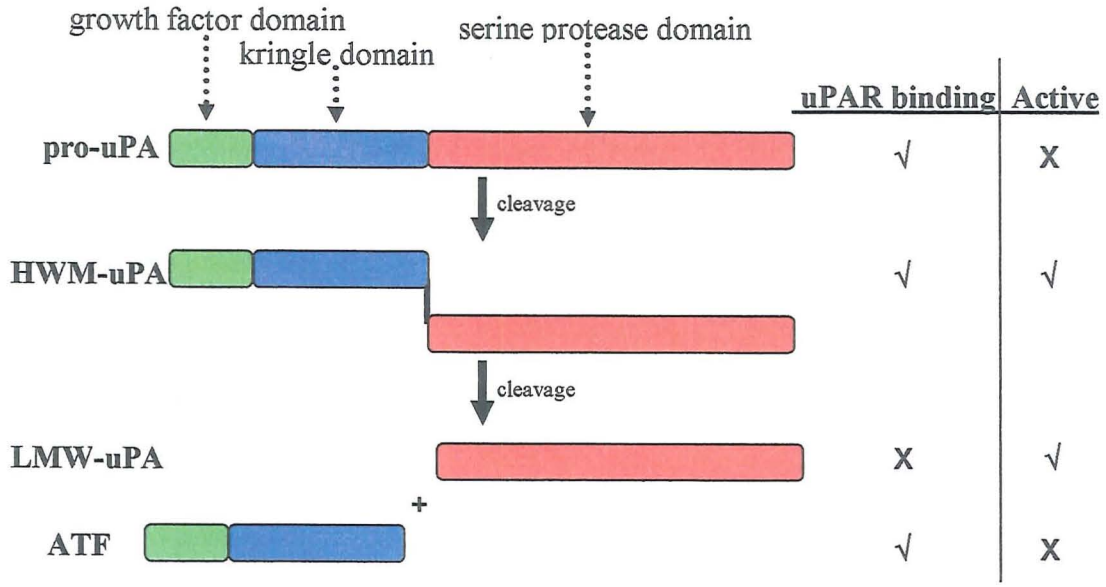

Figure 1.4
Structure of uPA
Pro-uPA is secreted as a single-chain zymogen containing an amino terminal fragment (ATF) consisting of a growth factor domain, a kringle domain and also a carboxy-terminal fragment containing a serine protease domain. Pro-uPA is activated and cleaved by plasmin to give high molecular weight uPA (HMW-uPA) which can be further cleaved into low molecular weight uPA (LMW-uPA) and ATF.

Transcriptional activation of the uPA gene can be obtained through various different stimuli e.g. phorbol esters, growth factors and signal transduction pathways, which mainly target the enhancer. These include NF-κB (Guerrini *et al.*, 1996; Sliva *et al.*, 2002a; Sliva *et al.*, 2002b; Lee *et al.*, 2006a), phosphoinositol 3-kinase (PI3K)/Akt (Lee *et al.*, 2006a), jun N-terminal kinase (JNK; Miralles *et al.*, 1998; Parra *et al.*, 2000; Benasciutti *et al.*, 2004), MEK/ERK and p38-MAPK pathways (Irigoyen *et al.*, 1997; Shin *et al.*, 2002; Shin *et al.*, 2003; Lee *et al.*, 2006b).

Cell-surface bound and soluble uPA have been shown to promote cell proliferation (Rabbani *et al.*, 1990; He *et al.*, 1991; Rabbani *et al.*, 1992; Stepanova *et al.*, 1999), stimulate migration (Fibbi *et al.*, 1998; Odekon *et al.*,

1992; Resnati *et al.*, 1996) and modulate adhesion of several cells such as smooth muscle cells (Chang *et al.*, 1998; Sidenius *et al.*, 2002). The mitogenic activity of uPA has been shown in several cell-types and uPA stimulates growth in human epidermal cells, normal and malignant renal cells and in melanoma cells (Kirchheimer *et al.*, 1988; Kirchheimer *et al.*, 1989). The ATF of uPA has also been identified as a mitogenic factor isolated from osteoblast-like cells (Rabbani *et al.*, 1990).

1.5.2 Urokinase plasminogen activator receptor

The specific binding of uPA to cell surfaces was first reported in 1985 and three years later Nielsen *et al.*, purified and characterised a membrane protein responsible for this binding and designated it uPAR (Nielsen *et al.*, 1988). Fully processed uPAR is heavily glycosylated (Behrendt *et al.*, 1990) and has a glycosyl-phosphatidyl-inositol moiety (GPI anchor) in its carboxyl-terminal region, integrating the receptor into the outer leaflet of the cell membrane (Ploug *et al.*, 1991a; Ploug *et al.*, 1993). Many cell types express uPAR including granulocytes (Nykjaer *et al.*, 1990), endothelial cells (Pepper *et al.*, 1993), B-lymphocyes (Plesner *et al.*, 1994), activated T-cells (Nykjaer *el al.*, 1994), fibroblasts (Anichini *et al.*, 1994) and epithelial cells (Limongi *et al.*, 1995).

The human 1.4kb cDNA has been cloned and mapped to chromosome 19q13.2 (Roldan *et al.*, 1990; Webb *et al.*, 1994) and gives rise to a 270 amino acid polypeptide with a molecular mass of 50 kDa (Nielsen *et al.*, 1988; Ploug *et al.*, 1991b). The gene is composed of seven exons, seperated by six introns and occupies approximately 21.23kb. The 5' region of the gene contains a CpG-rich

island and several putative *cis*-regulatory elements. These include two AP-1, an SP2, two SP1 and a novel NF-κB sequence (Wang *et al.*, 1995).

Various factors such as phorbol 12-myristate 13-acetate (PMA) (Langer *et al.*, 1993), cytokines such as TNF-α (Sitrin *et al.*, 1994; Wang *et al.*, 1994), TGF-β, IL-2,-3,-4,-7 (Nykjaer *et al.*, 1994), IFN-α, -γ (Kirchheimer *et al.*, 1988), as well as growth factors including VEGF (Mandriota *et al.*, 1995) have all been shown to modulate uPAR expression in a number of different cell types.

The ternary structure of uPAR is composed of three cysteine rich extracellular domains known as domains 1, 2 and 3 (D1, D2 and D3), connected by short linker regions (Ploug & Ellis, 1994). The uPA binding site is near to the N-terminus located within D1 (residues 1-87), leaving the whole external surface available for other interactions (Behrendt *et al.*, 1991; Ploug *et al.*, 1994). High affinity binding of uPAR to pro-uPA, HMW-uPA and ATF occurs (Figure 1.4; Cubellis *et al.*, 1986). As well as this, uPAR also associates with the ECM protein vitronectin (VN), integrin family members (Wei *et al.*, 1996; Wei *et al.*, 2001), and with several other co-receptors, including the EGF-R (Liu *et al.*, 2002) and endocytosis receptors of the low density lipoprotein receptor (LDLR) family (Strickland *et al.*, 2002; discussed in sections 1.5.2.1 and 1.5.5.1).

1.5.2.1 Cell signaling via uPAR

The binding of uPA to uPAR stimulates intracellular signaling (Kjoller, 2002). However, the mechanisms by which uPAR mediates signalling events remains to be fully elucidated as uPAR has no transmembrane domain. However, multiple transmembrane adapters and signaling receptors have been co-

immunoprecipitated or co-localised with cell surface uPAR suggesting they act as signal transducers (Resnati *et al.*, 1996). These include integrins such as β1 and β3 (Xue *et al.*, 1997; Wei *et al.*, 2001), β2 (Bohuslav *et al.*, 1995; May *et al.*, 1998) and β5 (Carriero *et al.*, 1999) which can transduce signals into the cell through the src-kinases activated by caveolin (Stahl & Mueller, 1995; Wei *et al.*, 1996; Chapman *et al.*, 1999; Wei *et al.*, 1999), a protein associated with intracellular signaling involved in cell adhesion (Okamoto *et al.*, 1998).

FPR-like receptor-1/lipoxin A4 receptor (FPRL-1) is a G-protein coupled receptor reported to interact with uPAR and activate NF-κB, MEK/ERK and Rho pathways (Behrendt *et al.*, 2000; Resnati *et al.*, 2002). In certain cells, the protein gp130 seems to mediate uPAR responses leading to activation of the janus kinase/ signal transducers and activators of transcription (JAK-STAT) signaling pathways (Koshelnick *et al.*, 1997). Epidermal growth factor receptor coimmunoprecipitates with uPAR and can be constitutively activated by ligand bound uPAR, activating ERK to promote proliferation and migration (Liu *et al.*, 2002; Jo *et al.*, 2003; Jo *et al.*, 2005). Non-receptor tyrosine kinases such as hck, fyn, lyn, jak1 any tyk2 (Bohuslav *et al.*, 1995; Resnati *et al.*, 1996; Dumler *et al.*, 1998) also coimmunoprecipitate with uPAR and other pathways shown to be activated downstream of uPAR ligand binding include fos, jun, myc and rac (Rabbani *et al.*, 1997; Nguyen *et al.*, 1998; Kjoller & Hall, 2001).

1.5.3 Plasminogen activator inhibitors (PAIs)

There are three specific serine protease inhibitors (serpins) of uPA known as plasminogen activator inhibitor (PAI) -1, -2 and -3 (Ginsburg *et al.*, 1986; Wilczynska *et al.*, 1995; Gettins, 2002). PAI-3 (also called SERPINA5, or protein C inhibitor, PCI) is a glycoprotein of 387 residues produced by several cell types and found in many tissues. PAI-3 is an inhibitor of activated protein C (APC) but also inhibits a wide range of other proteases including thrombin, kallikrein and prostate specific antigen (Espana *et al.*, 1989; Suzuki *et al.*, 1989; Espana *et al.*, 1991). Humans show a broad tissue expression pattern for PAI-3 (Laurell *et al.*, 1992; Radtke *et al.*, 1994; Elisen *et al.*, 1998) which functions primarily in the coagulation cascade and is involved in the process of reproduction demonstrated by male PAI-$3^{-/-}$ mice being infertile due to abnormal spermatogenesis (Uhrin *et al.*, 2000).

Although PAI-3 can inhibit uPA (Geiger *et al.*, 1989; Espana *et al.*, 1993), the major inhibitor of uPA is PAI-1 (SERPINE1) with PAI-2 (SERPINB2) being a less potent inhibitor. PAI-2 is a 47-kDa protein expressed in many cell types including gastric cancer cells (Ye *et al.*, 1989; Nakamura *et al.*, 1992), and can exist in two forms. Secreted PAI-2 modulates uPA activity but approximately 80% of PAI-2 is cytosolic acting within the cell to inhibit apoptosis (Kumar *et al.*, 1991; Dickinson *et al.*, 1995). Gene expression of PAI-2 is mediated by members of the CREB and AP-1 family of transcription factors (Schleuning *et al.*, 1987; Cousin *et al.*, 1991; Costa *et al.*, 2000). Within the PAI-2 promoter there are two putative AP-1 sites and a combined CRE/AP-1 site to which JunD has been shown to interact (Dear *et al.*, 1997; Costa *et al.*, 2000). Recently, PAI-2 expression has been shown to be regulated by gastrin in the AGS gastric cancer

cell line (Varro *et al.*, 2002b) and to be increased by *H. pylori* (Varro *et al.*, 2004).

1.5.4 Plasminogen activator inhibitor-1 (PAI-1)

PAI-1 was first discovered in rabbit endothelial cells in 1981 by Loskutoff *et al* (Loskutoff & Edgington, 1981). It is a single chain glycoprotein with a molecular mass of 50 kDA and is the principle physiologic regulator of plasminogen activation (Lawrence *et al.*, 1989; Fay *et al.*, 1992; Carmeliet *et al.*, 1993a; Carmeliet *et al.*, 1993b). The association of PAI-1 deficiency with clinical bleeding demonstrates that PAI-1 primarily functions to regulate haemostasis (Fay *et al.*, 1992).

The PAI-1 gene is an acute phase response gene approximately 12.2 kb in length, mapped to the long arm of chromosome 7 at 7q21.3-q22 and it is composed of 9 exons and 8 introns (Klinger *et al.*, 1987; Kruithof, 1988). Two distinct PAI-1 mRNAs (approximately 2.3 kb and 3.2 kb) are expressed by human cells, differing in the length of their 3' untranslated regions as a consequence of alternative polyadenylation. The 5'-flanking region of the PAI-1 gene has been extensively characterised and is considered to be the PAI-1 promoter containing the transcription initiation site, a TATA box, four Sp1 sites, an AP-1 and AP-2 binding site and several regulatory sequences.

PAI-1 can be rapidly induced suggesting it has a high biosynthetic rate and a variety of cultured cells synthesise PAI-1 due to the number of transcriptional elements within the PAI-1 promoter. These elements confer transcriptional responsiveness to a variety of mediators including growth factors, cytokines and

hormones (Keeton *et al.*, 1991; Fearns & Loskutoff, 1997; Stefansson *et al.*, 2003). Lipopolysaccharide (LPS) induces elevated PAI-1 which is mediated in part by the release of cytokines such as TNFα and IL-1 from inflammatory cells (Schleef *et al.*, 1988; van den Berg *et al.*, 1988; Sawdey & Loskutoff, 1991). Growth factors such as TGFβ also play a role in PAI-1 regulation (Sawdey & Loskutoff, 1991; Abe *et al.*, 1994; Dennler *et al.*, 1998) as does PMA through the induction of c-Jun/c-Fos. Glucocorticoids, glucose, very low density lipoprotein (VLDL), insulin and angiotensin as well as EGF and PDGF can induce PAI-1 expression (Loskutoff & Samad, 1998; Irigoyen *et al.*, 1999; Stefansson *et al.*, 2003; Hou *et al.*, 2004;).

Despite only 24% amino acid sequence identity between PAI-1 and PAI-2, both share a common tertiary structure consisting of three β-sheets, 8-9 α-helices and a reactive centre loop (RCL; Silverman *et al.*, 2001). The RCL is contained within a strained loop region at the carboxy terminus of the molecule and serves as the protease recognition site for uPA. The inhibition of uPA by PAI-1 occurs in a rapid, 1:1 stoichiometric manner leading to the formation of an SDS-stable complex in which PAI-1 adopts a more thermodynamically stable, relaxed conformation (Silverman *et al.*, 2001).

PAI-1 can exist in two different activity states, active and latent (Lawrence *et al.*, 1989; Loskutoff *et al.*, 1989), which is determined by conformational changes in the RCL (Levin *et al.*, 1987; Mottonen *et al.*, 1992). Active PAI-1 has the RCL exposed on the surface of the molecule which interacts with uPA. This unstable form is secreted by cells but spontaneously converts into the inactive (latent) form by insertion of the RCL (Levin *et al.*, 1987; Hekman *et al.*, 1988).

Human VN is a glycoprotein found in the circulation and in the ECM (Preissner, 1991) where it has a variety of functions dependent upon its ability to interact with humoral and cellular proteins. Physiologically, PAI-1 is present in plasma at 5-10 ng/ml (Declerck *et al.*, 1988a) and both here and in the ECM activated PAI-1 is in complex with VN (Declerck *et al.*, 1988b; Wiman *et al.*, 1988; Mimuro & Loskutoff., 1989). Binding to the somatomedin B (SMB) domain of VN stabalises PAI-1 in its active conformation (Lawrence *et al.*, 1994; Lawrence *et al.*, 1997), increasing its original half life of 90 minutes 2-10 fold (Hekman & Loskutoff, 1985; Declerck *et al.*, 1988b).

1.5.4.1 PAI-1 in disease states

Due to the fundamental importance of regulation of the uPA system it was originally speculated that PAI-1 deficiency would be inconsistent with normal development. Although PAI-1 deficient humans (Fay *et al.*, 1997) and mice (Carmeliet *et al.*, 1993a; Carmeliet *et al.*, 1993b) show a mild hyperfibrinolytic state and bleeding tendency, they are fertile, develop normally and do not display any gross abnormalities. Many studies, often using PAI-1 transgenic animals have related alterations in PAI-1 expression to the pathogenesis of a variety of disorders, including vascular disease, fibrosis, obesity and the metabolic syndrome and cancer.

The expression of PAI-1 is induced during inflammation (Quax *et al.*, 1990) and is increased in tissue injury. It is an acute phase protein important in epithelial cell wound healing (Providence *et al.*, 2000; Providence & Higgins, 2004), epidermal wound repair and in renal injury repair. Mice deficient in PAI-1 have inhibited vascular wound healing after injury (Swaisgood *et al.*, 2000). Increased

PAI-1 expression is associated with fibrosis such as experimentally induced glomerulosclerosis, liver and pulmonary fibrosis and is also increased in human fibrotic diseases such as glomernulonephritis, diabetic nephropathy and bronchoalveolar lavage fluid from respiratory distress patients (Bertozzi *et al.*, 1990; Yamamoto *et al.*, 1996; Loskutoff *et al.*, 2000; Oda et *al.*, 2001). Mice deficient in PAI-1 show an attenuated fibrinogenic response and develop less severe fibrosis after challenge than wild-type or PAI-1 over-expressing mice (Eitzman *et al.*, 1996).

A role for PAI-1 in vascular/cardiovascular disease has been demonstrated in several experimental settings. Spontaneous thrombi develop in the extremities and coronary arteries of PAI-1 over-expressing mice (Eren *et al.*, 2002) and lack of PAI-1 has been shown to protect mice from venous thrombi induced by endotoxin. Individuals with increased PAI-1 expression have an increased risk for thrombosis including myocardial infraction (MI; Thogersen *et al.*, 1998). A circadian rhythm causes an increase concentration of PAI-1 in the morning (Angleton *et al.*, 1989), corresponding to the circadian peak of MI (Muller *et al.*, 1989). Excess PAI-1 is seen in youths surviving MI and also in recurrent MI patients (Hamsten *et al.*, 1985; Hamsten *et al.*, 1987). An increase in PAI-1 is seen in atherosclerotic plaques which may contribute to the pathogenesis of diseases of vessel walls (Schneiderman *et al.*, 1992; Lupu *et al.*, 1993).

There is a positive correlation between PAI-1 expression in human adipose tissue and body mass index with the concentration of PAI-1 often increased five-fold in obese mice and humans compared with lean controls. Adipose tissue has an increased capacity for PAI-1 production and it is found to be increased in adipose

tissue from obese ob/ob mice and in human visceral fat (Samad & Loskutoff, 1996; Samad *et al.*, 1996; Alessi *et al.*, 1997; Bastelica *et al.*, 2002). Weight loss dramatically reduces the concentration of PAI-1 and ob/ob mice crossed into a PAI-1 deficient background have reduced body weight compared to controls, therefore suggesting PAI-1 itself might influence the development of obesity (Samad & Loskutoff, 1997). Following on from this, hyperinsulinaemia patients also have increased PAI-1 expression with improvements of this condition being made by weight loss associated with a decreased PAI-1 concentration (Nordt *et al.*, 1995; Samad *et al.*, 1998; Carmassi *et al.*, 1999; Mavri *et al.*, 1999; Morange *et al.*, 1999; Samad *et al.*, 2000; Juhan-Vague *et al.*, 2003).

Expression of PAI-1 is linked to tumour cell biology where it influences angiogenesis and is increased in many human cancers. A highly reproducible tumour model has been used to show that PAI-1 promotes tumor growth and invasion and angiogenesis which were inhibited using small molecule PAI-1 inhibitors (Bajou *et al.*, 1998; Bajou *et al.*, 2001; Brooks *et al.*, 2004). High PAI-1 expression levels correlate with a poor prognosis in many human cancers (discussed further in section 1.5.5.2; Foekens *et al.*, 2000; Dano *et al.*, 2005) and tumors in PAI-1 knock-out mice display lower proliferative and higher apoptotic indices compared to wild-type mice (Gutierrez *et al.*, 2000).

1.5.5 Pathophysiological functions of the uPA system

Although one of the principal identified functions of the uPA system is to regulate pericellular proteolysis and target the site of extracellular proteolysis at required sites on the cell surface (Blasi *et al.*, 1987; Blasi & Carmaliet, 2002), recent evidence has revealed that uPA binding to uPAR initiates intracellular

signaling events (discussed in section 1.5.2.1) affecting cell adhesion, migration, and proliferation in a variety of cell types, processes which are also influenced by PAI-1 (Chapman *et al.*, 1997; Ossowski & guirre-Ghiso, 2000; Preissner *et al.*, 2000; Blasi & Carmeliet, 2002; Kjoller, 2002).

1.5.5.1 Cell motility

Cell migration is crucial for normal and pathological processes including wound healing and cancer. Regulated changes in the affinity state of adhesion receptors are essential for optimal motility (Lauffenburger & Horwitz, 1996). It is now clear that this process is influenced by PAI-1 and other components of the uPA system (Deng *et al.*, 1996; Stefansson *et al.*, 1996; Kjoller *et al.*, 1997), resulting from direct interaction of system members with integrins and matrix proteins including VN.

As well as PAI-1, uPAR is also able to bind to the SMB domain on VN (Wei *et al.*, 1994; Okumura *et al.*, 2002) via an overlapping binding site to that of PAI-1 (Deng *et al.*, 1996) meaning both proteins compete for VN binding. The affinity of VN for PAI-1 is much higher than for uPAR (Deng *et al.*, 1996), and PAI-1 binding to VN blocks uPAR binding to VN (Deng *et al.*, 2001).

Integrins are a family of cell adhesion receptor heterodimers that interact with many ECM and cell-surface ligands, involving them in many biological and pathological situations (Hynes, 1992). Immediately adjacent to the SMB domain on VN is a distinct RGD site that binds $\alpha_5\beta_3$-integrins (Seiffert & Loskutoff, 1991). Binding of uPA to its receptor induces conformational changes in uPAR causing it to bind matrix-engaged integrins, with highest affinity for to the VN

receptors $\alpha_3\beta_1$ and $\alpha_5\beta_1$ (Chapman & Wei, 2001; Kugler *et al.*, 2003; Chaurasia *et al.*, 2006) which also induces activation of signalling molecules important for cell migration (Ossowski & guirre-Ghiso, 2000; Kjoller, 2002).

PAI-1 not only prevents cellular adhesion between uPAR and VN-rich ECM matrices (Deng *et al.*, 1996; Stefansson & Lawrence, 1996; Kjoller *et al.*, 1997), but also inhibits adhesive interactions between $\alpha_5\beta_3$-integrins and VN by steric hindrance (Okumura *et al.*, 2002; Stefansson *et al.*, 1996). The presence of PAI-1 can therefore inhibit VN-dependent cellular adhesion and migration independently of its inhibitory activity towards uPA and plasminogen activation. However, PAI-1 mediated cell attachment does have an absolute requirement for uPA and uPAR and is schematically outlined in Figure 1.5.

Besides the effect PAI-1 has on the adhesion of cells to VN, the above interaction may have other consequences as VN may function as a shuttle to transfer PAI-1 to uPAR bound uPA. When PAI-1 binds to uPA-uPAR, it undergoes a conformational change which destroys its affinity for VN and promotes its degradation as the PAI-1/uPA/uPAR/integrin complex is internalised from the cell surface (Cubellis *et al.*, 1990; Olson *et al.*, 1992; Czekay *et al.*, 2003) via the process of endocytosis. This is dependent on the α_2-macroglobulin receptor/ low-density lipoprotein receptor related protein (α_2M/LRP; Nykjaer *et al.*, 1997; Czekay *et al.*, 2003) or VLDL (Nykjaer *et al.*, 1992; Orth *et al.*, 1992; Conese *et al.*, 1995; Rodenburg *et al.*, 1998; Hussain *et al.*, 1999). Internalised uPAR is recycled to the cell surface (Nyjkaer *et al.*, 1997) and uPA and PAI-1 are targeted for lysosomal degradation (Figure 1.45). The

end result is the rapid disengagement of two adhesion receptors from their association with the ECM, and cell detachment.

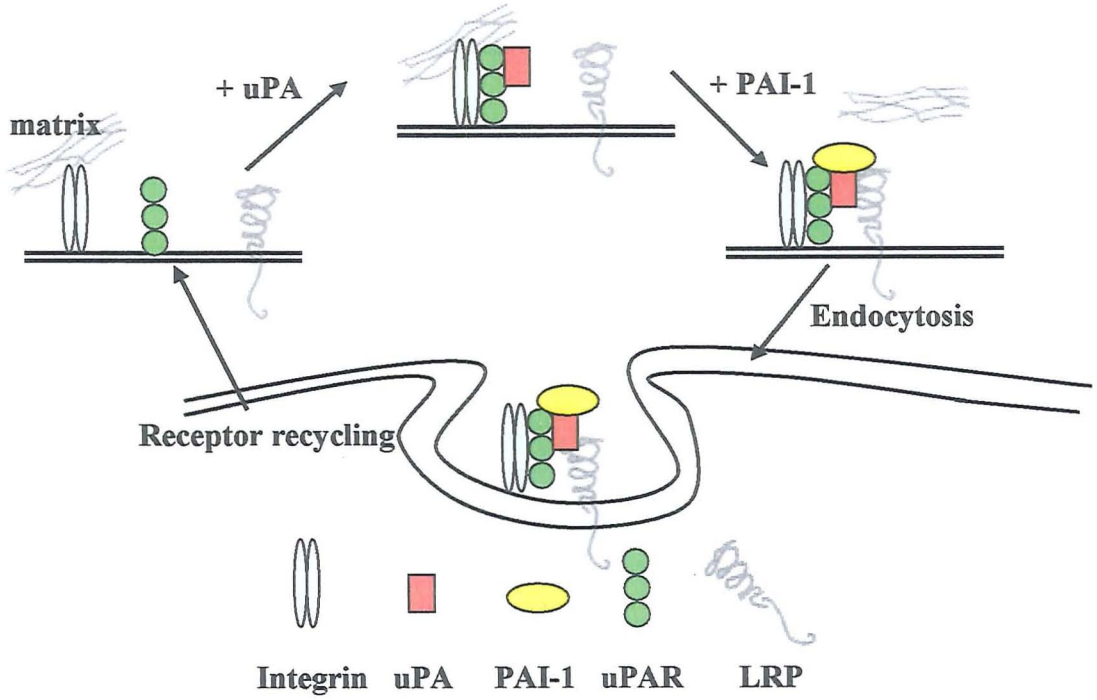

Figure 1.5
Model depicting proposed events in PAI-1-induced cell detachment
See section 1.5.6.1 text for details of interactions. (Diagram adapted from Czekay & Loskutoff, 2004). LRP, lipoprotein receptor-related protein.

PAI-1 stimulates cell migration of rat smooth muscle cells and human fibrosarcoma cells which is not dependent on the binding of PAI-1 to either VN or uPA, and is associated with LRP-induced activation of the Jak/Stat signaling pathway (Degryse *et al.*, 2004). A promigratory effect of PAI-1 is also seen in human breast cancer cells and melanoma cells (Stahl & Mueller., 1997; Chazaud *et al.*, 2002; Palmieri *et al.*, 2002).

1.5.5.2 Cancer

Many studies have focused attention on the involvement of the uPA system in cancer because it is heavily involved in matrix-proteolysis and uPA-initiated cell

signalling via uPAR activates many signal transduction pathways (as discussed in 1.5.2.1) effecting cell proliferation, migration and adhesion. For these reasons it is no surprise that uPA and uPAR have an adverse effect in cancer progression and are over-expressed in many epithelial tumours (Plesner *et al.*, 1994; Hudson & McReynolds, 1997; Morita *et al.*, 1998; De Petro *et al.*, 1998; Fisher *et al.*, 2000).

However, the finding that patients with increased PAI-1 concentrations also correlate with a poor prognosis for survival in a variety of metastatic human cancers including breast (Fisher *et al.*, 2000; Fox *et al.*, 2001; Duffy, 2002b), colorectal (Sakakibara *et al.*, 2005), lung (Pedersen *et al.*, 1994), renal (Hofmann *et al.*, 1996a; Hofmann *et al.*, 1996b; Ohba *et al.*, 2005), cervical (Kobayashi *et al.*, 1994; Hazelbag *et al.*, 2004), ovarian (Kuhn *et al.*, 1994; Chambers *et al.*, 1998) and endometrial cancer (Tecimer et *al.*, 2001) is suprising and unexpected given that PAI-1 is an inhibitor of extra-cellular proteolysis.

The expression of uPA, uPAR and PAI-1 has been shown to be increased in patients with gastric cancer (Herszenyi *et al.*, 1995; Ito *et al.*, 1996; Scicolone *et al.*, 2006). Using the technique of enzyme-linked immunosorbent assays (ELISA) both uPA and PAI-1 were found to be significantly increased in gastric cancerous tissue compared to tissue from control patients (Nekarda *et al.*, 1994). Immunohistochemical analysis was used to show expression of uPA, uPAR and PAI-1 to be significantly increased in primary gastric cancer patients and it has been suggested that these markers are used as new functional risk factors for gastric cancer prognosis (Heiss *et al.*, 1995; Kaneko *et al.*, 2003). Quantitative real-time PCR has been used to show that PAI-1 gene expression is substantially

increased in lymph node metastasis-positive gastric cancer patients (Sakakibara *et al.*, 2006) and ELISA has shown increased expression of uPA and uPAR in gastric cancer cell lines (MKN45 and KATO-III) after *H. pylori* infection (discussed further in section 3.4; Iwamoto *et al.*, 2005).

It has become evident that PAI-1 may influence the behavior of several cells through activities that are independent of its inhibitory effects on uPA and plasmin generation and the de-adhesive properties of PAI-1 (described in section 1.5.5.1) may begin to account for this observation. Angiogenesis is fundamental to tumour growth and PAI-1 may also influence this process (Stefansson *et al.*, 2003) depending on the concentration of the inhibitor and the specific experimental conditions. For example, one study observed no effect of PAI-1 in angiogenesis (Curino *et al.*, 2002), yet another showed impaired vessel formation in malignant keratinocytes transplanted into the skin of PAI-$1^{-/-}$ mice and increased tumour growth in mice that over-express PAI-1 (McMahon *et al.*, 2001). PAI-1 is expressed in endothelial cells of small vessels in human colon adenocarcinomas (Pyke *et al.*, 1991) and in proliferative vessels in high-grade gliomas and metastatic tumours (Kono *et al.*, 1994).

1.6 Overall aims and objectives

1.6.1 Objective 1: To determine the effect of *H. pylori* on PAI-1 expression in the gastric mucosa

Previous studies showed expression of a member of the uPA system, PAI-2 to be increased in *H. pylori* infection (Varro *et al.*, 2004). It is hypothesised that *H. pylori* infection increases expression of other key members of the uPA system including PAI-1, therefore the initial aims of this study were:

1. determine the effects of *H. pylori* infection on expression of PAI-1 within the gastric mucosa compared to uninfected controls;
2. study the cellular localisation of PAI-1 in *H. pylori* positive and negative subjects;
3. examine the relevance of studying PAI-1 expression using a mouse model of gastric cancer (INS-GAS).

1.6.2 Objective 2: To determine the effects of *H. pylori* on primary human gastric glands and the role of the uPA system in epithelial cell proliferation

Since the increased expression of PAI-1 by *H. pylori* was predominantly epithelial, and PAI-1 is increased in gastric cancer, primary human gastric epithelial gland cells were used to:

1. determine the effects of *H. pylori*, PAI-1 and uPA on gastric epithelial cell proliferation;
2. investigate the mechanism by which *H. pylori* can increase uPA system members using luciferase promotor-reporter constructs;
3. study the endogenous expression of uPA system members and expression of their respective luciferase promotor-reporter constructs;

4. determine the effects of epithelial and sub-epithelial derived growth factors associated with *H. pylori* infection on PAI-1-luc expression.

1.6.3 Objective 3: To determine the contribution of PAI-1 to gastric mucosal protection

A possible role for PAI-1 in the long-term protection of the gastric mucosa was demonstrated by the fact it restrains *H. pylori* and uPA mediated gastric epithelial cell proliferation. Transgenic mice were developed and used to assess the role of PAI-1 in an acute model of gastric epithelial damage and specifically to:

1. determine whether PAI-1 initially protects against, or aggravates gastric epithelial cell damage after challenge;
2. investigate an independent role of PAI-1 in tissue damage in mice deficient in uPA signalling.

Chapter 2

Materials and Methods

2.1 Materials and Methods

2.1.1 Tissue culture, plasmids and transfection

Tissue culture reagents were purchased from Life Technologies (Paisley, Glasgow, UK) with the exception of HAMS/F12 medium, antibiotic-antimycotic solution and dithiothreitol (DTT) which were purchased from Sigma (Poole, Dorset, UK). Fetal bovine serum (FBS) was obtained from Perbio (Cheshire, UK). Routine tissue culture vessels were purchased from TPP (Trasadingen, Switzerland) and 24-well plates from Corning Incorporated (NY, USA). Promoter-reporter constructs consisting of 4.5kb of the human PAI-1 promoter plus exon 1 coupled to luciferase (PAI-1-luc), approximately 2.9kb of the human uPA promoter plus 120bp exon 1 coupled to luciferase (uPA-luc), and also 2kb plus 207bp exon 1 of the human uPAR promoter coupled to luciferase (uPAR-luc) all in PXP2 vector were made by Professor Rod Dimaline (The University of Liverpool, Liverpool, UK). The generation of PAI-2-luc has previously been described (Varro *et al.*, 2002b) and contains 2.3kb of the human PAI-2 promoter together with 68bp of exon 1. The *Renilla* luciferase reporter phRL-SV40 was purchased from Promega (Southampton, UK) as were dual luciferase assay kits and TransFast reagent. CombiMag was obtained from Oz Biosciences (Marseille, France).

2.1.2 Growth factors

Routine chemicals were obtained from BDH Laboratory Supplies (Lutterworth, Leicestershire, UK) or Sigma Chemicals. Heptadecapeptide amidated gastrin (G-17) was obtained from Peninsula (St. Helens, Merseyside, UK). HGF was bought

from Genentech (CA, USA) and IGF-II from Calbiochem (Beeston, UK). Ethanol (99.7-100% v/v) was supplied by BDH Laboratory Supplies.

2.1.3 Bacteria

Bacteria were kindly routinely cultured by Debbie Sales (Medical Microbiology, The University of Liverpool) in a microaerophilic atmosphere at 37°C on fresh chocolatised Columbia blood agar. Wild-type *H. pylori* strain 60190 (American Type Culture Collection (ATCC) no.49503) was used unless otherwise stated. An alternative wild-type strain, 84-183 (ATCC no.53726) and isogenic *H. pylori* mutants that were *cagE*$^-$, *cagA*$^-$ or *vacA*$^-$ were kind gifts from John Atherton (Nottingham University, Nottingham, UK) and information regarding these strains has previously been published (Argent *et al.*, 2008). Cells were infected at a multiplicity of infection (MOI) of 1:150 as this was the optimal concentration found to give the best stimulation of luciferase constructs described in section 2.1.1. In murine infection experiments *H. felis* was used (ATCC 49179) which had been minimally passaged.

2.1.4 Antibodies and antisera

Antibodies used to identify differentiated cell types within gastric glands were obtained from a variety of sources and used at different concentrations (Table 2.1). Rabbit-anti-H^+,K^+-ATPase was obtained from Calbiochem and is raised against the α-subunit, C-terminal region sequence: GVRCCPGSWWDQGLYY. Rabbit-anti-chromogranin A was purchased from Abcam (Cambridge, UK) and rabbit-anti-pepsinogen was a kind gift from Dr. Mike Samloff (Centre for Ulcer Research, CA, USA). Mouse-anti-human spasmolytic peptide (HSP/TFF-2) was

purchased from NovoCastra (Newcastle-upon-Tyne, UK). Mouse-anti-trefoil factor 1 (TFF-1 or pS2) was obtained from Dako (Cambridgeshire, UK), guinea-pig-anti-vimentin purchased from Fitzgerald (MA, USA) and rabbit-anti-VMAT-2 was raised in-house and has previously been described (Hussain *et al.*, 1999). Antibodies used to detect PAI-1, uPA and uPAR were purchased from either Santa Cruz Biotechnology (Wiltshire, UK) or American Diagnostica (CT, USA). Details of these antibodies and optimal concentrations are given in Table 2.2. Goat-anti-luciferase antibody was purchased from Rockland Immunochemicals (Gilbertsville, PA, USA) and used at a concentration of 1:200. BrdU was detected using fluorescin isothiocynate-conjugated fast immune anti-BrdU/DNase obtained from Becton Dickinson (Oxford, UK) and was used at an optimal concentration of 1:50. FITC and texas-red (TRed)-conjugated secondary antibodies were purchased from Jackson ImunoResearch Laboratories (Soham, UK) and were all used at an optimal concentration of 1:400.

Cell type	Primary antibody	Titre	Secondary antibody	Titre
Mucous neck	Anti-TFF2	1:50	Anti-mouse IgM	1:400
Surface mucous	Anti-TFF1	1:100	Anti-mouse IgG	1:400
ECL	Anti-Chromogranin A	1:50	Anti-Rabbit IgG	1:400
ECL	Anti-VMAT-2	1:200	Anti-Rabbit IgG	1:400
Chief	Anti-pepsinogen	1:200	Anti-Rabbit IgG	1:400
Parietal	Anti- H^+,K^+-ATPase	1:200	Anti-Rabbit IgG	1:400
Myofibroblast	Anti-Vimentin	1:400	Anti-G.pig IgG	1:400

Table 2.1 Antibodies used to identify main cell types within human primary gastric glands.

