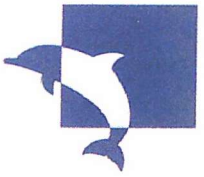

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THE BI-DIRECTIONAL RELATIONSHIP BETWEEN MAST CELLS AND HEPATIC STELLATE CELLS IN LIVER FIBROSIS

by

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"Science is a mechanism, a way of trying to improve your knowledge of nature. It is a system for testing your thoughts against the universe, and seeing whether they match."

Isaac Asimov (1920-92)

ABSTRACT

DIVISION OF CELL AND MOLECULAR MEDICINE
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Mast cell (MC) hyperplasia is commonly associated with tissue fibrosis. Within the liver during fibrosis and injury, MC accumulate in the fibrous septae within the portal tracts. Immunohistochemical studies have correlated MC hyperplasia with collagen deposition, which occurs in liver fibrosis. Hepatic stellate cells (HSC) are pivotal in the maintenance of matrix homeostasis within the liver. During injury HSC adopt a myofibroblastic- phenotype, synthesise and secrete a variety of extracellular matrix proteins. As activated HSC have similar characteristics to fibroblasts, HSC and MC may have a bi- directional relationship within the liver, as observed between fibroblasts and MC in other tissues. Fibroblasts synthesise stem cell factor (SCF), an important MC developmental factor, which can recruit MC into the surrounding connective tissue. MC, in return, release preformed mediators such as tryptase, that induce fibroblast proliferation and collagen synthesis. The mitogenic effect of tryptase has been suggested to be mediated by a novel receptor, namely proteinase activating- receptor 2 (PAR-2). PAR-2 is a member of a family of serine protease receptors that are activated by proteolysis.

In this thesis I have examined the hypothesis that HSC and MC have a bi- directional relationship in liver fibrosis This was examined by investigating a possible mechanism of MC recruitment within the fibrotic liver, the fibrogenic effects of MC mediators and their subsequent mechanism in HSC activation.

SCF expression in human and rat livers and HSC was investigated as a possible cytokine mediating the recruitment of MC to the fibrotic liver. By various techniques SCF mRNA and protein were detected in fibrotic livers and activated HSC. Addition of neutralising SCF antibodies to co-cultures of HSC and MC significantly inhibited MC adherence to HSC.

As MC numbers have been reported to increase during liver fibrosis, MC subtypes were examined in human diseased livers and in rat CCl_4 fibrotic livers by RT-PCR and immunohistochemistry in rat CCl_4 fibrotic livers. In human livers MC_T and MC_{TC} were found in normal and fibrotic livers, whilst MMC and CTMC were found in rat normal and fibrotic livers Immunohistochemistry illustrated MC hyperplasia in fibrotic rat livers within the portal tracts and fibrotic septae. The possible mitogenic effects of MC mediators on HSC were examined. Tryptase significantly induced HSC proliferation and in two independent experiments stimulated collagen synthesis.

To further support the existence of a relationship between MC and HSC, the expression of PAR-2 was investigated in rat fibrotic livers and cultured HSC. PAR-2 mRNA and protein was detected in rat livers and HSC by various techniques. As further confirmation of PAR-2 existence in HSC, the mitogenic effects of PAR-2 agonists on in-vitro rat HSC cultures were investigated. The PAR-2 activating peptide (SLIGRL) induced significant HSC proliferation and collagen synthesis, suggesting that tryptase acts via PAR-2. MAPK induction was examined in SLIGRL-stimulated cultured HSC in the presence or absence of a specific MAPK inhibitor. MAPK activity was maximally induced by SLIGRL after 15 minutes of stimulation, and was significantly inhibited by PD98059, a MAPK inhibitor as detected by ^3H- proliferation assays. MAPK therefore was a suggested signal transduction pathway involved in HSC stimulation via tryptase and SLIGRL.

To conclude, these results suggest that HSC may recruit MC to the fibrotic liver by the synthesis of SCF, in turn MC may upregulate HSC activation by tryptase stimulation mediated by PAR-2 . These findings may contribute to future studies involved in designing potential therapies to be used in controlling and possibly preventing liver fibrosis. Further investigation is necessary to understand the role of MC in liver fibrosis. With the recent discovery of PAR-2, and the induction of collagen synthesis by tryptase and SLIGRL possibly mediated by PAR-2, this may be an attractive target for future drug development.

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Dużo buźi.

ABBREVIATIONS

^{3}-H	tritiated thymidine
3T3	mouse skin fibroblast
5-HT	5- hydroxytryptamine
5-LO	5- lipoxygenase
A	adenosine
AA	arachidonic acid
AAF	2' acetylaminofluorine
AIDS	acquired immunodeficeincy syndrome
APMA	aminophenyl mercuric acetate
AP-1	activator protein-1
APS	ammonium persulphate
Arg	arginine
BAL	bronchoalveolar lavage fluid
BAPNA	N- benzyl- DL- arginine- p-nitroanilide
BD	bile ducts
BDL	bile duct ligation
bFGF	basic fibroblast growth factor
BMMC	bone marrow derived mast cell
bp	base pairs
BSA	bovine serum albumin
CCl_3	trichloromethyl radical
CCl_4	carbon tetrachloride
cDNA	complementary deoxyribonucleic acid
c-Ets	transcription factor for PEA-3 element
c-fos	transcription factor for AP-1 element
c-jun	transcription factor for AP-1 element
c-kit	tyrosine kinase receptor for stem cell factor
con-A	concavalin- A
CO_2	carbon dioxide
COX	cyclooxygenase
cpm	counts per minute
CRBP	cellular retinoid binding protein
C- term	carboxyl terminal
CTMC	connective tissue mast cell
CV	central vein
DAB	3,3- diaminobenzidine tetrahydrochloride
DAG	diacylglycerol
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
DEN	diethylnitrosamine
DEPC	diethylpyrocarbonate
dGTP	deoxyguanosine triphosphate
DMEM	Dulbecco's medium
DMEM +AA	Dulbecco's medium containing 25 µg/ml ascorbic acid and 0.01% BSA
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleotide triphosphate

dsDNA	double stranded deoxyribonucleic acid
dsRNA	double stranded ribonucleic acid
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
ECL	enhanced chemiluminescent detection system
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	epithelial growth factor
EHS	Englebreth-Holm Swarm
ELISA	enzyme linked immunobsorbent assay
EO	eosinophils
ER	endoplasmic reticulum
ERK	extracellular regulated-protein kinase
ESB	ethidium sample buffer
Fc	constant region of immunoglobulin
$Fc_{\varepsilon}IgE$	high affinity receptor for IgE
FCS	foetal calf serum
FGF	fibroblast growth factor
FITC	fluorescein isothiocyanate
FS	fibrotic septae
g	grams
GAGs	glycosaminoglycans
GDP	guanosine 5'diphosphate
GIT	*guanidinium isothiocyanate*
GM-CSF	granulocyte- macrophage colony stimulating factor
G-protein	guanidine protein receptor
GTP	guanosine 5' triphosphate
HA	hepatic artery
HBSS	Hank's buffered salt solution
HCl	hydrochloric acid
HEPES	hydroethyl piperazine ethane sulphonic acid
HMC-1	human mast cell line
HSC	hepatic stellate cells
IAA	isoamyl alcohol
ICAM-1	intercellular adhesion molecule-1
Ig	immunoglobulin
IgE	immunoglobulin E
IgG	immunoglobulin G
IFNα	interferon alpha
IFNγ	interferon gamma
IL	interleukin
IPTG	isopropyl-β–D- thiogalactopyranoside
IP_3	inositol triphosphate
kb	kilobase
KC	Kupffer cell
KCl	potassium chloride
kDa	kilodalton
kg	kilogram
Leu	leucine
LPS	lipoploysaccharide
LTB_4	leukotriene B_4
LTC_4	leukotriene C_4
LTD_4	leukotriene D_4

LTE_4	leukotriene E_4
LRAT	lecithin: retinol acyl transferase
Lys	lysine
M	Molar
MAPK	mitogen activated protein kinase
MC	mast cells
MC_T	mast cells containing tryptase only
MC_{TC}	mast cells containing tryptase and chymase
MCS	multiple cloning site
MCP	macrophage chemotactic protein
mg	milligram
$MgCl_2$	magnesium chloride
MIP	macrophage inflammatory protein
ml	millilitre
mm	millimetre
mM	milliMolar
MMC	mucosal MC
MMP	matrix metalloproteinase
M-MuLV	Moloney Murine Leukaemia Virus
MOPs	3-(N-morpholino) propanesulphonic acid
mRNA	messenger ribonucleic acid
MT1-MMP	membrane-type-1 matrix metalloproteinase
mU/ml	milliunits per millilitre of enzyme activity
mw	molecular weight
NaOH	sodium hydroxide
NEM	N- Ethylmaleride
NFκB	nuclear factor- kappa B
NF-1	nuclear factor 1
NF-AT	nuclear factor of activated T cells
ng	nanograms
NGF	nerve growth factor
nm	nanometres
nM	nanoMolar
NO	nitric oxide
N term	amino terminal
PAF	platelet activating factor
PAI	plasminogen activator inhibitor
PAR	proteinase activated- receptor
PBC	primary biliary cirrhosis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PEA-3	polyomavirus enhancer activator-3
pg	picogram
PGD_2	prostaglandin D_2
PGF_2	prostaglandin F_2
Phe	phenlylananine
PLA_2	phospholipase A_2
pM	picoMolar
PMA	phorbolmyristate acetate
PSC	primary sclerosing cholangitis
PSG	penicillin/ streptomycin/ gentomycin
PT	portal triad

PV	portal vein
PVDF	polyvinyl- difluoride
RA	retinoic acid
RAR	retinoic acid receptor
RARE	retinoic acid response element
RBP	retinol binding protein
RE	retinyl ester
REH	retinyl ester hydrolase
RER	rough endoplasmic reticulum
rhSCF	recombinant human stem cell factor
RMCPI	rat mast cell protease I
RMCPII	rat mast cell protease II
RNA	ribonucleic acid
RNase	ribonuclease
RNasin	ribonuclease inhibitor
RPA	ribonuclease protection assay
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute medium
RRNA	ribosomal ribonucleic acid
rrSCF	recombinant rat stem cell factor
rSCF	recombinant stem cell factor
RSF	rat skin fibroblast
RT	reverse transcription
RT-PCR	reverse transcription-polymerase chain reaction
rUTP	ribouridine triphosphate
RXR	retinoid X receptor
SCF	stem cell factor
SCG	sodium cromoglycate
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEC	sinusoidal endothelial cells
SEM	standard error of the mean
Ser	serine
SER	smooth endoplasmic reticulum
s-Ets	transcription factor for PEA-3 element
Sl	Steel locus
Sp1	stimulatory protein/ transcription factor for promoter carrying GC boxes
SSC	salt sodium citrate
ssDNA	single-stranded deoxyribonucleic acid
T	thymidine
TATA	promoter site (Hogness box)
Taq	*Thermus acquaticus*
TBE	tris borate/ EDTA
TEMED	N,N,N,N,-tetramethyl ethylenediamine
TGFα	transforming growth factor alpha
TGFβ	transforming growth factor beta
TIE	TGFβ inhibitory element
TIMPs	tissue inhibitor of MMPs
TNF-α	tumour necrosis factor
tPA	tissue plasminogen activator
tr⁻/⁻	PAR-1 knockout mice
tRNA	transfer ribonucleic acid
Tris	tris (hydroxymethyl)- methylamine

Trp	tryptophan
TSK	tight skin mouse
TTBS	tris- buffered solution containing Triton X-100
UK	United Kingdom
uPA	urokinase plasminogen activator
USA	United States of America
UTR	untranslated region
UV	ultra violet light
v	volts
Val	valine
v/v	volume/volume ratio
W	White- spotting locus
w/v	weight/ volume ratio
X-Gal	5-chloro- 4-bromo- 3-indolyl-β– galactosidase
α-SMA	alpha- smooth muscle actin
β-actin	beta actin
μCi	microCurie
μg	micrograms
μl	microlitres
μm	micrometre
μM	microMolar
°C	degrees centigrade
%	percent
+/+	wild type phenotype

CHAPTER ONE
GENERAL INTRODUCTION

1. GENERAL INTRODUCTION

1.1. INTRODUCTION TO LIVER FIBROSIS

Liver fibrosis is a result of hepatic injury from a variety of aetiologies, including viral infection (hepatitis), helminthic infection (*Schistosomiasis*), toxic damage (alcohol), biliary obstruction (biliary cirrhosis), autoimmune/ immune mediated injury (autoimmune hepatitis, primary sclerosing cholangitis) and metabolic diseases (haemachromatosis). Liver fibrosis is a major worldwide health problem, with approximately 6,000 deaths annually in the United Kingdom (UK) alone (Arthur and Iredale, 1994) and is the ninth highest cause of death in the United States of America (USA) (Dufour *et al,* 1993). Liver fibrosis is characterised as an imbalance in the 'physiological homeostasis of the synthesis (fibrogenesis), degradation (fibrolysis) and deposition of extracellular matrix (ECM) materials' (Clement *et al,* 1993), and occurs after recurrent and prolonged hepatocellular damage. To date there are few successful therapies available for treating liver fibrosis. At end stage fibrosis or cirrhosis, when fibrotic liver disease becomes irreversible, the only treatment available is liver transplantation.

There are two kinds of liver fibrosis: portal and perisinusoidal fibrosis (Cales, 1998). Portal fibrosis causes the most damage to liver architecture and is most abundant in cirrhosis. Perisinusoidal fibrosis is also observed in cirrhosis and is the cause of portal hypertension. Fibrogenesis is a part of the tissue repair response to hepatic injury and results in the classical characteristic increase in ECM proteins (Rojkind *et al*, 1979). This accumulation of ECM proteins results in the formation of dense hepatic scar tissue (Friedman, 1993), or nodules. The hepatic scar is made up of type I and III fibril- forming collagens and matrix glycoconjugates including: proteoglycans, fibronectin and hyaluronic acid (Gressner and Bachem, 1990). In liver cirrhosis there is a six to ten fold increase in collagen content in the diseased liver compared to the normal liver, which has approximately 5 milligrams (mg) collagen / gram (g) wet liver weight (Rojkind *et al*, 1979; Gressner and Bachem, 1990). Synthesis of hepatic scar tissue and excess collagen deposition results in abnormal liver architecture and the restriction of interhepatic blood flow that compromises liver function. Phenotypically this is observed in symptoms such as jaundice, portal hypertension, ascites, gastrointestinal bleeding and hepatic encephalopathy.

Hepatic stellate cells (HSC) (also known as fat- storing cells, Ito cells, lipocytes, or perisinusoidal cells), found within the space of Disse in the hepatic sinusoids, are the major cell within the liver involved in ECM synthesis and regulation (Friedman, 1993; Gressner

et al, 1996), and is pivotal in the pathogenesis of liver fibrosis/ cirrhosis. Hence much research into liver disease has been targeted specially at investigating the role of HSC in liver fibrosis.

1. 2. STRUCTURE OF THE LIVER

The liver is the largest mass of glandular tissue in the body. Weighing 1.2- 1.6 kilograms (kg), it is situated in the upper right region of the abdominal cavity. It is an unique organ as it has two different blood supplies, arterial blood from the hepatic arteries and a mixture of venous and arterial blood from the portal vein, direct from the digestive tract. The hepatic vein returns the blood from the liver back into the major blood circulation. Within the liver, blood from branches of the hepatic artery and portal vein enter specialised hepatic capillaries called sinusoids. Within the sinusoids, there is an exchange of substances between the blood and liver parenchyma. The blood leaves the sinusoids via a venous network that leads to the hepatic vein that drains into the inferior vena cava.

The liver has a variety of important and essential biological functions. Primarily the liver utilises absorbed foods from the digestive tract via the hepatic portal system. For example, it converts excess glucose from carbohydrate metabolism into glycogen which is stored and rapidly reconverted into glucose when energy is required (glycogenolysis); the liver stores and metabolises lipids; deaminates amino acids from metabolised proteins to be converted into glucose (gluconeogenesis) and any excess into urea to be excreted by the kidneys. The liver forms and secretes bile (500 to 1,000 millilitres (ml) a day) into the duodenum via the biliary system, an excretory medium for toxins, drugs, minerals, pigments and is an important substance for digestion of fats. The liver plays a major role in fighting infections via mobilising the macrophage system within the liver.

The liver is divided into four lobes, two main lobes (right and left, with the right lobe being the larger of the two) and two accessory lobes (quadrate and caudate). The lobes are further divided into lobules which are the structural and functional units of the liver. A cross section of the lobule shows that it has the shape of a polygon and portal tracts, comprising intrahepatic branches of the portal vein, hepatic artery, bile duct and lymphatic vessel, are found at angles of the polygon (figure 1.1). In the human, lobules are not clearly demarcated, however there are 3 to 6 portal triads per lobule. Traversing the long axis of the lobule is the central vein. Plates of hepatocytes radiate from the central vein to the perimeter of the lobule and constitute the parenchyma of the lobule. The sinusoids are found between the plates of hepatocytes and empty into the central vein. Blood flows from the hepatic artery and portal vein into the sinusoids and drain into two or three terminal

branches of the central vein. The sinusoids are lined by a discontinuous layer of fenestrated endothelial cells and Kupffer cells (resident liver specific macrophages). Occasionally in the sinusoids pit cells (hepatic granulated lymphocytes) are found, and are more numerous in the periportal region (Kaneda and Wake, 1985). They play a role in clearing opsonised particles and tumour cells from the hepatic blood. The perisinusoidal space between the endothelium of the sinusoids and the hepatocytes is known as the space of Disse. Numerous hepatocyte microvilli project into the space of Disse allowing hepatocytes to remove individual products from the blood and to discharge other unwanted products. HSC can be found within the space of Disse. In normal liver the sinusoid endothelium contains little or no collagens and basement membrane proteins (Gulubova, 1996) This allows the sinusoid wall to be highly permeable to allow for the transvascular exchange from the blood to the hepatocytes. However during liver injury collagens and basement membrane material accumulate in the space of Disse as will be described. This results in the impairment of transvascular exchange from the blood to the hepatocytes.

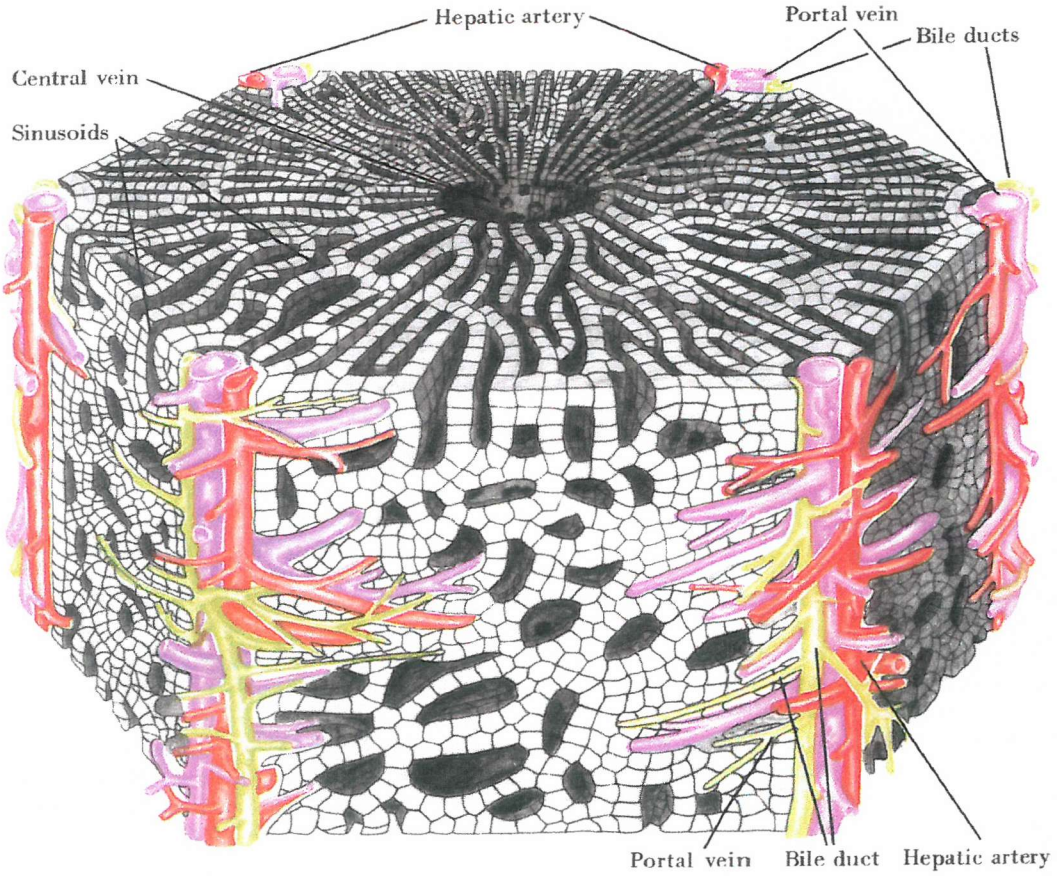


Figure 1.1. Schematic diagram outlining the liver lobule as a six- sided polyhedral prism with intrahepatic branches of the portal vein, hepatic artery and bile duct at each corner. The intrahepatic vessels branch along the sides of the lobule and open out into the sinusoids allowing for exchange and transfer of components within the blood. The central vein, found traversing the long axis of the liver lobule, receives blood from the sinusoids and returns it to the inferior vena cava where it rejoins the major circulatory system.
Adapted from Histology, a text and atlas. M.H. Ross and L.J. Romrell, 2nd edition, 1989, Williams and Wilkins.

1.3. CELLS OF THE LIVER

1.3.1. Hepatocytes

Hepatocytes make up 80% of the cell population within the liver. They are involved in the major functions of the liver such as exchange of substances from the blood, plasma protein synthesis, bile synthesis and secretion, glycogen storage, regulation and metabolism of cholesterol and urea formation from detoxified ammonia. Hepatocytes have a centrally placed spherical nucleus that is often binucleated. Within the cytoplasm of the cell are found mitochondria, rough endoplasmic reticulum (RER), smooth endoplasmic reticulum (SER) and Golgi apparatus that are involved in the regulation of hepatocyte function.

Within the liver lobule the polygonal hepatocytes are distributed as interconnecting sheets radiating out from the central vein. Microvilli project from the surfaces of the hepatocyte that face the space of Disse allowing for easier and more effective exchange of substances from the blood in the sinusoids. Bile canaliculi can be found between apposing hepatocytes (figure 1.2). The bile canaliculi form a mini network of ducts around the hepatocytes. This allows for the discharge of bile through a system of canaliculi and ducts into the duodenum.

1.3.2. Sinusoidal endothelial cells (SEC)

SEC are found in the liver lobule lining the sinusoids along with Kupffer cells (figure 1.2). The SEC are highly fenestrated (Wisse, 1970), forming sieve plates (Wisse *et al* 1985; Smedsrod *et al*, 1994), and discontinuously line the sinusoid. The fenestrae help the passage of solutes and particles between the sinusoid and the space of Disse. In hepatic fibrosis and during chronic alcohol consumption the SEC become defenestrated (Horn *et al*, 1987).

SEC contain endocytic vesicles within the cytoplasm and are involved in receptor mediated endocytosis of various substances such as serum proteins and low-density lipoproteins. This reflects their role as scavenger systems clearing blood of a variety of macromolecular waste products and connective tissue macromolecules (Smedsrod *et al*, 1994).