Antibody	Origin	Species	Titre	Specificity
PAI-1	Santa Cruz Biotechnology	Rabbit	1:200	Raised against a recombinant protein corresponding to amino acids 24-158 of mature human PAI-1.
PAI-1	American Diagnostica	Mouse	1:100	Raised against purified active PAI-1 secreted by human melanoma cell line, epitope specificity amino acid 110-145.
uPA	Santa Cruz Biotechnology	Rabbit	1:200	Raised against amino acids 136-275 of uPA.
uPA	American Diagnostica	Mouse	1:200	Directed against an epitope on the B-chain near the catalytic site. Recognises the single pro-enzyme, HMW, LMW and receptor bound uPA.
uPAR	Santa Cruz Biotechnology	Goat	1:100	Raised against a peptide mapping near the C terminus of uPAR.
uPAR	American Diagnostica	Mouse	1:100	Recognises domains 2 & 3 of the receptor. Binds with high affinity to both uPAR and uPAR/uPA complexes.

Table 2.2 Primary uPA system antibodies and dilutions used in immunocytochemistry to determine endogenous protein expression.

2.2 Animals

C57BL/6 were purchased from Charles River (MA, USA) at a specified age and sex as required. INS-GAS mice on a FVB/N background have previously been described (Wang *et al.*, 1996). PAI-1 KO and uPAR KO animals were initially purchased from JAX® MICE (Maine, USA) and are on a C57BL/6 background. Mice that are homozygous for targeted PAI-1 or uPAR mutation are viable, normal in size and do not display any gross physical or behavioural abnormalities. PAI-1 an d uPA over-expressing mice are also on a C57BL/6 background and the generation of these animals is discussed in Chapter 5 of this thesis. All mice were housed in an appropriately controlled environment with a strict light to dark light cycle (12:12 hr) in polycarbonate-bottomed cages in which there was environment enrichment. Animals were not specific pathogen free and were fed on a commercial pellet diet and water was provided *ad libitum*. In experiments where fasting took place this was for no longer than 17 hr and all animals still had full access to water.

Helicobacter felis infections took place when animals were between 6-8 weeks old. They were oral dosed three times over a period of 1-2 weeks with 0.5ml (for a 25g mouse) of an *H. felis* suspension in TBS broth. The optical density of the suspension was above 1.5 and *H. felis* were not used if they had been passaged more than five times. The *H. felis* status of animals was determined by antral urease test (Prontodry, Medical Instruments Corporation, Solothrum, Switzerland) or by antral histology.

Animals were humanely killed by increasing CO_2 concentrations followed by cervical dislocation according to schedule 1 methods as directed by the Home

Office. An abdominal incision was made down the linea alba and the stomach severed at both the oesophageal and pyloric sphincter, rapidly removed, and cut open along the greater curvature. Stomach contents were removed by careful washing in phosphate buffered saline (PBS) to allow the analysis of the gastric mucosa. For murine gastric gland isolation the stomach was ligated at both the oesophageal and pylori sphincters, removed and washed in Hanks Balanced Salts Solution (HBSS). The majority of the non-secretory epithelium was removed and the pyloric sphincter directed towards the newly made fundic opening, allowing complete inversion of the stomach. This was then ligated, washed in ice cold HBSS, injected with 0.5mg/ml collagenase A (Roche Molecular Biochemicals, East Sussex, UK) and placed on ice before culture (section 2.4.3).

2.3 Patients and ethical approval

Endoscopic pinch biopsies (maximum of 6) of the gastric corpus or antrum were obtained by oesophagogastroduodenoscopy from patients undergoing investigation for dyspepsia at the Royal Liverpool Gastroenterology Unit at Dr D.M Pritchard's clinic. Biopsies were placed in ice cold HBSS for primary culture (section 2.4.2). Blood samples were taken from patients to assay plasma gastrin and also for *H. pylori* serology. Both antral and corpus biopsies were fixed in formalin for histology at the Royal Liverpool Gastroenterology Unit and a rapid urease test carried out on antral biopsies of each patient (Prontodry, Medical Instruments Corporation). All patients gave informed consent and were included in the study if they were aged between 18 and 75 years of age, had no previous gastrointestinal (GI) surgery or GI malignancy, were not pregnant and did not have liver disease. Patients taking histamine (H_2) blockers and proton

pump inhibitors were included only if this medication had been stopped 7 days prior to endoscopy. All patients gave informed consent and local ethical committee approval was obtained from the Ethics Committees of Royal and Broadgreen Liverpool Hospitals NHS Trust. In addition, an experiment was performed on tissue obtained from patients with distal gastric cancer on a background of chronic *H. pylori* infection which was carried out in collaboration with Dr Suhail Ahmed, Aintree Hospital, Liverpool.

2.4 Tissue culture

2.4.1 Culture of AGS2 cells

AGS cells were obtained from the American Type Culture Collection (Manassas, VA) and have been stably transfected with the gastrin CCK2 receptor (AGS2) driven by the EF1-α promoter using TransFast reagent (Promega; Watson *et al.*, 2001). It has been shown that the receptor is functional (Varro *et al.*, 2002a). This cell line was cultured in Ham's F-12 medium supplemented with 10% FBS and 1% penicillin/ streptomycin in a humidified atmosphere of 5% CO_2 / 95% O_2. Medium was replaced every two days. AGS2 cells were maintained under selection by supplementing the medium with puromycin (2μg/ml).

2.4.2 Human primary gastric gland isolation and culture

Six endoscopic human pinch biopsies of gastric corpus were obtained with 2.4mm tip diameter, single-use, biopsy forceps (Diagmed healthcare, Thirsk, UK) from patients referred with dyspepsia. In all cases histology indicated superficial gastritis but not gastric atrophy or intestinal metaplasia and patients were excluded if there was a previous history of neoplastic disease, current

peptic ulcer or elevated plasma gastrin concentrations (>30pM). Results shown are from gastric glands isolated from *H. pylori* negative patients (determined as in section 2.3) unless otherwise stated.

Biopsies were placed in ice cold HBSS before being sliced into $2mm^2$ segments using a sharp razor blade and washed in 3 changes of 37°C HBSS. Biopsies were incubated in 5ml 1mM dithiothreitol (DTT; Sigma) for 15 min and were continuously gassed with 5% CO_2 / 95% O_2 at 37°C whilst shaking at 100 rpm. Tissue was washed 3 times in HBSS (37°C), incubated in 5ml 0.5mg/ml collagenase A (Roche Molecular Biochemicals) for 30 min before being washed 3 times with HBSS (37°C). Tissue was subjected to a second 5ml collagenase A digestion (0.5mg/ml 37°C), triturated for 45 sec using a wide mouthed pipette and transferred to a clean universal. Larger fragments were allowed to settle under gravity for 45 sec, supernatant was removed, transferred to another clean universal, shaken vigorously and left to sediment on ice for 45 min. The supernatant was carefully removed, discarded and the remaining mixture containing isolated glands was equally divided between six wells of 24-well cell culture plate. Glands were routinely cultured in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% FBS, 1% antibiotic-antimycotic solution (Sigma) and 1% L-glutamine at 37°C in a humidified atmosphere of 5% CO_2 / 95% O_2. Glands were allowed to adhere overnight and medium was changed every 24 hr.

2.4.3 Murine gastric gland isolation and culture

Glands were isolated using a modified method that was first described in 1976 (Berglindh & Obrink, 1976). A very similar protocol to that used for the isolation

of human primary gastric glands (section 2.4.2) was used to obtain gastric glands from the inverted mouse stomachs (obtained as described in section 2.2). The protocols only differ when it comes to collagenase A digestion as mouse stomachs are incubated in 5ml, 0.32mg/ml collagenase A (Roche Molecular Biochemicals) and watched during the first 30 min incubation for rupturing of the inverted stomach. The development of an opalescent medium indicates that the stomach has been sufficiently digested to yield isolated glands. If this did not occur then stomachs were washed (3 times HBSS) and incubated for a further 30 min in 0.32mg/ml collagenase A, checking every 10 min for ruptures thus avoiding over-digestion. Once sufficiently digested, the tissue was triturated for 45 sec using a wide mouthed plastic pipette and then transferred to a clean universal. The remainder of the protocol is identical to that of human gland isolation (section 2.4.2). Isolated gastric glands were routinely cultured in DMEM supplemented with 10% FBS, 1% L-glutamine and 1% antibiotic-antimycotic solution at 37°C in a humidified atmosphere of 5% CO_2 / 95% O_2. Glands were allowed to adhere overnight and medium was changed every 24 hr.

2.5 Transient transfection and luciferase assays

2.5.1 AGS2 cell transfection

200,000 cells/well were plated in 6 well culture plates and allowed to adhere 24 hr prior to transfection. Cells were transfected using TransFast (Promega) with 3 μg plasmid DNA/well and 5ng/well of a constitutively active *Renilla* luciferase reporter construct, phRL-SV40 in 840μl SFM (Hams-F12) for 1 hr at 37°C. Full medium (FM; 3ml) was added and cells incubated for 20 hr. Cells were washed twice in SFM and then incubated with G17 as indicated. Luciferase activity was

measured by Dual-luciferase Reporter Assay System (Promega) using a Lumat LB 9057 luminometer according to manufactures instructions (Berthold, Redbourne, Herts, UK). Results are displayed as fold increase over unstimulated control where 1.0 signifies no change in luciferase activity.

2.5.2 Human primary gastric gland transfection and luciferase assay

Human gastric glands (prepared as in section 2.4.2) were transfected 24 hr after plating in 12 well plates using CombiMag (Oz Bioscience) and TransFast (Promega) according to manufacturers instructions. 3μg of plasmid DNA/well (PAI-1-, uPAR- or uPA-luc) together with 5ng/well of a constitutively active *Renilla* luciferase reporter construct, phRL-SV40, 18μl Combimag and 18μl TransFast was added to 410μl SFM, vortexed and incubated at room temperature for 15 min before being added to cells for 20 min under an applied magnetic field (in the form of a magnetic plate). Full medium (2.5ml) was added and cells incubated 37°C overnight. Cells were washed twice in PBS and SFM added before the addition of *H. pylori* at a specified multiplicity of infection (MOI) or any other compounds as indicated. Luciferase activity was measured by Dual-luciferase Reporter Assay System (Promega) using a Lumat LB 9057 luminometer according to manufacturers instructions (Berthold). Results are displayed as fold increase over unstimulated control where 1.0 signifies no change in luciferase activity.

2.6 BrdU incorporation and anti-sense oligonucleotide treatment

Cells within human primary gastric glands in S phase of the cell cycle were labelled with 5'-bromo-2'-deoxyuridine (BrdU; Sigma) as outlined in Figure 2.1.

Human primary glands were prepared as in section 2.4.2, plated on glass coverslips and the following morning anti-sense oligonucleotides (ASO) added in FM. This was either control scrambled oligonucleotides (CSO) or PAI-1 antisense oligonucleotides (ASO; all at a final concentration of 2μM and details are given below; Biognostik, Germany). 8 hr later, cells were serum starved for 24 hr 1ml SFM and stimulated with either *H. pylori* MOI 1:150 or uPA (250 ng/ml; Chemicon), BrdU was added to cells at a final concentration of 3ng/ml, and a mouse monoclonal PAI-1 neutralising antibody (American Diagnostica) at a concentration of 10μg/ml. Throughout the experiment both CSO and ASO were replaced in the media when it was changed. Cells were fixed 18 hr later in 4% paraformaldehyde (PFA; Agar Scientific, Essex, UK) and immunocytochemistry carried out using a mouse-anti-BrdU antibody at a concentration of 1:50 (Becton Dickinson) and a guinea-pig-anti-vimentin antibody at a concentration of 1:400 (Fitzgerald) as described in section 2.7. An anti-vimentin antibody was used in order to exclude BrdU, vimentin positive myofibroblasts and 10 glands per patient per treatment were scored. BrdU labelled myofibroblasts were subtracted from the total number of BrdU labelled cells per gland to give percentage BrdU labelled epithelial cells.

Human gastric glands prepared
18 hr ↓ 3ml FM
+control ASO / +PAI-1 ASO
8 hr ↓ 1ml SFM
+control ASO / +PAI-1 ASO
24 hr ↓ 1ml SFM
+control ASO / + PAI-1 ASO
+ *H. pylori* or uPA
+BrDU
(+ PAI-1neutralising antibody)
18 hr ↓
Fix cells 4% paraformaldehyde for immunocytochemistry

Figure 2.1 Method for BrdU incorporation into human gastric glands

Most anti-sense experiments produce sufficient inhibition grades without using transfection reagents and anti-sense oligonucleotides can be taken up by the cells simply by adding them to the cell culture media. The sequence of human PAI-1 anti-sense oligonucleotide (Biognostik) is based on Accession number X04429 and shows no relevant cross homologies with genes found in the GenBank database. Sequence (5'-3'): GAG AGA GGC ACC TCT TTT. Control scrambled oligonucleotides (CSO) were also supplied and are designed to avoid toxic motifs and to be nonhomologues to any known sequence, therefore little effect should be caused by controls. However, the sequence of CSO was not stated by the supplier at time of purchase.

2.7 Immunocytochemistry of adherent gastric glands

Biopsies from one individual patient were prepared (as in section 2.4.2) and cultured on glass coverslips. Isolated glands were treated with various compounds/ bacteria as indicated, media removed, washed 3 times in PBS and fixed in 1ml 4% w/v PFA for 30 min at room temperature. Fixed glands were washed 3 times in PBS and permeabilised for 30 min using PBS containing 0.1% triton X-100. Permeabilised glands were washed again 3 times in PBS and incubated with 5% w/v bovine serum albumin (BSA; Jackson ImmunoResearch Laboratories) for 30 min. Glands were washed 2 times in PBS and non-specific binding of the secondary antibody was blocked by incubating glands in 10% donkey serum (Jackson ImmunoResearch Laboratories) for 1 hr at room temperature. Serum was removed, glands washed 2 times in PBS and primary antibody (diluted in PBS) was added to glands overnight at 4°C in a humidified atmosphere. Details of all primary antibodies used are given in section 2.1.4.

The following day a series of 5 min PBS/sodium chloride (NaCl) washes were carried out: 0.14M NaCl, 0.5M NaCl, 0.14M NaCl. The appropriate secondary antibody (diluted in 10mM Hepes) was added to glands for 1 hr in the dark. Secondary antibodies were raised in donkey and were either TexasRed (TRed) or fluorescein isothiocyanate (FITC) conjugated (Jackson ImmunoResearch Laboratories) and used at a titre of 1:400. Secondary antibody was removed and glands washed (3 times 10 min in PBS) and mounted with Vectashield containing DAPI (Vector Laboratories, Peterborough, UK). Slides were examined using a Zeiss Axioplan-2 microscope (Zeiss Vision, Welwyn Garden City, UK) and images captured using JVC-3 charge-coupled device camera using 40X magnification (air immersion) and KS300 software combined with deconvolution software (Imaging Associates, Bicester, Oxfordshire, UK). Specificity of immunostaining was determined by omitting primary antibodies.

2.8 Immunohistochemistry of paraffin embedded tissue

Immunohistochemistry was carried out on formalin-fixed, paraffin-embedded tissue sections by Andy Dobson and analysed and evaluated by Dr Fiona Campbell (Consultant Pathologist, Department of Pathology, Royal Liverpool University Hospital). Briefly, sections were cut at 4μm onto silane-coated glass microscope slides and dried overnight in an oven at 45-50°C. Sections were dewaxed through xylene and endogenous peroxidase activity blocked in methanol/H_2O. Immunohistochemical staining was performed on an Autostainer (DakoCytomation, Cambridgeshire, UK). Sections were incubated with diluted primary antibody for 60 min. PAI-1 was detected using a rabbit-anti-PAI-1 antibody at a concentration of 1:40 (American Diagnostica). In addition, primary

antibody was omitted and used as a negative control. Detection was performed using the ChemMate™ EnVision™ HRP detection system (DakoCytomation) according to manufacturers recommendations.

2.9 Western blot analysis

Protein extract was prepared in lysis buffer (20mM Tris pH 7.8, 150mM NaCl, 2mM EDTA, 0.5% Nonidet P-40, 10mM NaF, 15mg/ml benzamide, 8.8mg/ml sodium orthovanadate, 0.5mM DTT, 10mg/ml phenylmethylsulfonyl fluoride) containing 10µl/ml protease inhibitor cocktail set III and 10µl/ml phosphatase inhibitor cocktail set III (Calbiochem). Equal amounts (40 µg) of protein were electrophoresed and resolved on an 8% w/v SDS-polyacrylamide gel and electrotransferred onto a nitrocellulose membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK). After transfer, membranes were washed (3 times 10 min) in tris buffered saline (TBS) containing 0.1% Tween (TBST) then incubated in blocking buffer (TBST containing 5% Marvel) for 1 hr at room temperature. Membranes were incubated with primary antibody diluted in blocking buffer overnight at 4°C whilst shaking. The primary antibody used was mouse-anti-PAI-1 (1:100; American Diagnostica). Following this, membranes were washed in TBST (3 times 10 min) and incubated in blocking buffer containing horseradish peroxidase (HRP)-anti-mouse secondary antibody (1:5000; Sigma) for 1 hr at room temperature. Membranes were then washed (3 times 10 min) in TBST and protein was identified by incubating membranes in the chemiluminescent substrate SuperSignal® West Pico (Pierce, Illinois, USA) for 5 min. The signal was detected by exposure to HyperFilm (Amersham Biotech, Little Chalfont, UK), which was developed (Kodak D-19 developer)

and fixed (Kodak rapid fix). Samples were re-probed with goat-anti-β-actin (1:5000; Santa Cruz Biotechnology) to determine equal protein loading. This was identified using a HRP-anti-goat (1:5,000) secondary antibody.

2.10 Bacterial re-transformation with plasmid DNA

50 µl DH5α competent cells (Invitrogen, Paisley, UK) were incubated with 0.5µl plasmid DNA on ice for 30 min. Cells were heat shocked by subjection to 42°C, 50 sec and returned to ice for a further 2 min. 950µl SOC media (2% tryptone, 0.5% yeast extract, 10mM NaCl, 2.5mM KCL, autoclave, pH 7 then add 10mM $MgCl_2.6H_2O$, 20mM glcouse) was added and bacteria incubated at 37°C, 1.5 hr whilst shaking. Bacteria were streaked onto LB-agar plates (1.2% Agar added to Luria Bertani (LB) medium; 1% tryptone, 0.5% yeast extract, 1%NaCl, pH7 and autoclaved) containing ampicillin (100µg/ml).

2.11 Preparation of plasmid DNA

Single colonies were selected and cultured in LB medium containing 50µg/ml ampicillin at 37°C for 8 hr whilst shaking at 225rpm. The culture was transferred to 100ml LB medium and incubated overnight at 37°C whilst shaking at 225rpm. Plasmid DNA was recovered using QIAGEN Plasmid Maxi Kits (QIAGEN Ltd, West Sussex, UK) according to manufacturers protocol. Briefly, bacterial cells were pelleted by centrifugation (6000g, 15 min, 4°C), supernantant discarded and cells resuspended and lysed followed by neutralisation and precipitation. The precipitated material was centrifuged at 20000g, 30 min (4°C), the pellet discarded and the supernantant centrifuged again at 20000g, 30 min (4°C). The supernantant was applied to a resin and bound DNA was retained on the column.

This was eluted into a fresh tube and DNA was precipitated by adding 0.7 volumes room temperature isopropanol and pelleted by centrifugation at 15000g, 30 min, 4°C. The DNA pellet was washed with room temperature 70% ethanol and centrifuged at 15000g for 10 min. The supernantant was discarded and the pellet air-dried and redissolved in a suitable volume of Tris- EDTA (TE) buffer (Severn Biotech, Worcestershire, UK). Plasmid concentration was calculated using a Biowave II Optical Density reader (WPA, Cambridge, UK) by reading the absorbance at 260nm.

2.12 Real-time PCR

2.12.1 RNA extraction and cDNA preparation

Gastric corpus biopsies from male INS-GAS mice infected with *H. felis* as previously described (Wang *et al.*, 2000 and section 2.2) were obtained at 3, 6 and 9 months post-infection. Human antrum and corpus endoscopic pinch biopsies were taken from *H. pylori* infected and non-infected patients. Biopsy samples were stored in RNA-Later® (Ambicon, CA, USA) and tissue was later homogenised at 4°C using a Polytron homogeniser (Kinematica, Hessie, UK) in the presence of TRI® reagent (Sigma). A 20% volume of chloroform was added, mixed by inversion and centrifuged for 15 min at 14000rpm (4°C). Supernatant was transferred to a fresh tube, 50% volume of isopropanol added, mixed by inversion and left at room temperature for 15 min before centrifugation for 10 min at 14000rpm at 4°C. The supernantant was discarded and the pellet washed with 70% ethanol and air-dried.

RNA was resuspended in nuclease free water (MP Biomedicals, Cambridge, UK) or alternatively stored in 1ml 99.8% ethanol / 20µl 3M sodium acetate at -

80°C. RNA was recovered via centrifugation at 4°C for 15 min at 14000rpm, washed in 70% ethanol followed by ice-cold absolute ethanol. Supernantant was discarded and the pellet air-dried and resuspended in 50µl nuclease free water. RNA concentration was calculated using a Biowave II Optical Density reader (WPA) by reading the absorbance at 260nm. DNase treatment was performed on 30µg RNA by addition of RQ1-DNase (1µl per µg RNA; Promega), 10x buffer and DEPC-treated water were added according to manufacturers protocol. This was incubated at 37°C for 30 min and the volume made up to 200µl by the addition of DEPC-treated water. An equal volume of phenol/chloroform was added, the sample was mixed and centrifuged at 4°C for 5 min at 14000rpm. The supernantant was transferred to a fresh tube, 20µl of 3M sodium acetate added in addition to 1ml ice-cold 99.8% ethanol and samples were stored at -80°C. RNA was retrieved by 15 min centrifugation at 14000rpm (4°C), the pellet was washed in 70% ethanol followed by ice-cold absolute ethanol, air-dried and resuspended in 50µl nuclease-free water. RNA concentration was measured and 5µg reverse transcribed by the addition of 0.5µg of random primers (Promega) and incubation at 70°C for 5 min before slow cooling to below 40°C. Reverse transcription was carried out using AMV reverse transcriptase according to manufacturers instructions (Promega). The reaction mix was incubated at room temperature for 10 min followed by a 60 min incubation step at 42°C. Heating for 5 min at 95°C inactivated the enzyme and cDNA was stored at -20°C.

2.12.2 Quantitative real-time PCR

Real-time PCR was used to measure PAI-1 mRNA abundance using cDNA samples prepared from human and mouse gastric biopsies (prepared as in section

2.12.1) and standards (human or mouse cDNA). cDNA was added to a master mix containing QPCR Master Mix Plus (Eurogentec, Southampton, UK), forward and reverse primers, probes and ddH_2O (all volumes as in Table 2.3). The sequences of human and mouse PAI-1 forward and reverse primers as well as probe sequences are shown in Table 2.4.

	h-PAI-1	m-PAI-1	18S
cDNA	4µl	4µl	4µl
Forward Primer (10µM)	3µl	3µl	6µl
Reverse Primer (10µM)	3µl	3µl	
Probe (5nM)	2.5µl	3.5µl	2µl
ddH_2O	37.5µl	36.5µl	38µl
Master Mix Plus	50µl	50µl	50µl

Table 2.3 Primer and probe volumes for real-time PCR of human and mouse cDNA samples.

Each cDNA sample was analysed in triplicate and 25µl of the above mix pipetted into each well of 96-well Q-PCR plate (Applied Biosystems, Warrington, UK) which was then centrifuged at 2000G for 2 min. An ABI Prism^{TM}7700 Sequence Detector machine and Sequence Detector software (Applied Biosystems) was used to measure mRNA abundance. Taqman Technology was employed with the amplification cycles as follows: 50°C for 2 min, 95°C 10 min, 40 cycles of 95°C (15 sec) and 60°C (1 min). For each sample, the amount of 18S rRNA was assayed in a similar way using an 18S rRNA control kit (Eurogentec) and relative mRNA abundance was determined from the ratio mRNA/18SrRNA.

Primer/Probe	5'-3'
hPAI-1 forward	GGCTGACTTCACGAGTCTTTCAG
hPAI-1 reverse	CGTTCACCTCGATCTTCACTTTC
hPAI-1 probe	AAGAGCCTCTCCACGTCGCGCA
mPAI-1 forward	CCCTGGCCGACTTCACAA
mPAI-1 reverse	ACCTCGATCCTGACCTTTTGC
mPAI-1 probe	CAAGAGCAGCTCTCTGTAGCACAGGCAC

Table 2.4 Human and mouse PAI-1 specific forward and reverse primer and probe sequences for real-time PCR.

2.13 Haemorrhagic lesion model

Male or female C57BL/6, PAI-1-KO, uPAR-KO, PAI-1/ uPA over-expressing mice (10-12 weeks) were fasted overnight (17 hr) before being gavarged with 100µl of either 50% or 99.8% (absolute) ethanol. Animals were sacrificed after 1 hr and stomachs removed by cutting at both the oesophageal and pyloric sphincter. The stomach was cut along the greater curvature and briefly washed in PBS. Gastric tissue was opened up and stretched out mucosa side up and pinned out on a piece of cork. The number and length of haemorrhagic lesions was recorded using callipers and a ruler. Stomachs were also graded as possessing either 'weak', 'moderate' or 'severe' lesions. All animal procedures had full ethical approval and were carried out according to Home Office regulations.

2.14 Statistical analysis

Results are expressed as mean $\pm$ standard error about the mean (SEM), unless otherwise stated. One way analysis of variance (ANOVA) or Student's *t*-test was carried out to determine statistical significance of results and significance indicated (*) at $p<0.05$. *n* = individual patients/ mouse or individual experiments.

Chapter 3

Stimulation of PAI-1 expression in the gastric epithelium and its significance for epithelial cell function

3.1 Introduction

Approximately 15% of all cancers develop on a background of chronic inflammation linked to infection. A classic example of this is the development of gastric cancer in patients infected with the gram negative, oncogenic bacterium *Helicobacter pylori* (*H. pylori*). Infection with *H. pylori* is associated with inflammation of the gastric mucosa, which in a small percentage of people triggers the progression to gastric cancer (reviewed in Bodger & Crabtree, 1998; Peek & Crabtree, 2006). Stomach cancer is the second most common cause of death due to malignancy worldwide and proceeds via a progression of distinct histological steps (discussed in section 1.3.2; Correa, 1982; Hohenberger & Gretschel, 2003; Correa, 2004).

The response of gastric epithelial cells to *H. pylori* infection that may influence preneoplastic changes, cancer initiation and progression remains largely unknown, but may include altered expression of growth factors, cytokines and extracellular proteases, as well as involving both host and bacterial factors (El-Omar *et al.*, 2000; Machado *et al.*, 2003; Peek & Crabtree, 2006). Damage to epithelial cells such as that caused by bacterial infection can elicit a strong host response, causing the release of paracrine factors, ECM proteins, proteases and protease inhibitors. This can trigger a chain of events to occur, leading to remodelling of the epithelium and the development of a hyperproliferative state, ultimately contributing to malignant transformations (Lechago & Correa, 1993).

Preliminary studies showed that expression of a member of the urokinase plasminogen activator (uPA) system, plasminogen activator inhibitor-2 (PAI-2), is increased in *H. pylori* infection (Varro *et al.*, 2004). Increased expression of uPA system members including uPA itself, its receptor (uPAR), and its main

extracellular inhibitor (PAI-1) have been shown to correlate with a poor prognosis in gastric cancer patients (discussed in section 1.5.5.2; Nekarda *et al.*, 1994; Heiss *et al.*, 1995; Ito *et al.*, 1996; Plebani *et al.*, 1997; Kaneko *et al.*, 2003; Scicolone *et al.*, 2006). However, the direct effect of *H. pylori* on PAI-1 expression, and the involvement of the uPA system with the preneoplastic stages involved in gastric cancer development have been poorly studied. It is hypothesised that *H. pylori* infection increases expression of other key members of the uPA system including PAI-1, which can influence gastric epithelium remodelling during infection.

Plasminogen activator inhibitor-1 is an important physiological regulator of fibrinolysis and ECM homeostasis by inhibiting the activities of the serine protease uPA, which acts to control pericellular proteolysis through the regulation of the conversion of plasminogen into the proteolytic enzyme plasmin (Irigoyen *et al.*, 1999). However, although one of the physiological roles of PAI-1 is to protect the ECM from degradation by proteases it is also associated with many pathological conditions making it a very interesting protein to study (discussed in section 1.5.4.1).

Increased PAI-1 expression is known to contribute to tissue fibrosis in several organs such as kidney (Ingelfinger, 2003), lung (Kaikita *et al.*, 2001) and heart (Takeshita *et al.*, 2004). Fibrosis within the stomach is often regarded as a premalignant state associated with *H. pylori* infection (Matsukura *et al.*, 1980). An increase in PAI-1 expression is not only associated with a poor prognosis in gastric cancer but in multiple human cancers (discussed in section 1.5.5.2). Tissue remodelling involves epithelial cell and ECM interactions which is heavily dependant upon the regulated release of ECM-degrading proteases.

Dysfunction in these interactions is thought, at least in part, to promote tumourigenesis. It is therefore essential to consider the fine balance between the protease uPA and its inhibitor PAI-1 as a disruption to this could have pathological consequences. The mechanism by which *H. pylori* promotes cancer development involves several different host responses (Naumann & Crabtree, 2004), and it is therefore interesting to investigate if expression of uPA system members is influenced by *H. pylori* infection and if this has any role to play in the multi-step process of remodelling of the gastric mucosa.

3.1.1 Aims

The aims of this chapter are to study the expression of PAI-1 in the gastric epithelium and determine the role it has on epithelial cell function. The specific objectives were to:

1. determine the effects of *Helicobacter* infection on expression of PAI-1 mRNA- abundance within the stomach in both humans and mice;
2. study the difference in cellular localisation of PAI-1 protein expression between *H. pylori* positive and negative subjects;
3. investigate mRNA and protein expression of PAI-1 in gastric cancer patients;
4. study endogenous PAI-1, uPA and uPAR expression in a human primary epithelial gastric gland cell culture;
5. examine the effects of *H. pylori*, PAI-1 and uPA on gastric epithelial cell proliferation.

3.2 Materials and Methods

3.2.1 Patients

Endoscopic pinch biopsies of the gastric corpus and antrum were taken from human subjects as described in section 2.3. Samples from 10 *H. pylori* positive patients were compared to 15 *H. pylori* negative, control patients. Blood samples were taken to assay plasma gastrin (patients with levels greater than 30pM were excluded from this study) and also for *H. pylori* serology. In addition, a rapid urease test was carried out on antral biopsies of each patient. All patients gave informed consent and the study was approved by the Ethics Committee of Royal Liverpool and Broadgreen University Hospital NHS Trust. In addition, samples from 5 patients presenting with intestinal type, distal gastric cancer on a background of chronic *H. pylori* infection were also analysed. These samples were obtained from Dr Suhail Ahmed, Aintree Hospital, Liverpool, NHS trust.

3.2.2 Animals

Male INS-GAS mice on an FVB/N background were infected with *H. felis* as previously described (Wang *et al.*, 2000 and section 2.2) and infection status was determined by antral rapid urease test. PAI-1 mRNA abundance was measured from gastric corpus samples 3 and 6 months post-infection and compared to uninfected male INS-GAS mice. In a separate experiment, PAI-1 mRNA abundance was measured in uninfected INS-GAS mice and compared to uninfected FVB/N mice.

3.2.3 PAI-1 protein expression in *H. pylori* infection and gastric cancer

PAI-1 protein expression and localisation was determined via immunohistochemistry using paraffin embedded stomach sections from a *H.*

pylori negative and positive patient as well as on gastric cancer tissue (as described in section 2.8). This technique was performed by the Pathology department at The Royal Liverpool University Hospital using a rabbit-anti-PAI-1 antibody at a concentration of 1:400 (American Diagnostica) and a mouse-anti-α-SMA antibody at 1:400 dilution (American Diagnostica). Negative controls were carried out with the omission of primary antibodies.

3.2.4 Real-time PCR

Real-time PCR was performed on RNA extracted from biopsy samples taken from the corpus and antrum region of the stomach from either *H. pylori* negative or positive human subjects, *H. felis* positive or negative INS-GAS mice and *H. felis* negative FVB/N mice (as described in section 2.12.1). Real-time PCR was carried out as described in section 2.12.2 using either human or mouse PAI-1 primer and probe sets (volumes used and sequences given in Table 2.3 and Table 2.4 respectively). Relative mRNA abundance was determined from the ratio mRNA/18SrRNA.

3.2.5 Cellular localisation of endogenous PAI-1, uPA and uPAR

Human corpus gastric glands isolated from *H. pylori* negative patients as described in section 2.4.2 were cultured on glass cover slips in FM 48 hr and were stimulated with wild-type *H. pylori* M.O.I. 1:150 or left unstimulated in SFM 18 hr. The expression of uPA system members within gastric gland cells was assessed using dual immunofluorescence (as described in section 2.7). Endogenous PAI-1, uPA or uPAR protein was detected using antibodies listed in Table 2.2. Appropriate FITC conjugated secondary antibodies at a dilution of 1:400 (Jackson ImmunoResearch Labs) were used. Antibodies raised against cell

markers for the main terminally differentiated cell types of the gastric corpus were also used: parietal, chief, surface mucous, mucous neck and enterochromaffin-like cells with TRed labelled secondary antibodies (Jackson ImmunoResearch Labs) used as appropriate (details given in Table 2.1). A guinea-pig anti-vimentin antibody was also used to check for localisation of PAI-1, uPA and uPAR in myofibroblasts present in the preparation. Each cell specific antibody was used on at least 3 different patients before and after stimulation with *H. pylori* 18 hr, MOI 1:150 in SF media. Ten glands per antibody per patient were counted (minimum total of 30 glands) for the number of PAI-1, uPA and uPAR expressing cells per gland, the number of cells per gland expressing the cell specific marker, and the number of cells per gland expressing PAI-1, uPA or uPAR as well as the cell specific marker. The average number of cells per gland ranged from 60-250 cells.

3.2.6 Analysis of proliferation

Incorporation of BrdU into human gastric glands was carried out as outlined in section 2.6 and Figure 2.1. Cells were treated with PAI-1 ASO (sequences given in section 2.6) as described and stimulated with wild-type *H. pylori* MOI 1:150 or uPA (250mg/ml). Immunocytochemistry was performed on PFA fixed glands as described in section 2.7. Cells were dual-stained with a guinea-pig-anti-vimentin antibody (1:400; Fitzgerald) and FITC-conjugated fast immune anti-BrdU/DNase antibody (1:50; Becton Dickinson). Ten glands per patient, per treatment were scored for BrdU positive cells and BrdU positive cells that were also vimentin positive. Vimentin positive myofibroblasts which were also labelled with BrdU were subtracted from the total number of BrdU labelled cells per gland to give the percentage of BrdU positive epithelial cells.

3.3 Results

3.3.1 PAI-1 mRNA abundance is increased in *H. pylori* infection and in gastric cancer

Real-time PCR was used to determine if PAI-1 mRNA abundance is increased in the gastric corpus or antrum in *H. pylori* infection and in gastric cancer patients compared to *H. pylori* negative control subjects (Figure 3.1). A significant increase in PAI-1 mRNA abundance was seen in the corpus of infected subjects compared to non-infected subjects. This increase was enhanced in gastric cancer patients where PAI-1 mRNA abundance was further increased from than seen in *H. pylori* infection (*H. pylori* negative 100% $\pm$ 25, *H. pylori* positive 300% $\pm$ 90, gastric cancer 390% $\pm$ 111; $p<0.05$, ANOVA, n=5-15). Interestingly, real-time PCR was also performed on antrum samples from *H. pylori* positive (n=10) and negative patients (n=18) but no significant difference in PAI-1 mRNA abundance was seen in this region of the stomach. There was no significant difference in gastrin levels between *H. pylori* negative and positive patients used in this experiment and patients with gastrin levels higher than 30pM were excluded from the study.

3.3.2 *H. pylori* infection increases PAI-1 protein expression

To determine if PAI-1 protein expression and not just mRNA abundance is increased in *H. pylori* infection, immunohistochemical staining of human stomach sections was carried out on both a *H. pylori* negative and *H. pylori* positive patient using an anti-PAI-1 antibody (Figure 3.2). An increase in brown staining indicates an increase in protein expression and it is clear that there is a greater expression of PAI-1 protein in *H. pylori* infection compared to control, non-infected subject. The increase in expression is in the glandular region within

epithelial cells, including the large acid-secreting parietal cells determined by analysis of cell size and shape.

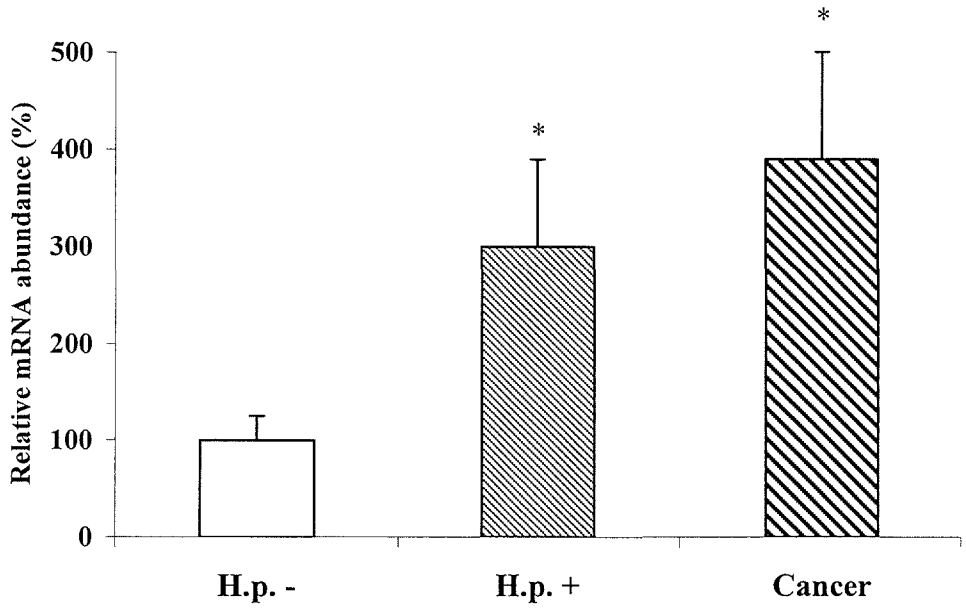

Figure 3.1

PAI-1 mRNA abundance is increased in *H. pylori* infection and gastric cancer.
Real time PCR normalised to 18s shows a significant increase in PAI-1 expression in the gastric corpus of *H. pylori* (H.p.) infected patients (n=10) and gastric cancer patients (n=5) compared to *H. pylori* negative subjects (n=15) (mean $\pm$ S.E. *$p<0.05$, ANOVA; Corpus real-time PCR was performed by John Sammut).

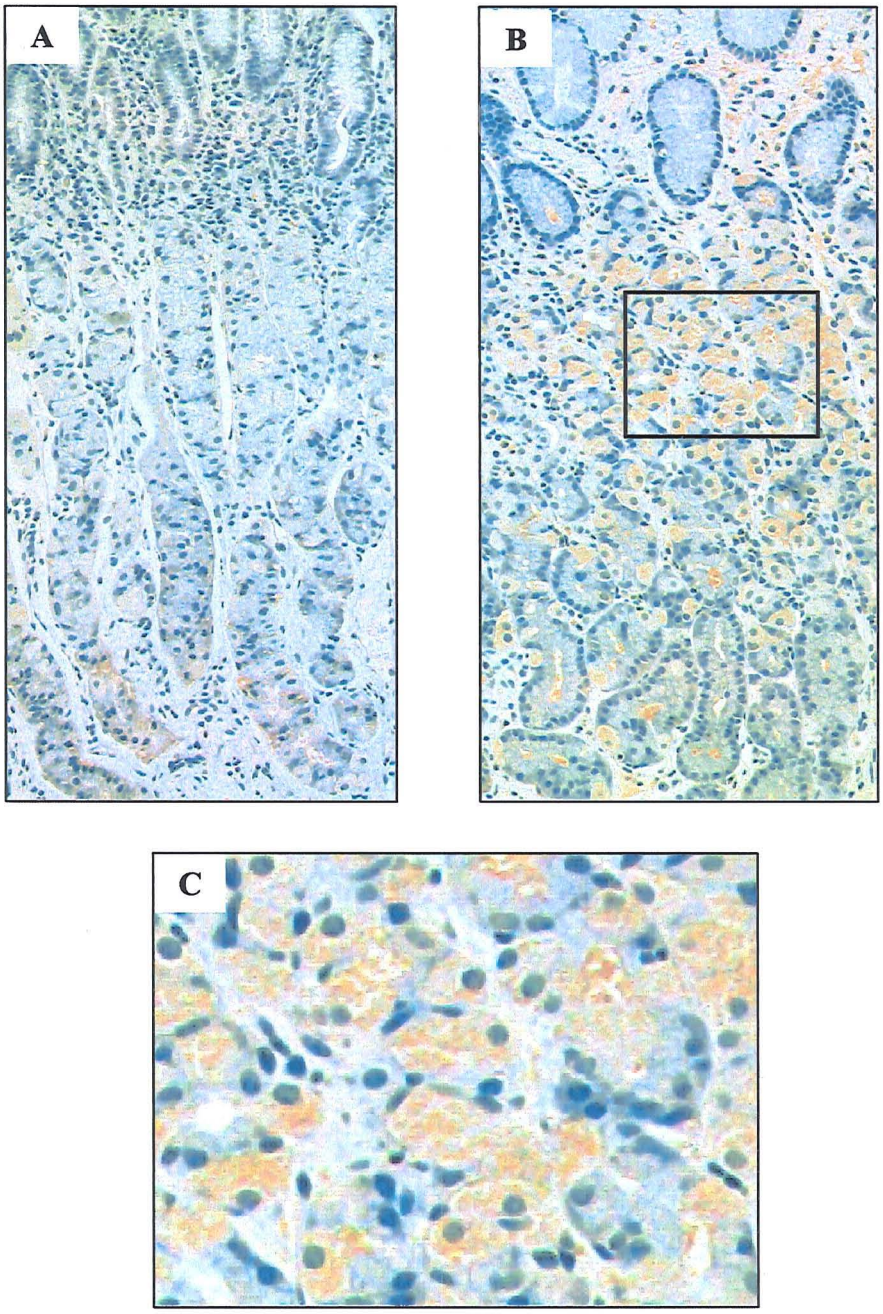

Figure 3.2

PAI-1 protein expression is increased in *H. pylori* infection.
Immunohistochemical staining of human stomach sections showed increased PAI-1 protein expression in *H. pylori* infected patients (B), compared to non-infected subjects (A). C, An enlarged area of B, showed increased PAI-1 staining is mainly localised to epithelial cells, possibly acid secreting parietal cells. (Carried out in collaboration with department of Pathology, The University of Liverpool).

3.3.3 PAI-1 protein is expressed in epithelial cells in gastric cancer

Immunohistochemical staining of human gastric cancer tissue showed PA I-1 protein to be expressed in epithelial cells (Figure 3.3). α-SMA staining showed the presence of myofibroblasts surrounding the cancer cells and PAI-1 protein is expressed to a much lesser extent in this cell type.

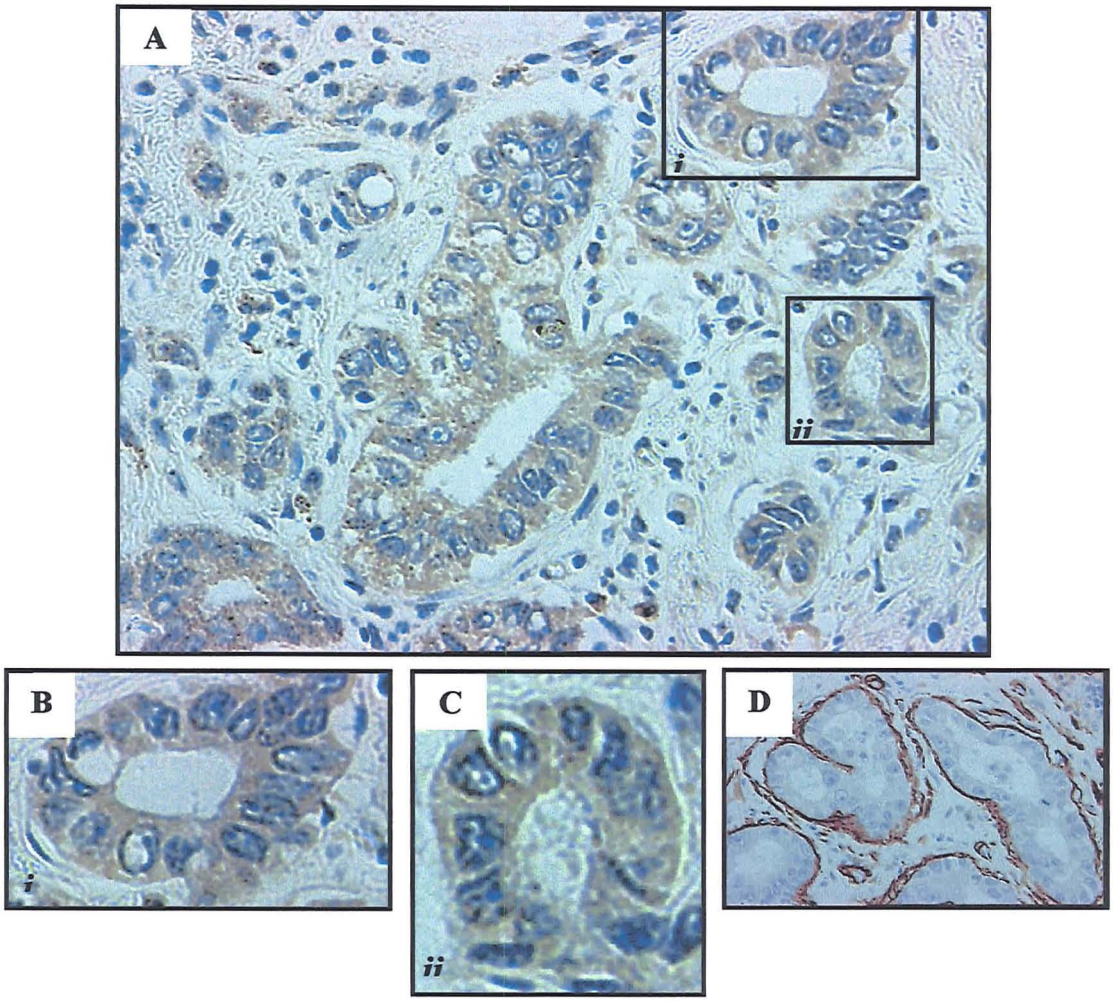

Figure 3.3

Increased PAI-1 staining in gastric cancer is localised to epithelial cells.
A, Immunohistochemical localisation of PAI-1 in human gastric cancer tissue. B & C, Enlarged sections of A showing PAI-1 is mainly localised to epithelial cells and to a lesser extent in surrounding stromal cells. D, α-SMA staining shows the myofibroblasts surrounding the cancer cells.

3.3.4 PAI-1 mRNA abundance is increased in a mouse model of epithelial remodelling

The finding that both PAI-1 mRNA and protein expression are up-regulated in humans infected with *H. pylori* was extended to a rodent model of transgenic (INS-GAS) mice infected with *Helicobacter felis* (*H. felis*). These animals have been documented to display a vast amount of tissue remodelling and show dramatic changes within the gastric architecture, eventually progressing to gastric atrophy and the development of gastric cancer approximately 6 months post-infection (discussed in section 1.2.5 and 1.3.7.1; Wang *et al.*, 2000). Real-time PCR indicated that PAI-1 mRNA abundance was significantly increased in INS-GAS mice infected with *H. felis* compared to uninfected INS-GAS animals 6 months post infection (*H. felis* negative 100% $\pm$ 18, *H.felis* positive 199% $\pm$ 41; $p<0.05$, *t*-test, n=6-7). There was no significant increase in PAI-1 mRNA abundance after 3 months of infection, and by 9 months post infection there was no further increase than that seen at the 6 month time-point (Figure 3.4-A). As INS-GAS mice are on FVB/N background, PAI-1 mRNA abundance was measured in uninfected mice of both groups of animals to be certain it was the *H. felis* infection and not that INS-GAS mice posses a transgene that was responsible for the increase in PAI-1 mRNA abundance. No significant difference between PAI-1 mRNA abundance was seen between uninfected INS-GAS and FBV/N mice (Figure 3.4-B). Also, although infected INS-GAS mice are known to display hypergastrinaemia there was no significant difference seen in plasma gastrin levels between *H. felis* infected and uninfected INS-GAS mice at the 6 month time-point (as published earlier; Steele *et al.*, 2007). This data

indicates that in mouse, as in humans, *Helicobacter* infection stimulates PAI-1 expression.

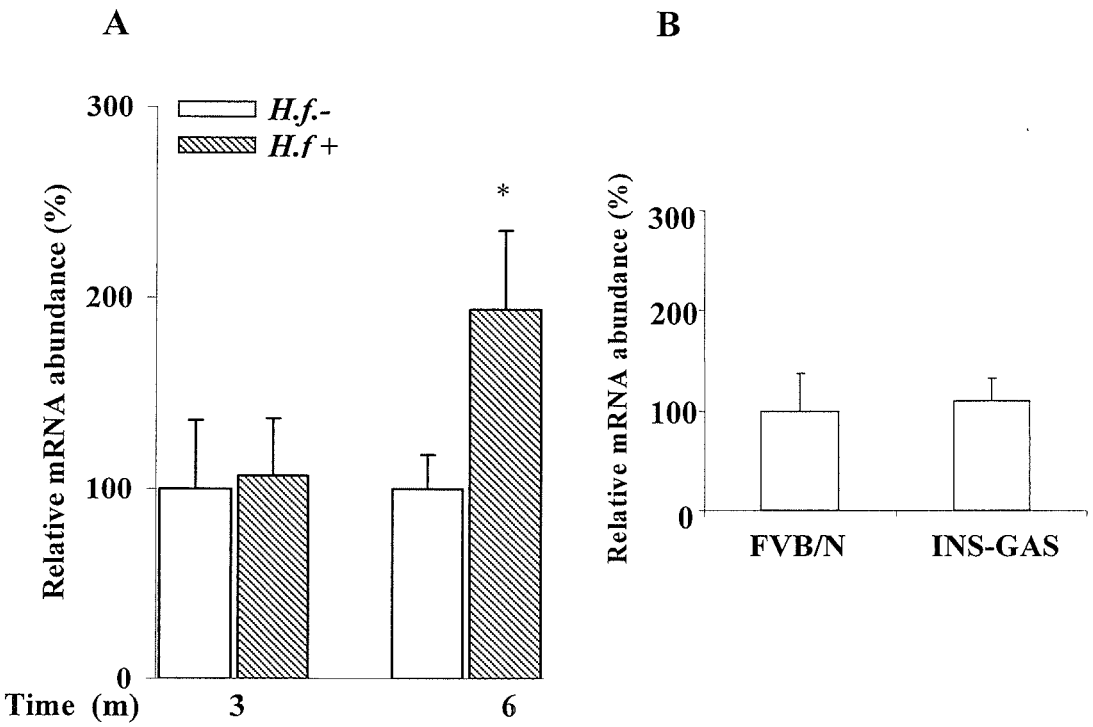

Figure 3.4

PAI-1 mRNA abundance is increased in *H. felis* infected mice 6 months post infection.

A, Real time PCR normalised to 18s showed elevated PAI-1 expression in the gastric corpus of INS-GAS mice (n=6) after *H. felis* (*H.f.*$^{+}$) infection 6 month post-infection compared to uninfected (*H.f.*$^{-}$) mice (n=7). B, Real time PCR normalised to 18s showed no significant difference in PAI-1 expression between uninfected control FVB/N and INS-GAS mice (n=5) (mean $\pm$ S.E, *P<0.05, *t*-test).

3.3.5 Cultured gastric glands contain the same epithelial cell types as *in vivo* glands

As PAI-1 was found to be up-regulated by *Helicobacter* in both human and murine gastric corpus this was the region of the stomach chosen for further investigation. This allowed the expression and regulation of PAI-1 and other members of the uPA system to be studied, and to determine if this increase in expression had any significance upon epithelial cell function. In order to study this, primary cultured gastric glands from *H. pylori* negative patients (and positive patients only were indicated) were used which are an epithelial cell culture obtained from human corpus endoscopic pinch biopsies (prepared as in section 2.4.2). The gastric epithelium consists of a single layer of cells that invaginate forming highly ordered tubular structures, containing a variety of distinct cell types which are collectively known as gastric glands. The main differentiated cell types within the gastric corpus are parietal cells, chief cells, mucous neck cells, surface mucous neck cells and various endocrine cell types such as enterochromaffin-like (ECL) cells (discussed in section 1.2.2). Each cell type possesses a different cell-specific marker to which anti-bodies have been raised enabling identification of that cell population by immunocytochemistry (Table 2.1).

Primary gastric glands in culture consist of the same key epithelial cell types as *in vivo* gastric glands making them an excellent model to use. Figure 3.5 depicts a schematic structure of a corpus gastric gland showing the various different epithelial cell types and their approximate location within the gland. Also shown is how an important a sub-epithelial cell type, the myofibroblast is which lies underneath and in close proximity to the epithelium. The right hand side of the

figure shows a freshly isolated gastric gland, which after approximately 24 hr of culture adheres to the culture dish and spreads out to form an adherent primary gastric gland.

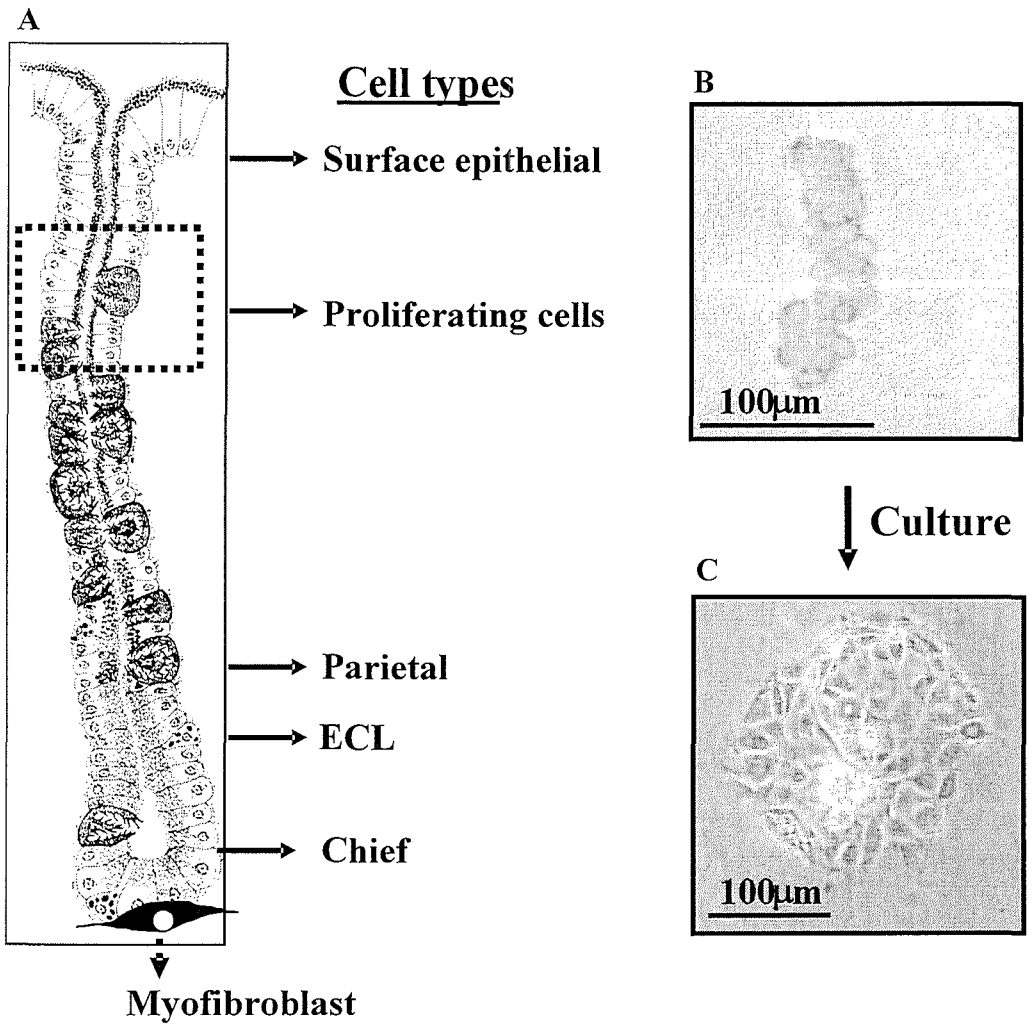

Figure 3.5

Schematic of the gastric corpus epithelium and the corresponding adherent cultured gland preparation.
A, Schematic representation showing the major differentiated cell types within a gastric gland of corpus origin (Adapted from Ito & Winchester, 1963). B, Freshly isolated gastric glands in suspension obtained from human corpus pinch biopsies. C, Adherent human primary gastric gland after 24 hr of culture.

3.3.6 Endogenous PAI-1 protein is expressed in multiple epithelial cell types within primary gastric glands

To determine the epithelial cell types which express PAI-1 protein, dual labelling immunocytochemistry was performed using an anti-PAI-1 antibody and various gastric cell specific markers (Table 2.1) on primary gastric glands which had been cultured for a total of 72 hr. PAI-1 was found to co-localised with TFF-1 (8.94 $\pm$ 1.48% of all PAI-1 positive cells), chromogranin A (51.83 $\pm$ 2.49% of all PAI-1 positive cells), pepsinogen (23.20 $\pm$ 1.86% of all PAI-1 positive cells) and H^+, K^+-ATPase (12.84 $\pm$ 1.72% of all PAI-1 positive cells; Table 3.1), indicating that PAI-1 is expressed in surface mucous, ECL, chief and parietal cells respectively (Figure 3.6). No significant difference in expression pattern was seen in the absence of *H. pylori* or when it was exogenously added to the gastric glands. PAI-1 was found not to co-localise with TFF-2 and is therefore not expressed in mucous neck cells.

3.3.7 Endogenous uPAR protein is expressed in multiple epithelial cell types within primary gastric glands

To determine the epithelial cell types which express uPAR protein dual labelling immunocytochemistry was performed using an anti-uPAR antibody and various gastric cell specific markers on primary gastric glands which had been cultured for a total of 72 hr. uPAR was found to co-localised with TFF-1 (11.91 $\pm$ 1.88% of all uPAR positive cells), chromogranin A (17.15 $\pm$ 1.66% of all uPAR positive cells), pepsinogen (28.30 $\pm$ 2.49% of all uPAR positive cells) and H^+, K^+-ATPase (22.41 $\pm$ 2.18% of all uPAR positive cells; Table 3.1), indicating that uPAR is endogenously expressed in surface mucous cells, ECL cells, chief cells

and parietal cells respectively (Figure 3.7). No difference in expression pattern was seen in the presence or absence of wild-type *H. pylori* and uPAR was found not to co-localise with TFF-2, and is therefore not expressed in mucous neck cells.

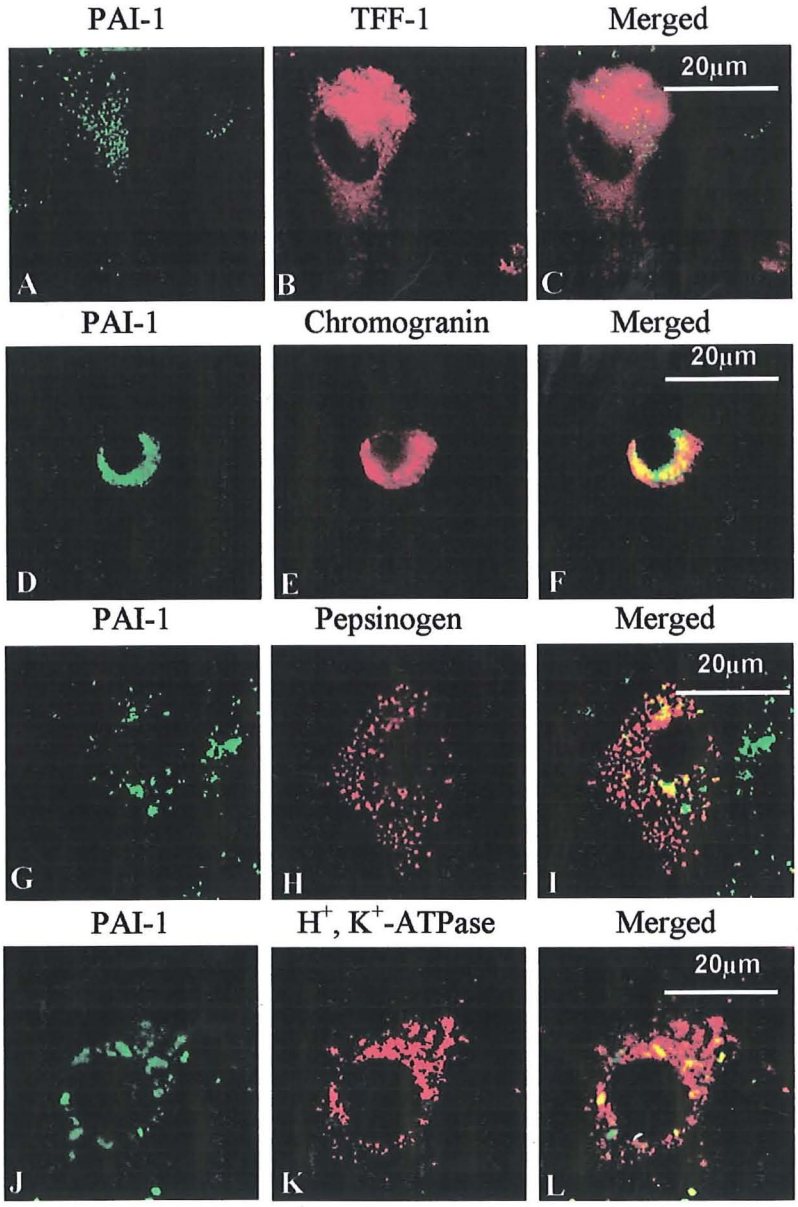

Figure 3.6

PAI-1 endogenous protein expression in human primary cultured gastric glands.
A-C, PAI-1 protein expression (green) in TFF-1 (red) positive cells. D-F, PAI-1 protein expression (green) in chromogranin A (red) positive cells ECL cells. G-I, PAI-1 protein expression (green) in pepsinogen (red) positive cells chief cells. J-L, PAI-1 protein expression (green) in H^+, K^+-ATPase (red) positive parietal cells.

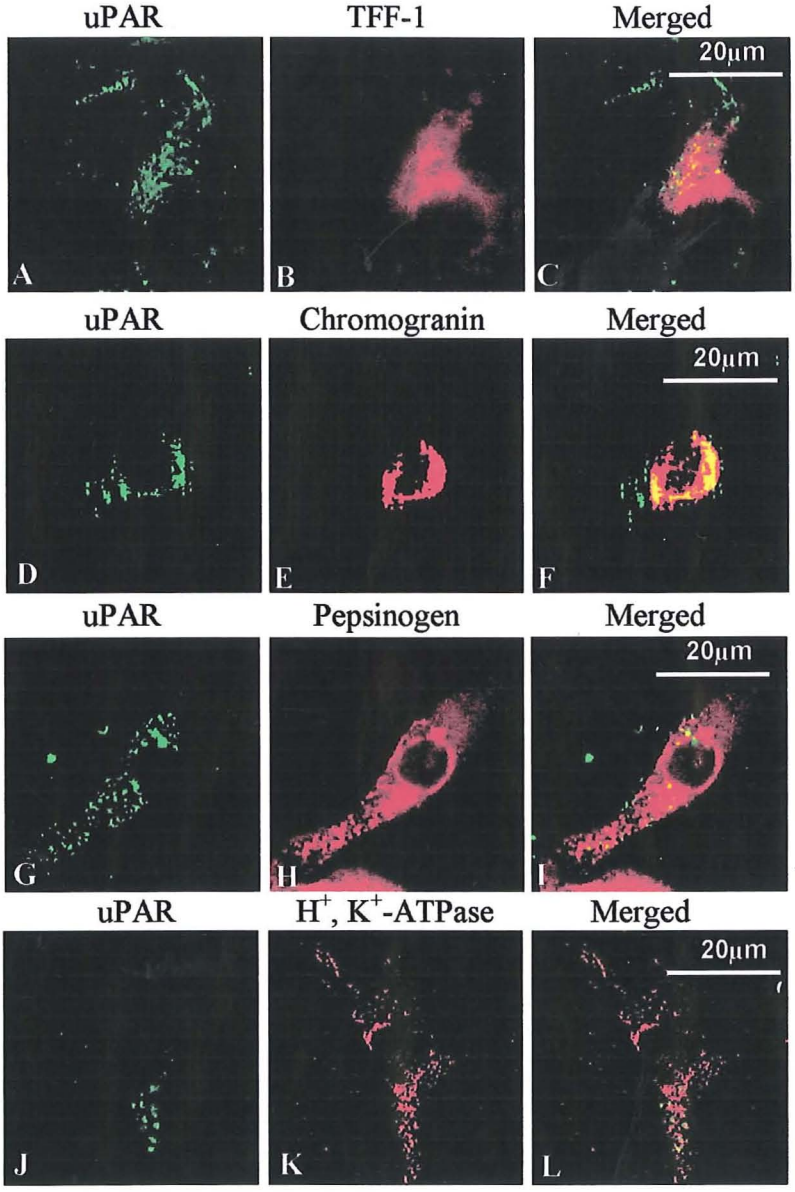

Figure 3.7

uPAR endogenous expression in human primary cultured gastric glands. A-C, uPAR protein expression (green) in TFF-1 (red) positive cells. D-F, uPAR protein expression (green) in chromogranin A (red) positive cells ECL cells. G-I, uPAR protein expression (green) in pepsinogen (red) positive cells chief cells. J-L, uPAR protein expression (green) in H^+, K^+-ATPase (red) positive parietal cells. The cell type in which endogenous uPAR is expressed in was the same in the presence and absence of wild-type *H. pylori*.

3.3.8 Both PAI-1 and uPAR are expressed in gastric myofibroblasts

Human primary gastric glands are primarily an epithelial cell culture but contain approximately a 3% population of gastric myofibroblasts. As both PAI-1 and uPAR are expressed within the epithelial cells, their expression within the present myofibroblasts was also tested. Dual-staining immunocytochemistry was performed on primary gastric glands which had been cultured for a total of 72 hr using an anti-PAI-1 or anti-uPAR antibody and a guinea-pig anti-vimentin antibody, which is a marker for myofibroblasts. PAI-1 was not found to be expressed in vimentin positive cells without stimulation with wild-type *H. pylori*. However, after stimulation with *H. pylori* some vimentin positive cells were found to express PAI-1 (Figure 3.8, Table 3.1). uPAR was found to be expressed in vimentin positive cells both before and after stimulation with wild-type *H. pylori* (Figure 3.8, Table 3.1).

3.3.9 Endogenous uPA protein is expressed in gastric myofibroblasts

To determine the exact cell types which express uPA protein, dual labelling immunocytochemistry was performed using an anti-uPA antibody and various gastric cell specific markers (Table 2.1) on primary gastric glands which had been cultured for a total of 72 hr. uPA was found to co-localised with vimentin only (Table 3.1) indicating that uPA is endogenously expressed in gastric myofibroblasts (Figure 3.9). No difference in expression pattern was seen in the presence or absence of wild-type *H. pylori* and uPA was found not to co-localise with chromogaranin A, H^+, K^+-ATPase, pepsinogen, TFF-1 or TFF-2.

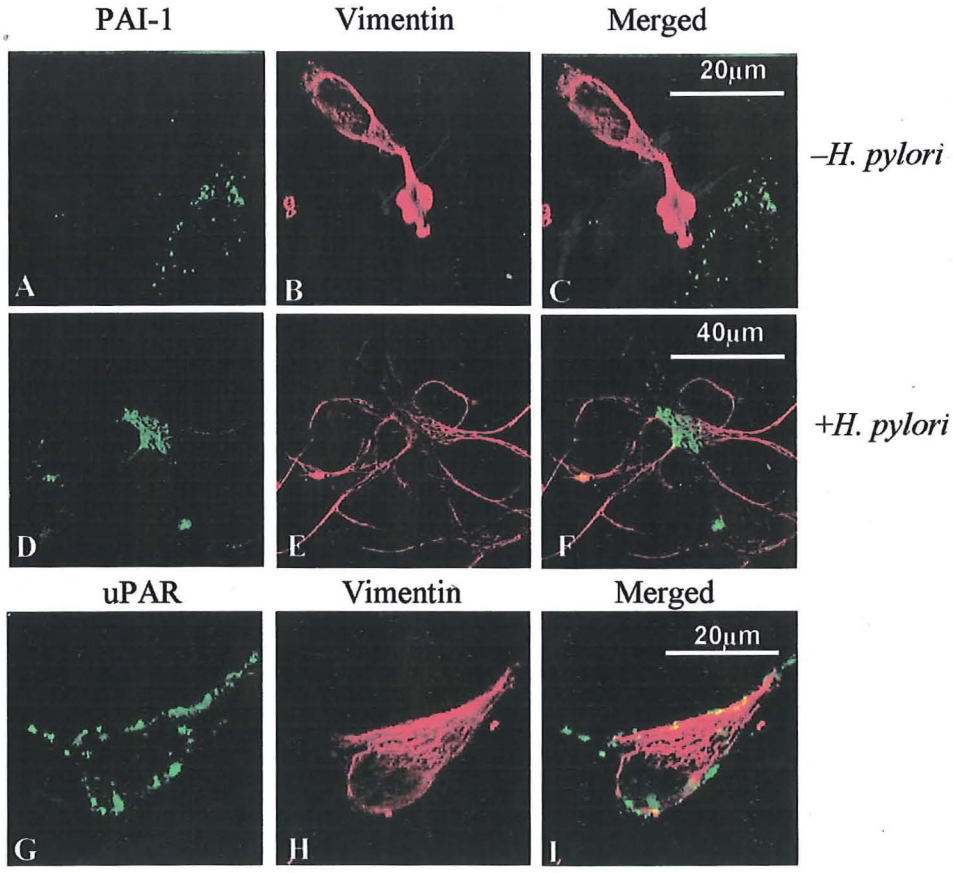

Figure 3.8

PAI-1 and uPAR endogenous protein expression in human primary cultured gastric myofibroblasts.
A-C, PAI-1 protein is not expressed in vimentin positive myofibroblasts in the absence of *H. pylori*. D-F, PAI-1 is expressed in vimentin positive cells after wild-type *H. pylori* stimulation. G-I, uPAR protein expression (green) in vimentin positive myofibroblasts (red) without *H. pylori* stimulation but uPAR is also expressed in myofibroblasts after *H. pylori* stimulation.

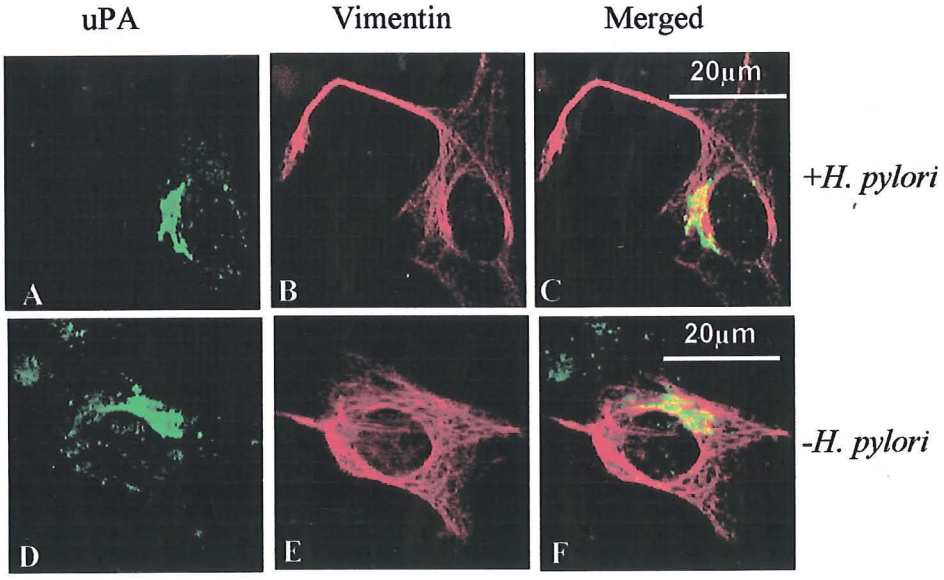

Figure 3.9

uPA endogenous protein expression in human primary cultured gastric glands.
uPA protein (green) is expressed in vimentin positive myofibroblasts (red) in the presence (A-C), and absence (D-F) of wild-type *H. pylori*.

	TFF-1	Chromogranin A	Pepsinogen	H^+, K^+-ATPase	Vimentin
% PAI-1 expressing cells	8.94 ± 1.45	51.83 ± 2.49	23.20 ± 1.86	12.84 ± 1.72	Only after *H. pylori*
% uPA expressing cells	<1	<1	<1	<1	100 ± 0.00
% uPAR expressing cells	11.91 ± 1.88	17.15 ± 1.66	28.30 ± 2.49	22.41 ± 2.18	14.7 ± 1.83

Table 3.1

Proportion of PAI-1, uPAR and uPA expressing cells in human cultured gastric gland cells after stimulation with wild-type *H. pylori*. Proportion of PAI-1, uPA and uPAR expressing cells that also express either TFF-1, chromogranin A, pepsinogen, H^+, K^+-ATPase or vimentin. Note <1% of PAI-1 and uPAR expressing cells also express TFF-2. *H. pylori* was added at an MOI 1:150 18 hr, SF media. Distribution of both proteins was found not to be significantly changed after *H. pylori* stimulation with the exception of PAI-1 only being expressed in vimentin positive cells after *H. pylori* stimulation (mean ± S.E, n=3-5 patients without *H. pylori* and n=3 with *H. pylori*).