1.3.3. Kupffer cells (KC)

Carl von Kupffer first described KC in 1898 as star- shaped fixed macrophages of the liver. KC are derived from recruited monocytes and accumulate in the liver sinusoids during hepatic inflammation (Bouwens *et al*, 1986). They make up 15% of the total cell population within the liver (Bioulac- Sage *et al,* 1996) and 30% of the total

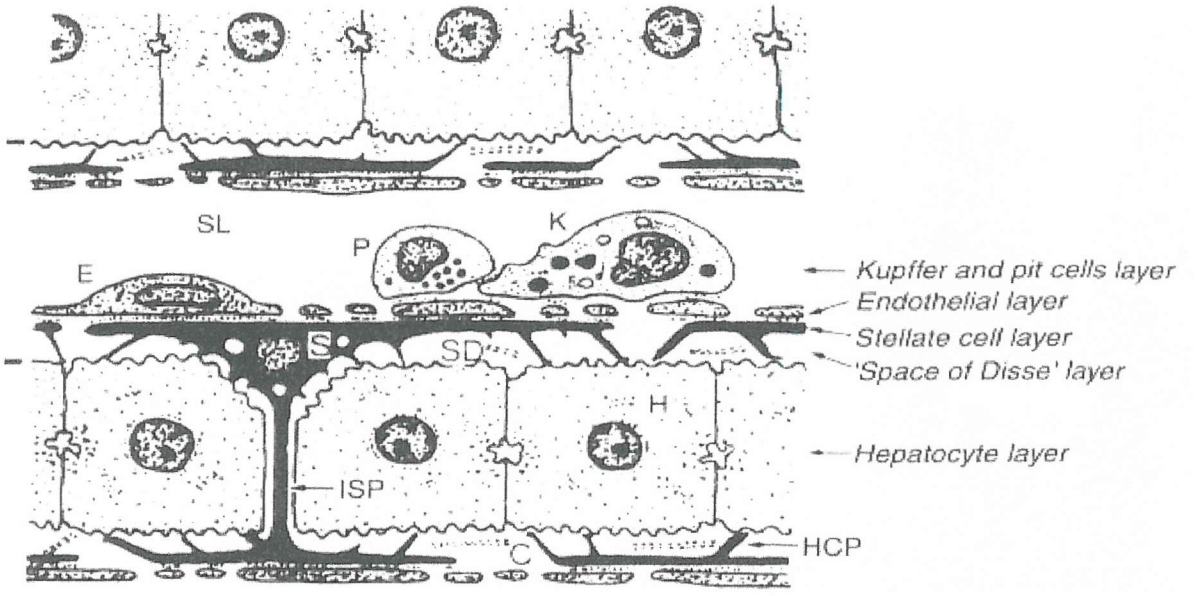


Figure1.2. Schematic representation of sinusoids within the liver and the cellular constituents, not to scale. **SD**- space of Disse, **SL**- sinusoidal lumen, **H**- hepatocyte, **E**- sinusoidal endothelial cell, **K**- Kupffer cell, **S**- hepatic stellate cell, **ISP**- intersinusoidal process, **HCP** - hepatocyte- contacting process, **P**- pit cell, **C**- collagen fibrils. Note the bile caniculi in-between the two apposing hepatocytes.
Taken from Hautekeete, M.L. and Geerts, A. The hepatic stellate cell: its role in human liver disease. Virchows Arch 1997; ***430:*** *195- 207.*

nonparenchymal cell population (Toth and Thomas, 1992). KC are found in the hepatic sinusoidal lumen on top or in between SEC and form a protective barrier removing potentially harmful materials from the circulation, such as viruses and bacteria (figure 1.2). They are irregular in shape and contain well-developed endoplasmic reticulum (ER) and Golgi and a large number of lysosomes and phagosomes associated with their phagocytic function. KC show heterogeneity interlobularly. In the periportal regions KC contain highly developed lysosomes compared to KC in the central region. This would suggest that KC within the periportal regions are more active.

The KC has many functions within the liver, most of which involve phagocytosis, for example erythrophagocytosis and iron metabolism. KC phagocytose erythrocytes and recycle iron in the form of ferritin which is taken up directly by hepatocytes (Toth and Thomas, 1992). Haemoglobin from erythrocytes is then converted into bilirubin before conjugation by hepatocytes and expelled into the bile. Clearance of foreign matter such as bacteria and viruses, immune complexes, endotoxins, tumour cells, cell debris and modified cholesterol (low- density lipoproteins) are all carried out by KC phagocytosis. In liver disease KC phagocytosis is known to be impaired (Drivas *et al,* 1976; Lough *et al.* 1989). Phagocytosis of endotoxins such as lipopolysaccharides (LPS) will result in the activation of KC cytokine production. Pro-inflammatory cytokines such as interleukin-6 (IL-6), IL-1, tumour necrosis factor α (TNFα) and interferon (INF) α and β are produced by LPS activated KC, and possibly exert their effect on transformed activated HSC and

induce chemotaxis of monocytes and neutrophils (Maher and Friedman, 1993; Bioulac-Sage *et al,* 1996). Nitric oxide (NO) is formed by KC by the action of nitric oxide synthase on L-arginine in response to LPS. This results in the inhibition of protein synthesis in hepatocytes and controls the contractility of HSC (see section 1.5.2). Synthesis of prostaglandin D_2 (PGD_2) and leukotriene B_4 (LTB_4) and LTC_4 is also due to LPS- KC activation (Bioulac- Sage *et al,* 1996).

1.3.4. Hepatic stellate cells (HSC)

HSC are stellate-shaped mesenchymal cells which contain characteristic cytoplasmic lipid droplets, have several dendritic processes and reside in the space of Disse (figure 1.2). They are in close contact with hepatocytes and SEC (Wake, 1980; Wake *et al*, 1988), by use of different cytoplasmic processes; intersinusoidal, that traverse along the sinusoid and hepatocyte- contacting processes that make contact with hepatocytes. HSC also make contact with other HSC and nerve endings with their cytoplasmic processes (Hautekeete and Geerts, 1997). HSC are distributed regularly in the liver lobule and comprise 5-8% of the total number of cells in the liver (Ramadori *et al,* 1990). In acute liver disease such as acute viral hepatitis and paracetamol- induced liver necrosis, and in chronic inflammation of the liver, HSC are found outside the space of Disse, often in close proximity to damaged parenchymal cells and KC.

Ultrastructurally, the cytoplasmic lipid droplets are the most prominent feature of HSC in the normal liver and are a characteristic trait used to identify and isolate HSC from other liver cells. The lipid droplets, containing vitamin A, make HSC buoyant so this feature is exploited when purifying HSC (Friedman *et al*, 1985). Use of ultraviolet light induces autofluorescence of vitamin A and results in a blue- green fluorescence at 328 nanometres (nm) allowing for the easy identification of HSC within liver cell preparations without fixation.

There are two types of lipid droplets found within HSC (Wake, 1980): membrane bound (Type I) droplets which are smaller than 2 (micrometres) μm in diameter and are derived from 'multivesicular bodies' which are similar to lysosomes (Wake, 1974) and non-membrane bound (Type II) droplets that are larger and up to 8 μm in diameter.

Also within the cytoplasm of the HSC mitochondria, Golgi apparatus and extensive RER complexes are found, which become markedly enlarged during liver injury. Microtubules and intermediate (10 nm) filaments are found within the cytoplasmic matrix (Kawada, 1997).

An extraordinary aspect of HSC is their ability to express a dual phenotype. In the normal healthy liver, the cells have a quiescent phenotype, are spherical in shape, show minimal proliferation and contain cytoplasmic lipid droplets containing vitamin A. However in the diseased liver HSC adopt an 'activated myofibroblast- like' phenotype (Friedman,1993; Gressner, 1996). The cells become dendritic in shape as they become myofibroblastic-like, lose their lipid droplets, become proliferative, express cytokine receptors such as platelet-derived growth factor (PDGF) (Friedman and Arthur, 1989b; Pinzani *et al,* 1996) and transforming growth factor-β (TGFβ) (Casini *et al,*1993), express intracellular α- smooth- muscle actin (α-SMA) (Ramadori *et al,* 1990) and most importantly synthesise various ECM protein components such as collagens type I and III, (McGee and Patrick, 1972; Maher *et al,* 1988; Milani *et al,* 1990; Rockey *et al,* 1992). This activation step can be mimicked *in-vitro* by culturing primary HSC on standard tissue culture plastic (Friedman *et al,* 1989a). In addition to using uncoated tissue culture plastic as a substratum for HSC in culture, a variety of extracellular matrices can be used to modulate HSC activation. Type I collagen a major matrix component produced during fibrosis, promotes HSC activation (Davis *et al,* 1987). Laminin- rich gel that resembles normal liver subendothelial matrix (first isolated from Engelbreth-Holm Swarm (EHS) tumours, a transplantable sarcoma (Timpl *et al,* 1979), but commercially known as Matrigel), downregulates HSC activation in culture (Friedman *et al,* 1989a; Gaça *et al,* 2000).

Activated HSC synthesise and secrete a wide range of profibrogenic cytokines that exert their effect via autocrine mechanisms. For example activated HSC upregulate the synthesis of TGFβ (De Blesser *et al,* 1997) and simultaneously upregulate their TGFβ receptor expression (Friedman, 1991). TGFβ is the principal profibrogenic growth factor in liver fibrosis and can modulate collagen expression (De Blesser *et al,* 1997). Activated HSC also synthesise and secrete other profibrogenic mediators such as PDGF, basic fibroblast growth factor (bFGF) and TGFα (Bachem *et al,* 1992; Pinzani *et al,* 1996, Tsukomoto, 1999)

Retinoids have been shown to influence HSC activation (Davis and Vuvic, 1988). The addition of retinol to passaged HSC cultures decreases HSC proliferation. Addition of retinoic acid to passaged HSC also decreases HSC proliferation and down regulates type I collagen expression and TGFβ synthesis (Davis *et al,* 1990). However, some recent studies have shown that addition of 9,13-di –cis-retinoic acid (a major metabolite of vitamin A) added to HSC cultures increased collagen expression and TGFβ synthesis (Okuno *et al,* 1997; Okuno *et al,*1999).

Wake and Sato (1993), have reported heterogeneity of HSC within the liver lobule. In the portal area of the liver, HSC are found to have cytoplasm that contains an abundance of vitamin A lipid droplets and have cytoplasmic processes that show intense desmin immunoreactivity. In the midzone area HSC have more elongated processes that appear to be more dendritic with thorn- like projections and vitamin A storage and desmin immunoreactivity is reduced. Within the centrilobular area HSC contain few or no vitamin A lipid droplets and the processes have irregular branching with very long and thin thorn-like projections. These HSC appeared to have desmin- negative immunoreactivity (Ballardini *et al*, 1994).The cause and functional significance of this heterogeneity is still uncertain but suggests that there may be sub-populations of HSC that have specific roles and functions in liver injury. Further immunohistochemical analysis of rat and human HSC have also shown possible heterogeneity of HSC between species. Mouse and rat HSC express desmin, a gold standard marker of HSC. However activated human HSC do not express desmin (Friedman *et al*, 1992), but α-SMA (Ramadori *et al,* 1990) which also can be found in rat HSC (Rockey *et al,* 1992).

1.4. ACTIVATION OF HEPATIC STELLATE CELLS

Activation of quiescent HSC is a two step process, initiation followed by perpetuation. Initiation involves early changes in HSC gene expression and cell phenotype. Initiation stimuli may come from alterations in ECM components and from paracrine stimuli by neighbouring cell types (figure 1.3). For example, KC can activate HSC by cytokine secretion which include TGFβ, IL-1 and TNFα (Chensue *et al,* 1991; De Blesser *et al,* 1997; Roth *et al,* 1998). Culture studies have demonstrated that conditioned medium from KC can accelerate activation of HSC, increase ECM production, release retinoids and promote the transition of activated HSC from their quiescent phenotype (Friedman and Arthur, 1989; Smedsrod *et al,* 1994).

SEC may play a role in initiation of HSC activation by converting latent TGFβ to the active form via paracrine interactions with HSC (De Blesser *et al,* 1995). Endothelial cells may also interact with HSC and control their contractility by the synthesis of endothelin I, a strong vasoconstrictor (Ortega Mateo and de Artinani, 1997). Hepatocytes may stimulate HSC activation by production of lipid peroxides (Bedossa *et al,* 1994), TGFα and FGF. Transportation of PDGF, epidermal growth factor (EGF) and TGFβ by platelets in the

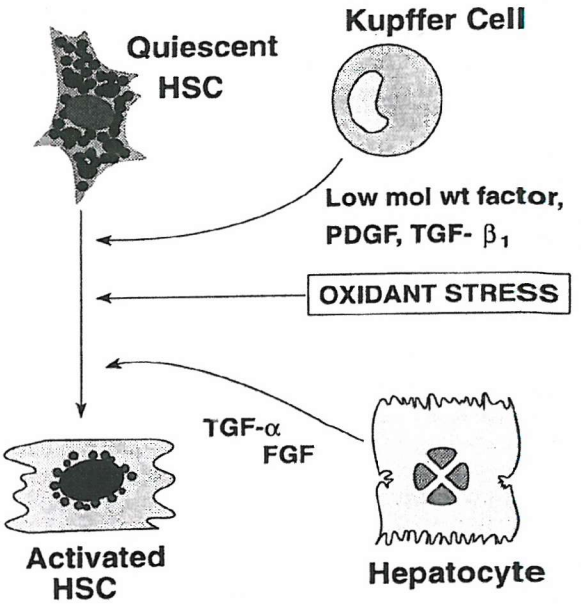


Figure 1.3. Diagrammatic representation showing the cellular and molecular mechanisms of HSC proliferation and activation. **HSC**- hepatic stellate cell, **low mol wt factor**- low molecular weight factor, **PDGF**- platelet-derived growth factor, **TGFβ$_1$**- transforming growth factor beta-1, **TGFα**- transforming growth factor alpha, **FGF**- fibroblast growth factor.

circulation may activate HSC via paracrine stimulation (Antoniades and Williams, 1983; Hwang *et al,* 1992).

Perpetuation, the second step in HSC activation, results from the change in the extracellular milieu and promotes the activation of HSC and involves five distinct phenotypic changes. **1). Proliferation**, which results in the increase in the number of collagen producing HSC. This is perpetuated by the autocrine and paracrine cytokine stimulation of HSC by PDGF and TGFβ and the increased responsiveness due to the upregulation of PDGF receptors. **2). Contractility** that is acquired by activated HSC. This contractility could lead to perisinusoidal constriction and total organ contraction by the increase in collagenous bands mediated by activated HSC. This contraction is controlled by endothelin I and NO synthesis by SEC and KC and perpetuated by thromboxane and prostaglandin F_2 (PGF_2). **3). Fibrogenesis** as a result of the increased synthesis of matrix components by activated HSC. **4). Cytokine release** from HSC and other hepatic cells is necessary for the perpetuation of HSC activation. Cytokines may also chemoattract other immunological cells to the liver such as leukocytes. **5). Matrix protease activity** accompanies liver injury and is a direct result of HSC activation. Upregulation of matrix protease activity (i.e. matrix metalloproteinase-2 (MMP2) and stromelysin in conjunction with tissue- inhibitors of matrix metalloproteinases (TIMPs)) is another feature of perpetuation of HSC activation. The net effect of perpetuation is the increased accumulation of ECM components by the activation of HSC.

1.5. FUNCTIONS OF HEPATIC STELLATE CELLS

1.5.1. Vitamin A metabolism

Up to 80% of total body vitamin A is stored in the liver (Blomhoff *et al,* 1990), 90% of which is stored by HSC as retinyl esters (predominantly retinyl palmitate) (Kawada, 1997). Vitamin A is also found in other tissues such as kidneys, lungs and adipose tissue in smaller amounts.

Vitamin A is derived from dietary sources in the form of provitamin A carotenoids (e.g. β-carotene) produced by plants, or preformed vitamin A such as retinyl ester (RE), retinol and retinoic acid derived from animal tissues. Vitamin A is hydrolysed in the intestinal lumen to free retinol and subsequently absorbed by gut enterocytes (Blomhoff *et al,* 1990). Retinol is then re-esterified to RE by the enzyme lecithin: retinol acyl transferase (LRAT) and together with triacylglycerol is incorporated into chylomicrons, the major intestinal lipoproteins. Chylomicrons then enter the circulation via the lymphatics (Blomhoff *et al,* 1985; Norum *et al,* 1992). In the liver, hepatocyte cell- surface receptors recognise the retinoid- chylomicron and are internalised. REs are hydrolysed within the hepatocyte to retinol and free retinol is bound to a retinol binding protein (RBP) (Blomhoff *et al,* 1988). RBP receptors on HSC recognise the RBP-retinol complex (holo- RBP) and subsequently bind and internalise the complex by receptor- mediated endocytosis (Senno *et al,* 1993). Some RE and retinols can be taken up by HSC in other ways in a non- RBP manner (Matsura *et al,* 1993). Retinol is taken up by HSC and bound to a cellular retinol-binding protein (CRBP), esterified in the presence of LRAT and stored in the HSC lipid droplets. When retinol is required within the body, the stored RE is hydrolysed by retinyl ester hydrolase (REH) to free retinol. Retinol binds to a serum RBP and is secreted into the circulation. Experiments have shown that in vitamin A sufficient rats, retinol is rapidly transferred from hepatocytes to HSC as RBP- retinol complexes from storage. However when vitamin A stores are low, retinol is retained by hepatocytes and less transferred to HSC.

HSC express two families of nuclear retinoid receptors through which retinoic acid mediates its effects, retinoic acid receptor (RAR) and retinoid X receptor (RXR). Both families express three different receptor subtypes α,β and γ (Weiner *et al,* 1992). The receptors are ligand- inducible transcription factors that bind to a cis- acting DNA sequence called retinoic acid response element (RARE), a retinoid response element found in the promoter region. However physiological roles and ligands have not been clearly defined in HSC.

Retinoids are lost from HSC *in- vivo* and in culture by hydrolysis of intracellular retinyl esters to retinol. HSC have been reported to synthesise apolipoprotein E (Friedman *et al,* 1991). Apolipoproteins are used to form stable lipoproteins and have been suggested to be an alternative means for retinoid, cholesterol and lipid transport from HSC into the plasma.

Liver fibrosis also occurs during vitamin A intoxication (Bioulac- Sage *et al,* 1988). In the early stages there is an increase in HSC numbers possibly to accommodate the uptake of excess lipids. This results in HSC hyperplasia within the sinusoids resulting in perisinusoidal fibrosis and sinusoidal dilation. This combination often results in non-cirrhotic portal hypertension. Electron microscopy identified HSC in vitamin A intoxication as being activated to a myofibroblast cell without the loss of vitamin A containing lipid droplets (Bioulac- Sage *et al,* 1988; Baker *et al,* 1990).

1.5.2. Regulation of blood flow through the liver sinusoids

As cytoplasmic HSC processes line the luminal surface of the sinusoid, they have been suggested to act and control sinusoidal blood flow by their contractility (Wake, 1988; Wake *et al,* 1992). The recent discovery of adrenergic and cholinergic nerve fibres making contact with HSC in the sinusoids suggests a possible neuronal control of sinusoidal blood flow. Akiyoshi and Terada (1998), found mast cells in association with HSC and nerve fibres within the sinusoids and suggested that adrenergic innervation may be as a direct result of mast cell degranulation, and release of 5- hydroxytryptamine (5-HT). Kawada *et al* (1993), demonstrated contraction of rat HSC by the use of a silicone rubber membrane (a method originally used to detect fibroblast motility). 30 seconds after addition of vasoconstricting ligands such as endothelin I and eicosanoids (PGF_2 and thromboxane A_2 (TXA_2)) contraction of HSC was observed by the creasing of the silicone rubber membrane with microscopy. This method also showed HSC relaxation in response to prostaglandin E_2 (PGE_2), prostacyclin (Kawada *et al,* 1992) and NO (Kawada *et al,* 1993; Rockey and Chung, 1995).

Upon HSC contraction, there appeared to be an increase in α-SMA expression by HSC (Housett *et al,* 1993; Rockey and Chung 1995). Relaxation of HSC was accompanied by the disruption of actin fibres. In fibrotic livers HSC upregulate α-SMA, therefore the increase in contractility observed by activated HSC and accompanied upregulation of α-SMA may result in the disturbance of sinusoidal microcirculation noticed in liver fibrosis.

1.5.3. Extracellular matrix (ECM) synthesis

The ECM has several roles within the liver. The main function is to maintain the structure of the liver tissue by providing a scaffold for the hepatic cells. However, ECM also plays an important role in regulating HSC phenotype and function. Changes in HSC phenotype are believed to be associated with the change in composition of the ECM in the liver. HSC cultured *in-vitro* on type I collagen result in the production of activated HSC (Davis *et al,* 1987), whilst HSC cultured on laminin- rich gel that resembles normal liver subendothelial matrix adopt the quiescent phenotype (Friedman *et al*, 1989a; Gaça *et al,* 2000). Therefore the replacement of basement membrane in hepatic fibrosis to interstitial collagen rich matrix may stimulate HSC activation and further synthesis of matrix components.

Liver fibrosis is characterised by the accumulation of ECM components, especially collagen I, II, IV, fibronectin, laminin and proteoglycans, in the space of Disse (Popper and Undenfield, 1970; McGee and Patrick, 1972). Activated HSC are a major source of ECM proteins in the fibrotic liver (Friedman *et al,* 1985; Friedman 1993; Gressner *et al,* 1996). The constituents of hepatic ECM include collagens, glycoproteins and proteoglycans.

1.5.3.1. Collagens

Collagens are made up of left-handed triple helical polypeptide chains of variable lengths consisting of repeating sequences of $(glycine\text{-}X\text{-}Y)_n$ where one third of the X and Y positions are proline and hydroxyproline respectively (Friedman *et al,* 1992). Glycine at every third position allows for regular folding to allow for the formation of a helix.

Collagen can be further divided into fibril forming (interstitial) collagens, I, II, III, V and XI, and non- fibril forming (non-banded) collagens, IV, VI, VII, VIII, IX and X. Collagen types I, III, IV and V have been identified in the liver. Fibril and non- fibril forming collagens differ in that non- fibril forming collagens do not undergo proteolytic processing during post-translational modification.

In the normal liver, fibrillar collagens type I, III and V make up 90 % of total liver collagen. They are found in the liver capsule, around large vessels and in portal tracts (Gressner and Bachem, 1990), where they give structural support to the liver. Non- fibrillar collagen type IV is found around large vessels and forms a discontinuous basement membrane in the subendothelial space of Disse. In the fibrotic liver, as detected by Northern blotting and *in-situ* hybridisation, there is an increase in fibrillar collagens type I and III (Weiner *et al,* 1989), especially type I that accounts for up to 70 % of total collagen in cirrhotic liver (Friedman *et al,* 1992). Type I and III collagens accumulate in the space of Disse and replace the basement membrane with dense interstitial matrix. This results in

the 'capillarisation' of the sinusoids and progressive deterioration of liver cell function (Schaffner and Popper, 1968). By use of immunohistochemical and *in- situ* techniques, the synthesis of type I collagen has been extensively studied. In fibrotic liver there is a six fold increase in collagen I deposition within the subendothelial space. Although activated HSC transcribe the collagen gene twice as fast as quiescent cells there is a sixty to seventy fold increase in collagen α1 (I) messenger ribonucleic acid (mRNA) expression due to an increase in collagen I mRNA stability. Stefanovic *et al* (1997), have shown that there is a sixteen fold increase in collagen α1 (I) mRNA stability in activated HSC to quiescent HSC which results in the increase of collagen I expression. This stability results from a novel RNA- protein interaction that binds to the C-rich sequence in the 3' untranslated region (UTR) of collagen α1 (I) gene (Stefanovic *et al*, 1997). Stability of most mRNAs is regulated by sequences in 3' UTRs and determined by proteins interacting with these sequences. Promoter analysis of collagen α1 (I) gene has identified four sites for protein binding, two of which are for transcription factors SP1 and nuclear factor 1 (NF1) (Nehls *et al,* 1991). DNA binding activity of SP1 is two fold higher in activated HSC than in quiescent HSC and is consistent with the high transcription rate of collagen α1 (I) mRNA in activated HSC (Rippe *et al,* 1995).

1.5.3.2. Glycoproteins and proteoglycans

These make up the non- collagenous component of the hepatic ECM and by weight are more abundant than collagens in normal and fibrotic liver (Friedman *et al,* 1992). Glycoproteins and proteoglycans are a heterogeneous family of carbohydrate/ protein molecules and are principally involved in the formation of basement membranes.