3.3.10 *H. pylori* stimulates epithelial cell proliferation which is increased by PAI-1 knock-down

The effect of *H. pylori* on epithelial cell proliferation was investigated using BrdU incorporation into human primary gastric glands. Exogenously added wild-type *H. pylori* M.O.I. 1:150, 18 hr was found to significantly increase the percentage of BrdU labelled cells (1.36 $\pm$ 0.12 fold) in 3 separate experiments, each consisting of 4 individual patients (Figure 3.10). The role of PAI-1 in this process was determined by using PAI-1 anti-sense oligonucleotides (ASO).

After PAI-1 knock-down and stimulation with wild-type *H. pylori,* a further increase in the percentage of BrdU labelled gastric gland cells was seen compared to *H. pylori* alone (control ASO 1.71 $\pm$ 0.17% BrdU labelled cells; control ASO & *H. pylori* 2.74 $\pm$ 0.36% BrdU labelled cells; PAI-1 ASO & *H. pylori* 3.44 $\pm$ 0.20% BrdU labelled cells. n=4 patients, ANOVA, *$p<0.05$). There was an increase (29.1 $\pm$ 3.6%) in BrdU labelled cells when cells were treated with PAI-1 ASO compared to control ASO. This data indicates that *H. pylori* stimulates gastric epithelial cells to proliferate and PAI-1 acts as positive regulator of proliferation. When PAI-1 is knocked-down the balance between protease and its inhibitor is disrupted and uPA can exert its effects on gastric epithelial cells causing an increase in proliferation.

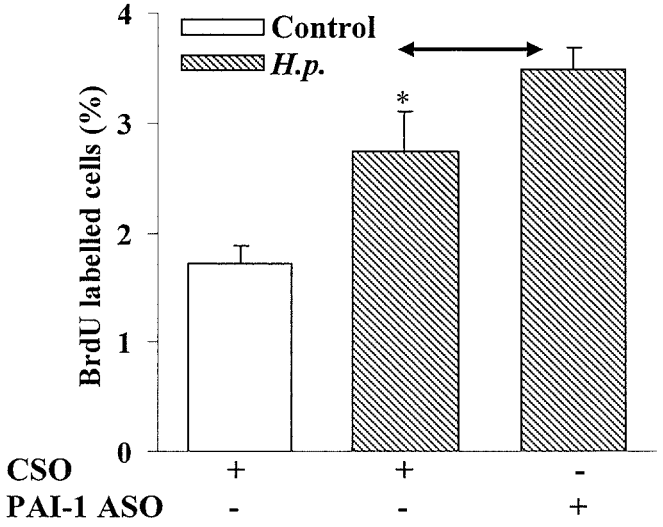

Figure 3.10

***H. pylori* stimulates proliferation of epithelial cells which is further increased by PAI-1 anti-sense oligonucleotide treatment.**
BrdU incorporation into human gastric epithelial cells showed that wild-type *H. pylori* increases proliferation of these cells. Knocking-down PAI-1 with anti-sense oligonucleotides resulted in a further increase in proliferation compared to *H. pylori* alone (mean $\pm$ S.E n=4 patients, ANOVA, * $p<0.05$).

3.3.11 Exogenous uPA stimulates proliferation of epithelial cells

As *H. pylori* was found to stimulate epithelial cell proliferation (Figure 3.10) and it is known to increase the expression of uPA within the stomach (Cédric Duval, PhD thesis, 2007) it was investigated whether exogenously added uPA could mimic the effect *H. pylori* has on proliferation of human primary cultured gastric glands. The role of PAI-1 in controlling uPA in this process was also studied by knocking-down PAI-1 using PAI-1 ASO. uPA alone was found to significantly increase the percentage of BrdU labelled cells within gastric glands which was further enhanced when the main secreted inhibitor of uPA, PAI-1 was knocked-down (Figure 3.11; control 1.72 $\pm$ 0.17% BrdU labelled cells; uPA 3.24 $\pm$ 0.18% BrdU labelled cells; uPA & PAI-1 ASO 4.46 $\pm$ 0.39% BrdU labelled cells; $p<0.05$, ANOVA, n=5). These data indicate that uPA can act to increase proliferation of epithelial cells but is restrained to an extent by PAI-1.

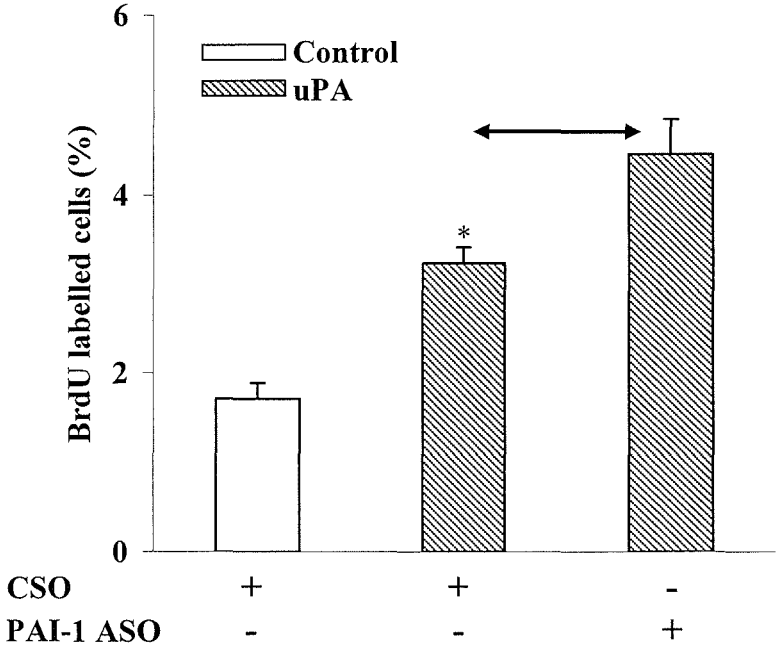

Figure 3.11

uPA stimulates proliferation of epithelial cells which is further increased by PAI-1 anti-sense oligonucleotide treatment.
BrdU incorporation into human gastric epithelial cells showed that exogenous uPA increased proliferation. Knocking-down PAI-1 with anti-sense oligonucleotides (ASO) resulted in a further increase in proliferation. (mean $\pm$ S.E n=5 patients, *$p<0.05$, ANOVA).

3.3.12 PAI-1 ASO reduce the percentage of PAI-1 expressing cells

To verify that PAI-1 ASO reduced the number of PAI-1 expressing cells within human primary gastric glands, immunocytochemistry using a goat anti-PAI-1 antibody was carried out (Figure 3.12). The average total number of cells per gastric gland expressing PAI-1 was quantified after treatment with either control ASO or PAI-1 ASO, before and after the addition of wild-type *H. pylori* M.O.I. 1:150 18 hr (Table 3.2). There was an approximate 60% decrease in the number of cells expressing PAI-1 in those glands treated with PAI-1 ASO compared to control ASO, both in the presence and absence of *H.pylori.* This confirms that PAI-1 ASO treatment is successful in knocking-down PAI-1 expression in human primary gastric glands.

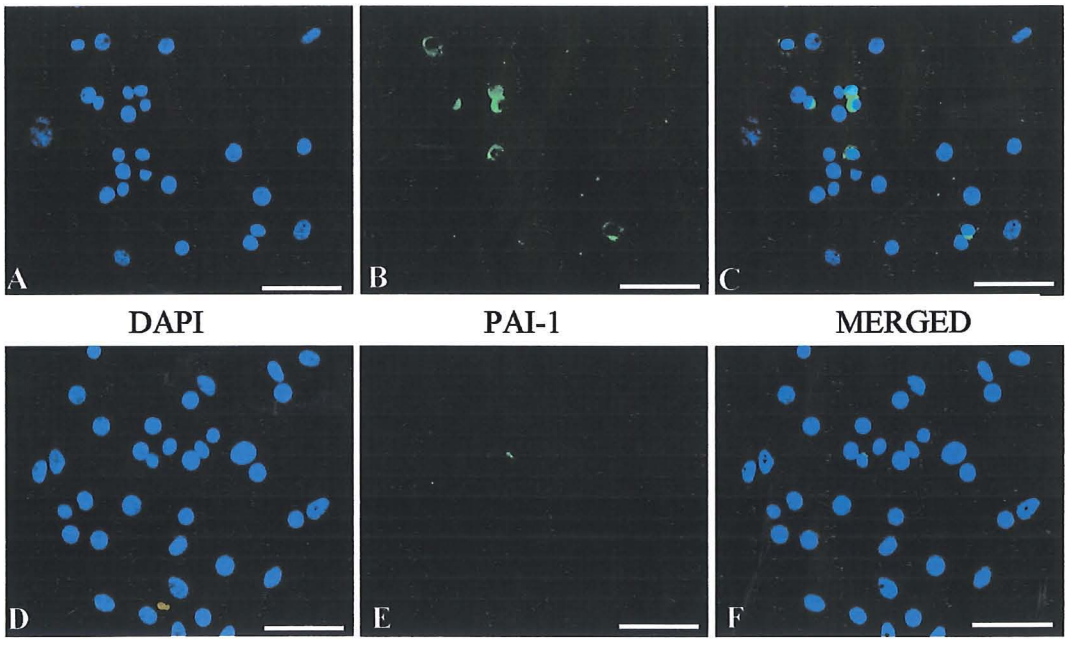

Figure 3.12

PAI-1 anti-sense oligonucleotides reduce the number of PAI-1 expressing cells in human gastric glands.
Immunocytochemistry to show PAI-1 expression in human gastric glands treated with control anti-sense oligonucleotides and wild-type *H. pylori* (A-C). Infected glands treated with PAI-1 anti-sense oligonucleotides and *H. pylori* showed a marked reduction in the number of PAI-1 expressing cells (D-F). Scale bar represents 63 μm.

	Control ASO	PAI-1 ASO	Control ASO + *H. pylori*	PAI-1 ASO + *H. pylori*
% PAI-1 expressing cells	8.71 ± 0.19	3.66 ± 0.22	10.04 ± 0.24	3.81 ± 0.21

Table 3.2

There is a decrease in PAI-1 expressing cells in human gastric glands treated with PAI-1 anti-sense oligonucleoides before and after the addition of *H. pylori*.
Average total number of cells per gastric gland expressing PAI-1 was quantified after treatment with control anti-sense oligonucleotides (ASO) and PAI-1 ASO before and after the addition of wild-type *H. pylori* M.O.I. 1:150. There was approximately a 60% decrease in the number of cells expressing PAI-1 in glands treated with PAI-1 ASO compared to control ASO in the presence and absence of *H. pylori* (n=30 glands, mean ± S.E).

3.4 Discussion

Results presented in this chapter have shown PAI-1 mRNA abundance to be increased by the oncogenic pathogen *H. pylori* in the gastric corpus, the region of the stomach where infection leads to glandular atrophy and is associated with elevated risk of gastric cancer (Mobley 1997; Correa, 2003). However, PAI-1 mRNA abundance was not increased in the antrum after *H. pylori* infection. An increase in PAI-1 protein expression was also seen in *H. pylori* infection which was predominately epithelial and localised in particular to the large acid secreting parietal cells. A critical stage in the development of intestinal metaplasia and gastric cancer is the induction of mature parietal cell loss (Canfield *et al.*, 1996; Correa, 2003). Therefore, it is an interesting finding that PAI-1 should be up-regulated in this cell type. However, it is not known at this stage if the increase in PAI-1 has any role to play in the loss of parietal cells.

Real-time PCR data showed *H. pylori* infection gives rise to a significant increase in uPA and uPAR mRNA abundance in the corpus region of the stomach (Cédric Duval, PhD Thesis, 2007). However, similar to PAI-1, there was no significant difference in mRNA abundance of these two genes in the antrum, and PAI-3 expression was unaltered in both regions with infection (John Sammaut, Msc Thesis, 2004). This highlights the fact that *H. pylori* up-regulates PAI-1 and other specific members of the uPA system.

It has previously been shown that PAI-2 is increased by *H. pylori* infection through the involvement of NFκB and RhoA (Varro *et al.*, 2004). However, the relevant signalling mechanisms by which *H. pylori* mediates PAI-1 expression remain to be elucidated. It is well recognised that *H. pylori* induces a variety of

proteins, growth factors and cytokines (Cox *et al.*, 2001; Mills *et al.*, 2001), which could ultimately be candidates for influencing PAI-1 induction. Given the extensive range of transcriptional response elements contained within the human PAI-1 promoter, and number of mediators known to stimulate PAI-1 transcription (discussed in section 1.5.4) this is likely be a difficult task and is discussed further in Chapter 4 of this thesis.

Real-time PCR showed PAI-1 mRNA abundance to be significantly increased in gastric cancer patients on a background of chronic *H. pylori* infection. The extent of this finding is limited by the small patient size sampled during this experiment. However, it is in good agreement with previous data demonstrating uPA, uPAR and PAI-1 expression to be increased by *H. pylori* in human gastric carcinoma cell lines (Kitadai *et al.*, 2003; Kim *et al.*, 2005; Iwamoto *et al.*, 2005). It is also in agreement with work relating to the fact that increased PAI-1 expression correlates with a poor diagnosis in gastric cancer patients (Nekarda *et al.*, 1994; Heiss *et al.*, 1995; Ito *et al.*, 1996; Plebani *et al.*, 1997; Kaneko *et al.*, 2003; Scicolone *et al.*, 2006), and might serve as a new and promising parameter for both prediction and prognosis of gastric cancer (Sakakibara *et al.*, 2006).

The paradoxical fact that PAI-1 acts to control extracellular proteolysis, yet is associated with poor prognosis in a variety of human cancers could be explained by the many other functions of PAI-1 which are independent of uPA inhibition. These include its involvement in cell motility via promotion of cell detachment both by inactivating integrins and binding to the ECM molecule VN (Stefansson & Lawrence, 1996; Czekay *et al.*, 2003) and also by encouraging cell migration through an interaction with the LRP (discussed in section 1.5.5.1; Degryse *et al.*,

2004). In addition to these alternative functions, PAI-1 has also been documented to influence processes involved in tumour cell biology such as angiogenesis (discussed in section 1.5.5.2) and inhibition of apoptosis via the inactivation of caspase-3 (Chen *et al.*, 2004). Alternatively, as PAI-1 expression can be induced by uPA itself (Shetty *et al.*, 2003) high PAI-1 expression seen in human gastric cancer could simply reflect high expression of uPA.

In this study, PAI-1 expression was found to be localised within gastric cancer epithelial cells and to a much lesser extent in surrounding stromal cells. Previous immunohistochemical studies have identified strong PAI-1 expression in both tumour cells and fibroblasts in breast carcinomas (Dublin *et al.*, 2000). However, in colon cancer tissue the majority of PAI-1 expression was seen in myofibroblasts with no PAI-1 immunoreactivity detected in cancer cells (Illemann *et al.*, 2004) therefore showing varying expression patterns in different tissue types.

In an INS-GAS mouse model of gastric epithelial remodelling, PAI-1 mRNA abundance was found to be increased 6 months post *H. felis* infection. Infected INS-GAS mice show initial moderate hypergastrinaemia, followed later by parietal cell loss, development of gastric atrophy, achlorhydria and gradual progression to dysplasia (discussed in sections 1.2.5 and 1.3.7.1). Some of these observations are similar characteristics to that seen in *H. pylori* mediated human disease (Wang *et al.*, 2000), making it a good model to help determine the role of PAI-1 in the regulation of the gastric epithelium and its involvement in gastric remodelling and carcinogenesis.

There is a well documented role for the involvement of PAI-1 in the process of tissue repair (Providence *et al.*, 2000). However, it is not yet defined if the increase in PAI-1 expression seen in *Helicobacter* infection is initially part of a mucosal defensive mechanism, protecting the host from bacterial damage, or if elevated PAI-1 has a pathophysiological involvement, effecting epithelial cell function and contributing to the process of tissue remodelling.

As PAI-1 was found to be increased in epithelial cells within the gastric corpus in *H. pylori* infection, and also in gastric cancer, a primary culture consisting of all the gastric epithelium cell types as *in vivo* gastric glands was used to further investigate the effects of PAI-1 on gastric epithelial cell function. The endogenous expression of PAI-1, uPAR and uPA in primary cells showed cell restricted expression. However, both PAI-1 and uPAR are expressed in the same cell types but to a different extent; PAI-1 is primarily expressed in ECL cells whereas uPAR is principally expressed in chief and parietal cells. No previous reports have investigated the specific gastric cell types in which uPA system members are expressed, but the different expression patterns may reflect variations in spatial regulation and control of the uPA system. Myofibroblasts in several experimental models have been shown to strongly express PAI-1, yet interestingly it was only expressed in gastric myofibroblasts after stimulation with *H. pylori*. Gastric sub-epithelium cells *in vivo* would not normally be directly exposed to *H. pylori*. However, it is possible that in culture the presence of the bacteria may stimulate myofibroblasts to express PAI-1 or epithelial cells may secrete factors which act upon myofibroblasts promoting the expression of the protein.

Immunohistochemical staining of human stomach sections showed increased uPA expression in epithelial cells in *H. pylori* positive patients, with an abundant expression in sub-epithelial cells compared to *H. pylori* negative patients (Cédric Duval, PhD Thesis, 2007). However, in primary cultured human gastric glands, uPA was found to be expressed only in sub-epithelial myofibroblasts and not in epithelial cells, both before and after stimulation with exogenously added *H. pylori*. A possible reason for this difference in uPA expression is that cultured gastric glands were only acutely stimulated with bacteria and the patients from which these glands were derived from did not have chronic *H. pylori* corpus infection. However, the stomach sections from patients in which immunohistochemical data were obtained from did have chronic *H. pylori* corpus infection. Unfortunately, this is clinical information obtained retrospectively after experiments had been conducted. Ideally it would be useful to culture gastric glands from chronically *H. pylori* infected patients to determine if uPA can be detected in gastric epithelial cells, and if so in what specific cell types.

Infection with *H. pylori* is not only associated with the induction and increased expression of many genes, including those of the uPA system, but it also associated with increased gastric epithelial cell proliferation in both humans (Moss *et al.*, 2001) and animals (Israel & Peek, 2001). This is also seen in the primary gastric epithelial cell cultures used in this study. Exogenously added *H. pylori* increased the percentage of BrdU labelled cells which was mimicked when cells were stimulated with exogenous uPA. A further increase in proliferation in both cases was seen after the knock-down of the protease inhibitor PAI-1. These data implicate activation of uPA in the preneoplastic hyperproliferative condition associated with *H. pylori* infection which is at least

partly held in check by PAI-1. This also demonstrates the need for a tightly regulated, evenly balanced relationship between protease/protease inhibitor as a disruption to this can result in pathophysiological consequences, contributing to the metastatic cascade (Choong *et al.*, 2003).

However, it is also important to consider the balance of uPA and PAI-1 at the individual patient level. Activity assays for uPA (Cédric Duval, PhD Thesis, 2007) showed that approximately 70% of human patients responded with an increased uPA activity after *H. pylori* infection when PAI-1 was knocked-down. It is probable that these individuals originally had an elevated concentration of PAI-1, restraining the activity of uPA, displaying endogenous protection from the action of the protease. In patients whose uPA activity did not alter much after *H. pylori* infection and PAI-1 knock-down, probably already had increased concentrations of uPA activity due to a decreased concentration of PAI-1.

The data presented in the current chapter shows that *Helicobacter* infection increases gene expression of PAI-1. This increase might function as part of a protective host response helping to combat infection by contributing to tissue repair after bacterial damage. By inhibiting the protease activity of uPA and the production of plasmin, PAI-1 not only controls fibrinolysis thereby promoting the process of wound healing, but also acts to restrain the proliferative effects of uPA. An increase in PAI-1 could also function to inhibit cell invasion, again by limiting the production of plasmin. However, sustained levels of elevated PAI-1 in chronic *Helicobacter* infection could have a negative effect for the host, contributing to gastric epithelium remodelling, influence metastasis, a process to

which there has been extensive experimental evidence supporting a major importance of the uPA system (reviewed Sidenius & Blasi., 2003).

3.5 Conclusions

1. PAI-1 mRNA abundance and protein expression are increased in *Helicobacter* infection and in gastric cancer.
2. The increased expression of PAI-1 by *H. pylori* is predominantly in epithelial cells, and seen especially in parietal cells.
3. In gastric cancer, PAI-1 expression is mostly epithelial.
4. Endogenous PAI-1, uPAR and uPA proteins are expressed by sub-sets of the main differentiated gastric epithelial cells.
5. Gastric epithelial cell proliferation is increased by *H. pylori* and uPA, which is further increased in both cases after PAI-1 knock-down.

Chapter 4

Transcriptional regulation of uPA, uPAR and PAI-1 in human gastric epithelial cells

4.1 Introduction

The previous result chapter showed that infection with *Helicobacter* is associated with increased expression of PAI-1 in the gastric epithelium. As *Helicobacter* has also been shown to increase expression of other key uPA system members including PAI-2 (Varro *et al.*, 2004), uPA and uPAR (Cédric Duval PhD thesis, 2007) it is important to understand and investigate the cellular mechanisms by which these changes in expression are regulated.

The clinical outcome o f *H. pylori* infection is dependent on several aspects including host (El-Omar *et al.*, 2000), environmental (Nomura & Stemmermann, 1989) and bacterial factors (Atherton, 1998). Many virulence determinants modulating its interactions with its host are produced by *H. pylori* with strains possessing the *cag* pathogenicity island (*cag*-PAI; encoding a type IV bacterial protein secretion system), and/ or an active form of VacA (a pore forming toxin) thought to be most virulent, and more strongly associated with disease (discussed in section 1.3.3; Blaser *et al.*, 1995; Kuipers *et al.*, 1995; Parsonnet *et al.*, 1997; Nomura *et al.*, 2002).

Previous studies have identified a role for *H. pylori* virulence factors in the up-regulation of certain proteins in epithelial cells such as matrix metalloproteinase-7 (MMP-7; Bebb *et al.*, 2003; Crawford *et al.*, 2003). The possible involvement of *H. pylori* virulence factors in the induction of PAI-1, uPA and uPAR has been investigated in this results chapter using a number of isogenic mutant bacterial strains which have previously been described (Argent *et al.*, 2008).

As well as its classical function in controlling acid secretion (discussed in section 1.2.3), the gastric hormone gastrin is now well recognised for regulating

expression of several genes in the gastric epithelium (Dockray *et al.*, 2001). These genes include chromogranin A (Dimaline *et al.*, 1993), histidine decarboxylase (HDC; Dimaline & Sandvik, 1991), vesicular monoamine transporter type 2 (VMAT2; Dimaline & Struthers, 1996), Reg1 (Steele *et al.*, 2007) and PAI-2 (Varro *et al.*, 2002). Gastrin also stimulates gene and protein expression in human cultured gastric cancer cells of several genes such as hepatocyte growth factor (HGF; Konturek *et al.*, 2003). Gastrin, produced by G-cells exerts its effects by binding specifically to the CCK2R which is expressed on epithelial cells within the human gastric mucosa (Schmitz *et al.*, 2001). It is generally known that *H. pylori* infection results in a mild hypergastrinaemia state (Levi *et al.*, 1989; Mulholland *et al.*, 1993) and the effects of gastrin on PAI-1 gene expression have been investigated in the current chapter.

Gastric subepithelial cells such as myofibroblasts produce a number of key growth factors thought to act on epithelial cells and play a role in epithelial-mesenchymal signalling (Powell *et al.*, 1999a). This process is important for normal mucosal organisation and occurs during development, wound healing and in progression to cancer (discussed in section 1.4). Infection with *H. pylori* has been shown to increase expression of a variety of growth factors in the human gastric mucosa including HGF (Kondo *et al.*, 1995; Taha *et al.*, 1996). The c-MET (mesenchymal epithelial transition factor) proto-oncogene encodes the MET protein, the high affinity receptor for HGF (Giordano *et al.*, 1989; Bottaro *et al.*, 1991; Naldini *et al.*, 1991). The MET receptor is expressed solely on epithelial cells (Prat *et al.*, 1991; Sonnenberg *et al.*, 1993; Fukamochi *et al.*, 1994) and is activated in a paracrine manner by HGF, produced and released from underlying mesenchymal cells.

Several reports have implicated HGF in the regulation of components of the uPA system and it has been demonstrated to mediate PAI-1 production in liver cells (Wojta *et al.*, 1994; Imagawa *et al.*, 2006), human keratinocytes (Wojta *et al.*, 1999), and has also been shown to increase expression of uPA and uPAR in kidney epithelial cells (Pepper *et al.*, 1992). The activation of HGF has been shown to require uPA (Naldini *et al.*, 1992), and uPA production has been found to be stimulated by HGF in bovine capillary endothelial cells (Grant *et al.*, 1993) and in human stomach cancer cell lines (Lee *et al.*, 2006c). Strikingly, hepatocyte cells transduced with activated MET show a prominent up-regulation of PAI-1 (Boccaccio *et al.*, 2005), providing a strong association between the two. Over-expression of *c-MET* has been found in many human cancers, including stomach cancer (Wu *et al.*, 1998; Huang *et al.*, 2001).

Work from our laboratory has shown *H. pylori* infection to increase the expression of MMP-7 in gastric epithelial cells (Wroblewski *et al.*, 2003) which stimulates myofibroblasts resulting in increased bio-availability of IGF-II (discussed in section 1.4; Hermes *et al.*, 2005; McCaig *et al.*, 2006). Acting as an autocrine growth factor, IGF-II influences migration, proliferation and adhesion of myofibroblasts, and proliferation of gastric epithelial cells (McCaig *et al.*, 2006; Varro *et al.*, 2007). As well as this, IGF-II is known to be increased in gastric cancer (Ajisaka *et al.*, 2001). The effects of gastrin, HGF and IGF-II on PAI-1 expression within the gastric epithelium were investigated as these are three epithelial or mesenchymal derived factors known to be important for epithelial cell function, and for gastric epithelial-mesenchymal interactions (Figure 4.1).

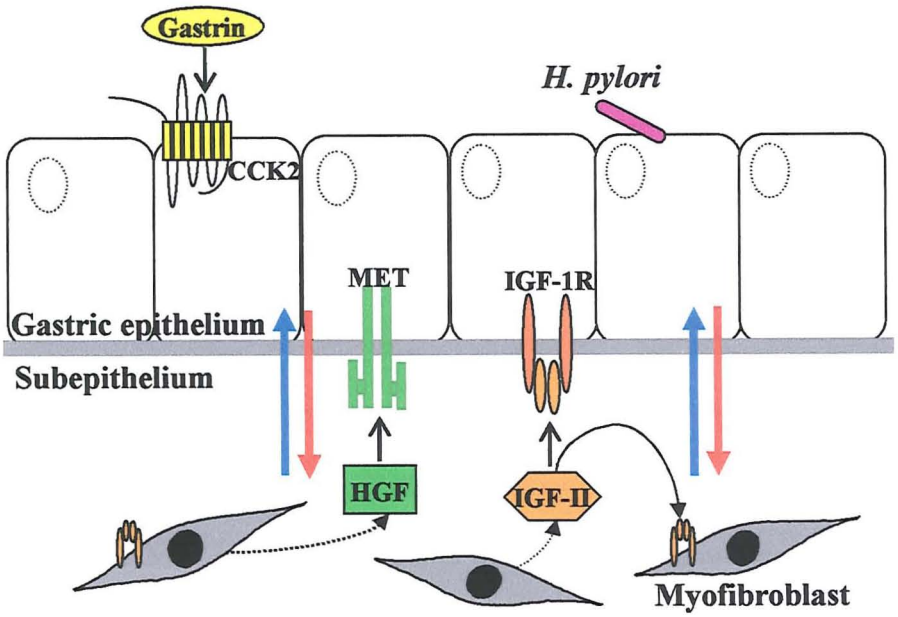

Figure 4.1

Simplified model of several epithelial and mesenchymal factors (and their respective receptors) which are known to play a role in epithelial-mesenchymal interactions within the gastric mucosa.

4.1.1 Aims

The aims of this chapter were to study the regulation of PAI-1 and other uPA system members in the gastric epithelium using luciferase promoter reporter consructs. The specific objectives were to:

1. study the cellular targeting and expression of uPA-, uPAR- and PAI-1-luciferase promoter-reporter constructs in primary gastric gland cells;
2. determine the effects of *H. pylori* on uPA-, uPAR- and PAI-1- luc expression;
3. investigate the involvement of *H. pylori* virulence factors on uPA-, uPAR- and PAI-1- luc expression;
4. examine whether gastric mucosa growth factors such as gastrin, IGF-II and HGF regulate PAI-1-luc expression within the gastric epithelium.

4.2 Materials and methods

4.2.1 Transient transfection and luciferase assays

AGS2 cells and primary gastric glands (prepared as in section 2.4.1 and 2.4.2) were cultured for 24 hr before being transfected (as described in section 2.5) with 3μg of DNA per well of uPAR-, uPA- or PAI-1-luc, together with 5ng per well of a *Renilla* luciferase reporter construct, phRL-SV40 (Promega) in SFM 20 min. Details of all plasmids used are given in section 2.1.1. Cells were stimulated with *H. pylori* at an M.O.I. of 1:150 (details of bacterial strains used are in section 2.1.3), 1nM heptadecapeptide amidated gastrin (G17), 40ng/ml HGF or 100ng/ml IGF-II in 1ml SFM. Luciferase activity was measured by dual luciferase assay (Promega) in a Lumat LB9507 luminometer (Berthold). Results are displayed as fold increase over unstimulated control, where 1.0 signifies no change in luciferase activity.

4.2.2 Cellular targeting of uPAR-, uPA- and PAI-1-luc in primary human adherent glands

To study the cellular targeting of the luciferase vectors, PAI-1-, uPA- and uPAR-luc, immunocytochemistry was carried out (as described in section 2.7) on transfected human gastric glands (as described in section 2.5.2). Cells were dual immunolabelled with a goat-anti-luciferase antibody (Rockland Immunochemicals), together with one of the cell specific markers for differentiated gastric gland cells (Table 2.1). The appropriate FITC or TRed conjugated secondary antibodies were used (Jackson Immunoresearch). Ten fields of cells from at least 3 patients were counted and results expressed as a percentage of total cell number as reported earlier (Wroblewski *et al.*, 2003).

4.3 Results

4.3.1 Gastrin stimulates PAI-1, uPA- and uPAR-luc expression in AGS2 cells

The functionality of 4.5kb of the human PAI-1 promoter, 1.94kb of the human uPA promoter and 2.3kb of the human uPAR promoter all coupled to a luciferase reporter (PAI-1-, uPA- and uPAR-luc) were tested by transiently transfecting these constructs into the AGS2 cell line. These were newly made promoter-reporter constructs so their activity in a cell line was verified before their use in experiments utilising valuable human primary cells. As AGS2 cells are stably transfected with the CCK2R, gastrin was the first choice of stimuli since it has previously been shown to stimulate PAI-2 expression (Varro *et al.*, 2002b). Stimulation with 1nM G17, 8 hr resulted in an increase in luciferase activity with all constructs compared to unstimulated controls (Figure 4.2; uPAR-luc 2.53 $\pm$ 0.19 fold increase; uPA-luc 2.40 $\pm$ 0.09 fold increase; PAI-1-luc 1.78 $\pm$ 0.06 fold increase. Mean $\pm$ S.E, n=9). This showed all three constructs to be functionally correct and transcriptionally active, enabling their use in further experiments.

4.3.2 Transient transfection of human gastric gland cells with PAI-1-, uPA- or uPAR-luc

To allow the transcriptional regulation of PAI-1, uPA and uPAR by both *H. pylori* and specific growth factors to be studied in primary gastric epithelial cells, a methodology was developed to transfect these cells with luciferase promoter-reporter constructs. The functionality of PAI-1-, uPA- and uPAR-luc had previously been tested in AGS2 cells (Figure 4.2), but never before in primary human gastric glands in culture. Transfection was carried out as described in

section 2.5.2 using Magnetofection and cells were stimulated with exogenously added *H. pylori* or growth factors as indicated. The technique of Magnetofection using CombiMag reagent is able to transfect every cell type within the gland as an adherent cell culture, is highly efficient and ideal for transfection of primary cells. Further details of this methodology can be found at www.ozbiosciences.com/magentofection.html. Briefly, Magnetofection associates nucleic acids/ plasmids with magnetic nanoparticles coated with cationic molecules. The resulting molecular complexes are targeted to and endocyted by cells using an applied magnetic field in the form of a magnetic plate. Magnetic nanoparticles are completely biodegradable and are not toxic to the cells. Nucleic acids/ plasmids are released into the cytoplasm by destabilisation of the endosome by cationic lipids (TransFast) coated on the particles which release plasmids by a flip-flop mechanism. Appropriate concentrations of reagents used are given in section 2.5.2. Figure 4.3 outlines the protocol used to identify the specific cell types within the gastric glands that express the luciferase-reporter constructs or alternatively, luciferase activity measured after *H. pylori* or growth factor stimulation.

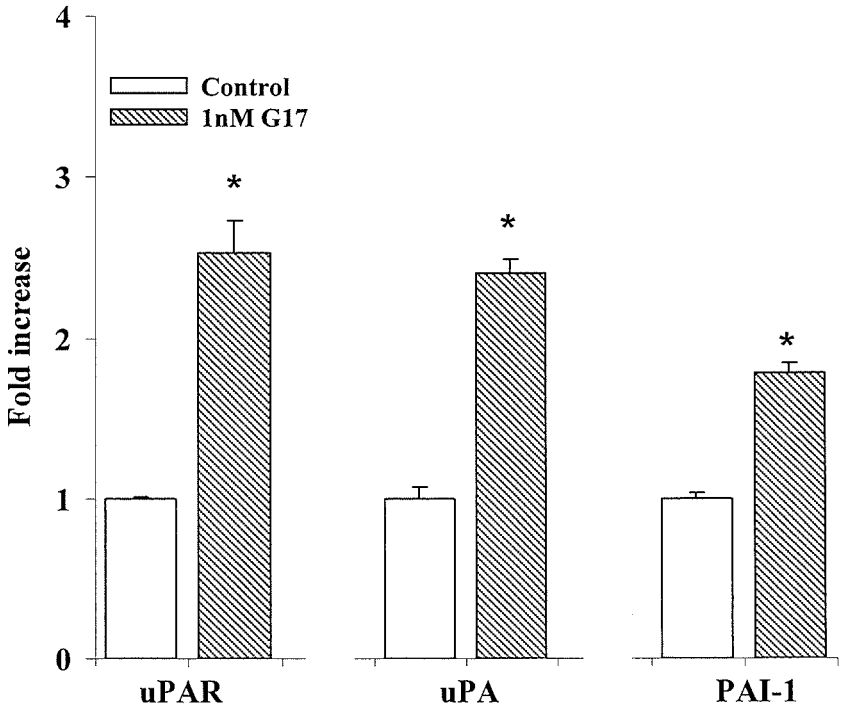

Figure 4.2

Gastrin stimulates expression of uPAR-, uPA- and PAI-1-luc.
Luciferase expression in AGS2 cells transfected with uPAR-, uPA or PAI-1-luc promoter-reporter constructs. Expression of all three constructs was significantly increased in response to 1 nM G17, 8 hr. Results are expressed as fold increase of luciferase expression after stimulation over unstimulated controls. (Mean ± S.E. n=9, *$p<0.05$, *t*-test).

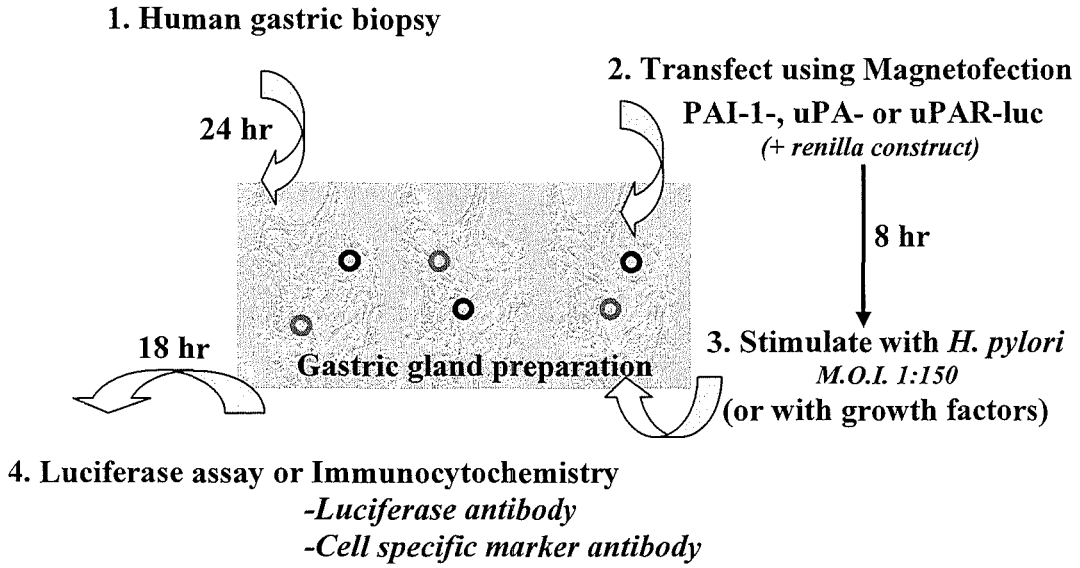

Figure 4.3

Schematic protocol for transfection of human gastric glands with uPAR, uPA- or PAI-1-luc.
Adherent gastric glands can be transfected with uPA system member luciferase promoter-reporter constructs using Magnetofection (see section 2.5.2 and 4.3.2 for details), allowing the transcriptional regulation of these genes by *H. pylori* or growth factors to be studied in luciferase assays. The specific cells types in which the constructs are expressed can be identified via immunocytochemistry using an anti-luciferase antibody and in addition various cell specific markers.