Glycoproteins possess a large number of short, but branched, oligosaccharide chains attached to a peptide chain. Many glycoproteins are rendered soluble by virtue of their carbohydrate content whilst others are membrane components possessing discrete hydrophobic and hydrophilic regions. In most glycoproteins, the oligosaccharide is joined to the protein component by *O*-glycosidic linkages. Laminin, fibronectin, nidogen, tenascin, undulin and elastin are glycoproteins. Laminin is a large 850 kilo-dalton (kDa) molecule and is a major constituent of basement membranes. It has a broad range of biological activities by itself and with other matrix constituents. The central region of the molecule mediates binding to cell types such as hepatocytes in the liver, and to matrix proteins such as entactin and heparin. Laminin has also been shown to have mitogenic effects on a number of cells in culture (Kleinman *et al,* 1985), stimulating neurite outgrowths (Edgar *et al,* 1984) mediating cell motility and tumour metastases (Beck *et*

al, 1990). Fibronectin, a 450 kDa glycoprotein, is found either as a circulating plasma protein where it functions as an adhesion protein or as a component of the ECM derived from mesenchymal cells. In normal liver, fibronectin is found in the subendothelial space of Disse and this is increased in fibrotic livers. Increase in fibronectin is one of the earliest detectable changes in fibrotic matrix composition. This suggests that fibronectin is synthesised and acts as a foundation for the following deposition of fibril collagens. Undulin, elastin, tenascin and entactin have also been identified in the liver and involved in hepatic matrix organisation during injury (Schafer *et al,* 1987; Knittel *et al,* 1992).

Proteoglycans too are a heterogeneous family that consist of linear sulphated polysaccharide chains (glycosaminoglycans (GAGs)) (95%), that are covalently attached to a protein core (5%). The GAGs are made up disaccharide repeating units of heparan sulphate, dermatan sulphate, chondroitin sulphate or hyaluronic acid. Proteoglycans are present either as intracellular granule constituents, as membrane associated species (receptors or adhesion molecules, for example for cytokines such as FGF or TGFβ and may regulate effects of these cytokines), or as ECM constituents (heparan sulphate is a major component of basement membranes). In the normal liver 80 % of GAGs are heparan sulphate and make up the liver connective tissue in association with protein fibres of collagen. In cirrhotic livers the proteoglycans increase 3- 7 fold with a dramatic increase in dermatan and chondroitin sulphates relative to heparan sulphate. Over 60 % of the increased synthesis of dermatan sulphate is from activated HSC (Schafer *et al,* 1987).

1.5.4. Extracellular matrix degradation

In addition to synthesis of ECM in fibrogenesis, HSC also play a role in matrix degradation by the synthesis and secretion of extracellular degrading matrix- metalloproteinases (MMPs). MMPs are zinc and calcium dependent endopeptidases and are able to degrade fibrillar and non-fibrillar macromolecular collagens. There are approximately 20 members of the MMP family which have similar structural and functional features. The most common members include gelatinases A and B, collagenases and stromelysin. Members of this family possess several common structural domains with highly conserved sequences (Matrisian, 1992). All mature MMPs possess an 80 amino acid pro-peptide domain which contains a highly conserved amino acid sequence 'proline- arginine- cysteine- glycine- any amino acid- proline- aspartic acid' (PRCGXPD) (figure 1.4), and is lost during activation. This region is known as the 'cysteine-switch' and maintains the proenzyme in an inactive state by blocking the active site. In the catalytic site a conserved zinc binding site can be found (amino acid sequence 'histidine- glutamic acid- any amino acid- glycine- histidine'

(HEXGH)). Mutations at this site have resulted in an inactive enzyme. The gelatinases also have a fibronectin-like gelatin binding sequence inserted into the catalytic domain. Gelatinase B has a further collagen- like insertion at the C-terminal of the catalytic domain. Some MMP members have additional vitronectin-like, fibronectin- like or collagen IV-like domains to determine substrate specificity, for example collagenase loses the ability to cleave triple helical collagen in the absence of C-terminal vitronectin-like domain (Murphy *et al,* 1992a). This domain mediates the interaction of MMPs to collagen fibres. It has been suggested that the C-terminal domain may also have a role in binding the active enzyme to tissue-inhibitors of matrix metalloproteinases-1 (TIMP-1) and TIMP-2, see section 1.5.4.2.

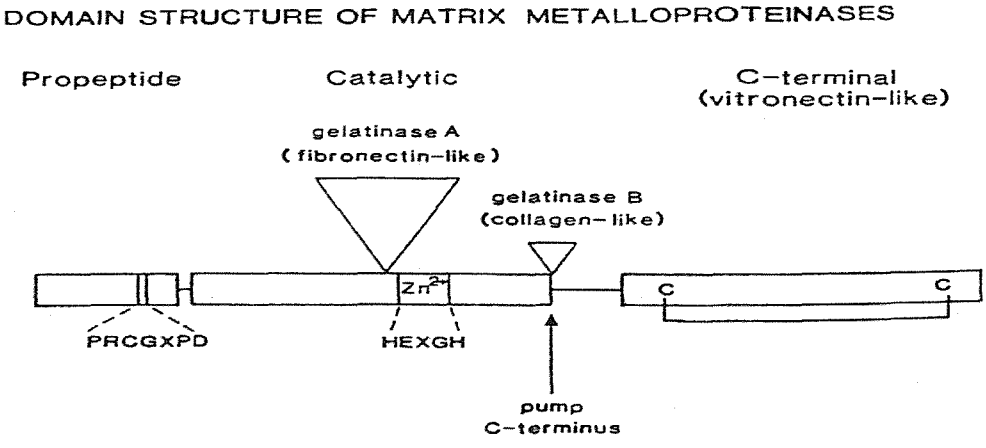


Figure 1.4. Domain structure of the matrix metalloproteinases. All members contain a propeptide that is lost during activation and contains the conserved motif PRCGXPD; a catalytic domain containing zinc binding region with conserved motifs HEXGH. Collagenases, stromelysins and gelatinases all have a third C-terminal domain which have additional vitronectin-like, fibronectin- like or collagen IV- like domains and is linked to the catalytic domain by a proline- rich sequence of 5 to 10 amino acids. Both gelatinases have an extra domain related to the collagen or fibronectin binding region that is inserted into the catalytic domain. *Adapted from Murphy et al. The matrix metalloproteinases and their inhibitors. Am J Respir Cell Mol Biol 1992b; 7: 120-125.*

The family of MMPs can be further sub-divided into 4 groups according to MMP substrate specificity (see table 1.1). Production of MMPs has been extensively examined in HSC activated by culture on plastic (figure 1.5). In early primary culture (1 to 3 days) when HSC still have the quiescent phenotype interstitial collagenase (MMP 1 in humans and MMP 13 in rats) and stromelysin (MMP 3) are transiently expressed. By day 5 in primary culture expression of MMP 1 (MMP 13) and MMP 3 are down regulated and are not detected at all in fully activated myofibroblastic-like HSC (day 7-21). However gelatinase A (MMP 2) has a different pattern of expression. In freshly isolated HSC (quiescent phenotype) the mRNA expression of MMP 2 is low.

Group/ Name	*MMP Number*	*Cellular origin in liver*	*Matrix degraded*
Collagenases			
Interstitial Collagenase	MMP 1	HSC, KC	Fibrillar collagens I
Collagenase 3	MMP 13	HSC (in rat)	II and III and collagensVIII and X
Gelatinases			
Gelatinase A	MMP 2	HSC	Basement membrane
Gelatinase B	MMP 9	HSC, KC	proteins; collagen IV, proteoglycans, laminin
Stromelysins			
Stromelysin 1	MMP 3	HSC	Basement membrane
Stromelysin 2	MMP 10		proteins; collagen IV, proteoglycans, laminin
Membrane- type MMP			
MT1-MMP	MMP 14	HSC	Activation of progelatinase A

Table 1.1. Matrix metalloproteinases (MMPs) in the liver.
*Taken from Arthur MJ. The pathogenesis of liver fibrosis. Connective Tissue 1998; **30:** 233-237.*

This is upregulated in activated myofibroblastic HSC and is a prominent feature of activated HSC (Arthur *et al*, 1992). Membrane type-1 matrix metalloproteinase (MT1-MMP) follows the expression of MMP 2. This enzyme mediates the conversion of progelatinase A to active gelatinase A (Sato *et al,* 1994), and activated gelatinase A (66 and 62 kDa) is detected in media from activated HSC (Benyon *et al,* 1999). HSC therefore have a large repertoire of MMP synthesis, which are used to degrade ECM proteins and destroy normal liver- cell interactions hence contribute to liver fibrosis.

Expression of MMPs has also been studied in normal and diseased liver by various techniques. By *in-situ* hybridisation and zymography, MMP 2 and MT1-MMP have been shown to increase in expression during progression of fibrosis (Takahara *et al,*1997). However although there is an increased expression of active gelatinase A in liver fibrosis, there is also an increase in type IV collagen deposition that appears contradictory. However as yet there is no clear explanation for this, but it has been observed within our laboratories that gelatinase A promotes proliferation and activation of stellate cells suggesting that it might have functions other than matrix degradation (Benyon *et al,* 1999).

MMPs are rigorously regulated by several different mechanisms allowing for the fine control of their enzymatic activity. For all members of the MMP family, regulation occurs at three levels that include, regulation of gene transcription, activation of proenzyme to catalytic forms, and activated enzyme by TIMPs.

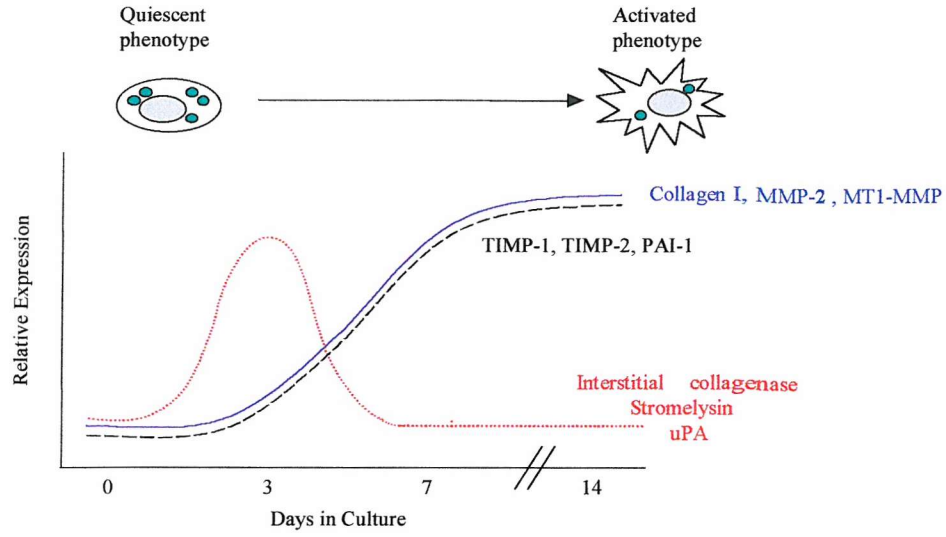


Figure 1.5. Graph outlining the expression of MMPs and TIMPs during activation of HSC *in vitro.*

1.5.4.1. Regulation of MMPs by gene transcription

Cytokines, hormones, growth factors and retinoids that stimulate the synthesis and secretion of MMPs regulate MMP gene transcription. Many of these can either co- regulate expression of several MMPs or specifically regulate individual members. Promoter regions for human stromelysin and interstitial collagenase have common features important to their transcriptional regulation. These genes contain thymine- adenine- thymine- adenine (TATA) consensus sequence elements 30 nucleotides upstream from the transcriptional start site and contain activating promoter- 1 (AP-1) and polyomavirus enhancer activator-3 (PEA-3) (originally identified as an oncogene- responsive element in the polyoma virus enhancer) transcription factor binding sites. AP-1 alone or in combination with PEA-3 can regulate indelibility of stromelysin and interstitial collagenase genes. Transcription factors *c-jun* and *c- fos* transmit through the AP-1 element and *s-Ets-1* and *c-Ets-2* through PEA-3 (Wasylyk *et al,* 1990; Brenner *et al,* 1989). For example, cytokines such as TNFα, PDGF, interferon -α (IFNα) and IL-1β have been shown to upregulate interstitial collagenase (MMP 1) gene expression by mediating the increased synthesis of *c-jun* and *c- fos.* These transactivating factors bind as heterodimers to the AP-1 transcription factor binding site in the gene promoter region enhancing transcription of the gene downstream (Brenner *et al,* 1989).

However, glucocorticoids and retinoids are able to downregulate MMPs by interacting with and blocking the binding of AP-1 transactivating factors (Lafyatis *et al,* 1990). Negative regulatory elements have been demonstrated for stromelysin (MMP 3). TGFβ decreases stromelysin gene expression in culture by a *c-fos* containing

transactivating factor binding to the TGFβ inhibitory element (TIE) present in the promoter region (Matrisian *et al,* 1992).

The urokinase plasminogen activator gene also has AP-1 and PEA-3 transcriptional elements that regulate transcriptional activity (Matrisian, 1992). The balanced expression of urokinase with MMPs may initiate a cascade of events that lead to matrix degradation. The promoter region for gelatinase A differs from stromelysin and interstitial collagenase in that it lacks an AP-1 binding site (Matrisian, 1992). This may explain the pattern of gelatinase A expression in activated HSC, see above.

1.5.4.2. Regulation of MMPs by proenzyme activation

MMPs are secreted as latent proenzymes. They become activated when there is disruption of the zinc-cysteine complex at the highly conserved catalytic site (cysteine switch mechanism). This results in the autoproteolysis and cleavage of the propiece and conversion to the active species (Murphy *et al*, 1991). *In-vivo* one of the major systems to effect this cleavage is the plasminogen-plasmin cascade (Murphy *et al,* 1992a), figure 1.6. Urokinase plasminogen activator (uPA) or tissue plasminogen activator (tPA) act to generate active plasmin from circulating plasminogen, which is a component of the plasma and interstitial fluids. Plasmin can cleave basic residues in the pro-domain of pro-collagenase and pro-stromelysin. Removal of the prodomain destabilised the cysteine residue associated in the conserved region with zinc. This process is known as the 'cysteine switch mechanism' (Van Wort *et al,* 1990). The intermediates then undergo autocatalytic cleavage to the final active form of collagenase and stromelysin. Collagenase is cleaved further by stromelysin to a highly active form. This process is inhibited by plasminogen activator inhibitors (PAI) which are produced by cells that synthesise MMPs. PAI acts by inhibiting the urokinase conversion of plasminogen to plasmin. The key components of the plasminogen- plasmin cascade are synthesised by HSC (Leyland *et al,*1996).

Pro- gelatinase A is not cleaved by uPA but becomes activated in association with the plasma membrane (Murphy *et al*, 1992b). Sato *et al* (1994), have identified an MMP that is an integral plasma membrane protein, MT1-MMP. Activation of progelatinase A occurs by binding to a TIMP-2 / MT1-MMP complex. TIMP-2 will bind to the carboxyl-terminal (C-terminal) domain of progelatinase A, which is then secreted from cells as an enzyme- inhibitor complex (Howard *et al,* 1991). This complex then acts with MT1-MMP and the trimolecular complex leads to progelatinase activation. Activated HSC express MT1-MMP so can regulate gelatinase A activation. There are four MT-MMPs which

activate gelatinase A (MT1, 2, 3 and 5 MMP) but MT4-MMP is relatively ineffective (Sato *et al,* 1997; Llano *et al,* 1999).

Other serine proteases have been identified in activating the pro-form of MMPs, for example mast cell tryptase and chymase (Gruber *et al,* 1988; Gruber *et al,* 1989; Lohi *et al,* 1992; Lees *et al,* 1994; Fang *et al,* 1996), cathepsins and elastase. *In vitro* organomercurials such as aminophenyl mercuric acetate (APMA) can activate MMPs.

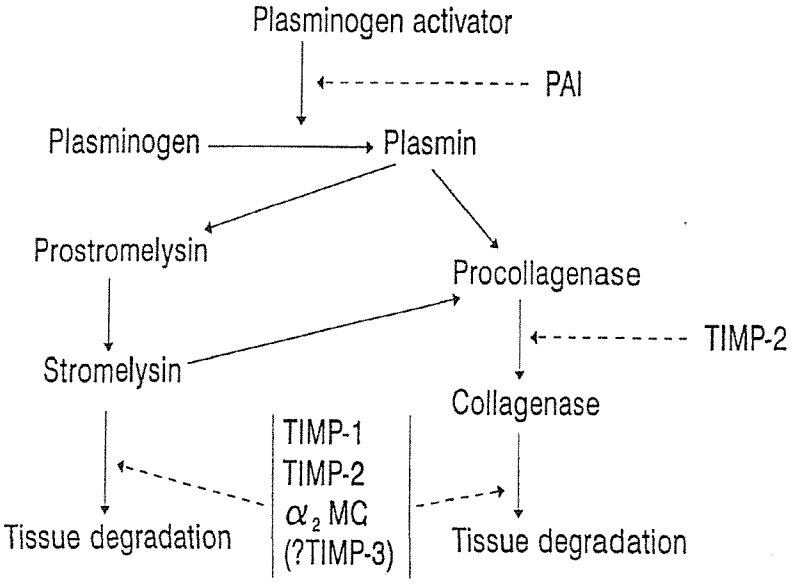


Figure 1.6 The cascade of regulatory control of MMP activity by proenzyme activation and TIMPs. Progressive fibrosis is associated with changes in the pattern of matrix synthesis and degradation. The key elements involved in this mechanism are synthesised by HSC in the fibrotic liver.
*Adapted from Iredale, J.P. Matrix turnover in fibrogenesis. Hepato-Gastro 1996; **43:** 56-71.*

1.5.4.3 Regulation of MMPs by TIMPs

Activation of MMPs can be inhibited by general proteinase inhibitors in the circulation such as α-2 macroglobulin. However specific inhibition of MMPs occurs by interaction with TIMPs. TIMPs bind non-covalently to MMPs and under normal physiological conditions this binding is irreversible. To date four types of TIMPs have been identified, 1 to 4 (table 1.2), and are products of separate genes. TIMP 1 and 2 have a 40% amino-acid homology and both have a three- looped structure stabilised by 6 disulphide bonds (Murphy *et al,* 1992c). Within the TIMP molecule, two functional domains exist. The amino- terminal (N terminal) is necessary for inhibitory activity against MMPs. The C-terminal is the key to the interaction with proMMPs, for example this enables TIMP-2 to form a trimer complex with progelatinase A and MT1-MMP (section 1.5.4.2).

	TIMP-1	TIMP-2	TIMP-3	TIMP-4
Molecular mass (kDa)	28	21.7	21.6	22.6
Inhibition of all MMPs	yes	yes	yes	?
Special properties	binds to proMMP 9	binds to proMMP 2	bound to matrix	in cardiac tissue
Role in liver disease	released by activated HSCs; prominent expression in fibrotic liver	released by activated HSCs; prominent expression in fibrotic liver	not known	not known

Table 1.2. TIMPs in the liver.
*Adapted from Arthur, M.J.P. Fibrosis and altered matrix degradation. Digestion 1998;**59:** 376-380.*

TIMPs inhibit MMPs by binding to the active site of MMPs in a way that under physiological conditions is irreversible (Murphy *et al,* 1992b). Like MMPs, TIMPs are regulated at the level of transcription by cytokines and growth factors that also control MMP activation. For example TGFβ upregulates TIMP-1 and gelatinase A expression whilst downregulating TIMP-2, interstitial collagenase and stromelysin. Mapping of TIMP promoter regions has resulted in the discovery of some common regulatory motifs, which differ in individual TIMPs/ MMPs in terms of their frequency and position in relation to transcription start site. For example, murine TIMP-1 and interstitial collagenase have AP-1 and PEA-3 binding sites but in different configurations (Schorpp *et al,* 1995). Human TIMP-2 is flanked by 5' AP-1 and AP-2 consensus sequences (De Clerck *et al,* 1994) and several SP-1 sites in association with a TATA box. The AP-1 site in TIMP-2 is located further upstream from the transcriptional start site than for TIMP-1 and is not associated with PEA-3. The TIMP-3 promoter region contains SP-1 site which too is found in TIMP-1 and 2 (Wick *et al,* 1995). The combination and frequency of regulatory elements related to each TIMP gene may explain the different patterns of TIMP expression observed in response to growth factors and cytokines, for example TGFβ which increases TIMP-1 expression and decreases TIMP-2 expression.

The onset of fibrosis and TIMP expression has been extensively studied in normal and diseased liver. Analysis of serum from patients with hepatic inflammation and cirrhosis revealed an increase in TIMP-1 level by enzyme linked immunobsorbent assay (ELISA) (Murawaki *et al,* 1993). Ribonuclease protection assays also detected an increase in TIMP-1 and TIMP-2 transcripts in fibrotic liver (Benyon *et al,* 1996). Studies of primary culture HSC have shown that activated HSC increased expression of TIMP-1 and TIMP-2 mRNA compared to quiescent HSC (Iredale *et al,* 1996; Benyon *et al,* 1996).

HSC, although they have many functions within the liver, principally synthesise and breakdown collagens and other ECM components. Liver fibrosis is characterised as an imbalance in the homeostasis of the fibrogenesis and fibrolysis of hepatic ECM by HSC. However, fibrosis in other organs of the body such as the heart, skin and lung is as a direct result of over expression of collagens, principally by fibroblasts.

Fibroblasts are differentiated cells of mesenchymal origin. During their development and in response to injury they respond by remodelling the ECM and epithelium. They have many functions and those include cell migration, chemotaxis, proliferation, attachment and most importantly the biosynthesis and degradation of various connective tissue components. Inflammatory cells such as MC and eosinophils (EO) can influence fibroblast properties through the release of a wide spectrum of biologically active components that can have fibrogenic or fibrolytic effects (Levi- Schaffer and Weg, 1997). Interactions between MC and fibroblasts are not surprising given their proximity to one another in connective tissue and because of the appearance of increased numbers of MC in sites of fibrous tissue production for example in scleroderma (Claman *et al,* 1990; Irani *et al,* 1992c), pulmonary fibrosis (Kawanami *et al,* 1979; Hunt *et al,* 1992; Pesci *et al,* 1993), cardiac fibrosis (Li *et al,* 1992) and liver fibrosis (Peng *et al,* 1994; Xu *et al,* 1994; Farrell *et al,* 1995; Rioux *et al,* 1996; Ambrust *et al,* 1997; Nakamura *et al,* 1997; Ramos *et al,* 1997).

1.6. MAST CELLS

In 1878 Paul Ehrlich first identified MC in human tissues due to the metachromatic staining properties of their cytoplasmic granules (Ehrlich, 1878). Metachromasia results from basic anilide dye molecules (from toluidine blue) interacting with the negative charges within the proteoglycan core of the MC granule, hence resulting in a colour change from blue to pink. Paul Ehrlich was the first to observe MC in fibrotic tissues and speculated that they had a role in fibrosis and tissue repair modelling. Since then morphological observations that focus on the dynamic changes in number and ultrastructural changes of MC during fibrosis have consistently reported an increase in MC numbers in several fibrotic diseases, such as scleroderma, lung fibrosis and wound healing (Kawanami *et al,* 1979;Claman *et al,* 1990; Hunt *et al,* 1992; Irani *et al,* 1992; Kawanami *et al,* 1979; Hunt *et al,* 1992; Pesci *et al,* 1993). However relevant functional and biochemical studies have yet to be established to assess if fibrosis occurs as a result of accumulation of activated MC within tissues.

1.6.1. Mast cell morphology

MC are 5- 18 μm in diameter and have a spherical morphology. The nucleus of a MC is large, single and unsegmented and often displaced to one side of the cell. The cytoplasm of a mature MC is packed with electron dense granules, approximately 50 – 200 per cell, each having a diameter of 0.2- 0.5 μm (figure 1.7). Ultrastructurally, in a resting state, the granules appear as ordered scrolls or crystals within the MC cytoplasm. Within these granules preformed proinflammatory mediators are stored, such as histamine, proteoglycans, proteases, and cytokines such as IL-4, IL-5, IL-6 and TNFα as detected by immunostaining (Walsh *et al,* 1991; Ohkawara *et al,*1992; Bradding *et al,* 1994; Bradding *et al,* 1995; Krüger-Krasagakes *et al* 1996). MC express plasma membrane receptors for important regulatory factors such as growth factors, receptors for adhesive molecules that enable MC to attach to certain sites, for example β1 and β3 integrins and receptors that bind to the constant region (Fc) portion for immunoglobulin E (IgE) with high affinity, $Fc_{\varepsilon}RI$ (Scharenberg and Kinet, 1995), section 1.6.4. Also within the cytoplasm are lipid bodies involved in arachidonate metabolism. In normal physiological conditions, MC are distributed throughout the connective tissue and are plentiful in the skin, lymphoid organs, bone marrow, intestine and respiratory tract (Kaliner, 1979). They are found adjacent to blood and lymphatic vessels, near to nerves and beneath epithelial surfaces.

1.6.2. Mast cell heterogeneity

Histochemically and functionally MC are a heterogeneous population and can be divided into subpopulations according to location in the tissue, i.e., connective tissue MC which are thought to be involved in angiogenesis and fibrogenesis, and mucosal MC that are involved in immunological roles (Castells *et al,* 1992; Dumitrašcu, 1996).

In humans there are two subsets of MC which are classified by the difference in their protease content (Irani *et al,* 1986; Schechter, 1990). The proteases found in MC are neutral proteases as they are active at a neutral pH (Schechter, 1995). Three types of neutral proteases have been isolated from human MC, tryptase, chymase and carboxypeptidase A, section 1.7.4. The MC_T subset contains only tryptase and is most plentiful in the small intestinal and lung mucosa (Irani *et al,* 1986; Irani and Schwartz, 1989). They are known as the immune- associated MC in that they increase in numbers at the site of T- lymphocyte cell activation; increase in allergic and parasitic diseases;

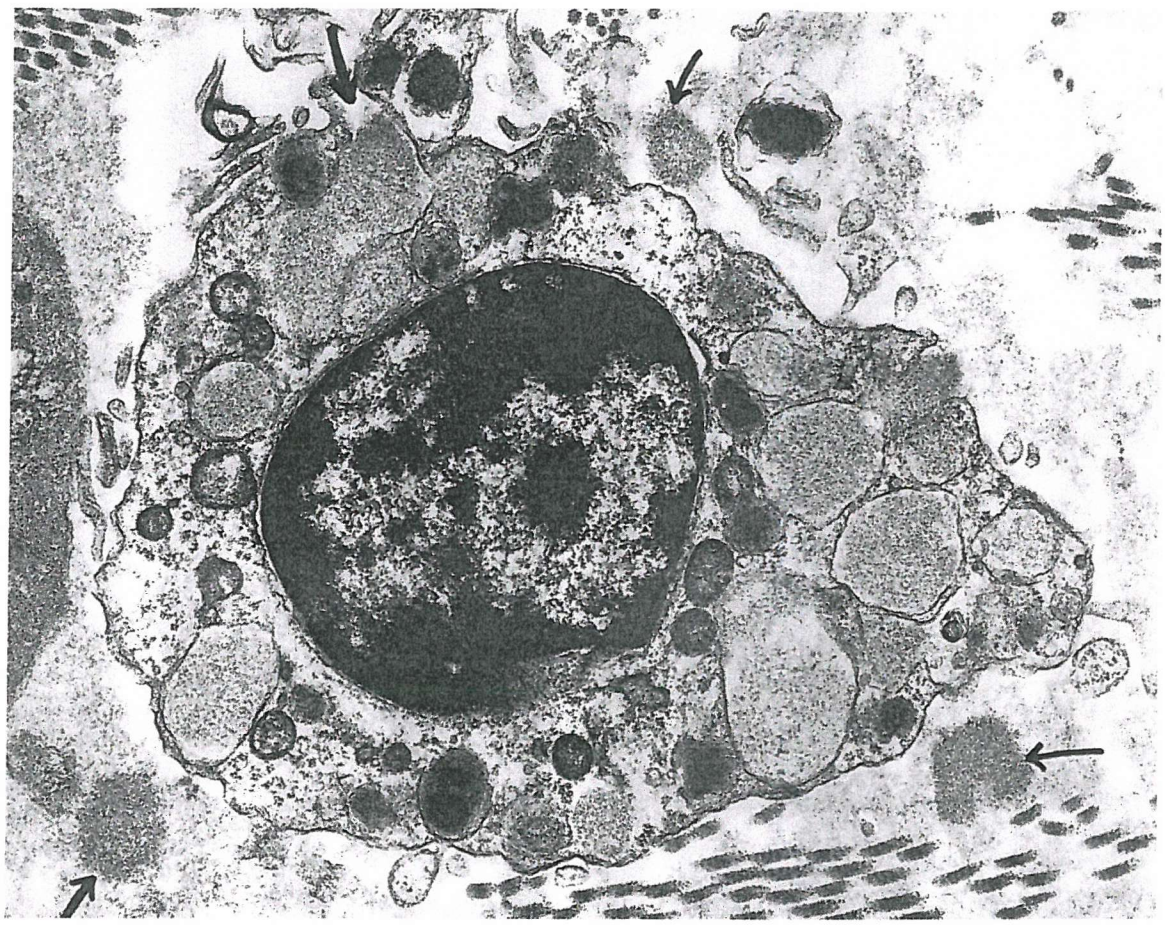


Figure 1.7. Ultrastructural analysis of a human skin mast cell undergoing degranulation, by electron microscopy. The arrows indicate empty MC granules, with their contents extruded into the extracellular environment. Notice the empty granules around the MC membrane.
Adapted from Dvorak, AM. New aspects of mast cell biology. Int Arch Allergy Immunol 1997; ***114:*** *1-9.*

decrease in number in acquired immunodeficeincy syndrome (AIDS) and chronic immunodeficeincy diseases (Dumitrašcu, 1996). The MC_{TC} subpopulation contains both tryptase and chymase and is predominant in the skin, heart, synovium and submucosa of the respiratory and intestinal tracts (Irani *et al*, 1986; Patella *et al*, 1995; de Paulis *et al,* 1996). They are known as non- immune system associated MC as they increase in numbers in fibrotic diseases, and are unchanged in number in allergic and parasitic diseases and in chronic immunodeficeincy diseases (Dumitrašcu, 1996). In humans MC from different anatomical sites have functional heterogeneity measured by their responsiveness to immunological and non- immunological stimuli (Benyon *et al,* 1989; Pearce *et al,* 1991).

Subpopulations of MC have been classified in rodents as connective tissue mast cells (CTMC) and mucosal mast cells (MMC) (Galli *et al,* 1990). These subpopulations may be distinguished from one another by staining properties due to their proteoglycan and serine protease content, tissue location and size. The predominant proteoglycan of CTMC is heparin and contain the protease, rat mast cell protease I (RMCPI) which is chymotryptic.

CTMC are found in the dermis, peritoneal cavity and muscularis propia of the stomach. MMC have chondroitin sulphate E as their principal proteoglycan and contain rat mast cell protease II (RMCPII) as their protease which also is chymotryptic. MMC are found in the respiratory and gastrointestinal tract.

The reason for this MC heterogeneity is still unclear. It may be that the different subtypes of MC exert specific functions during normal physiology and in disease states.

1.6.3. Activation of mast cells

MC can either be activated immunologically or non- immunologically. Immunological activation of MC requires IgE binding to the $Fc_{\varepsilon}IgE$. The cross- linkage of these receptors upon binding to IgE results in the activation and subsequent degranulation of MC (Ishizaka and Ishizaka, 1984). This activation results in distortion of the intracellular granule morphology causing them to fuse with one another, forming channels that fuse with the plasma membrane which allow for the release of mediators from the MC granule (Caulfield *et al,* 1980). Activation of MC results in the granules appearing as 'moth eaten', a process named 'piecemeal' necrosis. The most important immunologic triggers of MC secretion include allergen- induced IgE antibodies (and IgG antibodies in rodent MC and human MC line (HMC-1)) (Castells, 1994; Wedi *et al,* 1996) IL-1, granulocyte-macrophage colony stimulating factor (GM-CSF) and a family of histamine- releasing factors that are generated by neutrophils, platelets and macrophages.

Non-immunologic stimuli are also important in the activation of human mast cells and bind to MC by a non- IgE dependent mechanism. These include complement fragments including C3a, C4a and C5a (Fureder *et al,* 1995; Nilsson *et al,* 1996) various neuropeptides such as substance P; neurotransmitters (adenosine triphosphate); and various drugs such as opiates (Wasserman, 1990; Barke and Hough, 1993; Di Bello *et al,*1998).

1.6.4. Development and survival of mast cells by stem cell factor

MC, although exclusively found within tissues, belong to the hematopoietic system and originate from $CD34^{+}$ pluripotential cells that give rise to lymphocytes, erythrocytes, megakaryocytes, neutrophils, eosinophils, basophils and monocytes (Kirshenbaum *et al,* 1991). They originate from distinct precursors in the bone marrow (Saarien *et al,* 1994), foetal liver (Irani *et al,* 1992a; Irani *et al,* 1992b) and peripheral blood (Yamaguchi *et al,* 1996). In the bone marrow, the production of stem cell factor (SCF) by fibroblasts (Nocka *et al,* 1990; Galli *et al,* 1994; Linenberger *et al,* 1995) and various cytokines such as IL-3, IL-4, IL-9 and IL-10 derived from T lymphocytes, stimulate the division and commitment

of MC progenitor cells, figure 1.8. The blood then carries these MC precursors to final tissues of deposition where they mature into recognisable MC under the influence of the local tissue environment (growth factors and cytokines).

Several *in vitro* and *in vivo* studies have shown how fibroblast factors can stimulate the growth and development of MC progenitors to mature MC (Nicodemus and Austen, 1993). Ginsburg and Lagunoff (1967), cultured thymus cells on a monolayer of mouse skin fibroblasts (3T3) and observed the development of mature MC. *In vitro* developed immature mouse bone- marrow derived MC (BMMC) were shown to differentiate and mature into CTMC when cultured onto a monolayer of 3T3. (Levi Schaffer *et al,* 1986). If nucleated cells of human umbilical cord blood cells or foetal liver cells were cultured with 3T3, mature MC were seen to develop (Irani *et al* 1992a; Irani *et al* 1992b). From such *in vitro* studies, Nocka *et al* (1992), isolated a potent MC growth factor from the culture media of 3T3 fibroblasts, named SCF. Subsequent *in vitro* studies have shown that in the presence of recombinant SCF, mast cells develop from human bone marrow (Valent *et al,* 1992), foetal liver, (Irani *et al,* 1992a; Irani *et al,* 1992b), cord blood (Mitsui *et al,*1993) and peripheral blood (Valent *et al,* 1992). Recombinant human SCF (rhSCF) promotes human MC hyperplasia and activation *in vivo* (Costa *et al,* 1996).

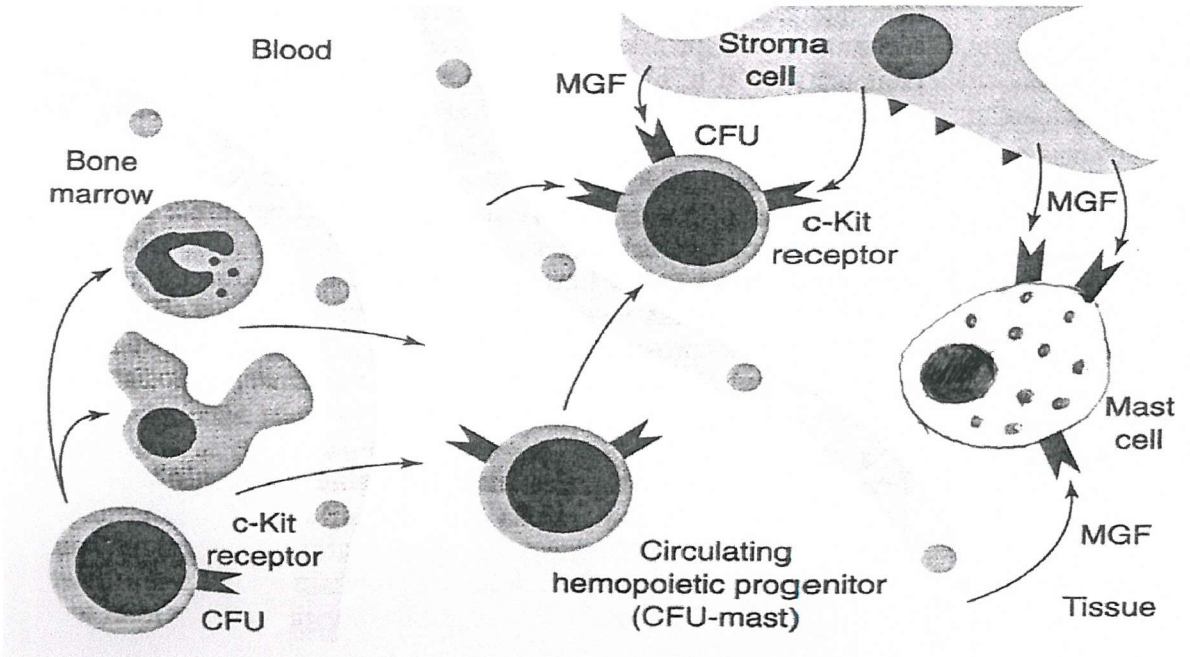


Figure 1.8. The development of mast cells. Mast cell originate from bone marrow-derived multipotent colony forming progenitor cells (CFUs). The circulating mast cell progenitor (CFU-mast) leave the bloodstream and enters distinct tissues. In the presence of mast cell growth factor (MGF), also known as stem cell factor, which is released by stroma cells, the mast cell acquires its functional maturity after activation of *c-kit. Adapted from Födinger et al. Molecular genetics and development of mast cells; implications for molecular medicine. Molecular Medicine Today 1997; **3**:131-137.*

SCF therefore is central to MC biology and has been reported as a principal growth factor (Zsebo *et al,* 1990), differentiation (Valent *et al,* 1992), chemotactic (Nilsson *et al,* 1994) and activating factor (Columbo *et al,* 1992; Bischoff and Dahinden 1992) of MC. SCF exerts its biological effect by the interaction with its receptor *c-kit*, which is a tyrosine kinase receptor and is found expressed on the membrane on all MC (Galli *et al,* 1993a).

1.7 STEM CELL FACTOR (SCF)

1.7.1. SCF gene structure

SCF is a gene product encoded by the Steel (*Sl)* locus (Huang *et al,* 1990; Matsui *et al,* 1990; Zsebo *et al,* 1990) and has been mapped to human chromosome 12 (Martin *et al,* 1990; Geissler *et al,* 1991) and to mouse chromosome 10 (Huang *et al,* 1990; Martin *et al,* 1990; Zsebo *et al,* 1990). It is synthesised as a biologically active transmembrane protein and can exist either as a cell- associated protein (membrane bound) or in the extracellular environment as a soluble factor (Anderson *et al,* 1990; Zsebo *et al,* 1990; Langley *et al,* 1993; Galli *et al,* 1994; Wypych *et al,* 1995). Soluble SCF is derived from the cell-associated SCF by proteolytic cleavage, by an unknown factor, at the cell surface (Martin *et al,* 1990; Zsebo *et* al, 1990; Pandiella *et al,* 1992). SCF is heavily glycosylated, with O and N- linked sugars and exists as non- covalently associated dimers (Huang *et al,* 1990; Arkawa *et al,* 1991).

The structure of the SCF gene has been characterised in a variety of species (figure 1.9). Location of introns in the coding region of the SCF gene is conserved in rats, mice and humans (Anderson *et al,*1990; Martin *et al,* 1990). The first 164 amino acid residues of the human and rat SCF sequence show 79% identity, with a conserved region occurring at the proteolytic cleavage site (exon 6) (Martin *et al,* 1990). Location of the promoter, transcription initiation site, and the detailed intron- exon structure of the SCF gene have not yet been reported. By post transcriptional and translational mechanisms, the SCF gene can produce alternative transcripts and protein products. For example, alternative splicing of exon 6 results in the production of 2 isoforms of SCF, cell-associated and soluble SCF (Miyazawa *et al,* 1995). It has been suggested that this is regulated in a tissue- specific manner that results in the different expressions of cell- associated and soluble forms of SCF protein.

In humans there are two alternatively spliced forms of SCF (Anderson *et al,* 1991). The full length mRNA encodes a 248 amino acid transmembrane protein, comprising a 189 amino acid extracellular ligand domain, 23 amino acid hydrophobic membrane spanning region and a 27 amino acid intracellular domain. The alternative SCF is a shorter species

that lacks exon 6 (a deletion of 84 base– pairs (bp) containing the proteolytic cleavage site for production of soluble SCF) and results in a cell- associated SCF protein that lacks 28 amino acids which include one of the four potential N- linked glycosylation sites, the C-terminal of soluble SCF (Ala 164 and Ala 165) and a protease recognition site. This form of protein yields soluble SCF less efficiently and may possibly contribute to the regulation of the abundance of alternative spliced variants of SCF.

In mice there are 3 alternatively spliced forms of SCF: the full length mRNA, an mRNA species that lacks exon 6 that also has a 28 amino acid deletion like the human transcript (Anderson *et al,*1990; Flanagan *et al,* 1991) and an alternative form with a smaller 16 amino acid deletion of exon 6 (Anderson *et al,*1990; Flanagan *et al,* 1991; Lev *et al,* 1991).

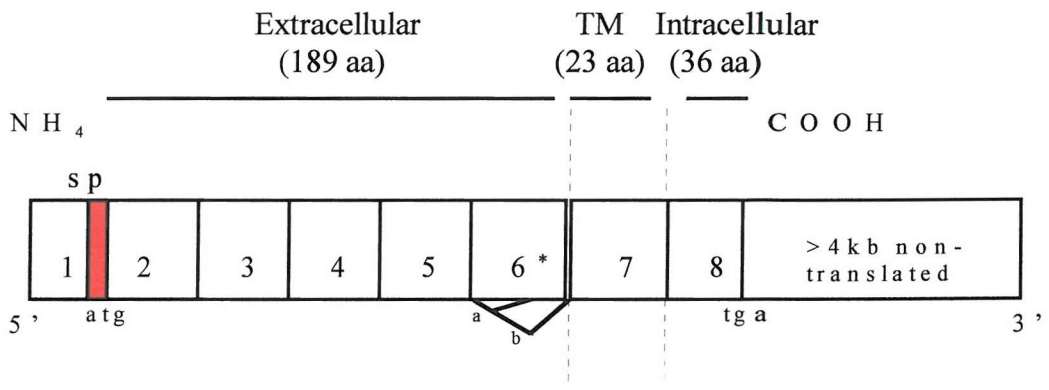


Figure 1.9. The structure of the stem cell factor gene (not drawn to scale).
This is a schematic map showing the locations of the exonic regions within the SCF cDNA that encodes the full-length membrane- associated SCF products SCF-1 and SCF-2. The locations of the ATG and signal peptide (sp) are in exon 1 (the promoter has not yet been characterised). An asterisk (*) in exon 6 indicates the site of proteolytic cleavage that gives rise to the soluble form of SCF. Letters (a) and (b) indicate the location of two alternative splicing events that result in two alternative transcripts that lack part of exon 6 (a) or all of exon 6 (band encodes a SCF product that lacks the peptidase cleavage site.
*Taken from Galli et al. The c-kit ligand, stem cell factor. Adv Immunol. 1994; **55:** 1-96.*

1.7.2. SCF signal transduction and actions

SCF exerts its effect by binding to the first three IgG- like domains of the amino- terminal half of the extracellular domain of the receptor *c-kit* (Lev *et al,* 1992; Blecham *et al,* 1993b). *c-kit* is found on the surface of MC and MC progenitors (Galli *et al,* 1993a). *c-kit* is a gene product of the *W* locus (Yarden *et al,* 1987; Matsui *et al,* 1990) and is found on human chromosome 4 and mouse chromosome 5 (Yarden *et al,* 1987). It is a tyrosine kinase receptor and the amino acid sequence displays extensive homology with the tyrosine kinase super- family, particularly type III transmembrane tyrosine kinase receptor

whose members include PDGF receptors (Besmer *et al,* 1986; Yarden *et al,* 1987; Qiu *et al,* 1988).

Regulation of *c-kit* expression is regulated by the *mi* locus (Ebi *et al,*1992). As a result of this, *W* and *mi* mutant mice produce defects in MC lineage, whilst *Sl* mutants impair the tissue microenvironment required for MC maturation. *W* and *Sl* mutant mice also exhibit macrocytic anaemia, hypopigmentation (i.e., white fur due to lack of melanocytes), sterility and deafness. Tono *et al* (1992), showed that in *W* mutant mice, MC depletion was associated with low *c-kit* expression on MC precursors. These precursors were found not to attach to fibroblasts in culture. Fibroblasts from *Sl* mutant mice could not stimulate the development of normal MC (Jarboe *et al,* 1989). In the *W* mutant mouse anaemia and MC deficiency can be cured by infusion of bone marrow from a wild- type donor, but not in *Sl* mutant mice. *mi/mi* MC cocultured with +/+ fibroblasts were found to be impaired in their binding to fibroblasts and did not further enhance the development of these MC (Ebi *et al,* 1990). These studies therefore highlight the important role SCF and fibroblast play in the development of mature MC.

Upon SCF binding, *c-kit* will subsequently dimerise (Blume- Jensen *et al,* 1991; Galli *et al,* 1993b; Kurosawa *et al,* 1996), auto-phosphorylate (Blume-Jensen *et al,* 1991; Herbst *et al,* 1991; Lev *et al,* 1991) and activate intrinsic protein kinases such as pp 70- and $pp90^{rsk}$- S6 kinases (Tsai *et al,* 1993).

This binding has a variety of resultant consequences on cellular activity. For example, cells expressing cell- associated SCF can mediate adhesion to cells expressing *c-kit* (Flanagan *et al,* 1991; Toksoz *et al,* 1992). SCF can also mediate adhesion of MC to extracellular matrix, such as fibronectin (Dastych and Metcalfe, 1994).SCF can induce chemotaxis of *c-kit* expressing cells. Subcutaneous or intravenous administration of recombinant SCF (rSCF) resulted in the recruitment and accumulation of MC (Zsebo *et al,*1990; Tsai *et al,* 1991b; Galli *et al,*1993a; Galli *et al,* 1993b). Differentiation and maturation of *c-kit* expressing cells are influenced by SCF, for example the development of MC from foetal liver (Irani *et al,* 1992a; Irani *et al,* 1992b), bone marrow (Toksoz *et* al, 1992) and cord blood cells (Yamaguchi *et al,* 1996). Tsai *et al,* (1991a), have reported that culturing BMMC with recombinant rat SCF (rrSCF) resulted in the development of CTMC and their respective protease RMCP1. CTMCs were the derived MC phenotype that resulted from the culture of immature MC from *Sl/Sl* mice and *Ws/Ws* mice in the presence of rrSCF (Tsai *et al,* 1991b; Tsai *et al,* 1994).SCF can activate *c-kit* expressing cells. *In vitro* studies of human lung MC (Bischoff *et al,* 1992), skin MC (Columbo *et al,*

1992) and mouse peritoneal mast cells (Coleman *et al* 1993) showed that SCF can induce MC secretion and mediator release.

Fibroblasts and SCF have been shown to be important in the survival of MC *in vivo* and aid in sustaining the viability of mature MC *in vitro.* Rat peritoneal MC (CTMC) cultured on 3T3 monolayers were shown to retain their viability, functional activity and phenotype for more than 1 month, whereas cells cultured with media alone died (Levi-Schaffer *et al,* 1985). Human lung and foreskin MC were also kept alive and functionally active when cultured with 3T3 and human foreskin fibroblasts for at least 13 and 8 day respectively (Levi-Schaffer *et al,* 1987; Levi-Schaffer *et al,* 1995). SCF not only acts as a developmental factor in MC maturation but also acts as a survival factor.

Another mechanism of regulating MC is apoptosis. SCF and IL-3 promote the survival of MC by suppressing apoptosis. Murine IL-3 dependent MC are 'rescued' upon withdrawal of IL-3 by the addition of SCF (Mekori *et al,* 1993). The action of SCF appears to be mediated by tyrosine kinase, as addition of tyrosine kinase inhibitor (herbimycin) inhibits the ability of SCF to prevent apoptosis in IL-3 dependent MC hence the rescue is mediated by SCF and on involvement of other cytokines. Another survival factor for rodent MC is nerve growth factor (NGF) which prevents rat peritoneal MC from undergoing apoptosis when cultured *in vitro* (Horigome *et al,* 1994).