4.3.3 PAI-1-luc is targeted to cell types which also express endogenous PAI-1

To elucidate the cell types which 4.5kb of the human PAI-1 promoter coupled to a luciferase reporter (PAI-1-luc) are targeted to, and expressed by, human primary gastric glands were transiently transfected and dual labelling immunofluorescence carried out using an anti-luciferase antibody and various gastric cell specific markers. PAI-1-luc was targeted to, and expressed by, parietal cells, ECL cells and myofibroblasts as luciferase co-localised with H^+, K^+-ATPase, VMAT2 and vimentin (Figure 4.4 and Table 4.1). PAI-1-luc was highly expressed in parietal cells, ECL cells and to a lesser extent in myofibroblasts. The expression pattern of PAI-1-luc was not significantly different when cells were stimulated with wild-type *H. pylori* (Table 4.1). As PAI-1-luc is expressed in the same cell population as the endogenous protein (shown in section 3.3.6) it is a good reporter construct to use to study the regulation of PAI-1 expression.

4.3.4 uPA-luc is targeted to cell types which also express endogenous uPA

To confirm the cell types which 2.9kb of the human uPA promoter coupled to a luciferase reporter (uPA-luc) is targeted to and expressed by, similar experiments to that described above for PAI-1-luc were carried out. uPA-luc was found to directly target to, and be expressed in, parietal cells, ECL cells, chief cells and myofibroblasts as luciferase co-localised with H^+, K^+-ATPase, VMAT2, pepsinogen and vimentin (Figure 4.5 and Table 4.1). The expression pattern of uPA-luc was not significantly different when cells were stimulated with wild-type *H. pylori* (Table 4.1).

4.3.5 uPAR-luc is expressed by the same cell types which express endogenous uPAR

Similar experiments to that described above for uPA-luc and PAI-1-luc were carried out to determine if 2kb of the human uPAR promoter coupled to a luciferase reporter (uPAR-luc) exhibits cell restricted expression within human primary gastric glands. uPAR-luc was found to have direct targeted expression to parietal cells, ECL cells, chief cells, surface mucous cells and myofibroblasts as luciferase co-localised with H^+, K^+-ATPase, VMAT2, pepsinogen, TFF-1 and vimentin (Figure 4.6 and Table 4.1). The expression pattern of uPAR-luc was not significantly different when cells were stimulated with *H. pylori* (Table 4.1). Endogenous uPAR was also found to be expressed in all five of the cell types in which uPAR-luc expressed in (shown in section 3.3.7), therefore making it a very suitable expression vector to use in further studies of uPAR.

4.3.6 PAI-2-luc is expressed by a variety of gastric epithelial cells

Endogenous PAI-2 has previously been shown to be expressed by a variety of gastric epithelial cells (Varro *et al.*, 2004) and the 2.3kb of the human PAI-2 promoter coupled to luciferase reporter (PAI-2-luc) has been extensively used in AGS2 cell line work. In the context of the other uPA system members expression patterns, it was also determined if PAI-2-luc exhibited cell restricted expression. Primary human gastric glands transiently transfected with PAI-2-luc showed that this reporter construct was expressed in parietal cells, ECL cells, surface mucous cells, neck cells and myofibroblasts. Luciferase was found to co-localised with H^+, K^+-ATPase, VMAT2, TFF-1, TFF-2 and vimentin (Figure 4.7). PAI-2-luc expression correlates well with endogenous PAI-2 protein expression which was

shown to be expressed in mucous cells, chief cells, parietal cells and ECL cells within primary human gastric glands (Varro *et al.*, 2004).

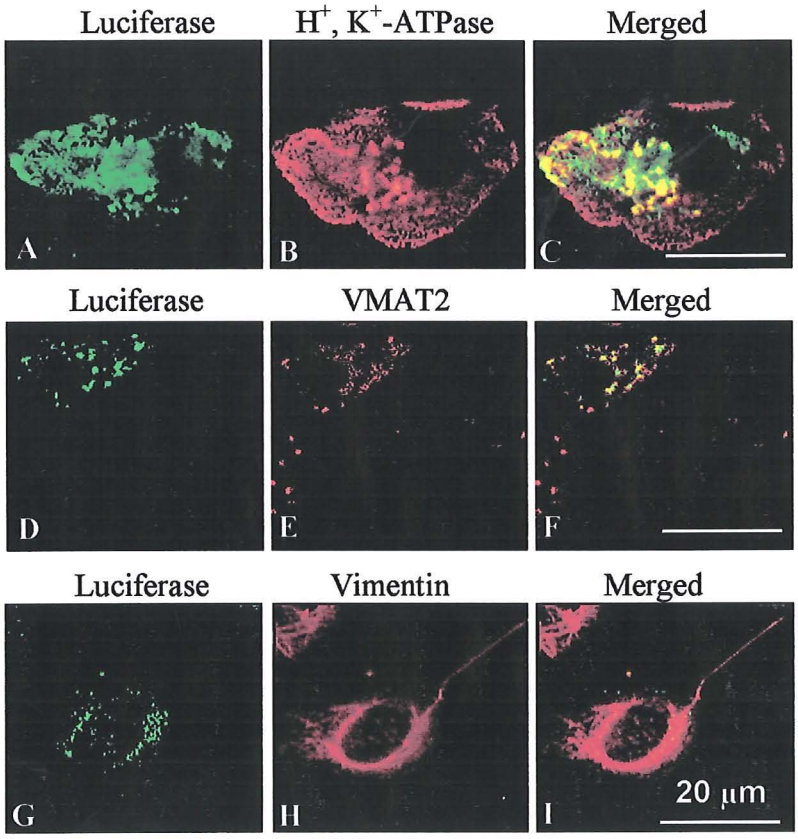

Figure 4.4

4.5kb of the human PAI-1 promoter targets expression of PAI-1-luc to specific cell types in human primary gastric glands.
A-C, PAI-1-luc (green) expression in H^+, K^+-ATPase (red) positive parietal cell. D-F, PAI-1-luc (green) expression in VMAT-2 (red) positive ECL cell. G-I, PAI-1-luc (green) expression in vimentin (red) positive myofibroblast.

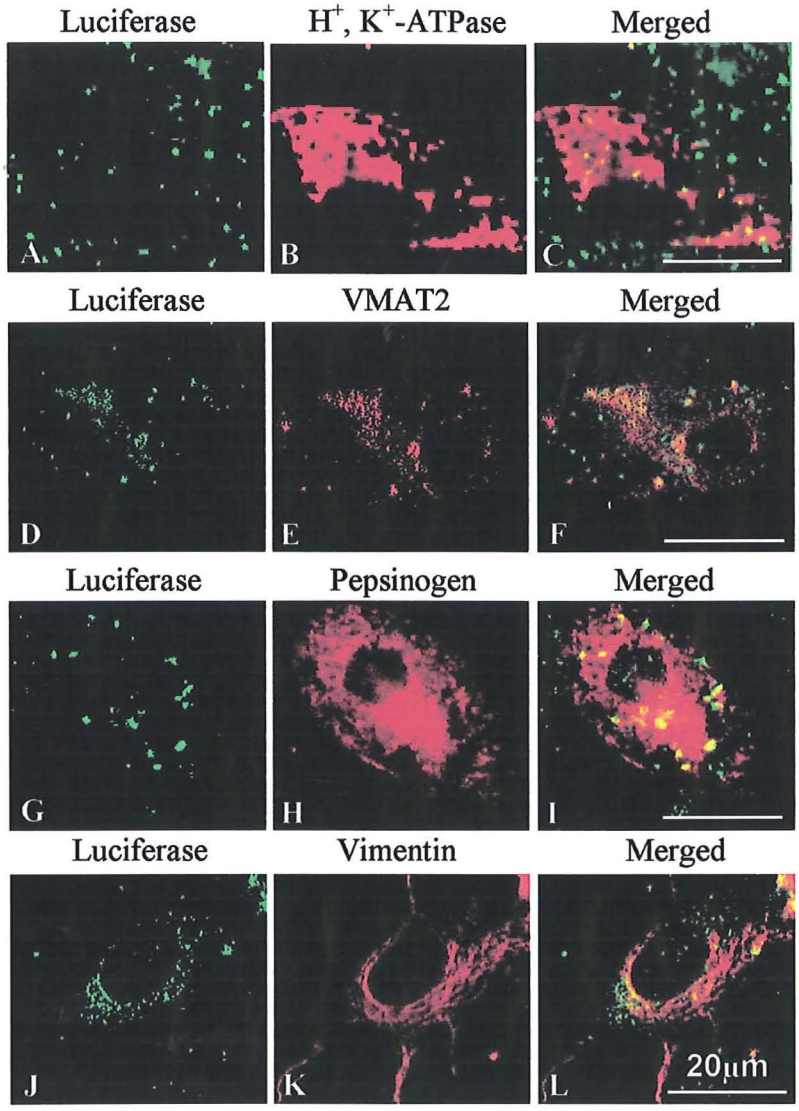

Figure 4.5

2.9kb of the human uPA promoter targets expression of uPA-luc to specific cell types in human primary gastric glands.
A-C, uPA-luc (green) expression in H^+, K^+-ATPase (red) positive parietal cell. D-F, uPA-luc (green) expression in VMAT-2 (red) positive ECL cell. G-I, uPA-luc (green) expression in pepsinogen (red) positive chief cell. J-L, uPA-luc (green) expression in vimentin (red) positive myofibroblast.

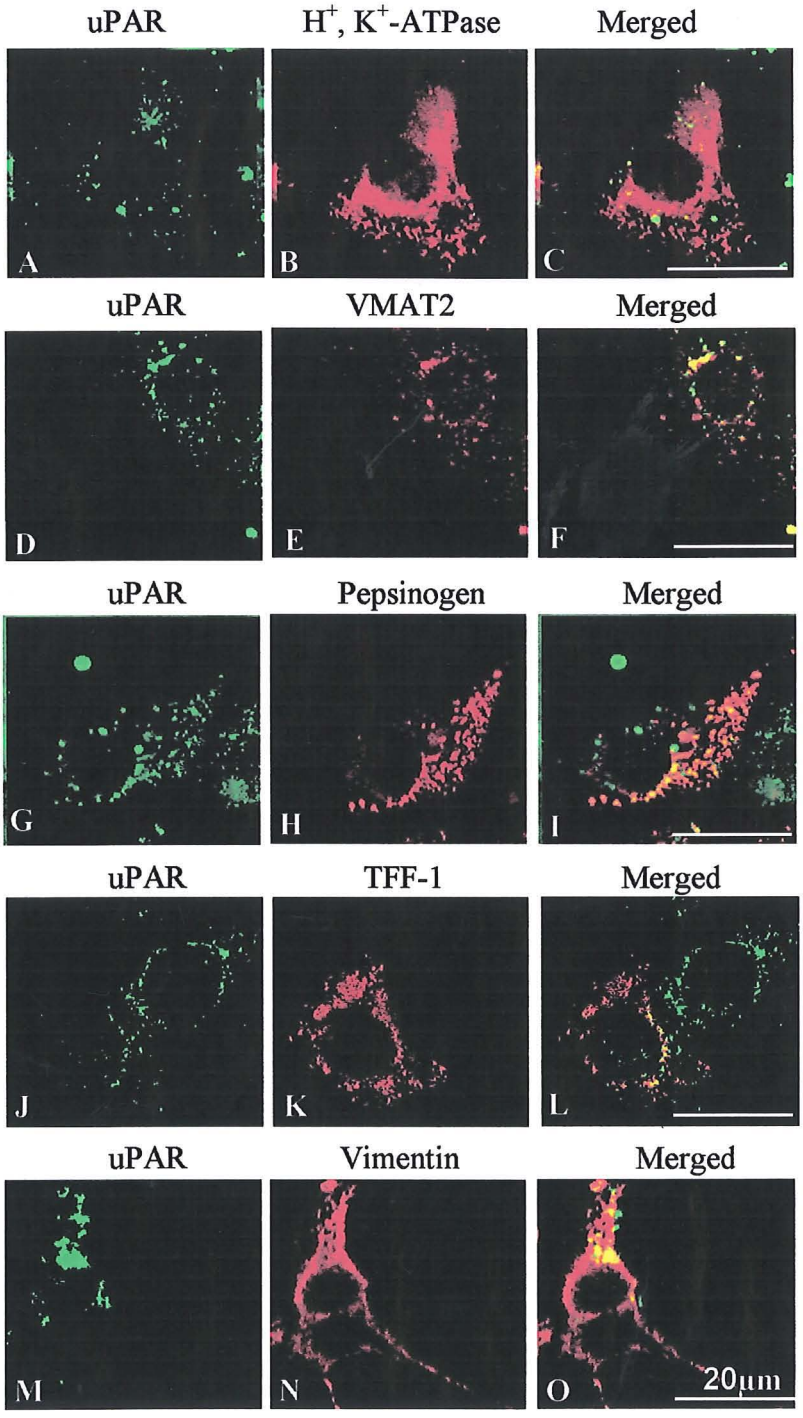

Figure 4.6

2kb of the human uPAR promoter targets expression of uPAR-luc to specific cell types in human primary gastric glands.
A-C, uPAR-luc (green) expression in H^+, K^+-ATPase (red) positive parietal cell. D-F, uPAR-luc (green) expression in VMAT-2 (red) positive ECL cell. G-I, uPAR-luc (green) expression in pepsinogen (red) positive chief cell. J-L, uPAR-luc (green) expression in TFF-1 (red) positive cell. M-O, uPA-luc (green) expression in vimentin (red) positive myofibroblast.

		TFF-1	VMAT-2	Pepsinogen	H^+, K^+-ATPase	Vimentin
% PAI-1-luc expressing cells	***-H.p***	<1	48.83 ± 8.50	<1	68.06 ± 7.58	6.91 ± 2.54
% uPA-luc expressing cells	***-H.p***	<1	36.55 ± 3.61	22.00 ± 3.93	39.94 ± 3.55	13.01 ± 3.92
% uPAR-luc expressing cells	***-H.p***	7.00 ± 3.59	47.67 ± 5.05	3.43 ± 2.33	34.04 ± 1.91	7.7 ± 2.66

Table 4.1

Proportion of PAI-1-, uPA- and uPAR-luc expressing cells in human primary cultured gastric glands.
Proportion of PAI-1-, uPA- and uPAR-luc expressing cells that also express either TFF-1, VMAT-2, pepsinogen, H^+, K^+-ATPase or vimentin. Note <1% of PAI-1-luc, uPA-luc or uPAR-luc expressing cells express TFF-2. Table shows quantification data in control, *H. pylori* negative glands. Stimulation with *H. pylori* MOI 1:150 18 hr gave no significant difference in distribution patterns. (mean ± S.E, n=3-5 patients).

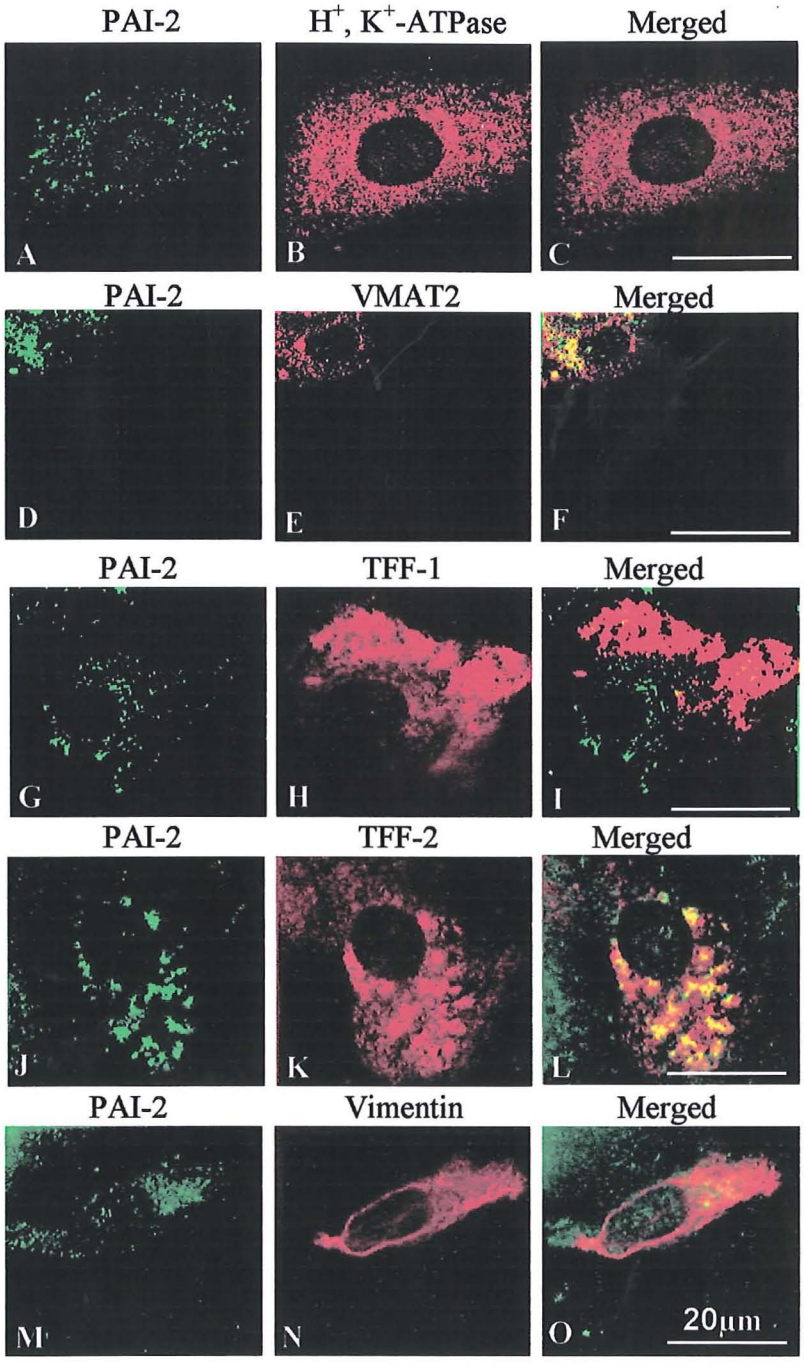

Figure 4.7

2.3kb of the human PAI-2 promoter targets expression of PAI-2-luc to specific cell types in human primary gastric glands.
A-C, PAI-2-luc (green) expression in H^+, K^+-ATPase (red) positive parietal cell. D-F, PAI-2-luc (green) expression in VMAT-2 (red) positive ECL cell. G-I, PAI-2-luc (green) expression in TFF-1 (red) positive cell. J-L, PAI-2-luc (green) expression in TFF-2 (red) positive cell. M-O, PAI-2-luc (green) expression in vimentin (red) positive myofibroblast.

4.3.7 Induction of uPAR-, uPA- and PAI-1-luc by *H. pylori* in gastric epithelial cells

The protocol outlined in Figure 4.3 was used to investigate if *H. pylori* directly stimulates expression of uPAR-, uPA- and PAI-1-luc in primary gastric epithelial cells. Results presented in Chapter 3 showed increased PAI-1 mRNA abundance and protein expression in gastric epithelial cells in response to *H. pylori* infection in human patients with the same being true for both uPA and uPAR (Cédric Duval, PhD thesis 2007). It is therefore interesting to study the regulation of these three proteins by *H. pylori* in primary epithelial cells in culture using promoter-reporter constructs thus determining if this *in vitro* model can be used to explain the cellular *in vivo* regulation mechanisms. Wild-type *H. pylroi* (strain 60190) significantly increased the expression of uPAR-, uPA- and PAI-1-luc in human gastric gland cells compared to non-stimulated controls (Figure 4.8; uPAR-luc 1.8 $\pm$ 0.2 fold increase; uPA-luc 2.1 $\pm$ 0.5 fold increase; PAI-1-luc 3.0 $\pm$ 0.3 fold increase. Mean $\pm$ S.E, n=6 patients).

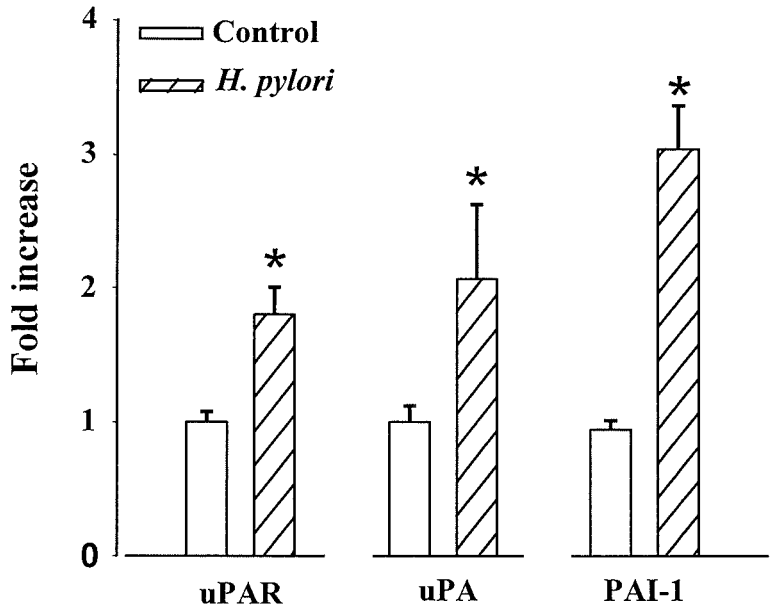

Figure 4.8

***H. pylori* stimulates uPAR-, uPA- and PAI-1-luc in human gastric gland cells.**

H. pylori M.O.I. 1:150 18 hr stimulated the expression of 2kb of the human uPAR, 2.9kb of the human uPA and 4.5kb of the human PAI-1 promoter in gastric epithelial cells. Results are expressed as fold increase in luciferase expression after stimulation compared to non-stimulated controls. (mean $\pm$ S.E, n=6 patients. *$p<0.05$, *t*-test).

4.3.8 Mechanism of *H. pylori* induction of uPAR-, uPA- and PAI-1-luc

To examine the mechanisms by which *H. pylori* up-regulates uPAR-, uPA- and PAI-1-luc, and to define the precise role of bacterial virulence factors in induction of these three genes, transiently transfected human gastric gland cells were stimulated with a series of isogenic *H. pylori* mutants. A similar response for the increase in uPAR-, and PAI-1-luc to that seen with the wild-type 60190 strain (Figure 4.8) was obtained when cells were stimulated with another wild-type *H. pylori* strain (84-183), and mutant strains that were *cagE* null, *cagA* null and *vacA* null (Figure 4.9 A and C). In contrast, uPA-luc expression was stimulated by the alternative wild-type strain (84-183) but expression was abolished by a *cagE* null *H. pylori* mutant (Figure 4.9 B).

4.3.9 Regulation of PAI-1-luc by growth factors

Previous studies have indicated that *H. pylori* induces epithelial cell release of various chemokine, cytokine and paracrine mediators. The possibility that *H. pylori* stimulates PAI-1-luc via paracrine mechanisms involving several growth factors was considered. Gastrin is known to stimulate PAI-2 expression in AGS2 cells (Varro *et al.*, 2002b) and real-time PCR data has previously shown PAI-1 mRNA abundance to be increased in patients with elevated gastrin concentrations (≥30 pM; John Sammut, Msc Thesis, 2004). The possibility of PAI-1 being a target of gastrin was investigated. As gastrin is both produced by, and known to act on epithelial cells the concept of PAI-1 expression being increased by growth factors such as HGF and IGF-II secreted from mesenchymal cells (such as myofibroblasts), but known to act on epithelial cells was explored (Sonnenberg *et al.*, 1993; McCaig *et al.*, 2006). PAI-1-luc expression was found

to be significantly increased by all three growth factors (Figure 4.10; IGF-II 2.02 $\pm$ 0.20 fold; HGF 2.33 $\pm$ 0.57 fold; G17 2.38 $\pm$ 0.74 fold; Mean $\pm$ S.E, n=3-9 patients).

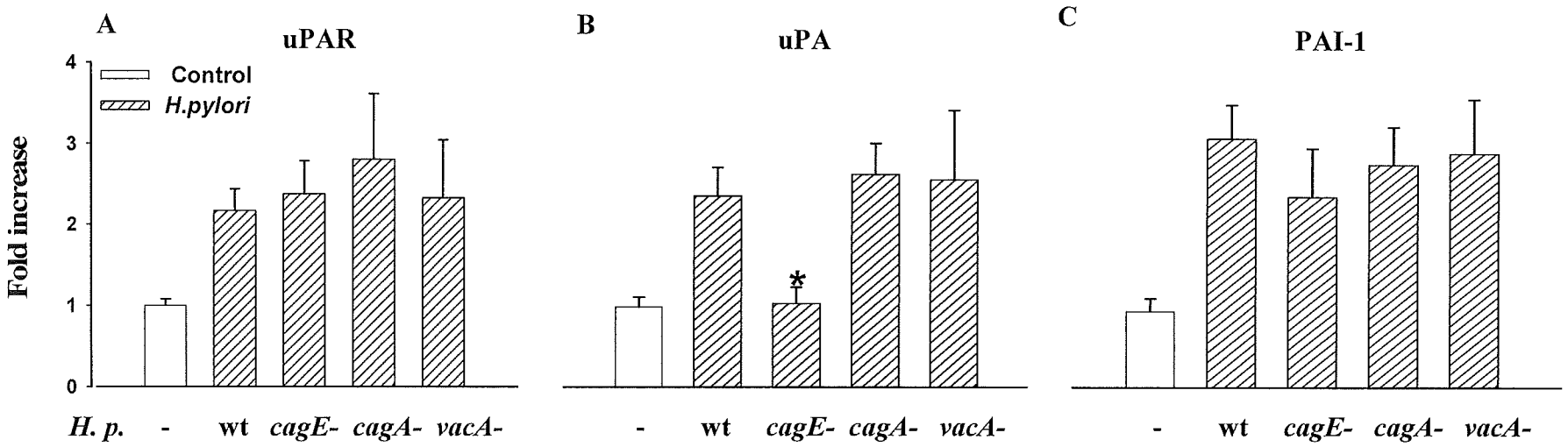

Figure 4.9

uPA but not uPAR or PAI-1 luciferase expression is abolished by a CagE null *H. pylori* mutant in human primary gastric epithelial cells. Luciferase expression in human primary gastric glands transfected with either uPAR-luc (A), uPA-luc (B), or PAI-1-luc (C) promoter-reporter constructs. Expression of all three constructs was significantly increased in response to wild-type (wt) *H. pylori* infection (M.O.I. 1:150). Deletion of *cagE*, *cagA* and *vacA* had no effect on PAI-1-luc or uPAR-luc expression. However, uPA-luc expression was abolished by a *cagE* null *H. pylori* mutant. Results are shown as fold increase in luciferase expression compared to non-stimulated controls. (Mean $\pm$ S.E, n=5 patients, * $p<0.05$, ANOVA).

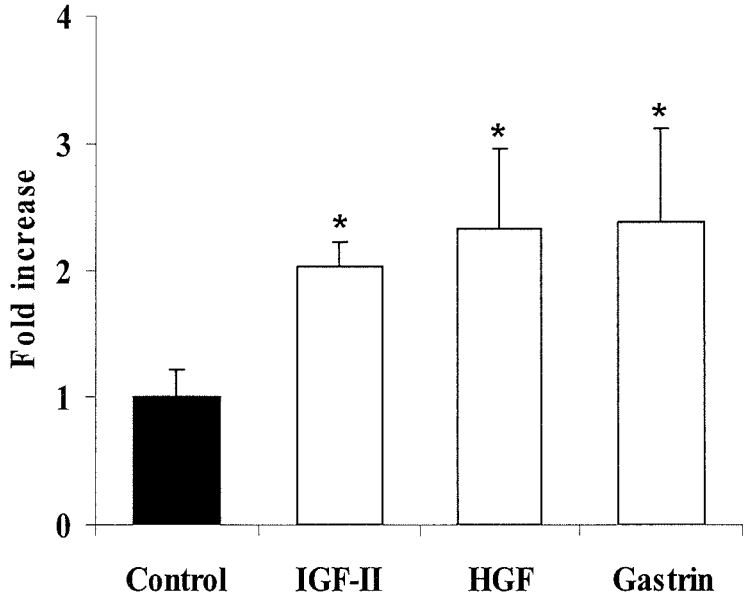

Figure 4.10

IGF-II, HGF and gastrin increase PAI-1-luc expression
IGF-II (100ng/ml), HGF (40ng/ml) and gastrin (1nM) 18 hr stimulated 4.5kb of the human PAI-1 promoter in gastric epithelial cells. Results are expressed as fold increase in luciferase expression after stimulation over non-stimulated controls. (Mean + S.E, n=3-9 patients, *$p<0.05$, ANOVA).

4.4 Discussion

Results presented in this chapter show that *H. pylori* can stimulate expression of luciferase promoter reporter constructs containing either 4.5kb of the human PAI-1 promoter, 2.9kb of the human uPA promoter or 2kb of the human uPAR promoter in human primary gastric epithelial cells. This is in excellent agreement with RT-PCR and immunohistochemical data displayed in Chapter 3 of this thesis (Figures 3.1 and 3.2), showing increased PAI-1 mRNA and endogenous protein expression in *H. pylori* infection. Endogenous uPA and uPAR protein and mRNA abundance have also been shown to be increased by *H. pylori* in infected human subjects (Cedric Duval, PhD thesis, 2007).

The promoter sequences of PAI-1-, PAI-2-, uPA- and uPAR-luciferase constructs were found to selectively target expression to specific cell types within human primary gastric gland cells. It is important to note that these luciferase vectors are generally expressed in cell types normally associated with expression of the corresponding endogenous protein.

Recent investigations into mechanisms underlying *H. pylori* induced gastric cancer have highlighted that disease involves well choreographed interactions between host and pathogen involving differences between bacterial strains, variations in individual hosts or environmental influences (discussed in section 1.3; Blaser & Berg, 2001). Patients infected with *H. pylori cag*+ strains are at a higher risk of developing distal gastric cancer (Crabtree *et al.*, 1993, Kuipers *et al.*, 1995, Blaser *et al.*, 1995; McColl *et al.*, 1997), and genes contained within the *cag*-PAI are required for

induction of several epithelial cell responses relevant to pathogenesis (Tummuru *et al* 1995; Sharma *et al.*, 1998; Naumann *et al.*, 1999). Stimulation of PAI-1-, uPA- and uPAR-luc by *H. pylori* occurs via distinct mechanisms as expression of all three were unaltered by isogenic mutants lacking CagA and VacA. However, uPA expression was virtually abolished by a CagE null mutant. This demonstrates a requirement for an intact and fully functional *cag*-PAI island and a role for CagE in the up-regulation of uPA by *H. pylori.*

Interestingly, INS-GAS mice infected with *H. pylori* with inactivated CagE show delayed progression to neoplasia compared to those animals infected with wild-type bacteria strains (Fox *et al.*, 2003a). The release of certain chemokines such as IL-8 from gastric epithelial cells has been found to require *cagE* (Keates *et al.*, 1999) and this gene is also needed for activation of several transcription factors in gastric cancer cell lines, including NF-κB and AP1 which are known to play a role in transcriptional regulation of uPA (Novak *et al.*, 1991; Kawasaki *et al.*, 1998; Mitsuno *et al.*, 2001; Guillemin *et al.*, 2002).

As well as *cag*-PAI and VacA there are at least 32 outer membrane proteins involved in bacterial adherence that have been identified on the cell surface of *H. pylori* which could also act as virulence factors (discussed in section 1.3.3.1). Possible important proteins include the blood group antigen-binding adhesin (BabA), the outer membrane inflammatory protein (OipA) and sialic acid-binding adhesin (SabA; Lu *et al.*, 2005a). *H. pylori* strains containing a functional copy of OipA are linked with more severe gastric

inflammation, increased levels of IL-8 and higher bacterial colonisation (Peek, 2002). It could be proposed that *H. pylori* virulence factors other than *cagE*, *cagA* or *vacA* are responsible for the increase in expression of PAI-1 and uPAR such as plasminogen binding proteins.

There is accumulating evidence that the transformation of the 'normal' *H. pylori* infected gastric mucosal cells into malignant cells and the development of gastric cancer requires the action of mucosal promoting factors such as gastrin, EGF, HGF, IL-8 and TGF-α (Crabtree *et al.*, 1993; Boyer *et al.*, 2000) and epithelial-mesenchymal interactions (discussed in section 1.4). The increase in PAI-1 expression after stimulation with gastrin and growth factors such as HGF and IGF-II demonstrates that both epithelial- and mesenchymal-derived factors are important for the regulation of this protease inhibitor.

The specific signalling pathways involved in the induction of PAI-1 gene expression in gastric epithelial cells have not been investigated but are likely to be complex and further work is needed to elucidate mediators which are responsible for *H. pylori*, HGF, IGF-II and gastrin induction of PAI-1. The human PAI-1 promoter has been extensively characterised and is known to contain consensus sequences for several known regulatory elements (discussed in section 1.5.4; Kunz *et al.*, 1995; Grubic *et al.*, 1996; Dennler *et al.*, 1998) and is induced by a number of controls (Emeis *et al.*, 1986; Alessi *et al.*, 1988; Hopkins *et al.*, 1991; Sperti *et al.*, 1992; Nordt *et al.*, 1995; Le Magueresse- Battistoni *et al.*, 1997; Pinsky *et al.*, 1998; Brown *et al.*, 2000).

There is a body of evidence linking PAI-1 expression to the processes of wound healing and epithelium repair (Providence *et al.*, 2000; Xia *et al.*, 2003; Providence *et al.*, 2004). Enhanced PAI-1 expression in the gastric epithelium by acute infection with *H. pylori* (and growth factors associated with infection) suggests this protease inhibitor could initially act to protect gastric epithelial cells by decreasing fibrinolysis in areas of damaged mucosa. Also, Chapter 3 of this thesis highlights a role for PAI-1 in the control of epithelial cell proliferation by inhibiting the proliferative effects of uPA (also shown to increase the proliferation, migration and adhesion of gastric myofibroblats which were further increased by PAI-1 knock-down; Cédric Duval, PhD thesis, 2007). This signifies that increased PAI-1 not only acts to restrain uPA within the gastric epithelium, therefore controlling cellular proliferation, but it could also participate in epithelial-mesenchymal interactions helping to protect the gastric mucosa from processes which are thought to play an important role in altering the 'niche' that favours premalignant changes.

In premalignant states such as long-term infection with *H. pylori,* associated with chronic atrophic gastritis, increased PAI-1 may act in an oncogenic manner, accelerating epithelial remodelling contributing to the progression of gastric cancer. Correlating with clinical data of high PAI-1 expression in patients suffering from a variety of tumours (Andreason *et al.*, 2000; Foekens *et al.*, 2000; Rakic *et al.*, 2003), PAI-1 can modulate cellular migration and have a promigratory effect (Deng *et al.*, 1996; Stefansson *et al.*, 1996; Kjoller *et al* 1997).

4.5 Conclusions

1. PAI-1-, uPA- and uPAR-luc exhibit cell restricted expression and are expressed in similar gastric gland cells as their endogenous proteins.
2. *H. pylori* stimulates expression of PAI-1-, uPA- and uPAR-luc in human primary gastric gland cells.
3. uPA, but not uPAR or PAI-1 expression is abolished by CagE null *H. pylori* mutant.
4. Both epithelial and myofibroblast derived growth factors such as IGF-II, HGF and G17 stimulate PAI-1-luc expression in gastric epithelial cells.

Chapter 5

Contribution of PAI-1 to the response of epithelial cell damage using uPA system transgenic mice

5.1 Introduction

Haemostasis is a complex interplay between platelet aggregation, coagulation and fibrinolysis and is a vital defence system that rapidly stops haemorrhage after injury. The uPA system is fundamental to this process as it is responsible for the activation of plasminogen to plasmin (discussed in section 1.5), the protease that degrades fibrin, the main component of clots. The importance of PAI-1 in the stabilisation of the primary haemostatic plug formed after injury is not fully established, but it is known that PAI-1 is the primary inhibitor of plasminogen activators, and the release of PAI-1 by aggregating platelets (Erickson *et al.*, 1984; Kruithof *et al.*, 1986), may provide a means to prevent premature fibrinolysis, therefore contributing to the stabilisation of the haemostatic plug in the early stages of haemostasis.

Gastric ulcer is a multifaceted disease with a complex pluricasual etiology that is not fully understood. To illustrate the complexity of gastric ulcer pathogenesis several experimental models of gastric mucosal damage exist which mimic ulcer disease and have been well characterised. These include an ethanol-induced acute gastric mucosal injury model (Mozsik & Javor, 1988), and a stress-induced mucosal injury model (Cho & Ogle, 1992). Examination of the former indicates that gastric damage is produced as a consequence of many interacting factors and is representative of human gastric ulcer disease (Silen, 1988). A hypothesis used to account for gastric mucosal injury or resistance to injury is that of an interaction and balance between aggressive and defensive factors.

The gastric epithelium is exposed to various physical and chemical factors such as infection with *H. pylori*, which is regarded as an aggressive factor and is

associated with epithelial cell damage, inflammation of the gastric mucosa (gastritis) and duodenal ulcerogenesis (discussed in section 1.3.2; Tytgat & Rauws, 1990). Defensive mechanisms within the gastric mucosa are represented by classic factors such as mucus (Wallace & Whittle, 1986) and bicarbonate (Kivilaakso, 1981) secretions, prostaglandins (Konturek & Pawlik, 1986) and NO (discussed in section 1.2.6; MacNaughton *et al.*, 1989).