The discovery of SCF has provided a powerful tool to examine the growth and differentiation of MC and enabled the classification of other cytokines also involved in this process. A number of other cytokines and growth factors appear to be able to induce viable effects on the development and phenotype of MC in culture. MC survival and differentiation in tissues is dependent upon the local cytokines and growth factors. Lymphocytes synthesise a variety of cytokines for MC development including IL-3 and IL-4. However, similar cytokines may differ in their function relative to human and rodent mast cells. IL-3 is a differentiation factor for rodent MC but has little effect on human MC differentiation (Nilsson and Metcalfe, 1996). IL-4 however, acts as a MC growth factor in rodents but inhibits SCF- dependent differentiation of human MC derived from foetal liver (Nillson *et al,* 1994).

1.8. THE MAST CELL IN DISEASE AND LIVER FIBROSIS

MC activation may result in the pathogenesis of various conditions including diseases that culminate in fibrosis. Detection of MC mediators and observations of MC hyperplasia in fibrotic tissues suggest that MC are involved in the fibrogenic process. For example, in idiopathic dilated and ischaemic cardiomyopathies, two conditions characterised by the

increase in fibrous components, MC hyperplasia and an increase in histamine and tryptase content was observed (Patella *et al,* 1997). In scleroderma patients with lesioned skin, there is extensive collagen deposition and upregulation of collagen gene expression (Hermann *et al,* 1991). Siebold *et al* (1990), showed that MC hyperplasia and increase in degranulation preceded dermal fibrosis in scleroderma. In fibrotic lung disorders fibrosis of the pulmonary interstitium occurs and an increase in tryptase and histamine concentrations are found in bronchoalveolar lavage fluid (BAL) (Crimi *et al,* 1991). In animal models, i.e., rat models of pulmonary fibrosis, there are also increases in MC numbers.

Within the normal liver, MC are sparsely distributed. In recent studies, by the use of immunostaining using antibodies to MC proteases and metachromatic dyes, MC numbers have been shown to increase during liver injury. In the normal human liver, MC density is sparse with approximately 1.2± 0.8 to 3.9± 3.3 MC/ millimetres squared (mm^2) (Farrell *et al,* 1995; Ambrust *et al,* 1997), 10% of which are distributed within the sinusoids and the rest found around the vessels within the portal tracts. During liver disease however, MC numbers are increased (Farrell *et al,* 1995; Ambrust *et al,* 1997; Nakamura *et al,* 1997). For example, in hepatitis C-induced cirrhosis MC density is increased to 10.2± 4.6 MC/ mm^2 (Ambrust *et al,* 1997). In human cholestatic disease (stage IV of primary biliary cirrhosis) MC density is increased to 31.5± 16.9 MC/ mm^2 of which 50% of the MC are found within the sinusoids (Farrell *et al,* 1995) and the rest periportally within the fibrotic septa (Farrell *et al,* 1995; Ambrust *et al,* 1997). The majority of MC present were of the MC_{TC} subtype (Ambrust *et al,* 1997). MC hyperplasia observed in the human liver correlated with collagen deposition, as assessed by Sirius Red staining and quantitated by image analysis (Farrell *et al,* 1995). As further evidence of MC hyperplasia in liver fibrosis, Gittlen *et al* (1990), have shown that in chronic human cholestatic liver disease, plasma histamine levels increase to 275 ±117 micrograms (μg) /ml, compared to normal livers 114 ±72 μg/ml, suggesting MC hyperplasia and MC activation.

In rodent livers the story is very similar. In normal rat livers MC are also sparsely distributed, 1.2± 1.0 MC/ mm^2 and found along the vessels of the portal tracts (Ambrust *et al,* 1997). In rat models of liver fibrosis MC numbers are increased (Miller *et al,* 1994; Peng *et al,* 1994; Ramos *et al,* 1994; Xu *et al,* 1994; Rioux *et al,* 1996; Ambrust *et al,* 1997). In rats treated with carbon tetrachloride (CCl_4), a potent fibrogenic agent, after 7 weeks from initial treatment numbers increased to 9.4± 4.6 MC/ mm^2 and after 11 weeks to 10.9± 4.6 MC/ mm^2 found within the portal tracts and fibrous septa (Ambrust *et al,* 1997). In cholestatic rats, treated for 21 days there was a two fold increase in MC numbers that showed a positive correlation with collagen deposition assessed by Sirius Red staining

(Rioux *et al*, 1996). In dimethylnitrosamine (DEN) -induced rat liver fibrosis, MC hyperplasia was observed in the fibrotic septa (Xu *et al*, 1994). MMC predominated as detected by alcian blue/ safranin staining (Rioux *et al*, 1996; Xu *et al*, 1994).

As further evidence for the recruitment of MMC in liver fibrosis, studies of rodents infected with *Schistosoma mansoni* parasites showed that during infection hepatic mastocytosis was observed. Miller *et al* (1994), found that in the infected rodent MC numbers within the liver increased to 147± 18.6 MC per 10x field from 2.6± 0.4 MC per 10x field. Quantification of RMCP II and I in rat infected liver showed there was a 330 fold and a 3-fold increase in protease expression respectively compared to control, suggesting that MC recruited to the infected liver were of the MMC subtype. Further studies have shown that MC undergo degranulation in the fibrotic liver (Rioux *et al,* 1996), and therefore increased hepatic levels of MC mediators such as histamine, tryptase and TNF-α, may contribute to collagen deposition and ultimately liver fibrosis

MC activation results in the degranulation of cytoplasmic granules and release of mediators into the extracellular fluid. These mediators will diffuse into the fluid and exert their effects on various cells, including fibroblasts, hence aiding the fibrotic process. Two general categories of mediators have been identified; preformed granule associated molecules and newly formed unstored mediators that are synthesised at the time of MC stimulation and activation.

1.8.1. Cytokine production by mast cells

Upon activation, MC are able to secrete an array of preformed proinflammatory or mitogenic cytokines. Cross-linking of the Fc_{ε}RIgE receptor and SCF stimulates a cascade of intracellular signalling events that lead to the activation of several DNA binding factors. Briefly this results in the breakdown of phosphatidyl inositol to inositol triphosphate (IP3) causing the generation of diacylglycerol (DAG) and subsequently an increase in intracellular Ca^{2+}. Calcineurin, a phosphatase becomes activated and induces the dephosphorylation of nuclear factor of activated T cells (NF-AT) which is involved in the transcription of most cytokine genes expressed in MC, including IL-3, IL-4, IL-5, and TNFα (Rao *et al,* 1994) from their promoter. IgE mediated signals also increase mitogen – activated protein kinase (MAPK) activation and the nuclear translocation of nuclear factor- kappa B (NFκB), which also induces cytokine gene expression. Non-immunological stimuli such as calcium ionophore or protein kinase C- active phorbol esters can mimic the activation of intracellular Ca^{2+}.

MC are therefore a potential source of vital cytokines involved in inflammatory responses, table 1.3. These cytokines have various effects on MC themselves and on other resident cells and tissues, for example fibroblasts, endothelial cells, epithelial cells and nerves. Cytokines have complex activity on fibroblast function; they can regulate fibroblast growth (Monroe *et al,* 1988), induce ECM synthesis (Rosenbloom *et al,* 1984; Jimenez *et al,* 1984; Varga *et al,* 1987; Fretin *et al,* 1991) and fibroblast chemotaxis (Postlethwaite *et al,* 1990). For example, IFNγ, PDGF and bFGF will induce fibroblast proliferation and protein synthesis. IL-8, macrophage chemotactic protein-1 (MCP-1), (macrophage inflammatory protein-1α (MIP-1α) and MIP-1β will attract lymphocytes, monocytes and neutrophils into the site of MC degranulation (Möller, *et al* 1993, Selvan *et al,* 1994). IL-5 and IL-6 are involved in regulating the activity of eosinophils and other cells in allergic responses (Bradding *et al,* 19934. TNF-α and TGF- β are constitutively present in MC and also synthesised upon MC activation (Gordon and Galli, 1990; Walsh *et al,* 1991). They increase the expression of adhesion molecules on endothelial cells, induce neutrophil chemotaxis and fibroblast proliferation and collagen synthesis. SCF and IL-4 are expressed by MC, perhaps acting as an autocrine factor in regulating activation of the MC (Bradding *et al,* 1992; Zhang *et al,* 1998; Welker *et al,* 1999).

Interleukins	IL-1, IL-3, IL-4, IL-5, IL-6, IL-8, IL-10
MIP Gene Family	MIP-1α, MIP-1β, MCP-1, TCA 3
Haematopoietic Factors	IFN- γ, GM-CSF, TNF-α, TGF-β, SCF, bFGF, PDGF

Table 1.3. Cytokine production by mast cells. IL = Interleukin; MIP= macrophage inflammatory protein; MCP= macrophage chemotactic protein; TCA= T cell activating antigen; IFN interferon; GM-CSF= granulocyte- macrophage colony- stimulating factor; TNF= tumour necrosis factor; TGF= transforming growth factor; SCF= stem cell factor; bFGF= basic fibroblast growth factor; PDGF= platelet derived growth factor.
*Adapted from Weber et al, Mast Cells, Internat J Derm 1995; **34:**1-10.*

MC are capable of expressing class II histocompatibility antigens on their surface and accessory molecules such as intracellular adhesion molecule-1 (ICAM-1). These molecules may allow for interaction between lymphocytes and MC (Banovac *et al,* 1989).

1.8.2. Histamine

Histamine is a basic amine, (2-(4- imidazolyl)- ethylamine) and is the major MC mediator stored preformed in the MC granule. MC contain 1 -5 μg of histamine per 10^6 MC, that is 5 – 10 % of the weight of the granules. In MC, histamine is held in the cytoplasmic

granules in a complex with an acidic protein and a glycosaminoglycan (heparin). Histamine release from MC *in vitro* following IgE- dependent stimulation is complete in 1- 3 minutes and its half-life in serum is 2 minutes. Histamine exerts its actions via specific histamine receptors, H_1, H_2 and H_3. Histamine released from MC has been shown to dose-dependently stimulate the proliferation of human skin and lung fibroblasts (Russell *et al,* 1977; Jordana *et al,* 1988) and induce collagen synthesis (Hatmochi *et al,* 1985). Addition of exogenous and MC released histamine can stimulate fibroblast growth in cultured guinea pig mesentery (Norrby, 1985) and is mediated via H_2 receptors on fibroblasts. Histamine is also profibrogenic in that it can stimulate the increase of collagen synthesis in guinea pig skin fibroblasts which is mediated through H_1 and H_2 receptors (Hatamochi *et al,* 1985). Histamine can also induce vasodilation, constriction of smooth muscles (bronchial and intestinal). In systemic sclerosis, there is an increase in circulating histamine plasma levels (Falang and Medsger, 1990). Clinically the release of histamine in the skin causes oedema, flushing, pruritis and the classic weal and flare reaction.

1.8.3. Heparin

Heparin is a sulphated glycosaminoglycan and is found in the MC granule in the form of large polymers of molecular weight (MW) 750 000. The main function of heparin is to stabilise the tryptase tetrameric complex. Heparin has anti-proliferative effect on fibroblasts, as addition of heparin to 3T3 fibroblasts significantly decreases fibroblast attachment and growth on collagen (San Antonio *et al,* 1992). Heparin has also been found to suppress the induction of *c-fos and c-myc* that encode proteins that are necessary for cell proliferation (Wright *et al,* 1989). Heparin may inhibit cell growth by binding to the extracellular matrix and disrupting fibroblast attachment.

1.8.4. Leukotriene and prostaglandin synthesis

Leukotrienes and prostaglandins are synthesised upon MC degranulation from arachidonic acid (AA) which is stored in the membrane phospholipids of human MC. During activation of MC via cross-linking of the $Fc_{\varepsilon}R$ receptor, AA is mobilised from storage pools by phospholipase A_2 (PLA_2). At least two major PLA_2 are involved in AA mobilisation in MC: a high molecular weight cytosolic PLA_2 (c PLA_2) provides AA that is mostly converted to leukotrienes by 5- lipoxygenase (5-LO), and a low molecular weight secretory PLA_2 ($sPLA_2$) that provides AA for conversion to prostanoids by cyclooxygenases (COX), (Chilton *et al,* 1996; Marone *et al,* 1997). When immunologically stimulated, MC predominately synthesise the proinflammatory prostaglandin PGD_2, leukotrienes LTC_4,

LTD_4 and LTE_4 and platelet activating factor (PAF) (Musch *et al,* 1987; Hogaboam *et al,* 1992; Colard *et al,* 1993; Dumitrašcu, 1996). PGD_2 and LTC_4 have vasoconstrictor effects on the surrounding microenvironment and cause chemotaxis of other inflammatory cells (Dumitrašcu, 1996). PAF, not only is a potent platelet aggregator but also can induce inflammatory cell chemotaxis and increase IL-1 and TNF-α synthesis.

1.8.5. Mast cell synthesis of matrix proteins

As well as degrading matrix proteins and stimulating collagen synthesis in other cell types, MC have been found to synthesise various ECM components. Laminin, type IV collagen, type VIII collagen and heparin sulphate have been shown to be synthesised by MC (Thompson *et al,* 1991; Rüger *et al,* 1994). Therefore MC could also contribute directly to fibrosis by the synthesis and secretion of basement membrane components.

1.8.6. Serine proteases

Proteases within the MC are further classified into families based on the amino acids within their active catalytic site. Tryptase and chymase are therefore also known as serine proteases. Tryptase is a tetrameric trypsin- like serine protease of 130 kDa and has a half-life of 2 hours in serum. It is found in abundance in MC, 35 μg per 10^6 MC (Remmington *et al,* 1988). Tryptase makes up approximately 25% of the dry weight of MC. Tryptase has been cloned and has been found to have 2 genes namely α and β. These encode for more than one peptide species of this enzyme with different N- linked glycosylation patterns (Vanderslice *et al,* 1990). Chymase is a monomeric chymotrypsin- like serine protease of 30 kDa. Chymase is found in smaller amounts, 4.5 μg per 10^6 MC (Remmington *et al,* 1988).

Tryptase and chymase have been reported to have many functions which are both fibrogenic and fibrolytic. Tryptase has been shown to be a potent mitogen for several cell types including human lung fibroblasts, Chinese hamster lung and rat fibroblasts (Ruoss *et al,* 1991; Cairns and Walls, 1997; Gruber *et al,* 1997), smooth muscle cells, and epithelial cells (Cairns and Walls, 1996) increasing cell proliferation and collagen synthesis (Cairns and Walls, 1997). Tryptase and chymase are also implicated in degradation of connective tissue by activating collagenase (Gruber *et al,* 1988;Gruber *et al,* 1989;Lees *et al,* 1994), urokinase (Stack *et al,*1994), stromelysin (Lees *et al,* 1994) and gelatinase (Lohi *et al,* 1992; Fang *et al,* 1996 ; Fang *et al,* 1999).

Tryptase has also been shown to work in an autocrine fashion to increase histamine release from activated MC (He *et al,* 1998). It has recently been suggested that tryptase can mediate its fibrogenic effects via a novel proteinase activated receptor, namely PAR2.

1.9. PROTEINASE ACTIVATED RECEPTORS (PARS)

Proteinase activated receptors are a new family of cell surface receptors for serine proteases. These receptors have similar structural characteristics of the classical guanosine (G) -protein coupled receptors, for example neuropeptide receptors such as substance P and substance K and also glycoprotein hormone receptors (figure 1.9). PARs consist of a long extracellular N- terminal domain, a seven transmembrane spanning domain consisting of 3 intra- and 3 extracellular domains and a long intracellular C-terminal tail which is made up of a large number of serine and threonine residues as potential phosphorylation sites for G-protein coupled kinases (Macey, 1998). However, unlike normal receptor-ligand binding which is reversible, activation of PARs is irreversible. Serine proteases activate PARs by a unique process that involves the recognition of a specific putative cleavage site on the PAR extracellular domain by the protease, followed by the cleavage of the receptor at the cleavage site and finally exposure of a new N-terminal (tethered ligand) which binds and activates the cleaved receptor molecule.

Specific amino acid residues within the tethered ligand domain, which comprise six or more amino acids, interact with the second extracellular loop of the transmembrane spanning region, causing changes in receptor conformation which results in the interaction of heterotrimeric G- proteins in the plasma membrane which transduce the signal (figure 1.10).

1.9.1. Discovery and evolution of the PAR family

PAR-1 was the first member of this family to be discovered. It is also known as the thrombin receptor as it is cleaved by this serine proteinase (Vu *et al,* 1991). It comprises 425 amino acids and since its discovery has been found distributed in many tissues such as the brain (Weinstein *et al,* 1995), endothelium of human arteries (Nelken *et al,*1992) and synovial membrane of patients with rheumatoid and osteoarthritis (Morris *et al,* 1994; Morris *et al,* 1996) and on various cells such as platelets, endothelial cells (Vu *et al,*1991), smooth muscle cells (McNamara *et al,* 1993) and fibroblasts (Chambers *et al,* 1998; Hou *et al,* 1998) and on HSC during chronic liver disease (Marra *et al,* 1998).

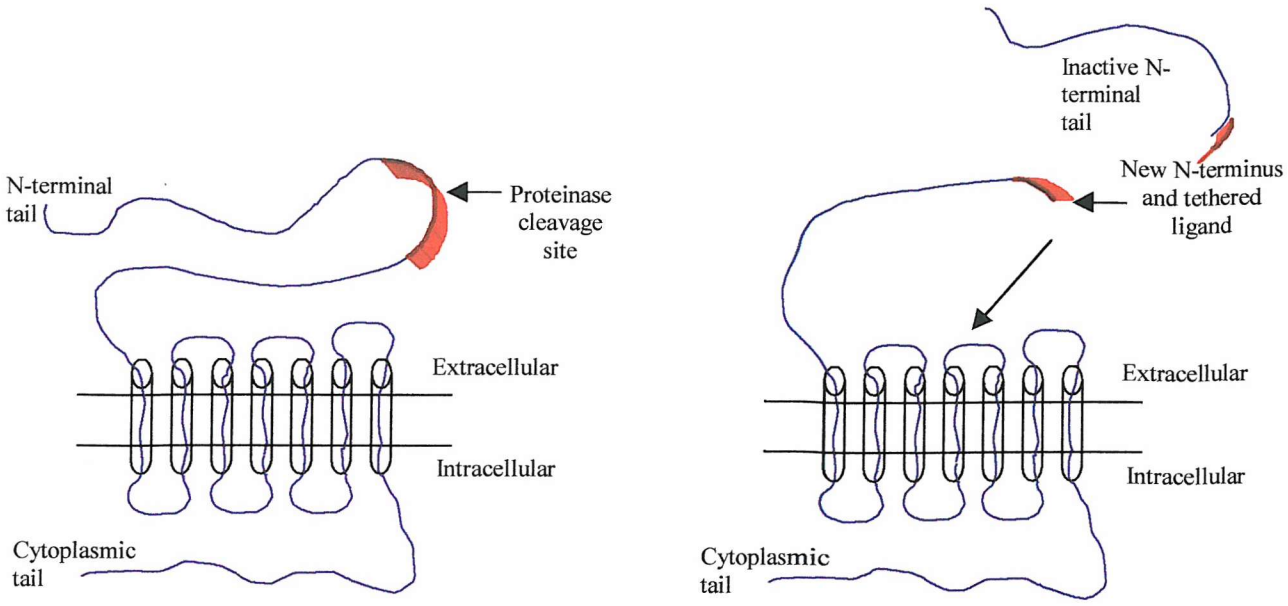


Figure 1.10. Activation of proteinase activated receptors. The serine protease will recognise the putative cleavage site (proteinase cleavage site) and will subsequently cleave the receptor at the N- terminal domain. The inactive N –terminal tail is released into the extracellular environment exposing a new N- terminal domain, or tethered ligand. The new N terminal will bind to the second extracellular domain causing receptor conformation and subsequent activation.

A second PAR member, PAR-2, was cloned by screening a mouse genomic library by filter hybridisation, a technique in which polymerase chain reaction (PCR) was employed using degenerate primers to the second and sixth transmembrane domains of the substance K receptor (Nystedt *et al,* 1994). This receptor consists of 395 amino acids and has 30% amino acid identity to the human PAR-1. Pancreatic trypsin and mast cell tryptase are the natural agonists for this receptor (Corvera *et al,* 1994; Nystedt *et al,* 1994; Molino *et al,* 1997). PAR-2 is found distributed in the gastrointestinal tract, pancreas, kidney, liver, prostate, ovary and eye (Böhm *et al,* 1996a; Nystedt *et al,* 1994; Nystedt *et al,* 1996). It is expressed on epithelial and endothelial cell lines (Nystedt *et al,* 1996), smooth muscle cells (Bono *et al,* 1997; Molino *et al,* 1998; Bretschneider *et al,* 1999), neutrophils (Howells *et al,* 1997) and fibroblasts (Schechter *et al,* 1998).

The discovery of PAR-2 suggested the existence of an extended family of PARs. Further investigation into the mechanisms of PAR-1 interactions with thrombin led to the discovery of the third member of the family, PAR-3 the second thrombin receptor. Platelets from transgenic PAR-1 knockout mice ($tr^{-/-}$) were found to respond strongly to thrombin, in contrast to $tr^{-/-}$ fibroblasts that lost their ability to respond to thrombin. This led to the cloning and characterisation of PAR-3 (Ishihara *et al,* 1997). PAR-3 is 374 amino acids long and has 27% amino acid homology to PAR-1 and 28% amino acid homology to PAR-2 and is cleaved by thrombin. It has been found expressed in the bone marrow, heart, brain, placenta, liver, pancreas, thymus, small intestine, stomach, lymph

nodes and trachea (Ishihara *et al,* 1997; Dery *et al,* 1998). Cell types expressing this receptor have yet to be identified.

Even more recently a fourth member has been discovered, PAR-4, identified in an expressed sequence tag database (Xu *et al,* 1998). It has been shown to be activated by thrombin or trypsin. It is found in the lung, pancreas, thyroid, testis and small intestine.

1.9.2. Activation of PARs

PAR-1 is the most extensively studied receptor of this family and its mechanism is well defined. The extracellular N- terminal of human PAR-1 contains the putative cleavage site for thrombin, 'cysteine- aspartic acid- proline- arginine^{41}↓ serine^{42} – phenylalanine- leucine- leucine- arginine- glutamic acid' (CDPR^{41}↓S^{42}FLLRN), followed by a sequence of negatively charged residues, 'aspartic acid^{51} lysine- tyrosine- glutamic acid-proline- phenylalanine^{56}' (D^{51}KYEPF^{56}). Thrombin will bind to the putative cleavage site and an anion- binding site on thrombin will interact with the negative charged domain on PAR-1. This induces a conformational change of the receptor to accommodate the cleavage site of PAR into the thrombin catalytic site, promoting efficient receptor hydrolysis (Dery *et al,* 1998).

Upon cleavage of the putative PAR-1 site, a new N-terminal domain is generated, beginning with the sequence SFLLRN. This will subsequently bind to the activation site of the receptor in the second extracellular loop of the transmembrane spanning region (figure 10). Synthetic peptides corresponding to this tethered ligand domain can activate PAR-1 without cleavage (Scarborough *et al,* 1992). Synthetic peptides to activate PAR-1 have proved useful biological tools to further investigate PAR mechanisms of activation. Several mutation analogues of SFFLRN , in which individual residues are substituted, have shown that the first 6 amino acids are vital for PAR-1 activation and binding to the second extracellular loop. Unfortunately, these synthetic peptides are relatively weak agonists compared with proteases. This may be due to inefficient presentation of these synthetic peptides to the binding domain of PAR-1.

PAR-2 is activated by trypsin or MC tryptase (Corvera *et al,* 1994; Nystedt *et al,* 1994; Molino *et al,* 1997), binding to the putative cleavage site in the N- terminal domain, 'cysteine- aspartic acid- proline- arginine^{34}↓ serine^{35} leucine- isoleucine- glycine- arginine- leucine' (CDPR^{34}↓S^{35}LIGRL) in mice and 'serine- lysine- glycine- arginine^{34}↓ serine^{35} leucine- isoleucine- glycine- lysine- valine' SKGR^{34}↓S^{35}LIGKV in humans.

Synthetic peptides corresponding to the tethered ligand domain (SLIGRL in mice and SLIGKV in humans) activate PAR-2 without PAR-2 cleavage (Böhm *et al,*1996).

Thrombin cleaves and activates PAR-1 and PAR-3 and pancreatic trypsin and MC tryptase PAR-2. However other proteases are capable of cleaving and activating these receptors but not as potently. Trypsin, plasmin, granzyme A and cathepsin G can activate PAR-1 by cleavage at the putative cleavage site $CDPR^{41}\downarrow S^{42}FLLRN$(Suidan *et al,* 1994; Molino *et al,* 1995). Coagulation factor Xa can activate PAR-2 at the putative cleavage site $SKGR^{34}\downarrow S^{35}LIGKV$ (Dery *et al,* 1998). The physiological relevance of PAR activation by different proteases is unknown. It may be that certain proteases are principal activators in certain tissues. For example, PAR-2 expressed by enterocytes in intestinal lumen is activated by pancreatic trypsin. However in intestinal smooth muscle and skin MC tryptase cleaves and activates PAR-2 (Dery *et al,* 1998).

Some proteases can cleave PARs at sites other than the putative cleavage site to render the receptor inactive. Cathepsin G can cleave PAR-1 at $phenylalanine^{45}\downarrow leucine^{46}$ and at $phenyalanine^{55}\downarrow tryptophan^{56}$ in addition to the activating cleavage site at $arginine^{41}\downarrow serine^{41}$ (Molino *et al,* 1995). Similarly, tryptase also cleaves PAR-2 at $lysine^{41}\downarrow valine^{42}$ site on PAR-2 in addition to the activating cleavage at site $arginine^{34}\downarrow serine^{35}$ (Molino *et al,* 1997). This unusual hydrolysis of PARs at sites to render them inactive may promote a regulatory mechanism for the downregulation of these receptors.

1.9.3. Signal transduction mechanisms of PAR

Upon activation of PAR, the binding of the tethered ligand to the second extracellular loop of the transmembrane region, results in conformational change of the receptor. This allows the receptor to interact with the heterotrimeric G proteins (α,β,γ) in the plasma membrane. The Gα protein will catalyse the conversion of guanosine 5'- triphosphate (GTP) to guanosine 5'- diphosphate (GDP) causing receptor activation. The receptor loses activity when hydrolysis of GTP occurs and the G-proteins return to their inactive states (Simon *et al.* 1991). PAR-1 signal transduction has been most extensively studied and has been found to couple to a variety of G-proteins and affect enzymes and signal pathways depending on the type of cell.

The principal mechanism of PAR-1 signal transduction in platelets and CCL-39 fibroblasts is via $G_{\alpha q}$ proteins, which are insensitive to pertussis toxin. This results in the activation of phospholipase C-β, the hydrolysis of phosphoinositide forming IP3 and DAG. This results in the mobilisation of Ca^{2+} and activation of protein kinase (figure 1.11). PAR-1 has also been shown to be coupled to G_i proteins that are pertussis toxin sensitive, which is coupled to phospholipase A2 in CCL-39 fibroblasts (Huang *et al,* 1992).

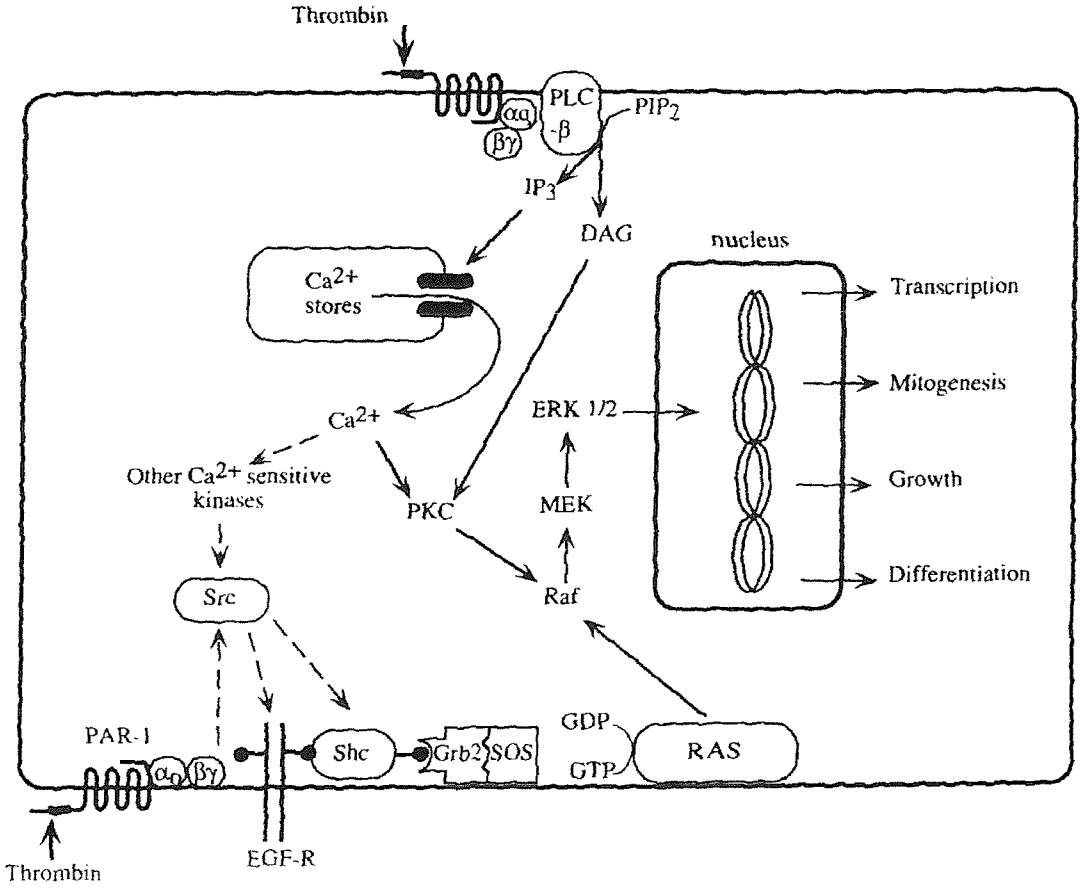


Figure 1.11. Mechanisms of signal transduction by PARs. Most of this information is derived from extensive PAR-1 signal transduction studies. **PLC:** phospholipase C;**PIP_2:** phosphatidylinositol 4,5,- bisphosphate; **IP_3:** inositol triphosphate; **DAG:** diacylglycerol; **ERK:** extracellular signal response kinase; **MEK:** mitogen activated protein kinase kinase; **PKC:** protein kinase C; **EGF:** epidermal growth factor.
*Adapted from Dery et al. Proteinase- activated receptors: novel mechanisms of signalling by serine proteases. Am J Physiol 1998; **274:** C1429- C1452.*

Thrombin is mitogenic in many cells due to PAR-1 activation and subsequent activation of MAPK (Molloy *et al,* 1996). MAPK may be activated through several pathways. One pathway has been studied in CCL-39 fibroblasts, which proliferate in response to thrombin, and is very similar to stimulation of growth via the epidermal growth factor pathway. The non- receptor tyrosine kinase *Src* is activated by PAR-1 agonists (Chen *et al,* 1994). This induces tyrosine phosphorylation of an adapter protein, *Shc*, which in turn is recruited to *Grb-2* (Chen *et al,* 1994). (Mutants of *Shc* that are

deficient in *Grb-2* binding suppress thrombin- stimulated activation of p44 MAP kinases and cell growth.) This led to the activation of *Ras* via the adapter molecule *Grb-2* coupled to the *SOS-Ras* exchange factor, which subsequently activates the MAP kinase pathway leading to cell growth and differentiation. Genistein (a tyrosine kinase inhibitor) and pertussis toxin can inhibit this *Ras* activation suggesting that it is mediated via G_o proteins. However in platelets, protein kinase C from diacyglycerol can activate MAPK via $G_{\alpha q}$ proteins and not by G_o proteins.

Agonists of PAR-2 can stimulate the production of inositol triphosphate and mobilisation of intracellular Ca^{2+} in smooth muscle and epithelial cells via $G_{\alpha q}$ or G_o proteins (Dery *et al,* 1998). In smooth muscle cells and enterocytes, PAR-2 agonists activate MAPK known as extracellular signal receptor kinase (ERK1/ERK2) and weakly stimulate the MAPK homologue p38 (Belham *et al,* 1996).

1.9.4. Mechanisms of PAR inactivation

Activation of PARs by serine proteases results in their rapid homologous desensitisation (Böhm *et al,* 1996). This is important to prevent the uncontrolled stimulation of cells that may result in their ultimate dysfunction. Desensitisation of the PARs can be mediated by two ways, their inactivation by protease cleavage and their internalisation.

1.9.5. Inactivation of PARs by proteolytic cleavage

With normal G-proteins, soluble agonists are removed from the extracellular fluid by diffusion, uptake and enzymatic degradation. However, with PARs this is quite different. Once the receptor has been cleaved and activated the tethered ligand is always available to further activate the receptor, so diffusion or uptake can not remove it. However, as mentioned in section 1.9.2., other proteases may inactivate the receptor by cleaving other than the specific protease cleavage site hence removing the tethered ligand before it is exposed. For example, cathepsin G removes the tethered ligand domain from PAR-1 so becomes unresponsive to thrombin stimulation (Molino *et al,* 1995).

The irreversible property of PAR activation renders the cleaved PAR resistant to further proteolytic activation, until the plasma membrane is replenished with new intact receptor.

1.9.6. PAR desensitisation

Within a minute of activation PARs are rapidly internalised (Hoxie *et al,* 1993; Brass *et al,* 1994; Böhm *et al,* 1996b). The receptor is sequestered from the cell surface into coated pits

in the plasma membrane and then into intracellular lysosomes (figure 1.12). This process takes place within 30 to 60 minutes of stimulation by proteases or activating peptides in transfected cell or cells naturally expressing PAR-1 or PAR-2 (Hoxie *et al,*1993; Böhm *et al,*1996b). During lysosomal targeting, 25 – 30% of the internalised receptor is recycled back to the plasma membrane, within 2 hours from original stimulus (Hoxie *et al,*1993), figure 1.12. These recycled receptors however are unable to respond to proteases but can respond to activating peptides, therefore the significance of this recycling is unknown. Hence this receptor-mediated endocytosis may contribute to receptor desensitisation.

Recent evidence shows that PARs are internalised constitutively even in the absence of ligands. The receptor appears to shuttle back and forth between plasma membrane and intracellular stores (Shapiro *et al,* 1993). Endothelial cells, platelets and fibroblasts have large intracellular pools containing PAR-1 within their Golgi apparatus (Hein *et al,* 1994; Horvat *et al,* 1995; Molino *et al,* 1997), and PAR-2 in transfected cells and enterocytes (Böhm *et al,*1996b; Kong *et al,* 1997).

1.9.7. PAR resensitisation

Receptor recycling and receptor dephosphorylation is required for PAR desensitisation. In endothelial cells and fibroblasts resensitisation is rapid and is associated with the loss of PAR-1 intracellular stores due to their subsequent mobilisation (Hein *et al,* 1994; Horvat *et al,* 1995). (Brefeldin A disrupts receptor mobilisation from intracellular stores and showed that resensitisation of PARs requires mobilisation of their pools). Once the pools are exhausted then new receptors are synthesised and has been reported to take up to 20 hours and can be inhibited with cycloheximide (Hoxie *et al,*1993). This restored the full thrombin response of PAR-1.

1.9.8. Importance of PARs in fibrogenesis

Serine proteases have been shown to be mitogenic for a variety of cells and also induce collagen synthesis. Thrombin, the PAR-1 agonist, is not only involved in blood coagulation but is also a mitogen for various cell types such as smooth muscle cells and fibroblasts (McNamara *et al,*1993; Chambers *et al,* 1998; Dabbagh *et al,* 1998). Thrombin has been implicated in several disease processes including fibrosis. Patients with systemic sclerosis, and also in corresponding animal models, have been reported to have an increase in thrombin levels in their BAL fluid (Tani *et al,* 1990; Ohba *et al,* 1994; Hernandez-Rodriguez *et al,* 1995). Thrombin is mitogenic for smooth muscle cells and fibroblasts and able to induce procollagen synthesis in these cells mediated via PAR-1 (Chambers *et al,*

1998; Dabbagh *et al,* 1998). In fibroblasts it was shown that thrombin could induce procollagen synthesis by up to 112% after 48 hour stimulation.

Tryptase, a PAR-2 agonist, has been shown to be mitogenic and to upregulate collagen synthesis in fibroblasts and epithelial cells (Rouss *et al,* 1991; Cairns and Walls, 1996; Cairns and Walls, 1997; Gruber *et al,* 1997).

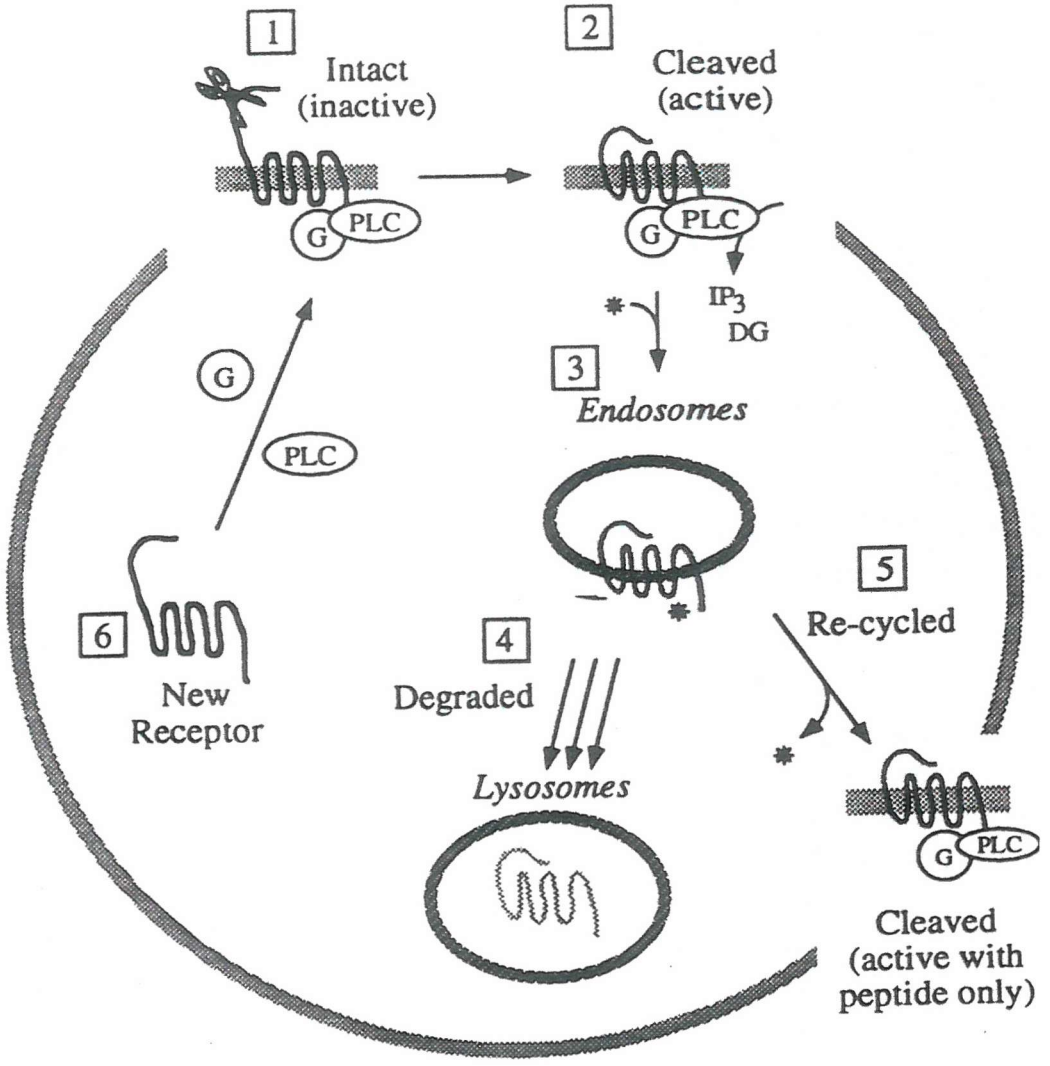


Figure 1.12. A schematic representation of the internalisation and recycling of PAR-1. Intact PAR-1 receptor on surface of cell (1) is cleaved by thrombin by proteolysis (2) initiating G-protein mediated events such as activation of phospholipase Cβ. PAR-1 is internalised and packaged into endosomes (3). Once internalised 75% of the receptors are transferred to lysosomes and degraded (4) whilst 25% are recycled to the cell surface (5). The recycled PAR-1 is unresponsive to thrombin for a second time but can be activated with activating peptide only. Responsiveness of thrombin is restored by the synthesis of new receptors (6). *Adapted from Dery et al. Proteinase- activated receptors: novel mechanisms of signalling by serine proteases. Am J Physiol 1998;* ***274:*** *C1429- C1452.*

1.10. SUMMARY

Liver fibrosis is characterised by the increase in ECM deposition and the decrease in its degradation. HSC are pivotal in liver fibrosis. During liver injury, HSC become activated, adopt a myofibroblastic phenotype (Gressner *et al,* 1996) and assume many features of fibroblasts in other tissues. Activation occurs due to extracellular stimuli such as changes in basement membrane and the release of inflammatory mediators (PDGF and TGFβ) from hepatic cells such as KC and hepatocytes. Activated HSC synthesise and secrete many factors important in liver fibrosis, such as ECM proteins (Maher *et al,* 1988; Milani *et al,* 1990; McGee and Patrick, 1992; Rockey *et al,* 1992) MMPs (Arthur *et al,* 1992; Benyon *et al,* 1997; Benyon *et al,* 1999) and TIMPs (Iredale *et al,* 1996; Benyon *et al,* 1996).

In human and rat liver disease, it has been shown by various experimental techniques that MC numbers accumulate especially within the sinusoids and the portal tracts (Miller *et al,* 1994; Peng *et al,* 1994; Ramos *et al,* 1994; Farrell *et al,* 1995; Xu *et al,* 1994; Rioux *et al,* 1996; Ambrust *et al,* 1997; Nakamura *et al,* 1997). MC and HSC may therefore have a bi- directional relationship within the liver, as observed between fibroblasts and MC in chronic inflammation and fibrosis of other tissues (figure 1.13). Receptor- ligand studies of mature MC or their precursors and fibroblasts resulted in the discovery that fibroblasts produce cell- associated and soluble forms of SCF (Nocka *et al,* 1990). This protein plays a central role in MC biology having multifunctional effects; promotes MC maturation from tissue resident precursors and MC progenitors (Nocka *et al,* 1990; Irani *et al,* 1992a; Rennick *et al,* 1995), prolongs MC survival in culture (Tsai *et al,* 1991a; Takagi *et al,* 1992), acts as a potent MC chemoattractant (Meininger *et al,* 1992; Nilsson *et al,* 1994) and induces MC mediator release (Bischoff *et al,* 1992; Columbo *et al,* 1992; Coleman *et al,* 1993). In return, MC mediators, such as tryptase and TNFα, can affect fibroblast properties by inducing their subsequent proliferation and collagen synthesis. The profibrogenic and mitogenic effects of tryptase are suggested to be mediated by a proteolytic activated receptor, namely PAR-2 (Corvera *et al,* 1994; Nystedt *et al,* 1994; Molino *et al,* 1997).

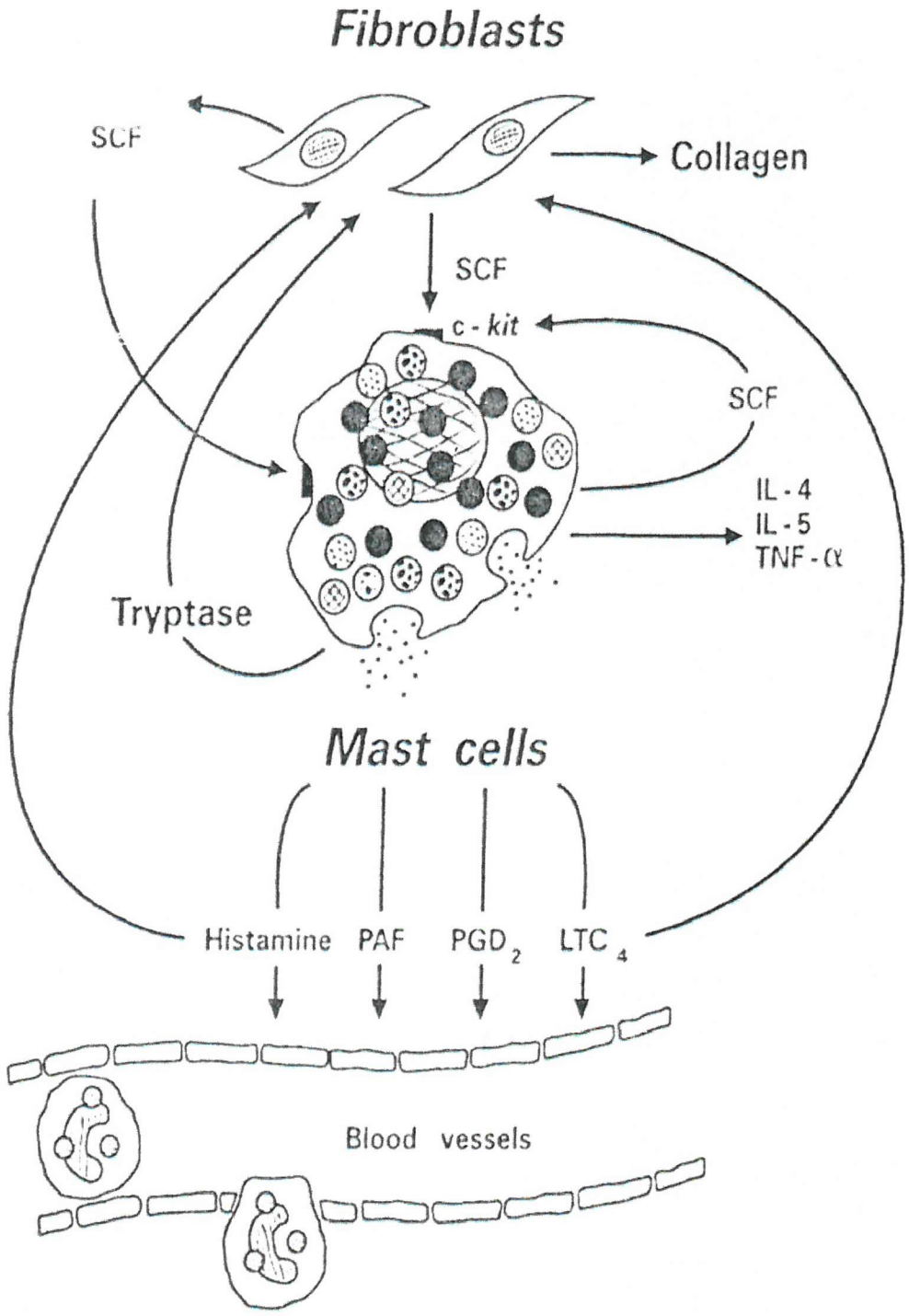


Figure 1.13 Diagram representing human mast cell and fibroblast interaction. MC activation immunologically or by fibroblast derived SCF results in their release of granule stored mediators (histamine, tryptase, chymase, proteoglycans) and newly synthesised mediators (cytokines such as IL-4, IL-5, TNF-α and lipid mediators). Mediators released by MC affect blood vessels (histamine, platelet activating factor (PAF), PGD_2 and LTC_4) and can influence fibroblast properties (proliferation and collagen synthesis). For example, tryptase and histamine can activate fibroblasts to stimulate collagen synthesis. This indicates direct MC involvement in the fibrotic process. SCF contained in MC granules exert an autocrine effect on these cells.

Adapted from Marone et al. Molecular and cellular biology of MC and basophils. Int Arch Allergy Immunol 1997; 114: 207-217

1.11. HYPOTHESIS AND AIMS

The hypothesis studied for this thesis is that HSC and MC have a bi-directional relationship, that is they interact in a manner which promotes liver fibrosis.