Gastric mucosal damage can be produced by direct application of ethanol where injury is limited to the gastric epithelium and involves mostly the inter-foveolar epithelium and gastric pits. However, deeper lesions involve intramucosal haemorrhage and vascular engorgement (Guth *et al.*, 1984). Due to damage, microvessels leak inflammatory mediators and vasoconstriction of submucosal arteries results in ischemia which eventually enhances the formation of more severe necrotic mucosal injury.

Ethanol-induced gastric mucosal lesions can be produced reliably and simply by intragastric administration of concentrated (50 - absolute) ethanol. Depending on the amount of ethanol given, 1-2 hr after administration between 10 and 40% of the glandular stomach of both rats and mice have been shown to display epithelial damage, characterised by mucosal edema, subepithelial haemorrhages, cellular exfoliation and inflammatory cell infiltration (Guslandi, 1987). Injury is attributed to congestion of the mucosa caused by constrictions of collecting venules (Ohno *et al.*, 1995; Ohno *et al.*, 1999; Saeki *et al.*, 2004). Time course studies have revealed that most gastric damage observed after ethanol administration occurs within 1-3 min of its instillation into the gut (Szabo, 1987).

Several studies have highlighted a tight correlation between the expression of uPA system components and wound re-epithelisation, with the majority of studies indicating PAI-1 as the major player (Pawar *et al*; 1995; Li *et al.*, 2000; Providence *et al.*, 2000; Providence *et al.*, 2002; Providence *et al.*, 2004). PAI-1 is an acute phase protein, can be induced during inflammation (Quax *et al.*, 1990; Swaisgood *et al.*, 2000; Luyendyk *et al.*, 2004; Lagoa *et al.*, 2005) and is increased in response to injury (Varela *et al.*, 2002). As well as its role in control of fibrinolysis, PAI-1 is also involved in the process of tissue repair in several different experimental settings (Providence *et al.*, 2000; Providence & Higgins 2004; Maquerlot *et al.*, 2006). The expression of PAI-1 in the gastric epithelium during mucosal injury has yet to be fully investigated although high levels of PAI-1 are found in duodenal ulcers (Herszenyi *et al.*, 1997).

5.1.1 Aims

The aim of this chapter was to investigate the involvement of PAI-1 in short term gastric mucosal protection using an acute *in vivo* model of gastric mucosal injury. The specific objectives were to:

1. develop transgenic mice with targeted over-expression of PAI-1 in the parietal cells of the stomach;
2. study the role of PAI-1 in gastric mucosal protection by examining the degree and severity of epithelial cell damage after challenge in wild-type, PAI-1 KO and PAI-1 over-expressing mice;
3. demonstrate a possible uPA independent role of PAI-1 in gastric mucosal protection, using mice in which the uPAR receptor is deficient.

5.2 Materials and methods

5.2.1 Induction of haemorrhagic lesions within the gastric mucosa

All animals were housed as described in section 2.2. Haemorrhagic lesions were induced within the gastric mucosa of male or female C57BL/6, PAI-1-KO, uPAR-KO, PAI-1 over-expressing and uPA over-expressing mice (10-12 weeks) using an acute ethanol model of epithelial damage (as described in section 2.13). Both the number and length of haemorrhagic lesions per animal was recorded using callipers and a small ruler. Individual mouse stomachs were also graded as possessing overall either 'weak', 'moderate' or 'severe' lesions and photographed using a Canon Digital IXUS iZoom camera with 5.0 mega pixels and a 2.4 x zoom lens. Lesion length per animal was determined by totalling both the number and length of each individual lesion. Average lesion length was calculated per group of animals.

5.2.2 Development and production of PAI-1 over-expressing mice

5.2.2.1 Expression of PAI-1

AGS2 cells were transfected with either pcDNA3 or pcDNA3-PAI-1 plasmids (as described in section 2.5.2) and left to express for 48 hr. Protein was extracted and western blot analysis carried out (as described in section 2.9). A mouse-anti-PAI-1 antibody (American Diagnostica) was used at a concentration of 1:100 and blots were re-probed with a goat-anti-β-actin antibody used at a concentration of 1:5000 (Santa Cruz Biotechnology).

5.2.2.2 Transfection of murine gastric gland cells

Murine gastric gland cells (isolated as in section 2.4.3) were transfected (3μg total DNA) with an expression plasmid in which murine the PAI-1 cDNA sequence had been coupled to the β-subunit of the H^+, K^+-ATPase promoter (HKB-PAI-1; as described in section 5.5.4 and plasmid map shown in Figure 5.6-A) using the same protocol as for human gastric gland transfection (as described in section 2.5.2). Cells were fixed 48 hr after transfection in 4% PFA. Dual-immunolabelling was carried out (as described in section 2.7) using a rabbit-anti-PAI-1 antibody at a concentration of 1:200 (Santa Cruz Biotechnology) and a goat-anti-ezrin (C-15) antibody at a concentration of 1:100 (Santa Cruz Biotechnology). Control, un-transfected murine gastric gland cells were also dual-immunolabelled with the above antibodies. More than 20 control and transfected glands were scored for the percentage of cells expressing, PAI-1, ezrin, or both. Results are displayed as percentage relative increase compared to control glands (taken to be 100%).

5.3 Results

5.3.1 PAI-1 KO mice develop more severe haemorrhagic lesions than wild-type or uPAR KO mice

To investigate the contribution of PAI-1 in the process of epithelial cell damage, an experimental *in vivo* model of gastric mucosal injury was carried out. Ethanol-induced gastric mucosal lesions effect epithelial cells within the stomach mucosa and can be produced reliably by intragastric administration of varying ethanol concentrations (50% and absolute ethanol). This model has previously been shown to induce gastric ulcers or what are termed 'haemorrhagic lesions' within the stomach of both rats and mice (Szabo *et al.*, 1987; Mozsik *et al.*, 1988; Pan *et al.*, 2005).

Commercially available PAI-1 KO mice, uPAR KO mice and wild-type C57BL/6 mice all showed the presence of haemorrhagic lesions within the gastric mucosa after challenge with 50% or absolute ethanol, although to varying extents (Figure 5.1). Untreated control animals showed the appearance of normal, unaltered tissue. However, 50% ethanol induced several small, pinpoint like haemorrhagic lesions termed petichae in C57BL/6 mice and slightly longer, thicker and more abundant lesions were seen in uPAR KO mice. The length, thickness and number of haemorrhagic lesions increased further in PAI-1 KO mice.

Wild-type mice developed more severe lesions when challenged with absolute ethanol compared to 50% ethanol, as did both uPAR KO and PAI-1 KO mice. However, the severity of the lesions formed in PAI-1 KO mice after absolute ethanol treatment were more severe than those seen in uPAR KO or C57BL/6

mice. These lesions were very thick, long and aggressive often covering the majority of the surface area of the mucosa.

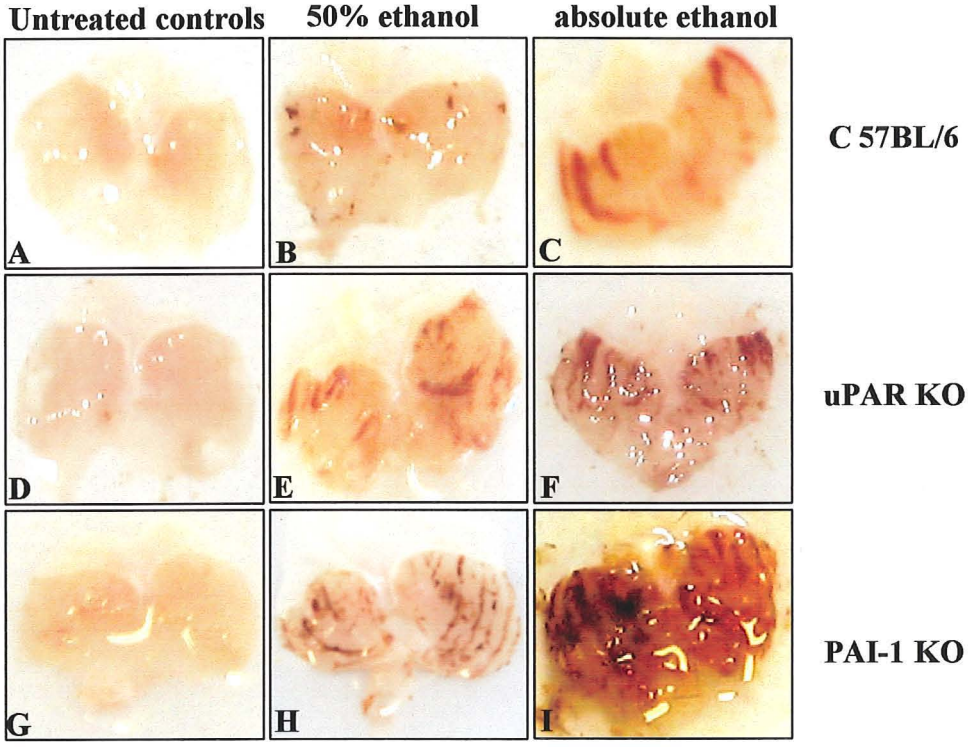

Figure 5.1

PAI-1 KO mice develop more severe haemorrhagic lesions than wild-type or uPAR KO mice after ethanol administration.
Representative photographs of haemorrhagic lesions formed inside the stomachs of C57BL/6, uPAR KO and PAI-1 KO mice after 1 hr treatment with 50% or absolute ethanol. A-C, C57BL/6 untreated control, 50% ethanol and absolute ethanol. D-F, uPAR untreated control, 50% ethanol and absolute ethanol. G-I, PAI-1 KO untreated control, 50% ethanol and absolute ethanol (n>10 animals).

5.3.2 PAI-1 KO mice display a more severe phenotype when challenged with ethanol that uPAR KO or wild-type mice

To quantify the extent of epithelial cell damage caused after ethanol administration, the gastric mucosa of each animal was graded as having the formation of 'weak', 'moderate' or 'severe' haemorrhagic lesions, depending on the number, length, aggressiveness and the area of the mucosa covered by lesions. The percentage of animals in each group with weak, moderate or severe lesions was recorded (Figure 5.2).

After challenge with 50% ethanol, 100% of C57BL/6 mice were graded as having weak lesions and no animals in this group displayed moderate or severe lesions. Weak lesions were developed by 72% of uPAR KO mice and the remaining 28% developed moderate lesions. Only 17% of PAI-1 KO animals challenged with 50% ethanol displayed weak lesions with a massive 83% graded as possessing moderate haemorrhagic lesions.

With absolute ethanol treatment fewer C57BL/6 mice developed weak lesions compared to with 50% ethanol, as more animals developed moderate lesions. However, only 10% of wild-type mice developed severe lesions after absolute ethanol treatment compared to 25% of uPAR KO mice and 55% of PAI-1 KO mice. This data shows that PAI-1 KO mice develop a more severe phenotype and display a greater extent of tissue damage after challenge than uPAR KO or wild-type mice.

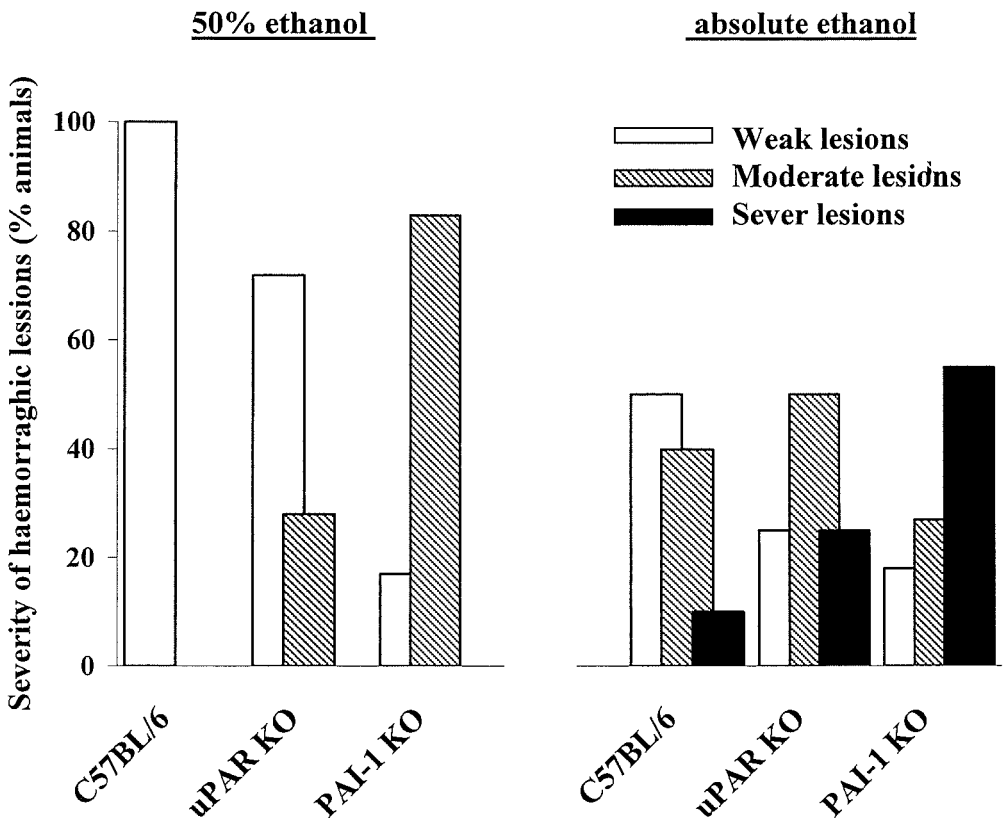

Figure 5.2

PAI-1 KO mice have a more severe phenotype when challenged with ethanol than uPAR KO or wild type mice.
Percentage of wild-type (C57BL/6), uPAR KO and PAI-1 KO mice graded as developing weak, moderate or severe haemorrhagic lesions after treatment with either 50% or absolute ethanol. There are no severe lesions after 50% ethanol insult in any strain of mouse, and no moderate lesions in C57BL/6 mice.

5.3.3 PAI-1 KO mice develop more severe haemorrhagic lesions with an overall greater lesion length than wild-type or uPAR KO mice

As another measure of quantification of tissue damage after ethanol challenge, both the number and length of lesions formed within the gastric mucosa of each animal was measured as accurately as possible and each animal given a lesion score. This corresponded to the total length of each individual lesion. The average lesion score for each group of animals (C57BL/6, uPAR KO and PAI-1 KO) was recorded after 50% and absolute ethanol treatment. When challenged with 50% ethanol (Figure 5.3), PAI-KO mice had a significantly greater average lesion length score (34.25 $\pm$ 4.74mm) compared to uPAR KO (13.86 $\pm$ 2.63mm) or C57BL/6 mice (7.64 $\pm$ 0.91mm). All three groups of animals displayed a greater lesion score with absolute ethanol than with 50% ethanol treatment, but the lesion score for PA I-1 KO mice remained signific antly higher (51.91 $\pm$ 5.11mm) than either uPAR KO (39.38 $\pm$ 5.37mm) or wild-type mice (33.42 $\pm$ 2.90mm). Although there was a slight difference in lesion length between uPAR KO and C57BL/6 mice with both concentrations of ethanol (significant only with 50% ethanol), it was not as substantial as the difference in lesion length between PAI-1 KO and wild-type mice. This data highlights that knocking out PAI-1 gives rise to more severe gastric mucosal injury after ethanol challenge.

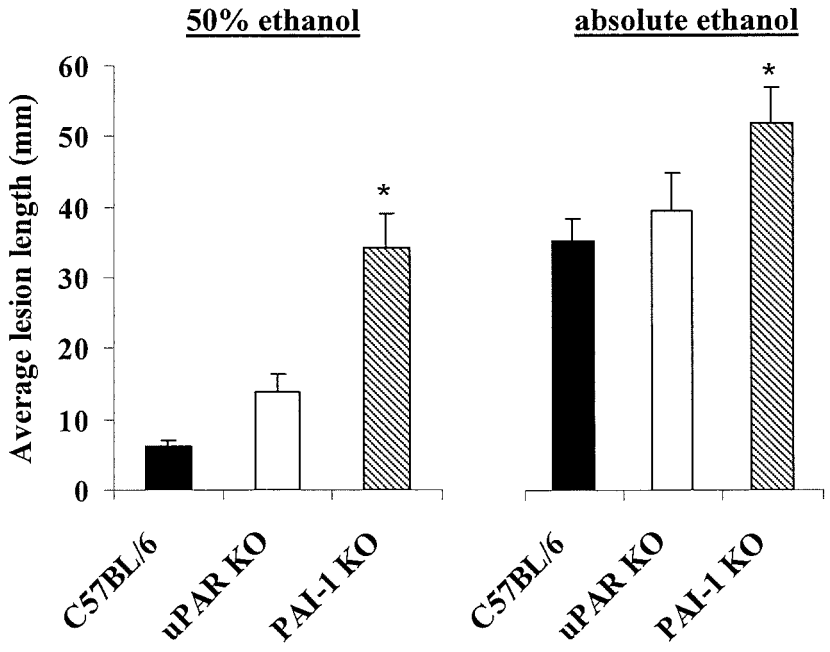

Figure 5.3

PAI-1 KO mice have a greater average lesion length after challenge with 50% or absolute ethanol compared to uPAR KO or wild-type C57BL/6 mice.

The lengths and number of lesions per animal was scored and summed up to give a lesion score per animal. Average lesion scores per group of animals was then calculated after challenge with 50% and absolute ethanol for C57BL/6, uPAR KO and PAI-KO mice (Mean ± S.E, n=7-12 animals, * $p>0.05$, ANOVA).

5.3.4 Generation of PAI-1 over-expressing transgenic mice

To investigate the contribution of elevated PAI-1 to the process of gastric mucosal protection after damage, and to compare this to results obtained from PAI-1 KO mice, over-expressing PAI-1 transgenic mice were made in collaboration with Dr Nikolina Vlatkovic, The University of Liverpool. In addition, uPA over-expressing mice were also developed simultaneously using the same strategy. It is important to note that these animals have over-expression of the given gene directly targeted to the stomach. More specifically, over-expression was targeted to a particular epithelial cell type within the stomach, the acid secreting parietal cell.

Parietal cells are one of the main differentiated cell type within the gastric corpus and in mice they undergo bilateral migration to be distributed throughout the gland (Karam, 1993e). The H^+, K^+-ATPase is the primary gastric proton pump and is situated on the apical membrane of a stimulated parietal (Forte & Yao, 1996). It is the product of 2 genes, *Atp4a* encodes the α-subunit and *Atp4b* encodes the β-subunit (Nguyen *et al.*, 2004). The murine β-subunit of the H^+, K^+-ATPase promoter sequence was used to drive expression of either PAI-1 or uPA to parietal cells. The β-subunit of the H^+, K^+-ATPase promoter has previously been used to successfully drive over-expression of other genes in the stomach of transgenic mice (Lorenz & Gordon, 1993), and has been used to direct expression of an attenuated diphtheria toxin or the herpes simplex virus 1 thymidine kinase to parietal cells (Canfield *et al* 1996; Li *et al.*, 1996). Parietal cells were chosen as a direct target as these are a major gastric epithelial cell type and are also the cell type in which up-regulation of uPA system members (including PAI-1) by *H. pylori* occurred (as shown in Figure 3.2).

There were several stages involved in the making of both transgenic animals. Due to the precision needed to specifically over-express the genes in the parietal cells of the stomach, as well as the cost involved in the project, it was important to ensure the correct expression constructs were used and that these were functionally sound. The molecular biology and production of constructs used in the making of these animals was performed by Professor Rod Dimaline, The University of Liverpool. The three different stages of plasmid generation are briefly outlined in Figure 5.4.

Initially, the β-subunit of the mouse H^+, K^+-ATPase promoter sequence was inserted into the pEGFP vector and was used to drive the expression of green fluorescent protein (GFP; Figure 5.4-A). This was transf ected into primary human gastric glands to ensure the promoter sequence was correct and that it directed GFP expression into parietal cells (Dr Islay Steele, personal communication).

Next, the cDNA sequence of murine PAI-1 was tested by inserting it into a pcDNA3 vector (pcDNA3-PAI-1) which uses a strong viral CMV promoter to drive gene expression (Figure 5.4-B). This expression plasmid or pcDNA3 as an empty vector were transfected into AGS2 cells. After 48 hr cell protein was extracted and western blot carried out. The directional orientation and sequence of PAI-1 cDNA was found to be correct as there was increased PAI-1 protein expression in cells transfected with pcDNA3-PAI-1 compared to when the empty pcDNA3 vector was used alone (Figure 5.5).

The final stage of plasmid production involved the removal of GFP from the original pEGFP vector, and the insertion of the murine PAI-1 cDNA sequence

(Figure 5.4-C). This resulted in an expression plasmids were PAI-1 was coupled to the β-subunit of the H^+, K^+-ATPase promoter (HKB-PAI-1). As a final precaution to verify plasmid expression in mouse parietal cells, HKB-PAI-1 was transfected into primary mouse gastric glands which were dual-immunolabelled with an anti-PAI-1 antibody and Ezrin (a marker of parietal cells). Gastric glands transfected with HKB-PAI-1 had a higher number of cells expressing PAI-1 and the percentage of parietal cells expressing PAI-1 was approximately 26% greater compared to control, un-transfected glands (Figure 5.7). HKB-PAI-1 transfected glands also displayed a stronger PAI-1 staining compared to control glands.

The detailed maps of HKB-PAI-1 and HKB-uPA are shown in Figure 5.6. The PAI-1 transgene was cut out from HKB-PAI-1 using the restriction enzymes *Hind III* and *Afl II* to yield a fragment of approximately 3.3kb containing a polyA sequence. The uPA transgene was yielded from HKB-uPA in a triple digest using the restriction enzymes *Afe I*, *Af II* and *Cla I* to give a fragment of approximately 3.3kb also containing a polyA sequence. Both transgenes were purified by agarose gel electrophoresis and were injected into pseudopregnant female C57BL/6 mice by pronuclear injection.

The overview described here for the generation of PAI-1/uPA over-expressing mice only outlines the stages prior to the actually establishment and breeding of true homozygous (+/+) PAI-1 and uPA over-expressing mice. Many stages of genotyping from DNA obtained from mouse tail biopsies were required and was performed by Cédric Duval, The University of Liverpool.

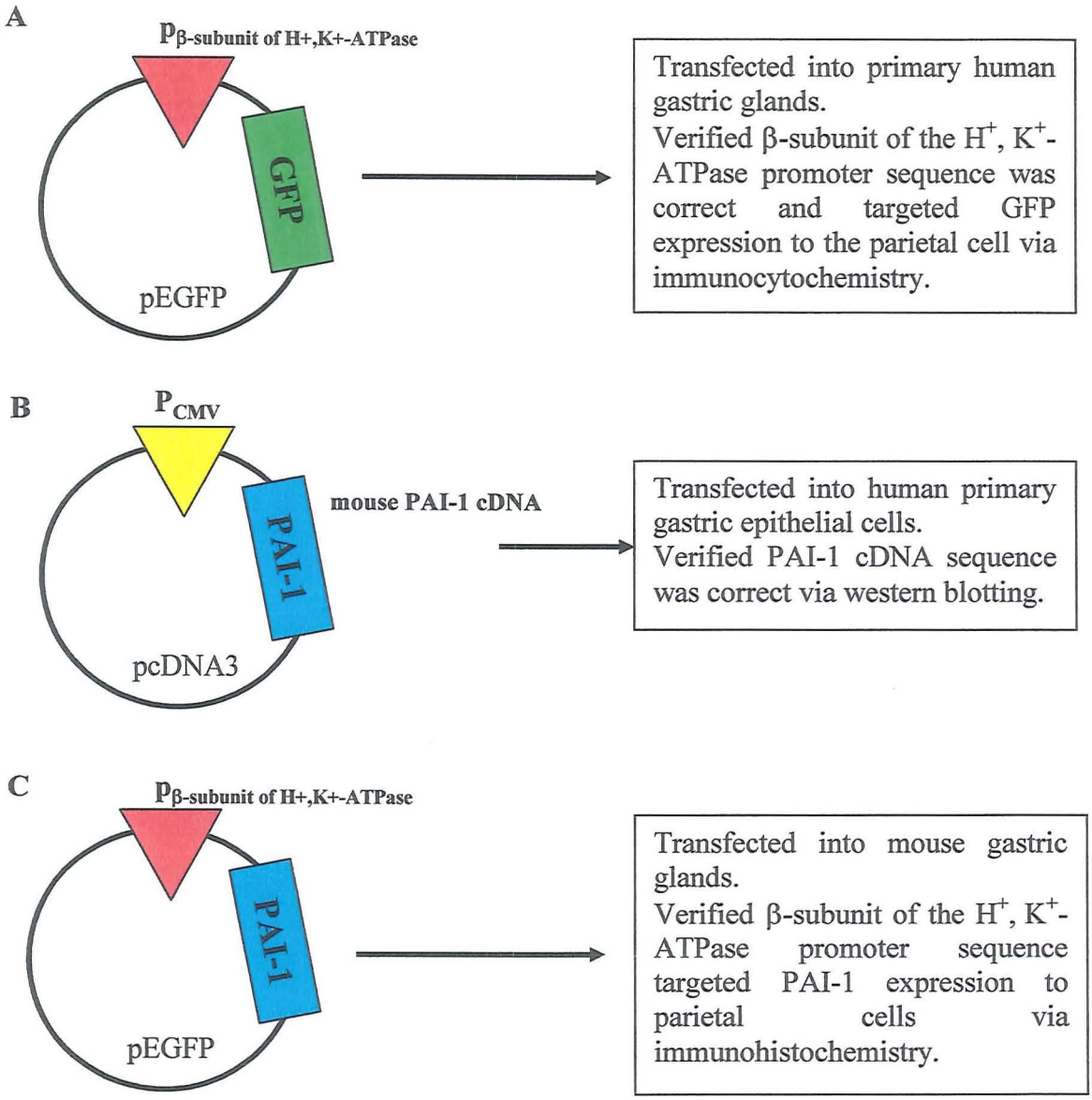

Figure 5.4

Schematic diagram of the stages and various expression plasmids involved in the generation of PAI-1 over-expressing mice.

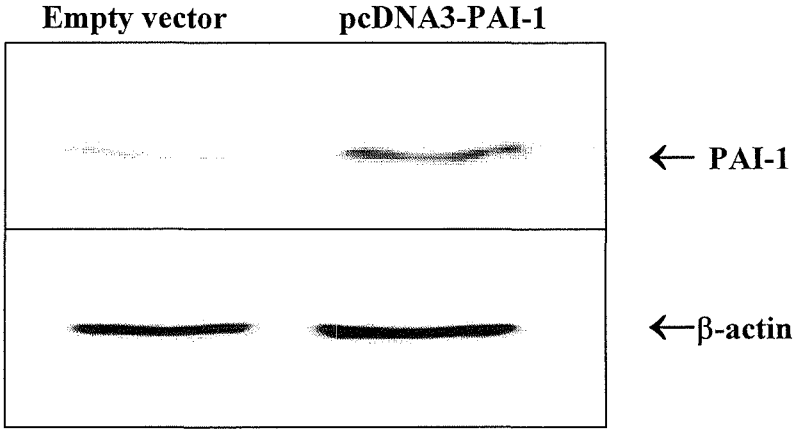

Figure 5.5

Increased PAI-1 expression in epithelial cells transfected with pcDNA3-PAI-1 expression plasmid.
Western blot showing the expression of PAI-1 (top panel) in AGS2 cells 48 hr after being transfected with either pcDNA3 empty vector or pcDNA3 vector in which the murine PAI-1 cDNA sequence had been inserted (pcDNA3-PAI-1). β-actin (bottom panel) shows protein loading controls.

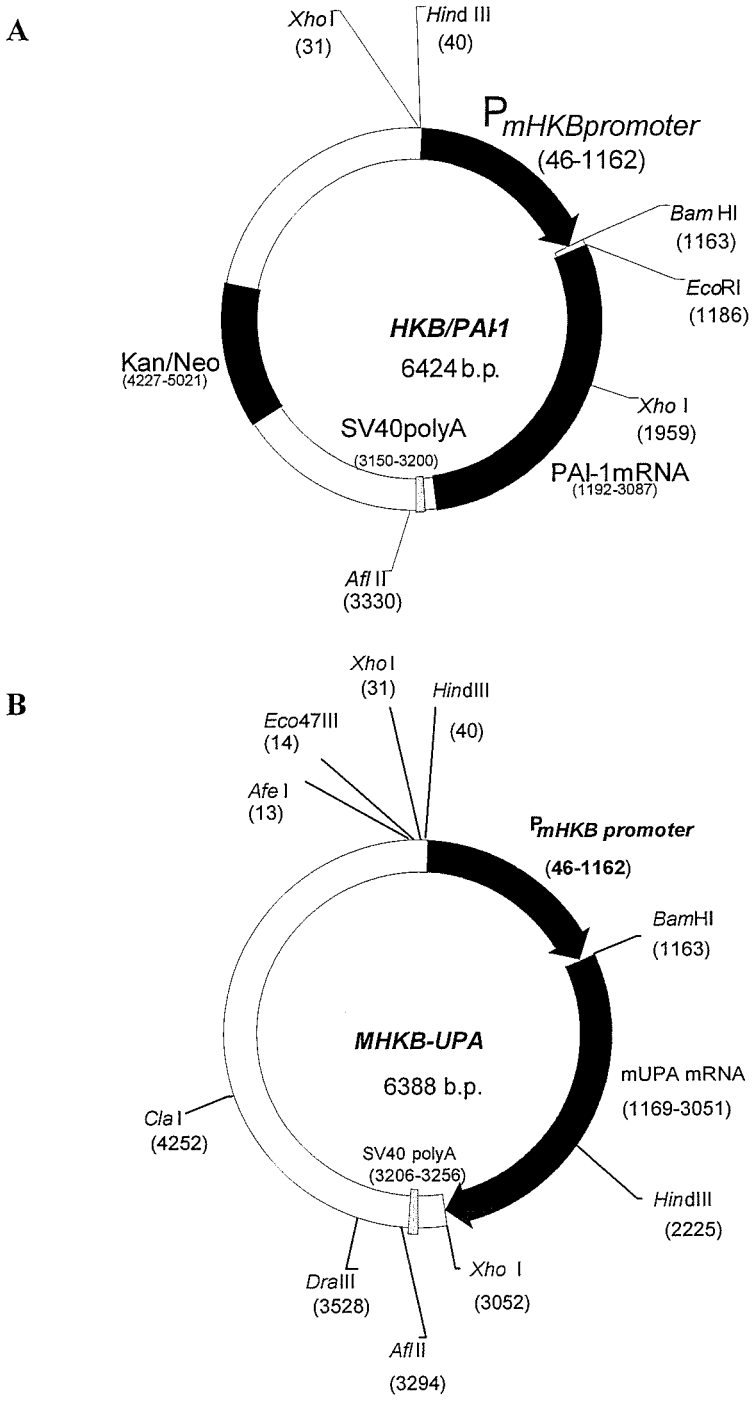

Figure 5.6

Map of expression plasmids used to over-express PAI-1 or uPA in murine gastric parietal cells.

A. HKB-PAI-1; Expression plasmid used to generate PAI-1 over-expressing transgenic mice. The promoter sequence of the β-subunit of the H^+, K^+-ATPase proton pump was used to drive PAI-1 gene expression in parietal cells. B. HKB-uPA; Expression plasmid used to drive uPA gene expression in murine parietal cells using the promoter sequence of the β-subunit of the H^+, K^+-ATPase proton pump.

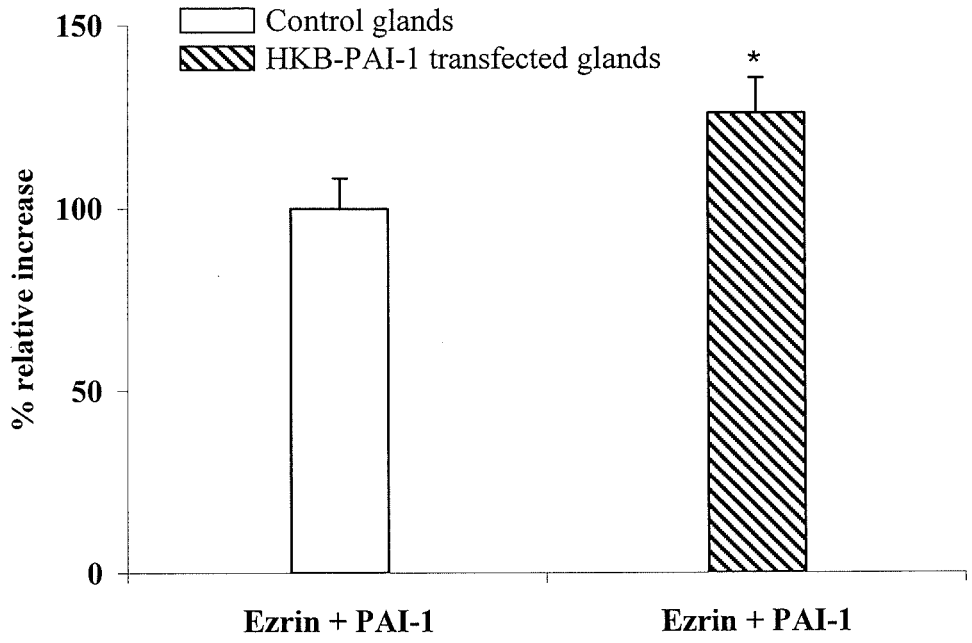

Figure 5.7

A higher percentage of parietal cells express PAI-1 in murine gastric glands transfected with HKB-PAI-1.
Dual-immunolabelled murine gastric gland cells transfected with HKB-PAI-1 showed that a higher percentage of parietal cells express PAI-1 compared to untransfected control glands (Mean ± S.E, n=20 glands. * $p>0.05$ ANOVA).

5.3.5 PAI-1 over-expressing mice develop less severe haemorrhagic lesions after ethanol challenge than PAI-1 KO mice

An acute model of gastric mucosal injury using ethanol administration was carried out on PAI-1 over-expressing mice (generated as described in section 5.5.4). This experiment had previously been carried out on PAI-1 KO mice which showed a more severe phenotype after challenge than wild-type mice (Figure 5.1). A comparison between PAI-1 KO and PAI-1 over-expressing mice was made to allow a thorough investigation into the involvement of PAI-1 in the process of tissue damage and gastric mucosal protection.

The over-expressing PAI-1 mice used in this experiment were from one founder and had 2-4 copies of the PAI-1 transgene at one integration site. After absolute ethanol administration, PAI-1 over-expressing mice had a smaller average lesion length score (20.25 $\pm$ 5.02mm) compared to C57BL/6 mice (30.17 $\pm$ 1.90mm; Figure 5.8). The average lesion score obtained for PAI-1 KO mice (Figure 5.3), was significantly greater (51.91 $\pm$ 5.11mm) than that obtained for PAI-1 over-expressing mice.

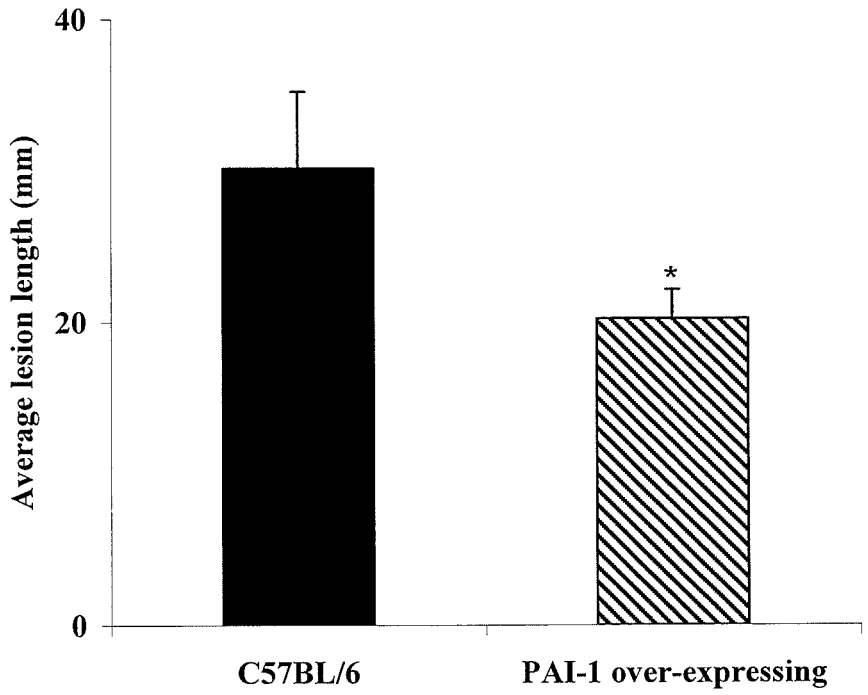

Figure 5.8

PAI-1 over-expressing mice show a less severe phenotype when challenged with ethanol compared to wild-type mice.
The lengths and number of lesions per animal was scored and totalled to give a lesion score per animal. Average lesion scores per group of animals (C57BL/6 and PAI-1 over-expressing mice) were then calculated after challenge with absolute ethanol. (Mean ± S.E, n=6-12 animals, * $p>0.05$, ANOVA).