To test this hypothesis it was fundamental to establish the possible mechanism of MC recruitment to the liver during injury and fibrosis. As activated HSC are phenotypically similar to fibroblasts, attempts to investigate SCF synthesis by HSC as a possible chemoattractant for MC were initially examined. In chapter 3, by using various experimental techniques, SCF RNA and protein expression were investigated in human and rat fibrotic livers and *in-vitro* cultured HSC. *In- vitro* adhesion studies of HSC and MC in the absence/ presence of a neutralising SCF antibody enabled further examination of SCF as a chemoattractant for MC in the liver.

MC hyperplasia has been reported to occur in fibrosis of various tissues and their role in fibrosis has yet to be established. During liver fibrosis MC numbers increase within the sinusoid and portal tracts. In chapter 4, the various MC subtypes in human and rat fibrotic livers were examined by RT-PCR and immunohistochemistry. To further test the hypothesis, the potential mitogenic effects of MC mediators on *in-vitro* rat HSC cultures were investigated. By use of ^{3}H –thymidine incorporation assays, the proliferative effects of tryptase, heparin, histamine, IL-4 and TNF-α on HSC were studied. The profibrogenic effect of tryptase on HSC collagen synthesis was examined by ^{3}H –proline incorporation assays.

Finally, in chapter 5, the expression of PAR-2 on HSC and activation of the MAPK signalling transduction pathway was further investigated to support the existence of a relationship between MC and HSC in liver fibrosis. PAR-2 RNA and protein expression in rat fibrotic livers and isolated HSC were examined by various techniques. To further confirm the existence of PARs in HSC, the mitogenic effects of PAR-1 and PAR-2 natural and synthetic agonists on *in-vitro* rat HSC cultures were investigated by use of ^{3}H – thymidine incorporation assays. MAPK induction by PAR 1 and 2 agonists were studied using Western blotting and further proliferation assays in the absence/ presence of a MAPK inhibitor.

CHAPTER TWO
MATERIALS AND METHODS

2.1. MATERIALS

All immunochemicals were supplied by Sigma Chemicals, Poole, UK. Molecular biology grade reagents were from Promega, Southampton, UK, unless stated. Reagents donated with an asterisk (*) are defined in the appendix (section 7).

2.2. METHODS

2.2.1 RAT MODELS OF LIVER FIBROSIS

It is difficult to study the process of liver fibrosis in humans primarily due to the disease presenting itself in the patient when it is highly developed. Therefore the study of early fibrotic change in the human liver is virtually impossible. Combined with the ethical limitations of biopsies, animal models of liver fibrosis are essential to liver research. These models provide the means of controlling the disease process and studying it from initiation through to fibrosis, although none accurately reproduce the disease in man.
In this thesis, two experimental models of liver fibrosis were used:

2.2.1.1. Carbon tetrachloride (CCl_4) induced fibrosis

CCl_4 is a commonly used hepatotoxic agent to induce liver fibrosis and cirrhosis in experimental animals (Tsukamoto *et al,* 1990; Wu and Norton, 1994). In this model the bioactivation of the haloalkane by oxidases such as microsome cytochrome P-450 yields a tricholoromethyl radical (CCl_3). This radical accelerates the formation of fibrosis/ cirrhosis by initiating lipid peroxidation by dissociating hydrogen atoms from unsaturated membrane lipids (Wu and Norton, 1994). In early stages of CCl_4 intoxication there is apparent hepatocyte damage and significant inflammation. After 6 to 8 weeks, major histology observed include hepatocyte necrosis, inflammatory infiltration, hepatocyte regeneration, proliferation of HSC and deposition of connective tissues. Perivenular and periportal fibrosis appears to predominate. Fully developed cirrhosis appears after 12 weeks of CCl_4 administration (Tsukamoto *et al,* 1990; Wu and Norton, 1994).

In this study male Sprague-Dawley rats were injected intra-peritoneal with 0.2 ml/100 g of sterile CCl_4 in a 1:1 ratio with olive oil, twice weekly for up to 8 weeks. Control rats were treated only with olive oil at the same time. Control and treated rats were sacrificed 6 hours, 24 hours, 1 week and 4 weeks after the initial treatment of CCl_4.

2.2.1.2. Bile duct ligation (BDL) induced progressive fibrosis

Common BDL induces cholestatic hepatic fibrosis leading to cirrhosis in the rat. However this model is hampered by many technical problems including recannulisation of the ligated bile ducts, the accumulation of bile in extraheptic biliary passages and a high mortality. In spite of these difficulties, this model is characterised by significant infiltration of connective tissues in the portal zone and enhanced proliferation of bile duct epithelial cells and hepatocytes (Wu and Norton, 1994).

Male Sprague-Dawley rats were anaesthetised and had their bile ducts double ligated and then divided. Control animals were sham operated. Control and treated rats were sacrificed 6 hours, 24 hours, 3 days and 7 days after bile duct ligation.

For each model of fibrosis, the animals were sacrificed and the livers snap- frozen in liquid nitrogen, until further used. Livers from the CCl_4 fibrotic model were immersed in formaldehyde, processed in graded alcohols and embedded in paraffin wax for immunohistochemistry.

2.2.2 HUMAN FIBROTIC LIVERS

Samples of snap frozen normal and fibrotic human liver (primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC)), removed at orthotopic transplantation were generously provided by the Liver Unit, Queen Elizabeth Hospital, Birmingham, UK.

2.2.2.1. Primary biliary cirrhosis (PBC)

PBC is a slow chronic autoimmune liver disease. The body's immune system attacks the bile epithelium causing destruction of the intra-hepatic bile ducts. Bile acids, normally drained through the bile ducts, build up forming pools of acid causing inflammation and scarring within the liver and subsequent cirrhosis of the liver. 90 % of people with PBC are female. The cause and why it occurs mainly in women is as yet unkown. There is presently no cure except liver transplantation.

2.2.2.2.Primary sclerosing cholangitis (PSC)

PSC is a bile duct disease. It results in the narrowing of the bile ducts within the whole biliary tree by chronic fibrosis and inflammation, causing obstruction of the biliary ducts and biliary cirrhosis. The cause of PSC is unknown however it often occurs in people with an inflammatory bowel disease, for example ulcerative colitis. Treatment includes surgery of the bile ducts to help reduce or halt the progression of the disease and at later stages of the disease liver transplantation.

2.2.3. CELL CULTURE

2.2.3.1. Hepatic stellate cell (HSC) isolation

Primary rat hepatic stellate cells (HSC) were obtained from normal rat liver by various techniques (Casini *et al,* 1993; Alpini *et al,*1994). Firstly, the liver tissue was digested by *in-situ* sequential pronase and collagenase perfusion via cannulation of the hepatic artery. Due to their vitamin A content HSC are more buoyant than other liver cells, therefore this property was used to isolate HSC from contaminating hepatocytes by density gradient centrifugation. Resuspended dispersed HSC were purified using a 11% Optiprep (Nycomed, Life Technologies, Renfrewshire, UK) gradient and purified HSC were collected from the top interface. Collected HSC were pelleted, resuspended and finally purified by use of centrifugal elutriation, to remove residual contaminating KC. This resulted in a highly purified HSC preparation (>95 % purity). Cell viability was detected by trypan blue exclusion test.

Primary HSC, at a plating density of 1.3 x10^6 cells/ml, were plated onto tissue culture plastic and maintained in culture in complete Dulbecco's medium (DMEM) (Life Technologies, Renfrewshire, UK), that is DMEM with 16% foetal calf serum (FCS) (Life Technologies, Renfrewshire, UK) and 4% antibiotics* (penicillin, streptomycin and gentomycin (PSG)), at 37 °C in 5% CO_2, in a humidifying incubator. Media were changed every 48 -72 hours. When HSC became confluent, at approximately 14 days, they were passaged using trypsin in calcium free medium. Once the cells had lifted off the culture flask, they were pelleted by centrifugation, resuspended in complete DMEM, diluted 3-fold and replated into tissue culture flasks and maintained as above.

2.2.3.2. Mouse skin fibroblasts (3T3)

Cells were obtained from Centre for Applied Microbiology and Research, (CAMR), Salisbury, Wiltshire, UK. Cells were maintained in culture in Roswell Park Memorial Institute medium (RPMI) with 10 % FCS and 4% PSG, at 37 °C in 5% CO_2, in a humidifying incubator. Media were changed every 48 -72 hours. These cells were maintained and used as positive control cells expressing SCF.

2.2.3.3. Rat skin fibroblasts (RSF)

Cells were obtained from rat skin outgrowths and were maintained in culture in identical conditions to that of HSC. Media were changed every 48 -72 hours. These cells were maintained and used as positive control cells expressing SCF.

2.2.4. MAST CELL DISPERSION AND PURIFICATION

MC were dispersed and purified from human foreskin tissues, which were obtained from circumcisions performed within our hospital, as previously described (Benyon *et al,* 1987). Briefly the tissue was chopped finely, digested using collagenase and hyaluronidase in the presence of 10 % bovine serum albumin (BSA) and 5% PSG for approximately 75 minutes. Digested tissue was separated from undigested tissue by sieving and washing in DMEM. Dispersed MC were resuspended and counted using Kimura* staining.

MC were further purified using a 65% Percoll gradient after lysis of erythrocytes in 1.7 mM ammonium chloride, pH 7.4. MC were pelleted, resuspended in DMEM and recounted using Kimura stain. MC represented 79 $\pm$ 2.7% of nucleated cells. Cell viability was detected by trypan blue exclusion test.

2.2.5. TOTAL RIBONUCLEIC ACID (RNA) EXTRACTION

Ribonucleic acid (RNA) is susceptible to degradation by ubiquitous ribonucleases (RNase), and to successfully isolate intact RNA by any procedure. The following measures were taken: all glassware was baked at 200° C for 5 hours; pipette tips and microfuge tubes were autoclaved; stock aqueous solutions were treated with diethylpyrocarbonate (DEPC)*, which inhibits RNases; gloves were worn at all times, as hands are a common source of RNases, when handling RNA products or reagents.

2.2.5.1. Preparation of 4M guanidinium isothiocyanate (GIT) lysates

In order to extract intact total RNA, effective disruption of cultured cells or liver tissue is necessary, followed by inactivation of endogenous RNase activity. This was achieved by solubilising the samples in guanidinium isothiocyanate (GIT), a strong chaotropic agent that disrupts nucleoprotein complexes, allowing RNA to be released into solution, and inhibiting endogenous RNase activity.

200 mg of snap- frozen livers from the two rat experimental models of fibrosis were homogenised in 5 mls of 4M GIT*, using a pre- autoclaved Polytron probe (Brutamann). The 5 ml lysates were centrifuged, at 2,000 g for 30 minutes, to precipitate out any large remaining particles. The lysates were made into 1 ml aliquots in 1.9 ml microfuges and stored at -70 °C.

Cultured cells were lysed by adding 900 µl of 4M GIT directly to the culture flask, after pouring off the culture media. The flask was left agitating on a shaker for 10 minutes

to mechanically disrupt the cells. The lysates were made into 1 ml aliquots in 1.9 ml microfuge tubes and stored at -70 °C.

2.2.5.2. RNA extraction

RNA was extracted from 4M GIT lysates by the acid-phenol method, adapted from Chomczynski and Sacchi (1987). 1 ml GIT lysate samples from liver and cell samples were thawed on ice. To each sample 90 µl of 2M sodium acetate, 700 µl phenol, 150 µl chloroform/ isoamyl alcohol (IAA) were added. The mixture was inverted several times and incubated on ice for 10 minutes, followed by centrifugation at 10,000 g for 10 minutes at 4° C. The top aqueous phase, containing RNA was removed and transferred to a clean 1.9 ml microfuge tube. Carbohydrates and proteins prefer hydrophobic conditions therefore will remain in the organic phase and at the interface. RNA was re- extracted from the aqueous phase by addition of 1 sample volume of phenol/ chloroform/ IAA. The mixture was inverted several times and incubated on ice for 10 minutes, followed by centrifugation at 10,000 g for 10 minutes at 4° C. The top aqueous layer was removed and transferred into a clean 1.9 ml microfuge tube. RNA was left to precipitate by adding 0.05 sample volume of 3M sodium acetate, to aid in the removal of any contaminating glycogen, and 1 sample volume of 100% isopropanol. The mixture was inverted a couple of times and left for a minimum of 90 minutes at -70 ° C. After incubation, samples were thawed on ice and the RNA was pelleted by centrifugation at 10,000 g for 15 minutes at 4° C. The supernatant was carefully aspirated and the pellet washed twice with ice-cold ethanol (75%). The RNA pellet was dried on a heating block at 65 °C and then resuspended in 20- 50 µl of DEPC water* (DEPCd'H_20). The RNA samples were stored at -70 ° C.

2.2.5.3. Analysis of RNA quantity and integrity

The RNA samples were quantified by UV spectrophotometry. 2 µl of RNA was added to 500 µl DEPCd'H_20, vortexed and heated for 10 minutes on a heating block at 65 ° C. Before loading into the spectrophotometer, the samples were vortexed and left to cool to room temperature. RNA was quantified at 260 nm and protein at 280 nm. The ratio of A_{260}/ A_{280} was used to assess sample purity. Ratios >1.7 indicate properly isolated RNA free of DNA and contaminating protein. Integrity of the purified RNA was determined by 1% denaturing agarose gel electrophoresis. 0.25 g of agarose was dissolved in 2.5 mls of 10x MOPS* stock solution and 22.5 mls of DEPCd'H_20, by heating in a microwave oven

(Panasonic). 1.3 mls of formaldehyde was added, to denature RNA secondary structures. The solution was poured into a casting tray with a comb (Biorad, Hemel Hempstead, Hertfordshire, UK), and allowed to set for 30 minutes. The tray was then placed in an electrophoresis tank (Biorad, Hemel Hempstead, Hertfordshire, UK) with 250 mls of 1x MOPS. 2 μg of each RNA sample was added to 3 μl of RNA loading buffer*, vortexed, centrifuged and heated for 10 minutes at 65 ° C. Before loading onto the gel, 1 μl of ethidium bromide (1mg/ml) was added to each RNA sample to enable the visualisation of 28S and 18S eukaryotic ribosomal bands under ultra-violet (UV) light. If 2 clear bands were visualised, it was judged that the RNA was intact.

2.2.6. REVERSE-TRANSCRIPTION POLYMERASE CHAIN REACTION (RT-PCR)

Reverse-transcription polymerase chain reaction (RT-PCR) is a relatively simple and sensitive technique used to detect the presence of mRNA (Saiki *et al,* 1985).

2.2.6.1 Reverse transcription (RT)

Reverse Transcription involves the production of double-stranded complementary deoxyribonucleic acid (cDNA) from an RNA template in a 5' to 3' direction, catalysed by a RNA dependent deoxyribonucleic acid (DNA) polymerase (reverse transcriptase). Random primers, diverse randomly generated oligonucleotides, will bind to the template and serve as primers for extension by the transcriptase enzyme.

For each RNA sample, from cultured cells or the liver models, to be reverse transcribed, the following reagents were added together in a sterile microfuge tube. A stock solution was made up consisting of: 4 μl 5x RT buffer* (Immunogen International Limited, Sunderland, Tyne and Wear, UK), 2μl 10 mM dideoxyribonucleotide mixture (dNTP), 3.2 μg ribonuclease inhibitor (RNasin), 3.2 μg random hexamer primers, 20 U Moloney Murine Leukaemia Virus Reverse Transcriptase (M-MuLV RT) (Immunogen International Limited, Sunderland, Tyne and Wear, UK). 9 μl aliquot of the stock solution was added to 1 μg of total RNA. The volume in each tube was made up to 20 μl by addition of DEPCd'H_20. The samples were incubated for 1 hour at 37 °C. The reaction was stopped by heating to 95 °C for 5 minutes. The resulting cDNA solution was diluted 5-fold by addition of 80 μl of water and stored at -20 °C.

2.2.6.2 Polymerase chain reaction (PCR)

Polymerase chain reaction PCR involves the amplification of target DNA by use of 2 synthesised oligonucleotides (primers) which are complementary to the sequences that flank the target DNA. During PCR, DNA is denatured by heating to 95 °C. The temperature is dropped to allow re-annealing of the single -stranded DNA (ssDNA), but due to the large concentration of primers present within the PCR mixture, the ssDNA will not re-anneal with itself but with the complementary primer. The temperature is then increased to allow the extension of the primer in the presence of the 4 dNTPs and the heat stable *Taq* Polymerase enzyme. Finally the end product is denatured again as the temperature is increased and the cycle starts again, resulting in the exponential increase in target sequence. A 'hot-start' PCR method was used to amplify cDNA products obtained from the RT step. Hot -start helps to reduce the appearance of non-specific products by physically isolating dNTPs and primers from cDNA and *Taq* Polymerase enzyme.

PCR was employed to detect mRNA of interest in cultured HSC and total liver homogenates. In general for each reaction, 50 µl of the following master mix was made for each sample to be amplified, 1.5 mM $MgCl_2$, 5 µl of 10x *Taq* Buffer* (Flowgen, Staffordshire, UK) and water to make master mix to a volume of 50 µl. Firstly, a 'lower layer' was formed. A wax bead was placed in the bottom of each 0.5 ml microfuge tube. 13 µl of master mix, 1 µl of dNTP mix and 50 pmol of primer combination (Perkin Elmer, Cheshire, UK) (table 2.2.1) were added to each microfuge tube. The mixture was vortexed briefly and the wax melted in a thermal cycler for 2 minutes at 95 °C. The samples were put on ice to allow the wax to solidify to form an impermeable layer between the reagents in the two layers. The 'upper layer' was then added to each microfuge tube and consisted of 37 µl of master mix, 2.5 U of *Taq* DNA polymerase enzyme (Flowgen, Staffordshire, UK) and 5 µl of diluted cDNA. The mixture was then vortexed, centrifuged and placed on a thermal cycler that was preheated to 94 °C and underwent the following cycling conditions: 94 °C for 2 minutes, 1 cycle only, 94 °C for 1 minute, corresponding annealing temperature for the primer combination for 1 minute (see table 2.2.1), 72 °C for 1 minute for 35 cycles followed by a final extension at 72 °C of 6 minutes. The samples were then analysed on a 1.5% DNA agarose/ TBE gel (see section 2.2.6.3). During any PCR run, a negative control was set up consisting of water instead of cDNA. Also a control PCR was set up, to detect β-actin a house-keeping gene, to monitor efficiency of the PCR.

mRNA investigated	primer sequence	annealing temperature °C	product size (bp)
β-actin	5'- tgt acg tag cca tcc agg ct + 3'- ttc ttc agg gag gaa gag ga	54	319
chymase	5' –aat tca act ttg tcc cac ctg cg + 3' –tcg acc gtc cat agg ata cga tgc	55	277
PAR-1	5'- ccc gct cat ttt ttc tca gga a + 3'- caa tcg gtg ccg gag aag t	54	400
PAR-2	5'- cac cac ctg tca cga tgt gct + 3'- ctc agt agg agg ttt taa cac	54	600
RMCP I	5'- aac act gag aga ggt tac aag gcc ac + 3'- ctt ata atg aaa ata gtt ttt aca ggc ctc	56	423
RMCP II	5'- gtc act gag aaa ggt tta agg gtc at+ 3'- gta ttc ata ata cct gta gtc cac aca ggc ctt ttc	56	426
SCF	5'- tgt acg tag cca tcc agg ct + 3'- aca tcc atc ccg gcg aca tag ttg aag ggt	54	259
tryptase	5'- gga aaa cca cat ttg tga cg + 3'- att cac ctt gca cac cag gg	55	151

Table 2.2.1. Sequence of primers, corresponding annealing temperatures and product size, used in PCR. β-actin primers (Thompson *et al,* 1998); chymase and tryptase primers (Xia *et al,* 1995); PAR-1 and PAR-2 primers (Saifeddine *et al,* 1996); RMCP I and RMCPII primers (Rouleau *et al,* 1994); SCF primers (Gaça *et al,*1999; Zhang, *et al,* 1994).

2.2.6.3. 1.5 % agarose/ TBE analysis gel

0.37g of agarose was dissolved in 2.5 mls of 10x TBE* and 22.5 mls of water, in a microwave. 10 µl of ethidium bromide (1 mg/ml) was added and the solution was poured into a casting tray with a comb and left to set for 30 minutes. The tray was placed in an electrophoresis tank containing 250 mls of 1x TBE. 10 µl of PCR product was added to 2 µl of DNA loading buffer* and 4 µl of PCR marker was added to 2 µl of DNA loading buffer. The gel was run at 120 volts (v). Bands were visualised under UV light.

2.2.7 RIBONUCLEASE PROTECTION ASSAY (RPA)

RPA is a sensitive technique in detecting specific mRNA and used to investigate the expression of SCF mRNA in CCl_4 development models of liver fibrosis. Briefly a radiolabelled anti-sense riboprobe was synthesised from purified SCF cDNA PCR products, by a process that involved plasmid ligation, *E.coli* cloning and amplification, restriction enzyme digestion and template labelling with radioactivity. The radiolabelled anti-sense riboprobe was prehybridised with the target RNA in solution. After hybridisation, single-stranded RNA (ssRNA) (non-hybridised RNA) were digested with RNase resulting in protected dsRNA fragments that can be separated by RNA/ urea gel electrophoresis and visualised by autoradiography.

2.2.7i. Purification of SCF PCR product

SCF PCR products were amplified by further PCR and analysed on a 1.5% low melting-point agarose gel. Under UV light, the band of interest was excised from the gel and the PCR product was purified using Promega's Wizard PCR Preps DNA purification system, in accordance with the manufacturer's protocol.

2.2.7ii. Cloning of the insert into a pGEM-T vector

By a ligation reaction, purified SCF PCR product was inserted into the multiple cloning site (MCS) of a pGEM-t vector, which is flanked by a T7 and a SP6 RNA polymerase promoter, restriction enzymes and the α- peptide coding region of the enzyme β-galactosidase. Briefly, all components were allowed to thaw on ice, vortexed, centrifuged and left on ice until needed. The ligation reactions were set up as outlined in table 2.2.2. The reactions were mixed by pipetting and incubated over night at 4 °C.

	standard reaction	positive control	background control
T4 DNA Ligase 10x Buffer	1 µl	1 µl	1 µl
pGEM-T Vector	1 µl	1 µl	1 µl
PCR Product	x µl*	--	--
Control Insert DNA	--	2 µl	--
T4 DNA Ligase	1 µl	1µl	1 µl
water to a final volume of	10 µl	10 µl	10 µl

Table 2.2.2. How ligation reactions were set up.
* Molar ratio of PCR product: Vector, i.e., 3:1, 1:1, 1:3

2.2.7iii. Transformation of *E. coli.* bacteria

As only small quantities of plasmid were available (µgs), the cloned plasmids needed to be amplified to have enough template available to synthesise a riboprobe. The process of plasmid amplification involved transformation of bacteria with the cloned plasmid, culture of the bacteria and then selection of the bacterial colonies containing the ligated plasmid.

Competent *E. coli* were used during the transformations as they multiply rapidly. The bacteria were temporarily permeabilised by heat shocking at 42 °C, allowing the insertion of the ligated pGEM-T vector. Further transformation reactions were carried out following the Promega Standard Transformation Protocol. The transformed cells were plated onto indicator plates*, containing isopropyl- β-D- thiogalactopyranoside (IPTG) and 5-chloro- 4-bromo- 3-indoyl -3- d-galactoside (X-Gal), overnight at 37 °C and selected by blue/ white screening. That is insertion of the PCR product in the MCS of the plasmid will cause the inactivation of the α-peptide by interrupting the coding sequence of β-

galactosidase. Hence clones containing the PCR product will produce white colonies as X-Gal has not been hydrolysed. Clones without PCR products will produce blue colonies as X-Gal has been hydrolysed by the enzyme β- galactosidase, liberating a colour component 4-bromo- 4-chloro 3-indole. White colonies were therefore selected.

2.2.7iv. Small-scale purification of plasmid DNA (Miniprep)

Before the main plasmid amplification was undertaken, a plasmid miniprep was performed to confirm the presence of ligated vector.