5.3.6 uPA over-expressing mice develop a more severe phenotype when challenged with ethanol compared to wild-type mice

An acute model of gastric epithelial damage was carried out on uPA over-expressing mice (generated using the same strategy as described for PAI-1 over-expressing mice in section 5.5.4) to establish if uPA has any significant role to play in the process of gastric mucosal protection after tissue injury. The over-expressing animals were from one founder and had 10-20 copies of the uPA transgene at one integration site. Both the number and length of lesions formed within the gastric mucosa of each animal was measured as accurately as possible after 50% ethanol administration and each animal given a lesion score. This corresponded to the total length of each individual lesion. The average lesion score for both group of animals, C57BL/6 and uPA over-expressing was calculated. After challenge, uPA over-expressing mice had a significantly greater average lesion length score compared to C57BL/6 mice (Figure 5.9).

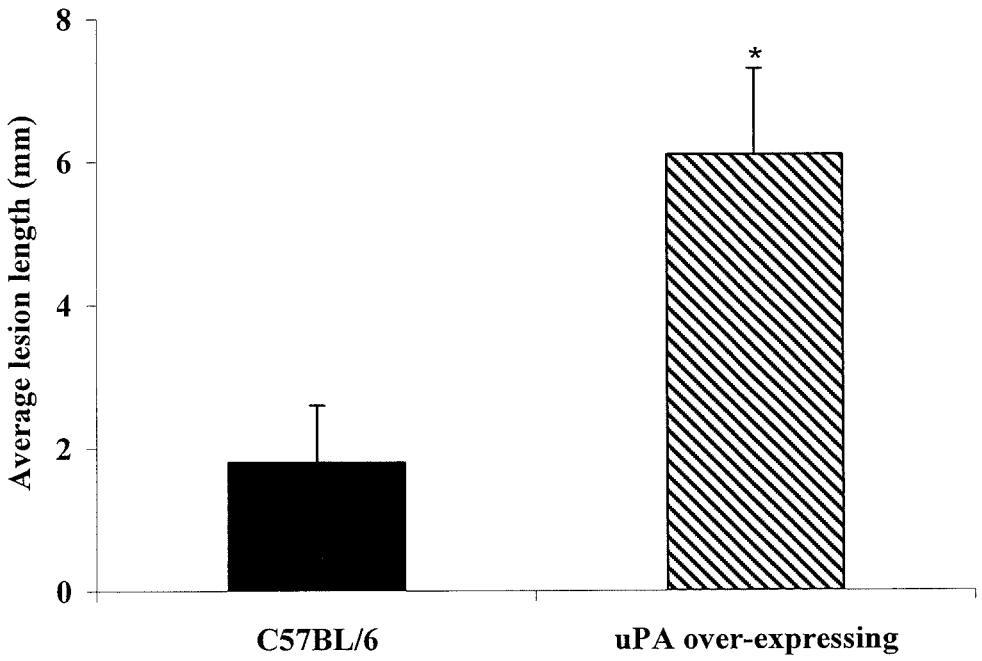

Figure 5.9

uPA over-expressing mice have a greater average lesion length after challenge with ethanol compared to wild-type mice.
The length and number of lesions per mouse was scored and summed up to give a lesion score per animal. Average lesion scores per group of mice (C57BL/6 and uPA over-expressing mice) were then calculated after challenge with 50% ethanol (Mean $\pm$ S.E, n=5 animals, * $p>0.05$, ANOVA).

5.4 Discussion

The results presented in this Chapter show that members of the uPA system have a possible involvement in the process of gastric mucosal protection after acute epithelial cell damage. In particular, it has been demonstrated through the use of transgenic animals that PAI-1 has a protective role to play in the gastric epithelium during acute tissue injury, as knocking out this gene gives rise to a more severe phenotype compared to wild-type or uPAR KO mice after ethanol administration. Not only is it evident macroscopically that the gastric mucosa of PAI-1 KO mice is dramatically different after challenge with 50% and absolute ethanol (Figure 5.1) compared to uPAR KO and wild-type mice, but careful analysis of the formation of haemorrhagic lesions confirmed this result (Figure 5.2 and Figure 5.3).

However, over-expression of PAI-1 seems to provide protection to the gastric mucosa after the epithelium is damaged, as the extent of tissue damage caused after ethanol administration resulted in the development of a less severe phenotype compared to PAI-1 KO mice. Interestingly, the effect of ethanol administration seen in PAI-1 KO mice was reversed in PAI-1 over-expressing mice which developed a phenotype less severe than wild-type mice (Figure 5.8).

In addition, it is likely that the observed effects of ethanol administration in these transgenic mice are dependent on the actions of PAI-1 in the control of uPA. Comparison of haemorrhagic lesions formed between uPAR KO and wild-type mice revealed very little difference with absolute ethanol. When uPAR is deleted, the action of uPA in fibrinolysis is inhibited as it is unable to bind to its receptor and ultimately plasmin is unavailable to degrade fibrin. However, when

PAI-1 is deleted, it can no longer inhibit uPA, and as a result a significant effect on the degree of severity of lesions formed in PAI-1 KO mice is seen. This could possibly be a consequence of plasmin over-production and degradation of fibrin within the fibrin plug. The importance of PAI-1 in the stabilisation of the haemostatic plug has previously been demonstrated in a 36 year old patient who showed a lifelong bleeding disorder which was associated with a deficiency of PAI-1 and the inability to form fibrin clots (Dieval *et al.*, 1991).

This is also reflected in uPA over-expressing mice which show more severe haemorrhagic lesions than wild-type mice, again possibly caused by the inability of these animals to form a fibrin plug at the site of injury. Over-expression of PAI-1 reversed these effects as uPA is now inhibited, fibrin is not degraded thus haemorrhagic lesion development is reduced. It can be concluded that in part, the protective effects of PAI-1 in an ethanol induced model of acute gastric epithelial injury are dependent on not only a vital balance between protease and protease inhibitor, but the presence of uPAR and ultimately the down stream effects of uPA binding to its receptor are necessary.

In addition to the pivotal role of PAI-1 in haemostasis, the possibility of well established agents which are involved in gastric mucosal protection (such as those discussed in section 1.2.6) mediating PAI-1 production could also help explain the protective effect of PAI-1 in the gastric epithelial after damage. However, this has proved difficult to evaluate due to a current lack of literature. For example, the role of the potent vasodilator NO on PAI-1 production is still under debate as some authors have found that NO suppresses PAI-1 expression and synthesis in vascular smooth muscle cells (Wolfsgruber *et al.*, 2003), and

reduces PAI-1 release from platelets (Numano *et al.*, 1995). Instead, other researchers found a dual effect of NO on PAI-1 expression in endothelial cells (Swiatkowska *et al.*, 2000). In particular, NO endogenously generated by NOS can potentiate PAI-1 expression, while NO exogenously released from NO donors inhibits its generation. However, no *in vivo* data are available to confirm these findings, leaving the question of whether NO up- or down-regulates PAI-1 synthesis unconfirmed.

Cyclooxygenase is a rate-limiting enzyme for PG synthesis which plays a crucial role in protecting the gastric mucosa against injury. In the gastric mucosa, COX-1 is constitutively expressed in normal tissues, whereas there is a low but significant expression of COX-2 (Jackson *et al.*, 2000) which is induced under pathogenic conditions, such as inflammation (Vane *et al.*, 1994). The induction of PAI-2 expression in gastric epithelial cells by *H. pylori* has been shown to be mediated by COX-2 activation (Varro *et al.*, 2004). Anti-inflammatory drugs such as NSAIDs are widely used to inhibit the action of the COX pathway (Vane *et al.*, 1998). Cyclooxygenase-2 could be a possible mediator of PAI-1 expression within the gastric epithelium since six different NSAIDs have been shown to down-regulate the expression of COX enzymes and also PAI-1 (Yang *et al.*, 2008).

The ethanol induced haemorrhagic lesion model used was chosen as it acutely induces epithelial damage. However, the gastric mucosa is frequently exposed to a wide range of other aggressive insults, and numerous stimuli such as oxidative stress, mechanical trauma and inflammation which all induce injury. Superficial damage heals rapidly via the migration of epithelial cells recruited from the

adjacent mucosa onto the denuded basement membrane through a process known as restitution (Lacy & Ito, 1984; Silen, 1985; Lacy *et al.*, 1993). Gastric myofibroblasts can also migrate through the basement membrane to aid this process (Wu *et al.*, 1999). However, deeper damage such as the formation of erosions and ulcers within the gastric mucosa (which are often a result of chronic *H. pylori* infection) requires a complex sequence of events for re-epithelialisation to occur (Miyake *et al.*, 1980).

Restoration of epithelial integrity and remodelling of the ECM after injury is an essential feature of wound repair and for it to be successful the net result of the expression of proteases and inhibitors must allow for the formation and deposition of new ECM proteins, while at the same time permitting cell migration and remodelling (Wysocki *et al.*, 1999). There is a well documented role for PAI-1 in influencing cell migration (discussed in section 1.5.5.1) and in the process of tissue repair in several different experimental settings. PAI-1 has been found to be an important protein in epithelial alveolar cell wound healing (Maquerlot *et al.*, 2006), is required for epidermal wound repair (Providence & Higgins, 2004), and also participates in renal epithelial injury repair (Providence *et al.*, 2000). PAI-1 may also act by influencing restoration of epithelial integrity in the gastric epithelium during injury and repair.

5.5 Conclusions

1. PAI-1 KO mice develop a more severe phenotype after an acute model of gastric epithelial mucosal injury induced by ethanol administration compared to uPAR KO or wild-type C57BL/6 mice.

2. PAI-1 over-expressing mice develop a less severe phenotype than PAI-1 KO mice and wild-type mice after an acute model of gastric epithelial mucosal injury induced by ethanol administration.

3. uPA over-expressing mice develop a more severe phenotype after induction of gastric epithelial cell damage than wild-type mice.

Chapter 6

Conclusions and Future Work

6.1 Major Findings

The main findings of this thesis are: PAI-1 mRNA abundance and protein expression are increased in *Helicobacter* infection and gastric cancer where expression is predominantly epithelial; Both *H. pylori* and uPA increase gastric epithelial cell proliferation which is further increased after PAI-1 knock-down; Expression of endogenous PAI-1, uPAR and uPA proteins show cell restricted expression in human primary gastric glands as do their respective luciferase reporter constructs; *H. pylori* stimulates expression of PAI-1-, uPAR- and uPA-luc with the later requiring the CagE virulence factor; PAI-1 KO mice and uPA over-expressing mice develop a more severe phenotype after acute gastric mucosal damage compared to wild-type mice.

Previous data has shown *H. pylori* infection of the human gastric corpus to be associated with increased expression of uPA and its receptor, uPAR (Cédric Duval, PhD Thesis, 2007). In addition, the present data show that *H. pylori* infection of the human gastric corpus mucosa is also associated with increased expression of the main extracellular inhibitor of uPA, PAI-1. Expression of PAI-1 was also found to be increased in gastric cancer patients, and in a mouse model leading to gastric cancer. The expression of PAI-1 in *H. pylori* infected patients appears to be predominantly epithelial. Within the epithelium, both parietal and ECL cells have the capacity to increase uPA, uPAR and PAI-1 expression in response to *H. pylori*, while pepsinogen containing cells can also express uPA and surface epithelial cells may express uPAR. Moreover, the CagE virulence factor is required for uPA responses to *H. pylori* but not PAI-1 or uPAR, therefore it seems probable that separate mechanisms are associated with increased expression of uPA, uPAR and PAI-1 during infection. Both *H. pylori*

and uPA stimulate epithelial cell proliferation which is at least partly held in check by PAI-1. To elucidate the role of the uPA system in the initial response of gastric epithelial cells after insult, an acute model of ethanol induced epithelial injury was used in uPA system transgenic mice. Expression of PAI-1 was found to protect the gastric epithelium from the damaging effects of ethanol, while uPA expression gave rise to the development of a more severe phenotype compared to wild-type mice.

The data implicate increased expression of PAI-1 during both acute and chronic *H. pylori* infection to be part of a defensive host response; it is vital for the restraint of uPA in allowing the initial formation of a haemostatic fibrin plug, thus helping to protect and repair the gastric epithelium after damage. In addition to its role during initial infection, PAI-1 also acts to control the activation and proliferative effects of uPA on gastric epithelial cells which could be associated with the preneoplastic hyperproliferative condition seen during *H. pylori* infection. However, in chronic *H. pylori* infection, associated with gastric cancer development, a sustained increase in PAI-1 expression could have a pathophysiological role explaining its correlation with a poor prognosis. The control of uPA system activity may therefore present a new target in arresting *H. pylori* stimulated progression to cancer.

6.2 Conceptual advances and future work

Previous work has established that uPA, uPAR and PAI-1 are increased in gastric cancer and in chronic atrophic gastritis (Nekarda *et al.*, 1994; Ito *et al.*, 1996; Plebani *et al.*, 1997; Kawasaki *et al.*, 1998; Kaneko *et. al.*, 2003), and that increased expression in cancer is associated with an adverse outcome. Expression

of uPA, uPAR and PAI-1 occurs in both stromal and tumour cells, and it is generally thought that the expression of these proteins is associated with progression of the disease via increased cell invasion, metastasis, and angiogenesis (Ito *et al.*, 1996; Johnsen *et al.*, 1998; Peper, 2001). In the case of uPA, binding to uPAR generates a focal point of proteolytic activity that facilitates cell invasion (discussed in section 1.5.2.1; Heiss *et al.*, 1995). In addition to inhibition of uPA, through accelerated internalisation of the uPA/uPAR complex, PAI-1 also exhibits uPA-independent actions. Specifically, by binding to VN it can increase cell dissociation from the ECM and also accelerate invasion (discussed in section 1.5.5.1; Deng *et al.*, 1996). It has been suggested that the latter effects account for the association of increased PAI-1 in gastric and other human cancers with an adverse outcome. This is often referred to as "paradoxical" given that uPA is also associated with disease progression (discussed in section 1.5.5.2). In spite of the interest in uPA and PAI-1 in gastric cancer, little attention has been given to how this system might be implicated in the response of the gastric epithelium to insult and in preneoplastic conditions.

It has previously been shown that PAI-2 is increased in gastric epithelial cells in response to *H. pylori* via activation of NFκB (Varro *et al.*, 2004). A pool of PAI-2 is retained within cells and is associated with inhibition of apoptosis (Kumar *et al.*, 1991), while some is secreted and has been shown to be involved in inhibition of invasion of a cancer cell line (Andreason *et al.*, 1997). The expression of uPA and uPAR have also been shown to be increased in *H. pylori* infection (Cédric Duval, PhD Thesis, 2007) and the present data now make it clear that acute infection with *H. pylori* also induces another main member of the uPA system, the main extracellular inhibitor of uPA, PAI-1. No evidence is

available that a third inhibitor, PAI-3, is changed with *H. pylori* infection and it was not studied further.

Previous work using microarrays to identify *H. pylori* regulated genes, demonstrated increased uPA in gastric cancer cell lines (Kitadai *et al.*, 2003), and there has been work using gastric cancer cell lines that indicates *H. pylori* stimulation of uPAR expression (Kim *et al.*, 2005; Kim *et al.*, 2007). Some work has also been carried out in cell lines regarding the effect of *H. pylori* on PAI-1 expression (Iwamoto *et al.*, 2005). However, given the nature of cancer cell lines, it remained uncertain how these observations related to expression of members of the uPA system in primary gastric epithelial cells. Data presented here show that with acute infection there is the capacity to increase uPA, uPAR and PAI-1 expression in two cell types associated with the gastric corpus namely the acid-secreting parietal cell and the histamine releasing enterochromaffin-like cell; the restriction of both cell types to the gastric corpus is consistent with the observation that there was no difference in expression of PAI-1 (or uPA/uPAR; Cédric Duval, PhD Thesis, 2007) in the antral region of the stomach (where these cells types are absent). In contrast, PAI-2 is prominently expressed in mucin and pepsinogen-expressing cells and is only occasionally found in parietal or ECL cells (Varro *et al.*, 2004); it is also increased in the antrum in *H. pylori* positive patients (personal communication, Andrea Varro; unpublished observation).

H. pylori increases plasma gastrin concentrations, and parietal and ECL cells express the CCK2R at which gastrin acts (discussed in section 1.2.3 and 1.3.5; Levi *et al.*, 1989; Dockray *et al.*, 2001). However, increased abundance of PAI-1 transcripts were found in *H. pylori* infected patients with plasma gastrin concentrations in the normal range and *H. pylori* stimulation of promoter-

reporter luciferase constructs occurred in primary epithelial cells *in vitro* (in the absence of gastrin), therefore the effects of *H. pylori* are not secondary to increased gastrin. However, gastrin was found to be able to stimulate expression of PAI-l-luc in primary epithelial cells *in vitro*, and uPA-, uPAR-, and PAI-1-luc in a gastric cancer epithelial cell line. While stimulation of uPAR and PAI-1 was independent of the pathogenicity island, a *cagE* isogenic mutant was defective in uPA induction. Disruption of *cagE* means that *H. pylori* cannot produce a functional type IV secretory apparatus. This has two effects as it blocks Nod1-NFκB signalling (discussed in section 1.3.3.2) and also blocks delivery of CagA into epithelial cells, which ultimately has consequences on cell signalling events (discussed in section 1.3.3.3). As disruption of *cagA* had no effect on uPA induction, it is likely that the lack of induction of uPA by the *cagE* mutant is due to lack of stimulation of Nod1-NFκB. There is an already described role of NFκB in the regulation of uPA transcription and it is known that the human uPA promotor contains an NFκB binding site (discussed in section 1.5.1; Novak *et al.*, 1991; Hansen *et al.*, 1992; Mitsuno *et al.*, 2001; Guillemin *et al.*, 2002). There are many different ways that bacteria are known to interact with the uPA system (Bergmann & Hammerschmidt, 2007). In the case of *H. pylori,* two plasminogen binding proteins have been identified that are proposed to coat the organism and with the subsequent activation of bound plasminogen to plasmin these proteins are thought to increase virulence (Jonsson *et al.*, 2004).

Both *H. pylori* and exogenous uPA stimulate proliferation of primary gastric epithelial cells. These data link *H. pylori* induction of the uPA system to one of the key characteristics of preneoplastic, *H. pylori*-infected epithelium, namely increased cell proliferation which was enhanced further by blocking PAI-1

expression, compatible with a dynamic equilibrium between enzyme and its inhibitor. It is unknown at this stage if the pr oliferative response of gastric epithelial cells to *H. pylori* is mediated as a direct consequence of increased uPA expression, or indeed how the response of gastric epithelial cells to uPA is mediated. The involvement of uPA binding to uPAR in this process also warrants further investigation and all three questions can be addressed by carrying out proliferation assays on primary gastric epithelial cells treated with various inhibitors, anti-sense oligonuclceotides and neutralistaion antibodies.

Previous work has shown that *H. pylori* stimulates proliferation of epithelial cells and that this includes transactivation of the EGF-R and stimulation of HB-EGF cleavage (Keates *et al.*, 2001; Wallasch *et al.*, 2002; Ashktorab *et al.*, 2007). The main ligands of EGF-R produced in the gastric epithelium, are HB-EGF, amphiregulin and TGF-α. It is known that HB-EGF is cleaved from its cell bound precursor via proteases that include TACE (ADAM-17; Sunnarborg *et al.*, 2002). It has also been shown in prostatic DU-145 cells that uPA stimulated invasion is mediated by HB-EGF in an autocrine mechanism (Caceres *et al.*, 2005). Epidermal growth factor receptor is known to coimmunopreciptate with uPAR and can be constitutively activated by ligand bound uPAR, activating ERK to promote proliferation and migration of a variety of cell types (Liu *et al.*, 2002; Jo *et al.*, 2003; Jo *et al.*, 2005). Whether the effects of uPA on gastric epithelial cell proliferation involves EGF-R, or if any of the ligands for EGF-R are required for this response remains to be determined but is an interesting avenue to explore.

The primary function of uPA is the cleavage of plasminogen to plasmin and hence control of fibrin degradation. It is possible that with acute *H. pylori*

infection, the induction of PAI-1 is part of a defense mechanism initiated to control the protease activity of increased uPA, therefore preventing premature fibrin lysis in order for a stable haemostatic fibrin plug to be formed. Fibrinolysis is a reparative process that occurs in response to a haemostatic plug (Loskutoff *et al.*, 1993). The involvement of PAI-1 in this process is well established (Dieval *et al.*, 1991), and studies have also shown that PAI-1 can bind to the fibrin clot either directly or via VN (Murayama *et al.*, 1987; Wagner *et al.*, 1989). However, the fact that PAI-1 protects the gastric epithelium from the formation of haemorrhagic lesions after ethanol administration is novel, and is therefore difficult to discuss in the context of other literature.

The fibrin clot formed during haemostasis must be remodelled and removed during tissue repair to restore normal structure and function. The fibrinolytic system is the principle effecter of clot removal as it controls the enzymatic degradation of fibrin and is therefore important for tissue repair. It has been shown here that PAI-1 expression is associated with gastric mucosal protection; however, its role in gastric epithelium repair after acute injury has yet to be determined. To explore this, a cryoinjury ulcer model (Ashurst *et al.*, 2008) could be used comparing the repair process of the gastric mucosa between wild-type, PAI-1 KO and PAI-1 over-expressing mice. It has been previously shown that wild-type, C57BL/6 mice show a progressive repair of the ulcer crater at 6 and 9 days postinjury, and by 13 days the mucosal ulceration is barely discernible macroscopically (Ashurst *et al.*, 2008). As PAI-1 has an already well documented role in tissue repair in several experimental settings (discussed in section 5.4; Providence *et al.*, 2000; Providence & Higgins, 2004; Maquerlot *et al.*, 2006), it could be hypothesised that the repair process would be retarded in PAI-1 KO mice, but accelerated in PAI-1 over-expressing mice.

So far it has been highlighted that PAI-1 expression is not only pivotal in the control of fibrinolysis during initial gastric mucosal injury but increased expression is required to restrain the proliferative effects of *H. pylori* and uPA during infection. However, aside from the uPA-independent, 'paradoxical' actions of PAI-1 (described above and discussed in section 1.5.5.1) which accounts for the pathophysiological association of increased PAI-1 in gastric and other human cancers with an adverse outcome, sustained, increased anti-fibrinolytic concentrations of PAI-1 could contribute to fibrosis, a process intimately tied to chronic infection. Analysis of PAI-1 KO mice have offered an insight into the relative contributions of PAI-1 in fibrosis; in a mouse model of cholestatic liver injury, PAI-1 KO mice are robustly protected against hepatic fibrosis (Bergheim *et al.*, 2006) and mice genetically deficient in PAI-1 are protected from developing fibrosis after lung injury (Eitzman *et al.*, 1996; Hattori *et al.*, 2000; Chuang-Tsai *et al.*, 2003) whilst fibrosis is worsened in mice that have a constitutively active PAI-1 transgene (Eitzman *et al.*, 1996). PAI-1 KO mice also have attenuated interstitial fibrosis after unilateral ureteral obstruction compared to wild-type mice (Oda *et al.*, 2001).

The role of PAI-1 in gastric remodelling and cancer progression can be further studied in *H. felis* or *H. pylori* infected PAI-1 KO and PAI-1 over-expressing mice and comparing the extent of tissue remodelling to that observed in wild-type, C57BL/6 mice. *H. felis* infection in the C57BL/6 mouse model reproducibly results in the classic sequence of histological changes seen in human infection (discussed in section 1.3.2) with adenocarcinoma occurring in 100% of mice by 15 months of infection (Wang *et al.*, 1998; Houghton *et al.*, 2004; Rogers *et al.*, 2004; Cai *et al.*, 2005). As there is an association between high PAI-1 expression and a poor outcome for gastric cancer patients, it could be

suggested that PAI-1 KO mice display a less severe phenotype after *H. felis* infection with PAI-1 over-expressing mice developing a more aggressive phenotype.

The present data suggest a role for PAI-1 in gastric mucosal protection during acute injury which could account for the initial increase in expression during acute *H. pylori* infection. However, in prolonged infection increased PAI-1 expression acts to restrain the proliferative effects of increased uPA expression, helping to inhibit hyperproliferation of gastric epithelial cells. During chronic infection, associated with gastric cancer development, it is suggested that a sustained increase in PAI-1 expression has a pathophysiological role by contributing to fibrosis and by acting independently of uPA to influence cell migration and invasion. In addition to this it can be suggested that increased proliferation favours the acquisition of mutations leading to cancer. The present data therefore raise the possibility that intervention of the uPA system might be therapeutically useful in those patients at risk of progression from gastric atrophy to cancer.

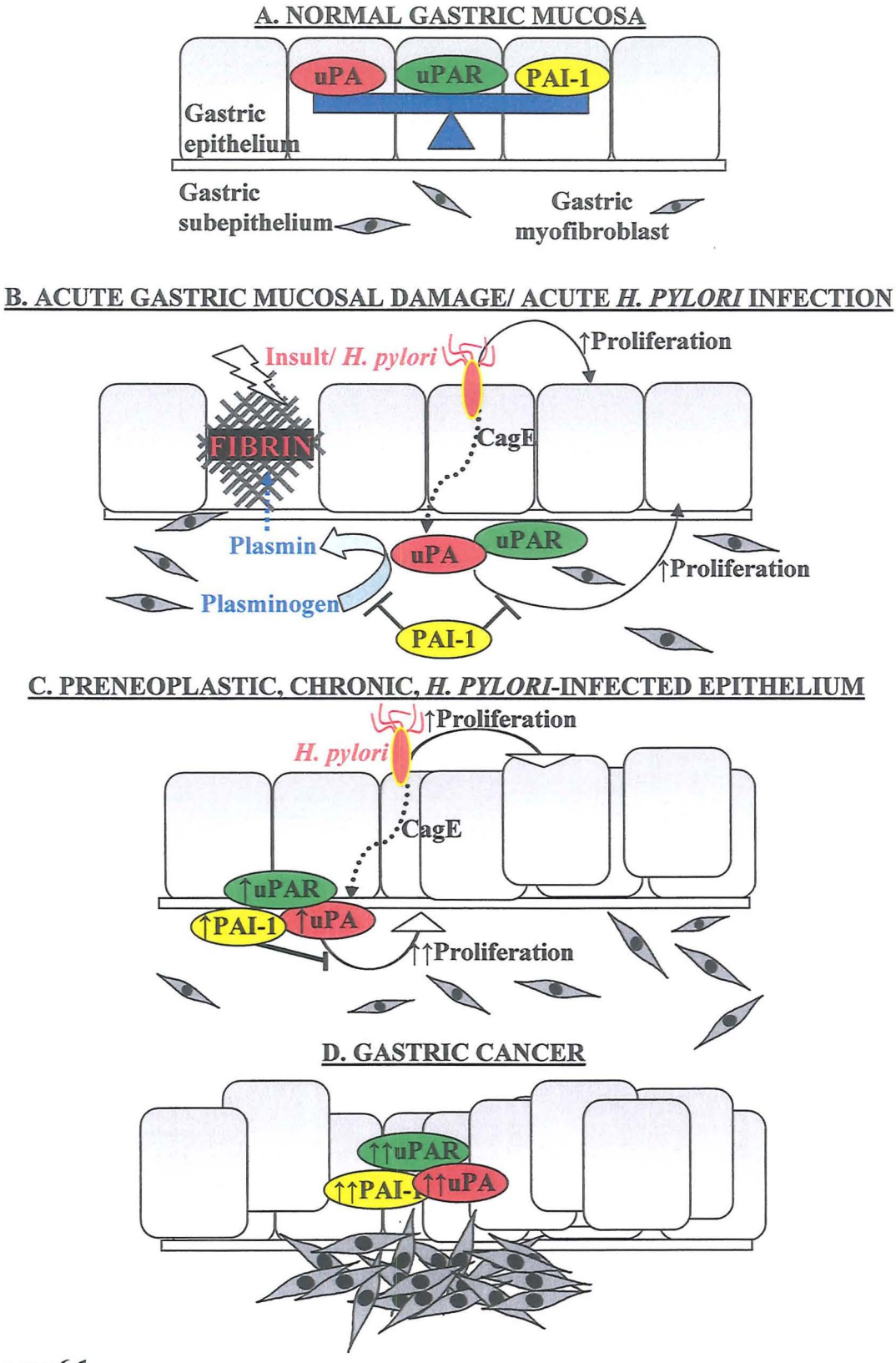

Figure 6.1
(See opposite page for legend).

Chapter 7

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Increased gastric expression of MMP-7 in hypergastrinemia and significance for epithelial-mesenchymal signaling

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Varro A, Kenny S, Hemers E, McCaig C, Przemeck S, Wang TC, Bodger K, Pritchard DM. Increased gastric expression of MMP-7 in hypergastrinemia and significance for epithelial-mesenchymal signaling. *Am J Physiol Gastrointest Liver Physiol* 292: G1133–G1140, 2007. First published January 11, 2007; doi:10.1152/ajpgi.00526.2006.—Chronic hypergastrinemia is associated with enterochromaffin-like (ECL) cell hyperplasia, which may progress to gastric carcinoid tumors. The latter consists of epithelial cells and stroma, and both compartments usually regress after normalization of hypergastrinemia. We previously showed that matrix metalloproteinase (MMP)-7 in gastric epithelial cells was upregulated by *Heliobacter pylori* and described MMP-7-dependent reciprocal signaling between the epithelium and a key stromal cell type, the myofibroblast. Here, we describe the regulation of gastric MMP-7 by gastrin and the potential significance for recruiting and maintaining myofibroblast populations. Biopsies of the gastric corpus and ECL cell carcinoid tumors were obtained from hypergastrinemic patients. Western blot analysis, ELISA, immunohistochemistry, and promoter-luciferase (luc) reporter assays were used to study MMP-7 expression. Gastric myofibroblasts were identified by α-smooth muscle actin (α-SMA) expression, and the effects of MMP-7 on myofibroblast proliferation were investigated. In hypergastrinemic patients, there was an increased abundance of MMP-7 and α-SMA in gastric corpus biopsies and ECL cell carcinoid tumors. In the latter, MMP-7 was localized to ECL cells but not stromal cells, which were nevertheless well represented. Gastrin stimulated MMP-7-luc expression in both AGS-G_R and primary human gastric epithelial cells. Conditioned medium from gastrin-treated human gastric glands stimulated myofibroblast proliferation, which was inhibited by neutralizing antibodies to MMP-7. MMP-7 increased the proliferation of myofibroblasts via the MAPK and phosphatidylinositol 3-kinase (PI3K) pathways. In conclusion, stimulation of gastric MMP-7 by elevated plasma gastrin may activate epithelial-mesenchymal signaling pathways regulating myofibroblast function via MAPK and PI3K pathways and contribute to stromal deposition in ECL cell carcinoid tumors.

matrix metalloproteinase-7; pernicious anemia; multiple endocrine neoplasia type 1; enterochromaffin-like cell carcinoid tumors

MUCOSAL ORGANIZATION throughout the gastrointestinal tract depends on interactions between the epithelium and subepithelial cells including fibroblasts and myofibroblasts as well as blood vessels, immune cells, and neurons. Recent work has suggested an unexpected diversity in the relevant signaling systems (13). In particular, in addition to growth factors and cytokines, it is now recognized that proteolytic enzymes may also play a role in these signaling processes (13, 20). Matrix metalloproteinases (MMPs) are involved in remodeling of the extracellular matrix and the liberation of growth factors and are frequently increased in inflammatory conditions, injury, and cancer (10, 24). Most MMPs are produced in subepithelial cells, but an exception is MMP-7 (also known as matrilysin or PUMP), which is predominantly expressed in epithelial cells both of the gut and other organs including airways, mammary glands, and the urogenital tract (9, 19, 25). Recent work has shown that in the stomach, there is increased MMP-7 with *Heliobacter* pylori infection (2, 4, 33). This is associated with increased myofibroblast abundance, involving both cell proliferation and migration via increased IGF-II bioavailability through cleavage of its binding protein, IGF binding protein (IGFBP)-5, which is released from myofibroblasts (13, 20).

It is well established that a prolonged elevation of plasma gastrin is found in achlorhydria, e.g., pernicious anemia (PA), as well as in gastrinoma, including on a background of multiple endocrine neoplasia (MEN) type-1, and in some patients on prolonged proton pump inhibitors (5, 7, 17, 30). Elevated plasma gastrin is associated with hyperplasia of histamine-secreting enterochromaffin-like (ECL) cells in the gastric corpus (3, 17, 18). In some hypergastrinemic patients, there is a progression via dysplastic ECL cells to ECL cell carcinoid tumors (11, 23); thus, the latter are reported to occur in patients with hypergastrinemia on a background of either gastric inflammation, as occurs in PA, or MEN-1 but are generally uncommon in patients with sporadic gastrinoma (5, 17). In the case of patients with hypergastrinemia of antral origin, resection of the antrum to lower plasma gastrin has been reported by several groups to lead to regression of ECL cell carcinoid tumors (12, 14, 15). Interestingly, both ECL cells and the stroma regress, suggesting that gastrin acts via ECL cells to trigger a stromal reaction. The present study was based on the specific hypothesis that gastrin stimulates MMP-7 expression, which, in turn, stimulates proliferation of a key stromal cell type, the myofibroblast. We therefore asked whether *1*) there is increased expression of MMP-7 in gastric biopsies of hypergastrinemic patients and in a transgenic mouse model overexpressing human gastrin, *2*) there is also increased MMP-7 in ECL cell carcinoid tumors, *3*) gastrin stimulates expression of a MMP-7 promoter/luciferase reporter construct in cultured cells in vitro, *4*) increased MMP-7 is accompanied by increased myofibroblast abundance, and *5*) MMP-7 stimulates myofibroblast proliferation through mechanisms involving MAPK and phosphatidylinositol 3-kinase (PI3K) pathways.

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MATERIALS AND METHODS

Cells, plasmids, and drugs. The gastric cancer cell line AGS-G_R, permanently transfected with the CCK-2 receptor, was routinely cultured in Ham's-F-12 medium as previously described (27). Human gastric adherent glands and myofibroblasts were prepared as previously described (20, 33). A vector consisting of 2.3 kb of the human MMP-7 promoter coupled to firefly luciferase (i.e., MMP-7-luc) was kindly donated by Lynn Matrisian (Vanderbilt University, Nashville, TN). Human recombinant (r)MMP-7, MMP-7 fluorogenic substrate, AG-1478, AG-825, Ro-340432, and mouse purified EGF were obtained from Calbiochem (Nottingham, UK). Heptadecapeptide amidated gastrin (G17) was purchased from Peninsula (Merseyside, UK). All other chemicals were obtained from Sigma (Dorset, UK).

Patients. Six endoscopic pinch biopsies of the gastric corpus were obtained from 12 patients with PA and 7 patients with MEN-1 with hypergastrinemia. Five patients with MEN-1 and six patients with PA had macroscopic ECL cell carcinoid tumors, as confirmed by histology. Dyspeptic patients ($n = 41$) with normal endoscopy, normal plasma gastrin concentrations (<30 pM), negative *H. pylori status* by serology, antral urease test, and antral and corpus histology were used as controls. In addition, some experiments were made on dyspeptic subjects ($n = 8$) with normal endoscopy and negative *H. pylori* status but mild fasting hypergastrinemia (30–100 pM). Myofibroblasts were obtained from the macroscopically normal gastric corpus mucosa resected at least 3 cm from the tumor margin in patients undergoing surgery for gastric cancer as previously reported (20). The study was approved by the Ethics Committees of the Salford and Trafford Health Authority (Manchester, UK), South Sefton, and Royal Liverpool and Broadgreen University Hospitals National Health Service Trusts. All patients gave informed consent.

Human gastric gland and myofibroblast preparations. Isolated human gastric glands were prepared from endoscopic biopsies of *H. pylori*-negative patients as previously described (33). Human gastric myofibroblasts were prepared as previously described (20, 34).

Animals. The gastric corpus was taken at 3 mo of age from sex-matched FVB/N, gastrin-overexpressing (InsGas), gastrin knockout (GKO), and C57Bl/6 [wild type (WT)] mice for protein extraction and histology as previously described (20). Some GKO mice fed ad libitum were treated with G17 (20 nmol ip at 09.00 and 16.00 hours on *day 1* and 09.00 hours on *day 2*), and the gastric corpus was taken for extraction for Western blot analysis 3 h later. Procedures were approved by the Local Animal Welfare Committee and were in accordance to United Kingdom legislations.

Western blot analysis. Protein extracts of human gastric biopsies and gastric myofibroblasts were prepared, and Western blot analysis was performed as previously described (13, 20). Samples were probed with antibodies to MMP-7 (Chemicon, Chandlers Ford, UK), α-smooth muscle actin (α-SMA; RDI, Flanders, NJ), phospho-p42/44 MAPK (New England BioLabs, Hertford, UK), and phospho-Akt (Thr^{473}) (Cell Signaling Technology), followed by horseradish peroxidase-conjugated secondary antibody and detection by an incubation with SuperSignal West Pico Chemilminescent Substrate (Pierce) and HyperFilm (Amersham) as previously described (28). Samples were reprobed for GAPDH (Biodesign, Saco, MA) or β-actin (Santa Cruz Biotechnology) and total MAPK using anti-ERK1 (BD Transduction Laboratories, Bedford, MA) or anti-Akt antibodies (BD Transduction Laboratories) as appropriate.

MMP-7 ELISA. Plasma samples were processed for the detection of MMP-7 using a Quantikine Human MMP-7 (total) Immunoassay kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

Gastrin RIA. Plasma samples from fasted control, MEN-1, and PA patients and from mice were assayed for total amidated gastrin concentrations using antibody L2 (which reacts with G17 and G34 but not progastrin or Gly-gastrins) as previously described (8). The upper limit of normal fasting gastrin concentrations in this assay was 30 pM (5, 26).

Fluorogenic substrate assays. Medium from gastric glands treated with G17 was incubated with specific MMP-7 fluorogenic substrate, and fluorescence was measured using excitation at 280 nm and emission at 360 nm according to the manufacturer's instruction as previously reported (20).

Immunohistochemistry. Formalin-fixed, paraffin-embedded tissue sections were processed for the immunohistochemical detection of MMP-7 (Chemicon) and α-SMA (RDI) after antigen recovery as previously described (33).

Transfection and luciferase assays. AGS-G_R cells (2×10^5) were plated in full medium. The following day, the medium was removed, and cells were cotransfected with MMP-7-luc (1.0 μg/well) and *Renilla* luciferase (0.1 μg/well; Promega, Madison, WI) using TransFast (Promega) as previously described (33). Medium was then replaced with 2 ml serum-free medium, and cells were incubated with G17 for 8 h. Luciferase activity was measured with Bright-Glo or Dual Glo (Promega) using a LumiCount Platereader (Packard BioScience) (33). In addition, to explore the control of MMP-7-luc in primary gastric epithelial cells, we developed the following new method. Primary human gastric glands (33) were transfected using CombiMag (OzBiosciences, Marseille, France) on a magnetic plate according the manufacturer's instructions, and cells were incubated with G17 for 20 h. Luciferase activity was measured by a dual-luciferase assay (Promega). Results in both cases are presented as fold increases over the unstimulated control, so 1.0 signifies no change in luciferase activity. The protein concentration was determined when appropriate using a Bio-Rad detergent compatible protein assay (Bio-Rad, Herts, UK) to monitor the plating efficiency.

Cellular targeting of MMP-7-luc in primary adherent glands. To study the cellular targeting of MMP-7-luc expression, transfected human glands were double immunostained with goat anti-luciferase antibody (Rockland Immunochemicals, Gilbertsville, PA) together with one of the following antibodies: rabbit anti-pepsinogen (a gift from Mike Samloff, Center for Ulcer Research, Los Angeles, CA), anti H^+-K^+-ATPase (Calbiochem), anti-vesicle monoamine transporter type 2 (VMAT-2) (16), mouse anti-trefoil factor-1 (TFF-1; Dako, Glostrup, Denmark), and mouse anti-TFF-2 (NovoCastra, Newcastle-upon-Tyne, UK) antibodies, with the appropriate FITC- or Texas red-conjugated secondary antibodies, raised in the donkey (Jackson Immunoresearch, Soham, UK), using Vectashield mounting medium with 4′,6-diamidino-2-phenylindole (Vector Laboratories, Peterborough, UK) to counterstain nuclei. Slides were examined using a Zeiss Axioplan-2 microscope (Zeiss Vision, Welwyn Garden City, UK), and images were captured using a JVC-3 charge-coupled device camera and KS300 software combined with deconvolution software (Imaging Associates, Oxfordshire, UK). Ten fields of three patients were counted in each case, and results are expressed as percentages of the total cell number as previously reported (33).

Proliferation assays. The incorporation of [^{3}H]thymidine into human gastric myofibroblasts was studied using methods previously described (29). Cells (25,000) were cultured in six-well plates in serum-free medium for 48 h and stimulated for 18 h with conditioned medium from G17-treated glands with or without mouse monoclonal MMP-7 neutralizing antibody (4 μg/ml, Chemicon). [^{3}H]thymidine (2 μCi/ml) was added for the last 2 h; cells were processed as previously described (29).

Statistics. Results are presented as means ± SE; comparisons were made using ANOVA or Student *t*-tests where appropriate and were considered significant at $P < 0.05$.

RESULTS

Increased gastric MMP-7 in both the human and mouse gastric corpus with hypergastrinemia. In initial studies, we found elevated circulating MMP-7, as determined by ELISA,

in patients with hypergastrinemia due to PA and MEN-1 compared with control subjects (Fig. 1*A*). To determine whether the increase of plasma MMP-7 was reflected in the gastric corpus, we then performed Western blot analysis of gastric biopsies from both patient groups. The abundance of MMP-7 was indeed significantly increased in the gastric corpus of both PA and MEN-1 patients compared with control subjects, compatible with the idea that elevated plasma concentrations of gastrin stimulated MMP-7 expression in the stomach (Fig. 1, *B* and *D*). Subsets of both PA and MEN-1 patients exhibited ECL cell carcinoid tumors, which are recognized to be gastrin dependent, and when these were separately sampled, we again found increased MMP-7 abundance compared with control subjects (Fig. 1, *C* and *D*). Increased gastric MMP-7 was not, however, associated with mild hypergastrinemia, because in biopsies from subjects with fasting plasma gastrin concentrations in the range of 30–100 pM, MMP-7 abundance was not different from controls (gastrin: 8.7 ± 1.3 pM, $n = 9$, compared with 58.6 ± 8.4 pM, $n = 8$; control MMP-7 abundance: 100 ± 20.2% compared with 101.6 ± 21.9% in hypergastrinemic patients).

To explore a causal link between gastrin and MMP-7, we then examined a mouse model of hypergastrinemia (InsGas), in which there are increases in ECL cell markers at 3 mo (6). We found increased abundance of MMP-7 in the stomach of InsGas mice compared with the parental (FVB/N) strain (Fig. 2, *A* and *B*). Moreover, treatment of mice null for the gastrin gene with exogenous G17 also increased MMP-7 abundance, whereas there were no significant differences in MMP-7 between GKO mice and their WT counterparts (Fig. 2, *C* and *D*).

Gastrin stimulates MMP-7 promoter activity in primary cells. To determine whether gastrin might regulate MMP-7 transcription, we initially studied the response of a promoter-luciferase reporter vector (MMP-7-luc) transfected into AGS-G_R cells; these cells were chosen because they have been widely used in the past as an experimental model in studies (32, 37) of gene expression in ECL cells (Fig. 3*A*). In response to concentrations of gastrin in the range of 100 pM–10 nM, there was a progressive increase in MMP-7-luc expression; the concentration for half-maximal stimulation was ~2 nM. We then asked whether the same stimulation occurs in primary human gastric gland cells. To this end, we developed a novel method for transfection of adherent glands with MMP-7-luc as described in MATERIALS AND METHODS. In primary human gastric glands, G17 (1 nM, 20 h) also significantly stimulated MMP-7-luc expression (Fig. 3*B*). Using these methods, 5.7 ± 1.2% of all cells expressed the construct. Importantly, expression of MMP-7-luc was targeted to subsets of TFF-1-positive surface epithelial, chief (pepsinogen), and ECL (VMAT-2) cells (Fig. 4, *A–I*, and Table 1). There was no targeting of the construct to parietal cells (H^+-K^+-ATPase) (Fig. 4*J*) or to TFF-2-positive neck cells (Fig. 4*K*). The former could, however, be transfected with other constructs, for example, the promoter of the β-subunit of H^+-K^+-ATPase coupled with green fluorescent protein (GFP). Moreover, a control GFP-vector was expressed in all cell types in this system (not shown).

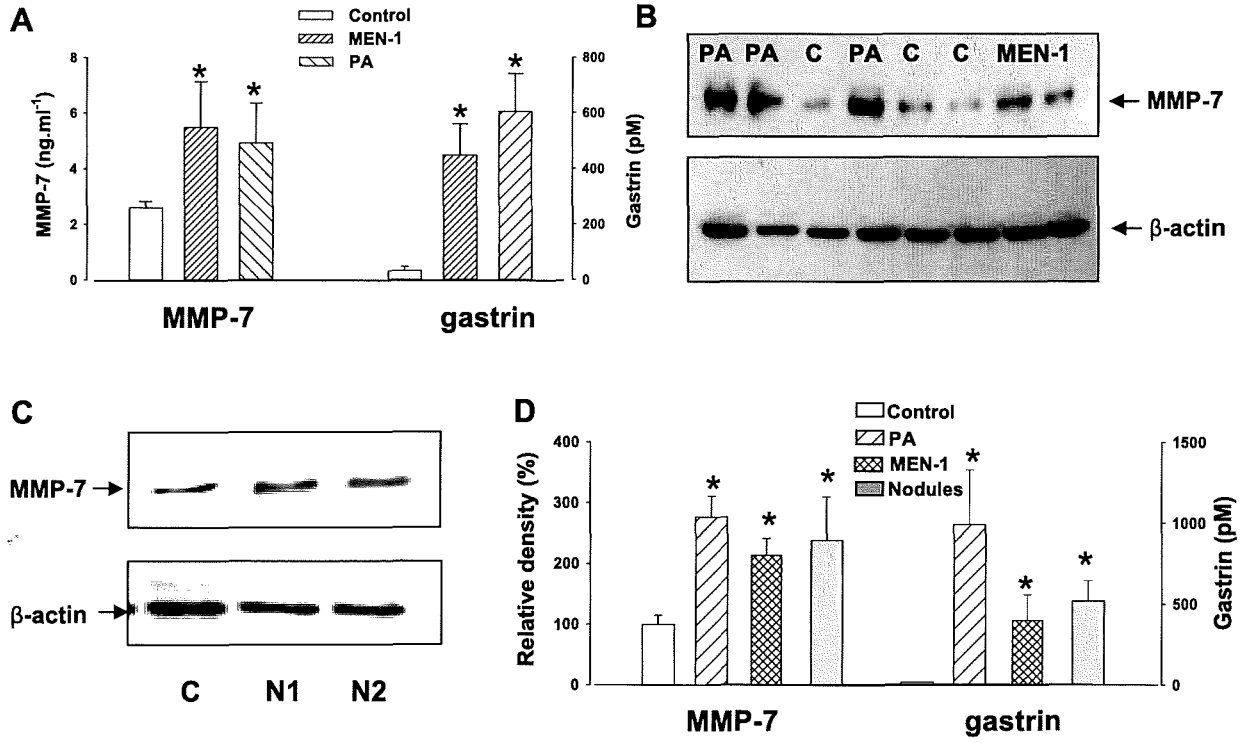

Fig. 1. Increased circulating and gastric matrix metalloproteinase (MMP)-7 in hypergastrinemia. *A*: ELISA showed a modest elevation of circulating MMP-7 in patients with pernicious anemia (PA; $n = 6$) or multiple endocrine neoplasia type 1 (MEN-1; $n = 5$) compared with controls ($n = 8$). *B*: Western blots of the gastric corpus from PA, MEN-1, and control subjects probed for MMP-7. *C*: Western blots of gastric carcinoid tumors from PA (N1), MEN-1 (N2), and control (C) subjects probed for MMP-7 and β-actin. *D*: quantification of Western blots showed an increase in MMP-7 in the corpus of PA patients ($n = 9$) and MEN-1 patients ($n = 7$) and in the carcinioid nodules of PA and MEN-1 patients ($n = 5$). In *A* and *D*, plasma gastrin concentrations are shown for the relevant patients used for Western blots or ELISA in each case. *$P < 0.05$ vs. control.

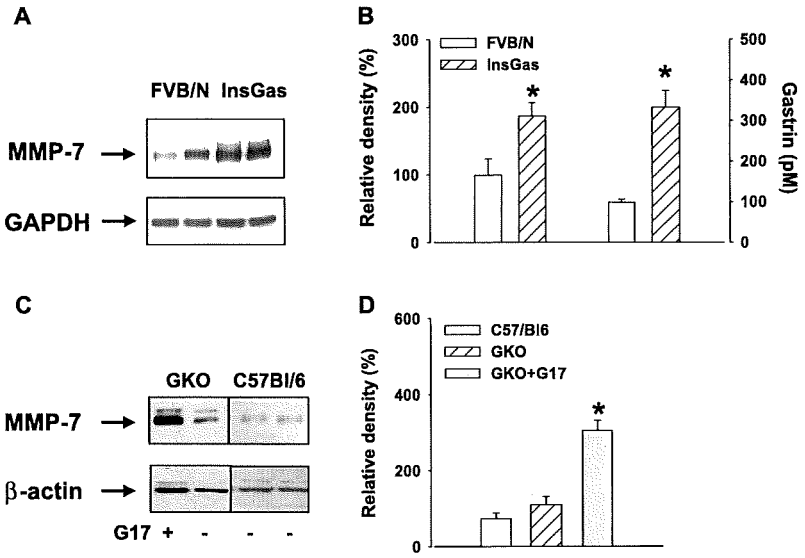

Fig. 2. Increased MMP-7 in mice with hypergastrinemia. *A*: Western blots of the gastric corpus from gastrin-overexpressing (InsGas) and FVB/N mice probed for MMP-7 and GAPDH. *B*: quantification of Western blots showed an ~2-fold increase in MMP-7 in the corpus of InsGas (n = 5) compared with FVB/N (n = 7) mice normalized to GAPDH. There was also an ~3-fold increase in plasma gastrin in these mice. *P < 0.05 vs. FVB/N mice. *C*: Western blots of the gastric corpus from C57Bl/6 and gastrin knockout (GKO) mice treated with or without G17 and probed for MMP-7 and β-actin. *D*: quantification of Western blots showed an ~2–fold increase in MMP-7 in the corpus of GKO mice treated with G17 (n = 3) compared with vehicle-treated control (n = 3) mice, whereas there were no differences in MMP-7 in the corpus of C57Bl/6 mice compared with untreated GKO (n = 3) mice normalized to β-actin. RIA confirmed high circulating plasma gastrin concentrations (~5 nM) in gastrin-treated GKO mice, whereas plasma gastrin concentrations in wild-type mice were normal (56.7 ± 7.2 pM). *P < 0.05 vs. C57Bl/6 mice.

Increased abundance of the myofibroblast marker α-SMA in both the human and mouse gastric corpus with hypergastrinemia. Previous work has established that MMP-7 is localized mainly to epithelial cells but not to stromal cells in the gastric mucosa (33). In line with this, in the present study, we found strong immunostaining of MMP-7 in ECL cells in gastric carcinoid tumors (Fig. 5*A*). In contrast, there was little or no staining of stromal cells. Even so, myofibroblasts identified by staining with antibody to α-SMA were abundant in gastric ECL cell carcinoid tumors and were often dispersed throughout the tumor (Fig. 5*B*). There was also increased α-SMA abundance, as detected by Western blot analysis in the corpus of both PA and MEN-1 patients, including ECL cell nodules, compared with the mucosa from normal subjects (Fig. 6, *A* and *B*). Similarly, there was increased MMP-7 and α-SMA in mice overexpressing gastrin (Fig. 6, *C* and *D*).

MMP-7 released by gastrin from cultured gastric glands stimulates human gastric myofibroblast proliferation. The presence of abundant myofibroblasts in gastric carcinoid tumors suggested that there might be a gastrin-activated signaling pathway from the epithelium to myofibroblasts. To examine whether MMP-7 might be one such mediator, we first treated gastric gland cultures with G17 and measured MMP-7 enzyme activity. G17 (1 nM, 20 h) stimulated MMP-7 enzyme activity by 272.2 ± 36% compared with control (100 ± 19.9%, P < 0.05). Moreover, conditioned medium from cultured human gastric glands treated with G17 stimulated human gastric myofibroblast proliferation, and this was significantly reduced by MMP-7 neutralizing antibodies (Fig. 7*A*), whereas the proliferative effect of another growth factor, EGF (50 ng/ml), was not affected (EGF: 199.4 ± 23.7%; EGF + MMP-7 antibody: 177.6 ± 15.8%). G17 itself had no effect on myofibroblast proliferation (control: 100 ± 5.56%; 1 nM G17: 89.1 ± 10.3%). These data are, therefore, compatible with the idea that native MMP-7 released from epithelial cells by gastrin stimulates myofibroblast proliferation.

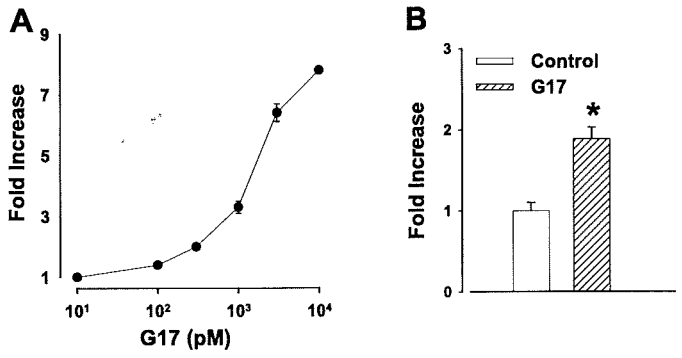

Fig. 3. Gastrin regulation of MMP-7-luc in both AGS-G_R and primary gastric epithelial cells. *A*: in AGS-G_R cells transiently transfected with MMP-7-luc, G17 (8 h) produced a concentration-dependent increase in luciferase activity. *B*: G17 (1 nM, 20 h) also stimulated MMP-7-luc expression in human primary cultured gastric glands. *P < 0.05 vs. control.

MMP-7 stimulates human gastric myofibroblast proliferation via MAPK and PI3K. To examine the cellular mechanisms that mediated the effects of MMP-7 on myofibroblast proliferation, we then considered the involvement of MAPK and PI3K pathways, as these have been previously implicated in the proliferation of colonic myofibroblasts (13). The MEK inhibitor U-0126 (10 μM) reversed the proliferative effect of recombinant MMP-7 in a concentration (2 μg/ml) that had been previously shown to be optimal for myofibroblast proliferation (20) and so too did the PI3K inhibitor LY-294002 (50 μM; Fig. 7*B*). Compatible with the idea that MMP-7 activates the MAPK pathway, we found increased phosphorylation of p42/44 ERK, which peaked within 10 min of the application of rMMP-7 (Fig. 7*C*). Similarly, there was also increased phosphorylation of Akt, which is a known target of PI3K; the time course was distinct from that of p42/44 ERK activation with a progressive increase in phosphorylation after up to 240 min of incubation in MMP-7 (Fig. 7*D*). The activation of ERK and Akt pathways occurred in parallel, since the MEK inhibitor U-0126 inhibited MMP-7-induced p42/44 but not Akt phosphorylation and, vice versa, the PI3K inhibitor LY-294002 inhibited MMP-7-induced Akt but not p42/44 phosphorylation. In contrast, the PKC inhibitor Ro-320432, the EGF receptor tyrosine kinase inhibitor AG-1478, and the Erb-2 receptor tyrosine kinase inhibitor AG-825 had no effect on MMP-7-stimulated proliferation, suggesting that these signaling molecules are not implicated in the actions of MMP-7.

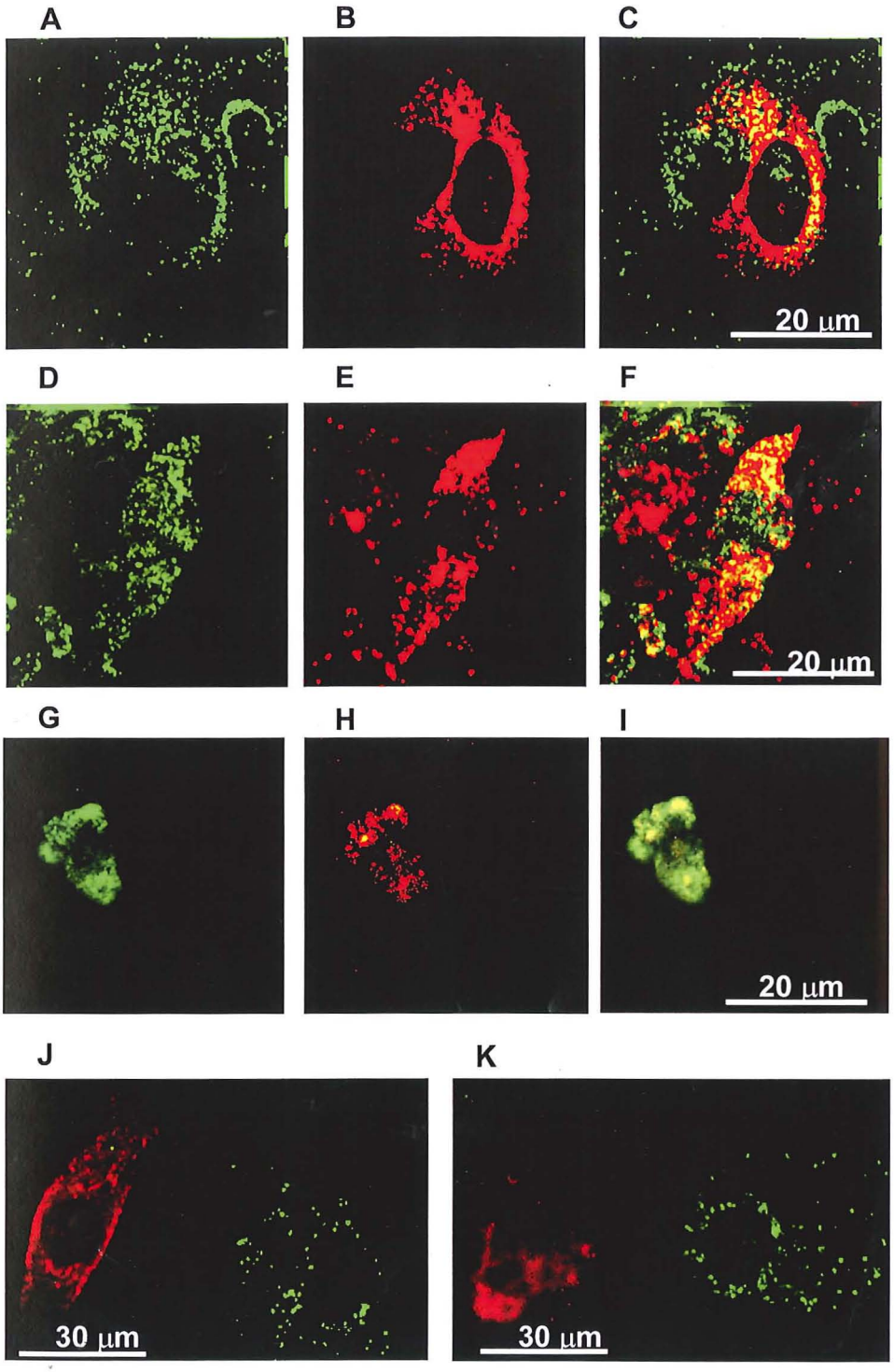

Fig. 4. A 2.3-kb portion of the human MMP-7 promoter targets expression to specific cell types in primary human epithelial cells. *A–C*: MMP-7-luc (green) expression in trefoil factor (TFF)-1 (red)-positive cells. *D–F*: MMP-7-luc (green) expression in pepsinogen (red)-positive chief cells. *G–I*: MMP-7-luc (green) expression in vesicle monoamine transporter type 2 (VMAT-2; red)-contaning enterochromaffin-like (ECL) cells. *J* and *K*: MMP-7-luc (green) expression was not colocalized with H^+-K^+-ATPase (*J*; red) or TFF-2 (*K*; red).

DISCUSSION

The present study was based on the hypothesis that elevated plasma gastrin concentrations stimulate gastric MMP-7 expression, which, in turn, increases myofibroblast proliferation. The main findings of the study were that MMP-7 is increased in ECL cell carcinoid tumors and the gastric corpus of hypergastrinemic patients and that gastrin acts on gastric glands to release MMP-7 in sufficient concentrations to trigger myofibroblast proliferation. ECL tumors provide an interesting model for studies of epithelial-stromal interactions since their growth is dependent on gastrin, which, in turn, is thought to act exclusively on epithelial cells. When gastrin is removed in PA patients by antrectomy, there is tumor regression, including the stromal component, suggesting that ECL cells release factors regulating stromal cell abundance and deposition of extracellular matrix proteins (14, 15). We suggest now that gastrin activates a sequence of events that includes ECL cell proliferation and increased expression of MMP-7, which then stimu-

Table 1. *Percentages of MMP-7-luc-expressing cells that also express TFF-1, VMAT-2, or pepsinogen*

	TFF-1	VMAT-2	Pepsinogen
Percentage of MMP-7-luc-expressing cells	12.3±0.7	36.8±3.9	34.4±0.7

Values are means ± SE. MMP, matrix metalloproteinase; luc, luciferase; TFF, trefoil factor; VMAT, vesicle monoamine transporter. Note that <1% of MMP-7-luc-expressing cells also expressed TFF-2 or H^+-K^+-ATPase (n = 3).

lates myofibroblast proliferation, expanding the extracellular matrix around ECL cells.

Previous studies (2, 4, 33) have shown increased gastric epithelial expression of MMP-7 with *H. pylori* infection. Recently, we (13, 20) reported the consequences of increased epithelial MMP-7 production by *H. pylori* on the gastric microenvironment, namely, rapid cleavage of IGFBP-5 by secreted MMP-7, followed by increased bioavailability of IGF-II from myofibroblasts. IGF-II stimulated the proliferation and migration of myofibroblasts and increased the proliferation of epithelial cells. The present data suggest that hypergastrinemia is also associated with increased MMP-7 expression. Our data suggest that gastrin increases MMP-7 expression at concentrations only observed in frank hypergastrinemia, since it was observed in groups with plasma gastrin concentrations of >100 pM but not in subjects with fasting plasma gastrin concentrations of 30–100 pM. Moreover, in a transgenic mouse model of hypergastrinemia (31), we also found increased MMP-7. In both humans and mice, circulating gastrin concentrations above 100 pM appeared to be required for increased MMP-7. The available data do not, therefore, suggest that there is physiological control of MMP-7 by gastrin. Importantly, however, patients exhibiting increased MMP-7, i.e., with prolonged plasma gastrin concentrations of >100 pM, include those most at risk of developing ECL cell carcinoid tumors (5). It is possible that the apparent elevation of MMP-7 simply reflects increased ECL cell numbers (3, 18, 23) without changed expression at the level of single cells. Set against this, however, it should be noted that exogenous gastrin increased MMP-7 in mice null for gastrin over a time course that could not be attributed to increased ECL cell numbers, and, in studies of cultured cells, we observed direct stimulation of MMP-7 expression in response to gastrin.

Thus, in addition to describing increased MMP-7 in vivo, we have also shown in an in vitro system that gastrin was able to stimulate the expression of an MMP-7-promoter/luciferase reporter construct at concentrations similar to those in the plasma of hypergastrinemic patients. For these experiments, we first made use of AGS-G_R cells as this system has been intensively used for similar previous studies (32, 37). However, to establish whether primary cells might also express MMP-7-luc, we developed a novel methodology to transfect primary human gastric epithelial cells using cultured gland cells in which all the main cell types are preserved and in which cell-cell junctions are intact. In this system, MMP-7-luc was expressed in a cell-restricted manner. Importantly, the pattern of expression of luciferase driven from the MMP-7 promoter resembled that of the WT gene (33). In particular, subsets of TFF-1-, pepsinogen-, and VMAT-2-expressing cells all exhibited luciferase activity after transfection, whereas TFF-2- and H^+-K^+-ATPase-expressing cells did not. Further work will be necessary to define in detail the molecular basis of this expression. It is, however, important to note that luciferase expression in primary cells was increased by gastrin in at concentration of 1 nM. In the future, this method should be generally applicable to studies of other genes normally expressed in cell-restricted patterns in the gastric epithelium. In the present context, the main conclusion was that 2.3 kb of the MMP-7 promoter is sufficient to determine the physiological pattern of expression in the stomach and to support increased expression in response to gastrin.

It is increasingly clear that myofibroblasts play an important role in determining the organization of epithelia during development, in wound healing, and in cancer. The identification of MMP-7 as a novel target of gastrin in this regard therefore raises new possibilities for manipulating epithelial-stromal interactions both experimentally and therapeutically in ECL cell carcinoid tumors. Our data suggest that MMP-7 is able to stimulate MAPK and PI3K pathways in myofibroblasts, leading to an expansion in cell numbers. These pathways are often activated in inflammation, injury, and cancer due to increased growth factor production or mutations leading to accelerated cell proliferation.

Previous work has established the expression of MMP-7 in gastric cancer and an association with poor outcome (1, 21, 35). The possible cellular mechanisms are still largely unexplored. They may include remodeling of the extracellular matrix leading to fibrosis (36, 38), increased cell invasion, or suppression of apoptosis (10, 25). Our data suggest that there is also increased stimulation of stromal cells, and, because these in turn produce growth factors acting on the epithelium, an aggressive cycle of stimulation may be established. The proliferative responses of myofibroblasts to MMP-7 appear to depend on its proteolytic activity, since we have found that heat-inactivated MMP-7 has no effect (data not shown). While the available data suggest that MMP-7 works via cleavage of

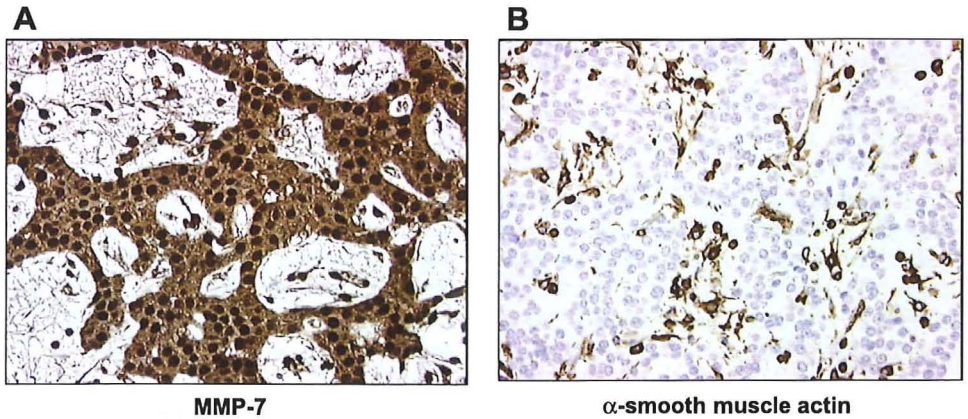

Fig. 5. Immunohistochemical localization of MMP-7 and the myofibroblast marker α-smooth muscle actin (α-SMA) in an ECL cell carcinoid tumor from a PA patient. *A*: MMP-7 immunoreactivity was localized to endocrine cells but not stromal cells in the carcinoid tumor. *B*: α-SMA was localized to myofibroblasts that surround tumor cells. Magnification: ×40.

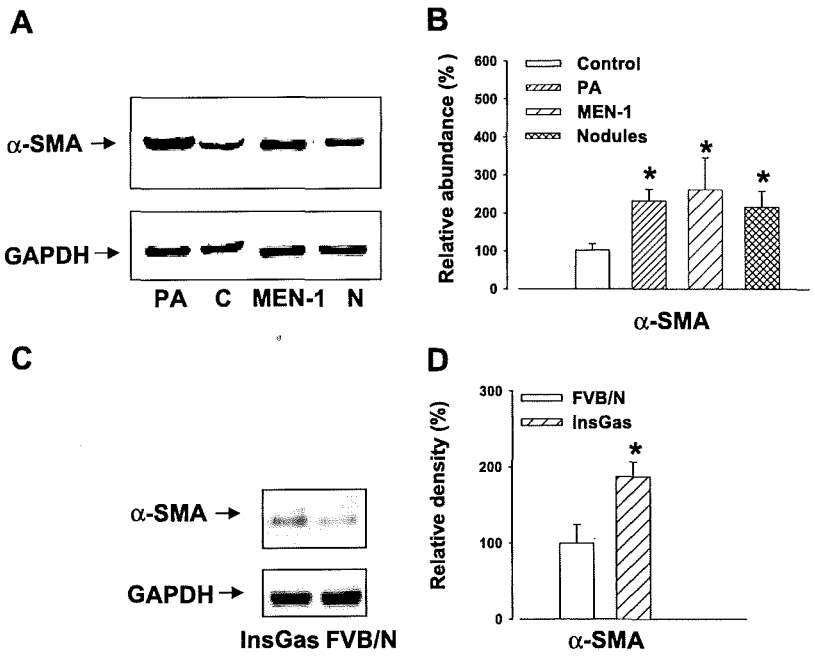

Fig. 6. Increased α-SMA abundance in both humans and mice with hypergastrinemia. *A*: Western blot of the gastric corpus from both PA and MEN-1 patients and an ECL cell carcinoid biopsy (N) from a PA patient showed increased α-SMA compared with the control sample. *B*: quantification of Western blots showed increased α-SMA in both the gastric corpus of PA (n = 11) and MEN-1 (n = 6) patients and in the ECL cell carcinoid tumors itself (n = 6) compared with controls (n = 14) normalized to GAPDH. *$P < 0.05$ vs. control. *C*: Western blot of the gastric corpus from InsGas mice showed increased α-SMA compared with FVB/N mice. *D*: quantification of Western blots showed increased α-SMA in the gastric corpus of InsGas (n = 5) compared with FVB/N (n = 7) mice normalized to GAPDH. *$P < 0.05$ vs. FVB/N mice.

IGFBP-5 and liberation of IGF-II, it is worth noting that at least in colonic myofibroblasts, there is some evidence that MMP-7 activates other MMPs that participate in the response (13); further work will be need to determine whether comparable protease interactions occur in the present system. Moreover, the direct evidence for MMP-7 stimulation of myofibroblast cell proliferation comes from studies in vitro, and there is now a need to extend this work to include direct studies of the effects of MMP-7 on myofibroblast cell numbers in vivo.

Taken as a whole, the present data identify MMP-7 as a previously unsuspected target of gastrin that is increased in hypergastrinemic conditions. We also show that in at least one condition, ECL cell carcinoid tumors, MMP-7 is a potential regulator of the stromal compartment by action on myofibroblast cells via activation of MAPK and PI3K pathways. Previous studies (13, 20) have established that MMP-7 degrades IGFBP-5 released by myofibroblasts, thereby liberating IGF-II. Moreover, there is already evidence that in different cellular systems, gastrin is able to activate distinct paracrine pathways stimulating EGF receptors (29) and FGF receptors (22). However, whereas previous studies were based on a cancer cell line (AGS cells), the present work focused on primary epithelial cells and myofibroblasts. The relative importance of these different pathways in the normal stomach remains to be established; indeed, it should be noted that the gastric myofibroblasts used in our study were obtained from tissue adjacent to gastric tumors and may well not be representative of myofibroblasts in normal gastric tissue. Even so, the present study indicates that, in the future, it will be profitable to determine the precise molecular mechanisms by which MMP-7 contributes to the maintenance of the stromal compartment in epithelial tumors.

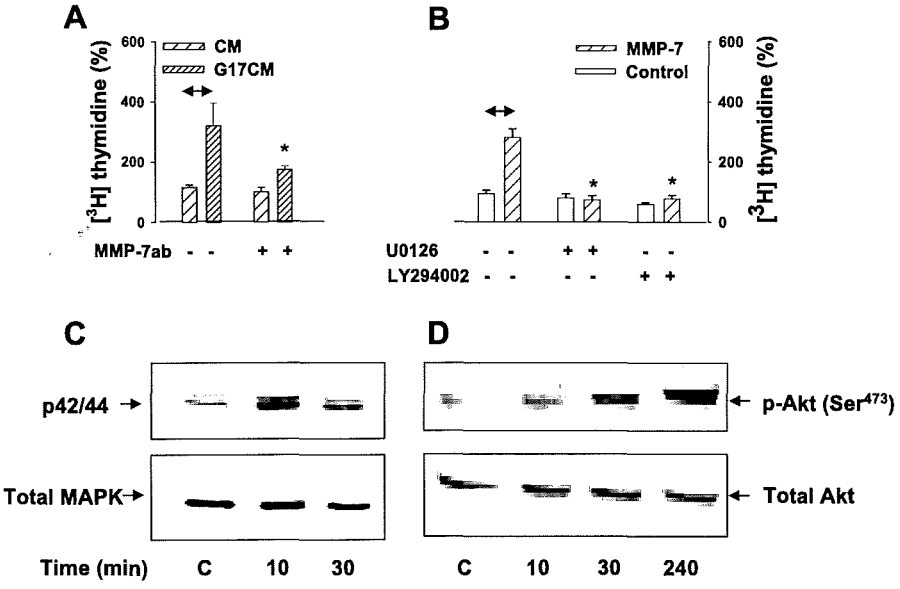

Fig. 7. Conditioned medium (CM) from gastrin-treated human gastric glands stimulates proliferation of human gastric myofibroblasts by MMP-7 via MAPK and phosphatidylinositol 3-kinase (PI3K). *A*: proliferation of human gastric myofibroblasts was stimulated by coculture with CM media from G17 (1 nM, 20 h)-treated gastric glands. Treatment of glands with neutralizing mouse monoclonal MMP-7 antibody (Ab) partially inhibited proliferation. Treatment of myofibroblasts with nonimmune mouse IgG had no effect (n = 3). *B*: stimulation of [^{3}H]thymidine incorporation by recombinant MMP-7 (3 U/ml) in primary human gastrin-treated myofibroblasts was virtually abolished by the MEK inhibitor U-0126 and the PI3K inhibitor LY-294002. *C* and *D*: Western blots showed increased abundance of phospho-p42/44Erk 10 min after the addition of recombinant MMP-7 (*C*) and increased abundance of phosphorylated Akt (p-Akt) 240 min after the incubation with recombinant MMP-7 (*D*).↔, significant differences between MMP-7-treated and control samples. *Decreases in MMP-7-stimulated proliferation following application of the compound specified.

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