1 colony of transformed cells were added to 12.5 mls of warm LB broth*, and incubated overnight at 37 °C. Plasmid DNA was purified using a Promega Miniprep Kit following manufacturer's protocol. UV spectrophotometry and restriction enzyme digestion (section 2.2.7v), were used to analyse the purified ligated plasmid.

2.2.7v. Restriction enzyme digestion to confirm the presence of cDNA insert

Restriction enzymes are used to cut a plasmid to check inserts cloned into the vector, and to linearise a plasmid. For each μg of plasmid, 5U of restriction enzyme, 0.1 sample volume of 10x restriction enzyme buffer and 1 sample volume of water were used. For example, to 30 μg of pGEM-T SCF plasmid were added 75 U of Spe I restriction enzyme, 5 μl of 10x restriction enzyme buffer and 50 μl of water. The mixture was incubated overnight at 37 °C. 6 μl of product was analysed on a 1.75% agarose / TBE gel with 6 μl of uncut plasmid, to ensure cutting was complete.

2.2.7vi. Amplification and large scale purification of plasmid DNA (Maxiprep)

After confirming there was ligated plasmid within specific colonies chosen from the transformed *E.coli* cells, a large-scale grow-up was performed and the plasmid then purified. 5 mls of the overnight culture from the small-scale purification was added to 250 mls of LB broth overnight at 37 °C. Plasmid DNA was isolated and purified using Promega's Maxiprep kit in accordance with manufacturers protocol. Concentration of plasmid DNA was determined by UV spectrophotometry (section 2.2.5.3). Restriction enzyme digestion was used to check the plasmid for the insert.

2.2.7vii. Confirmation of alignment of cDNA insert using T7 and SP6 RNA polymerase promoter, linerisation of plasmid and purification

In order to make an anti-sense riboprobe, the template needs to be in an orientation such that the positive strand is the strand being copied therefore the clone selected is one that

had an insert in the anti-sense orientation. By use of PCR, the orientation of the insert can be determined. Within the pGEM-T vector, flanking the MCS, are found the T7 and SP6 RNA polymerase promoters. T7 is a sense primer and SP6 and anti-sense primer. These primers, when used in combination with sense and antisense primers in a PCR, (P5 and P2 respectively) can help elucidate the orientation of the cloned insert. For example, if the insert is in the sense orientation, there will be a product with T7 and the antisense primer or SP6 and sense primer. If the insert in anti-sense then there will be a product with T7 and sense or SP6 and antisense primer. PCR was employed as in 2.2.6.2 using a combination of T7 and SP6 primers with SCF primers. Products were analysed on a 1.5% DNA agarose/ TBE gel (section 2.2.6.3).

Once the correct template has been elucidated, restriction enzymes are used to cut the insert at the 5' end to remove the unwanted polymerase resulting in linearised plasmid. The linearised plasmid was further purified to remove any unwanted contaminant proteins by acid/ phenol extraction and ethanol purification as in section 2.2.5.2. The concentration of cDNA was determined by UV spectrophotometry.

2.2.7viii. Preparation of an anti-sense radiolabelled riboprobe

An anti-sense radiolabelled riboprobe was synthesised *in vitro* by use of a DNA dependent RNA Polymerase, primed by RNA Polymerase promoter at the 3' end of the cloned insert (T7 in this case), incorporating [α-^{32} P]- rUTP, as a label.

1 µg of the linearised plasmid, 2 µl of rNTP mix* , 0.5 µl of dithiothreitol (DTT) and 2.7 µl of 5x transcription buffer* were incubated for 10 minutes at 65 °C. This incubation helps to enhance the inhibition of RNases by DTT, a reducing agent. 1 µl (20,000 U/ul) of the appropriate RNA Polymerase (T7 in this case), 0.5 µl (20 U) RNase Inhibitor and 50 µCi [α-^{32} P]- rUTP were added to the transcription mix, and incubated for 30 minutes at 37 °C to allow transcription to occur followed by 10 minutes at 65 °C to inactivate the polymerase. 3 µl of RNase-free DNase and 25 µl DEPC'd$H_2$0 were added to the transcription mixture and incubated for 45 minutes at 37 °C. During this time any DNA present was digested leaving only radiolabelled RNA.

RNA transcripts were then precipitated by addition of 20 µg tRNA, 500 µl 0.3M sodium acetate and 550 µl isopropanol at -70 °C overnight. This was followed by two washes in 1 ml 75% ice-cold ethanol. The riboprobe was dried on a heating block at 65 °C and resuspended in 50 µl of DEPC'd$H_2$0. Riboprobe integrity was assessed by electrophoresis of the probe on a denaturing 1% MOP gel (method 2.2.5.3) and visualised

by autoradiography. Scintillation counting was used to determine the radioactivity of the probe.

2.2.7ix. Ribonuclease protection assay

In this method, the radiolabelled riboprobe was hybridised with total RNA samples from rat CCl_4 development model of liver fibrosis. To each 30 µg RNA sample (30 µg yeast tRNA was used as a negative control), 30 µl of RPA hybridisation buffer* containing 10^5 cpm of radiolabelled riboprobe was added. The samples were vortexed and incubated for 5 minutes at 95 °C and then left to hybridise overnight at 45 °C.

After hybridisation, 270 µl of ribonuclease digestion buffer containing RNase ONE was added to the samples and left incubating for 45 minutes at 37 °C to digest any unhybridised probe and non-target RNA. The reaction was stopped by the addition of 30 µl stop solution* and 825 µl 100% ethanol, and incubated for 15 minutes at -70 °C

The RNA- probe fragments were pelleted by centrifugation at 14,000g for 15 minutes at 4°C. In each sample, the pellet was washed in 1 ml of ice-cold 75 % ethanol. The pellet was resuspended in 8 µl of RPA loading buffer*. 10^5 cpm of probe was added to 8 µl of RPA loading buffer, to be used as a molecular weight reference when loaded onto the gel. All the tubes were briefly centrifuged and heated for 5 minutes at 95 °C before being loaded onto a 5% polyacrylamide gel, (method 2.2.7x). The gel was run for 45 -60 minutes at 55 °C at 1,800v, transferred to 3MM chromatography paper, covered with cling film and dried out on a gel vacuum drier at 80 °C for 1 hour. The bands were visualised by autoradiography.

2.2.7x. Polyacrylamide/ urea gel

This vertical gel was used for electrophoresis of protected RNA fragments in a RPA. Urea will denature the nucleic acids. The gel was set up in RNase- free conditions. All apparatus was washed with 1% SDS, DEPC'dH_2O and 100% ethanol, sequentially. The back plate was wiped with 'Sigmacoat' to ensure that the gel would stick only to the front plate when the plates were separated.

A blocking gel was made to seal the bottom of the glass plates. 10 ml of 6% PAGE solution*, 60 µl 10% ammonium persulphate (APS) and 4.5 µl TEMED were mixed and then inserted between the plates using a syringe and needle and left for 30 minutes to polymerise. A 5% resolving gel was made using 42 ml of 6% PAGE solution*, 8 ml 7M urea , 400 µl 10% ammonium persulphate (APS) and 20 µl TEMED, and poured between

the plates. The combs were inserted at the top of the gel and the gel was left for 60 minutes to polymerise. The set gel was deionised and warmed to 55 °C by pre-running at 1,800 v for 1 hour with 1x TBE running buffer. Before loading the samples, the wells were flushed out with 1X TBE to remove any unpolymerised acrylamide.

2.2.8. DETECTION OF mRNA BY NORTHERN BLOTTING

This method was used to detect and determine specific mRNA in total cellular RNA. RNA from various activated rat HSC preparations was isolated and separated by size by electrophoresis on a 1% RNA MOPS gel (method 2.2.4.3). The separated RNA was then transferred to a nylon membrane. The membrane was then hybridised with a radiolabelled cDNA probe and mRNA visualised by autoradiography.

2.2.8.1. Transfer of RNA from gel to nylon membrane

Before the RNA can be transferred from the gel to a nylon membrane, it has to be washed to remove the formaldehyde from the gel. The gel was subsequently washed in 200 ml DEPC'dH_20 for 10 minutes on the shaker, followed by washes in 100 ml 50 mM sodium hydroxide for 20 minutes, to partially hydrolyse the RNA, then a second wash in DEPC'dH_20 for 10 minutes and then a final wash in 10x SSC* for 10 minutes.

RNA was transferred from the gel to the nylon membrane (Amersham International, Amersham, Buckinghamshire, UK) overnight by capillary elution in 10x SSC. After transfer, the nylon membrane was dried between two sheets of chromatography paper (Whatmann, Maidstone, Kent, UK) and exposed to UV light to immobilise the RNA to the membrane. The UV light causes cross-links between RNA bases and amide groups on the surface of the membrane. The membrane was stored in a cool dark place in cling film until probed.

2.2.8.2. Prehybridisation of the Northern blot

Before addition of the probe, the membrane was pre-hybridised to block any non-specific sites on the nylon membrane, using hybridisation buffer*. The hybridisation buffer is a solution of high ionic strength (5x SSC) and is used to maximise annealing of the probe with its target. Herring sperm DNA and Denhardt's reagent will help the probe to bind to the relevant RNA on the membrane. The membrane was pre-hybridised with 25 ml of buffer for 3 -4 hours at 42 °C.

2.2.8.3 Random primed radiolabelled cDNA probe preparation

Radiolabelled cDNA probes were synthesised by random priming, in which random hexamer oligonucleotides are used as primers for initiation of DNA synthesis from ssDNA templates by DNA polymerases. The oligonucleotides form hybrids along the template, which are then primed to second strand synthesis by the Klenow fragment of the *E.coli* DNA polymerase, and incorporates [α- ^{32}P] adenosine- triphosphate (ATP) as a label.

100-200 ng of purified cDNA template (purified from PCR cDNA products) of interest was added to 5 ul primer (random hexamer) (Amersham International, Amersham, Buckinghamshire, UK) and 26 µl DEPCd'H_20. The mixture was heated for 5 minutes at 95°C to allow disannealing of the double stranded DNA (dsDNA). Using a megaprime DNA labelling kit (Amersham International, Amersham, Buckinghamshire, UK) 4 µl of deoxycytosine triphosphate (dCTP), deoxythymidine triphosphate (dTTP), deoxyguanosine triphosphate (dGTP), 5 µl reaction buffer, 2 µl Klenow fragment and 5 µl [α- ^{32}P] ATP (Amersham International, Amersham, Buckinghamshire, UK) were added to the mixture which was then incubated for 60 minutes at 37 °C to allow transcription of radiolabelled dsDNA. The probe was purified using a Sephadex G50 column to remove any un-incorporated dNTP's.

2.2.8.4 Hybridisation of the Northern blot

After purification, the random primed cDNA probe was heated for 5 minutes at 95 °C to denature the DNA and added to 25 ml of preheated hybridisation buffer*. The pre-hybridisation buffer was discarded and the radioactive probe and hybridisation buffer were added to the membrane and left to hybridise overnight at 42 °C.

2.2.8.5. Stringency washing of the Northern blot

After overnight hybridisation, the membrane was washed to remove any unbound probe. Non-specific links between the probe and RNA involving hydrogen bonds can be disrupted by high temperatures and low salt conditions.

The hybridisation solution was discarded. The membrane was washed once by addition of 0.2% SDS /0.2% SSC* v/v for 15 minutes at 42 °C followed by 2 subsequent washes for 15 minutes at 55 °C. After the final wash, the membrane was wrapped in cling film and the mRNA bands visualised by autoradiography.

2.2.9 DETECTION OF PROTEIN BY WESTERN BLOTTING

2.2.9.1. Normalising DNA content of cell cultures by DNA assay

Before samples could be employed further they had to be assessed for DNA content so that samples could be normalised for cell number. This is a fluorometric assay that is based on the ability to measure the enhanced fluorescence of bisbenzanidazole (Hoechst 33258 reagent) when bound to adenosine= thymidine (A=T) base pair of dsDNA. This can be detected on a spectrophotometer at an excitation of 360 nm and emission of 460 nm.

Cells were lysed in a recorded volume of phosphate-buffered saline (PBS) and sonicated in a water bath for 10 minutes prior to assay. 50 µl of each lysed cell sample were analysed in triplicate on a 96 well plate (Nunc Maxisorb, Life Technologies, Renfrewshire, UK). 50 µl of Hoechst solution (1µg/ml) was added to each sample and fluorescence was read. To quantitate the amount of DNA per sample, a standard curve was plotted using herring sperm DNA concentrations ranging from 0 -5 µg, in triplicate.

2.2.9.2. Separation of proteins on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transfer to polyvinyl- difluoride (PVDF) membrane

Proteins can be separated out by sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE) to enable examination of protein expression within a given sample. The samples are lysed in a buffer containing SDS and a reducing agent and are heated to ensure protein disruption. The denatured protein will bind to the SDS, in a manner that is proportional to the molecular weight of the protein, and become negatively charged. The SDS- polypeptide complexes will migrate through the gel upon application of an electric current and separate due to size, as larger complexes will migrate slowly and become trapped in the polymerised acrylamide gel.

For each culture condition, cells were lysed in sample buffer*, heated to 95 °C for 5 minutes and loaded onto a 10%/ 12% polyacrylamide gel* corresponding to equal DNA content. The gel was run at 110 v for approximately 90 minutes in electrode buffer* in gel apparatus (Biorad, Hemel Hempstead, Hertfordshire, UK).

The separated samples were transferred to PVDF membranes by use of Western blotting. The gel was placed in direct contact with the PVDF membrane (pre- soaked in methanol) and sandwiched between filter paper. The 'sandwich' was placed in a Western blotting tank (Biorad, Hemel Hempstead, Hertfordshire, UK), the membrane facing the anode side, and ran at 100 v for 60 minute During this time proteins were stimulated to migrate from the gel towards the membrane.

2.2.9.3. Immunodetection of proteins on PVDF membranes

After transfer of proteins to membrane from gel, membranes were blocked for 1 hour in TTBS* 1% dry milk (Marvel) at room temperature. After 1 wash in TTBS, the membranes were incubated overnight with primary antibody of interest in 0.01% Marvel in TTBS at room temperature. Following 3 washes in TTBS, the membranes were incubated in a peroxidase- conjugated secondary antibody (1:10,000) in 0.01% Marvel in TTBS for 1 hour and then developed using a commercial enhanced chemiluminescent detection system (ECL) (Amersham International, Amersham, Buckinghamshire, UK). Briefly, ECL detection reagent 1 forms a substrate for the peroxidase- conjugated secondary antibody. The reduction of hydrogen peroxide by the antibody is coupled to luminol, found in detection reagent 2, which on oxidation produces blue light. The light output is intensified by the presence of an enhancer and can be detected on blue-light sensitive film.

As negative control, membranes were probed with non-immune IgG in place of primary antibody.

2.2.10. IMMUNOFLUORESCENT DETECTION OF SCF PROTEIN IN CULTURED HSC

Three stage fluorescence immunocytochemistry was employed to determine presence of SCF protein in rat HSC. Third passaged HSC were seeded onto glass slides (Lab- Tech from Life Technologies, Renfrewshire, UK) and maintained in culture as previously described for 3 days. Before further processing, cells were washed in PBS to remove any excess DMEM and FCS that could interfere with the staining procedure. The cells were fixed in 4% para-formaldehyde for 10 mins and permeabilized in 100% ice-cold methanol for 7 minutes. The cells were incubated for 1 hour with primary antibody (10 μg/ml polyclonal anti mouse SCF). Following 3 washes in TBS*, the cells were incubated with a biotinylated- conjugated secondary antibody (20 μg/ml) for 1 hour. After 3 washes in TBS, the cells were further incubated with ExtrAvidin fluorescein isothiocyanate (FITC) - conjugated antibody (7.5 μg/ml) for 30 minutes. Cells were counter-stained using propidium iodide (25 μg/ml) for 10 minutes prior to 3 final washes in TBS. Cells were independently stained with anti-desmin antibody (10 μg /ml) as a positive control and rabbit non- immune IgG (10 μg/ml) as a negative control, in place of primary antibody. Slides were viewed using a Leica fluorescence microscope and by confocal microscopy.

2.2.11. IMMUNOSTAINING OF PAR-2 PROTEIN IN CULTURED HSC

Three stage immunocytochemistry was employed to determine the presence of PAR-2 protein in culture- activated HSC. As described above in method 2.2.9, passaged HSC were seeded onto glass slides (Lab- Tech from Life Technologies, Renfrewshire, UK) and maintained in culture for 7 days. The cells were fixed in water-free acetone at room temperature for 10 mins. The cells were incubated for 30 minutes with primary antibody (10 μg/ml monoclonal anti human PAR-2, a gift from Professor L Brass, University of Pennsylvania, USA). Following 3 washes in TBS, the cells were incubated with a biotinylated- conjugated secondary antibody (Dako Ltd, High Wycombe, Buckinghamshire, UK) (1:200 dilution) for 30 minutes at room temperature. After 3 washes in TBS, the cells were further incubated with a streptavidin-biotin horseradish peroxidase complex (1: 200 dilution) (Dako Ltd, High Wycombe, Buckinghamshire, UK) for 30 minutes at room temperature. Cells were stained using 3,3- diaminobenzidine tetrahydrochloride* (DAB) (Biogenex from Biomen Ltd, Berkshire, UK) for 5 minutes prior to a final wash in running tap water for 2 minutes. The cells were then counter-stained using Harris haematoxylin for 2 minutes. Sections were dehydrated by passing them through a series of alcohols ranging from 70% to 100%. Slides were mounted and viewed using a Leica light microscope. Cells were independently stained with anti-SMA antibody (1:400 dilution) as a positive control and mouse non- immune IgG (10 μg /ml) or TBS as a negative control, in place of primary antibody.

2.2.12. IMMUNOSTAINING OF SCF, RMCPI AND RMCPII PROTEIN IN RAT CCL_4 FIBROTIC LIVER SECTIONS

6 μm paraffin- embedded tissue sections from rat normal and CCl_4 fibrotic livers were mounted onto slides. Sections were de-paraffinised by two 5 minute incubations in xylene. The sections were re –hydrated through a series of alcohols, that is 100%, 90% and 70% alcohols and then into distilled water at 5 minute intervals. Endogenous peroxidase within the sections was inhibited (as can cause high background staining) in inhibitor solution* for 10 minutes, followed by two 5 minute washes in distilled water. It was then necessary to further unmask the antigen by microwaving the slides in a citric buffer* for 30 minutes (Cattoretti *et al,* 1993). Polyclonal RMCPI and monoclonal RMCPII antibodies were a gift from Drs. G Newlands and H Miller, Moredun Research Institute, Edinburgh, UK, and suggested dilutions for immunohistochemistry were 1:100 and 5 μg/ml respectively. SCF polyclonal antibody (Genzyme, UK) was used at a dilution of 10 μg/ml. Primary antibody was diluted in TBS and applied to the sections and left to incubate overnight at 4

°C. Following 3 washes in TBS, the cells were incubated with a biotinylated- conjugated secondary antibody (Dako Ltd, High Wycombe, Buckinghamshire, UK), (1:200 dilution) for 30 minutes at room temperature. After 3 washes in TBS, the cells were further incubated with a streptavidin-biotin horseradish peroxidase complex (1: 200 dilution) (Dako Ltd, High Wycombe, Buckinghamshire, UK) for 30 minutes at room temperature. Cells were stained using DAB (Biogenex from Biomen Ltd, Berkshire, UK) for 5 minutes prior to a final wash in running tap water for 2 minutes. The cells were then counter-stained using Harris haematoxylin for 2 minutes. Sections were dehydrated by passing them through a series of alcohols ranging from 70% to 100%. Slides were mounted and viewed using a Leica light microscope. Control rat normal and fibrotic liver tissues were independently stained with anti-SMA antibody (1:400 dilution) as a positive control and non- immune IgG (5 μg /ml) or TBS as a negative control, in place of primary antibody.

2.2.13. *IN-VITRO* CO-CULTURE OF HUMAN MC AND RAT HSC

Passaged rat HSC were seeded onto a 96 well-plate (Nunc Maxsorb from Life Technologies, Renfrewshire, UK) at a plating density of 0.5x 10^6 HSC/ ml and left to adhere to the culture vessel overnight. Before MC were to be co-cultured with HSC, various concentrations of a neutralising SCF antibody ranging from 0- 10 μg/ml were added to the HSC and left for 3 hours at 37 °C in 5% CO_2 in a humidifying incubator.

The MC were left to adhere for 1 hour at 37 °C in 5% CO_2 in a humidifying incubator. After incubation, non-adherent cells were washed off in DMEM. The plates were air-dried for 30 minutes at 37 °C in an incubator. 100 μl of 95% methanol and 5% acetic acid v/v were added to fix the cells, which were left at -20 °C. The cells were washed three times in PBS to remove the fixative. The co-cultured cells were left at 4°C in PBS until counted. The MC were stained using Kimura and counted. Numbers of MC adherent to HSC were assessed in triplicate wells for each treatment by counting nine low power fields. As a negative control, co-culture was set up as previously described and non-immune IgG was used in place of the neutralising SCF antibody. MC adhesion in the presence of SCF antibody was compared to that in the absence of the antibody using paired *t*-test and confirmation by Wilcoxon signed rank test with p values < 0.05 being considered significant.

2.2.14. ENZYMATIC ACTIVITY ANALYSIS OF TRYPTASE

Before addition of tryptase to HSC in culture it was analysed for enzymatic activity. Tryptase was assayed using the peptide substrate N- benzyl- DL- arginine- *p*- nitroanilide*

(BAPNA). 10 µl of enzyme was added with 10 µl of BAPNA and 80 µl tryptase assay buffer* on a microtitre plate. The hydrolysis of the substrate was monitored spectrophotometrically at 410 nm. Tryptase activity was expressed in milliunits (mU) per millilitre, where 1 U represents that required to hydrolyse 1 µmol of substrate per minute at 25 °C.

2.2.15. PURITY OF TRYPTASE USED IN PROLIFERATION STUDIES AS ASSESSED BY SDS-PAGE

Samples of tryptase were analysed by a 10% SDS-PAGE gel, method 2.2.8. The gels were stained using Sigma silver staining reagents, following manufacturer's protocol.

2.2.16. MEASUREMENT OF DNA SYNTHESIS AND CELL PROLIFERATION BY ^{3}H- THYMIDINE INCORPORATION

Proliferation assays were set up to determine if MC mediators such as tryptase, heparin, histamine, IL-4 and TNF- α could induce HSC proliferation as previously described in fibroblasts (Sempowski *et al,* 1994; Gordon and Galli, 1994). Proliferation was measured and assessed by examining the incorporation of ^{3}H- thymidine into cellular DNA during the S phase of the cell growth cycle.

HSC were cultured in 24 well plates (Griener, Stonehouse, Gloucester, UK) until they reached 80-90% confluence (generally day 6 to day 7 of culture), after which they were quiesced for 24 hours in the presence of 0.5 % FCS to arrest all the cells at G_0 of the cell cycle. HSC were then exposed to MC mediators at various concentrations, in triplicate for 24 hours. The cells were pulsed with 1µCi ml^{-1} ^{3}H- thymidine (Amersham International, Amersham, Buckinghamshire, UK) overnight (16 hours). After incubation HSC were washed twice in Hank's buffered salt solution (HBSS) to wash off any unincorporated radioactivity and were then fixed in 100% methanol at -20°C for a minimum of 1 hour. Following fixing, methanol was washed off the cells by washing three times in HBSS on ice. The cells were lysed with 500 µl of cell dissociation solution (0.25 M sodium hydroxide and 0.1 % SDS) and neutralised with 30 µl 5N hydrochloric acid. Incorporation of ^{3}H- thymidine was determined by scintillation counting (Wallac 1257 Rack-beta liquid scintillation counter). Proliferation was assessed by calculating the resulting counts per minute, averaged for each mediator concentration added to HSC, in relation to untreated HSC (control)..

2.2.17. DETECTION OF GELATINASE IN HSC CULTURE MEDIA BY ZYMOGRAPHY

The presence of pro- and active forms of gelatinase in HSC cultured media were analysed by gelatin zymography after SDS- PAGE in 8% polyacrylamide gels containing 20 μg/ml gelatin as previously described (Benyon *et al,* 1996). Any gelatinase activity in the media can be observed as a clear band after staining the gel with Coomassie blue. Under experimental conditions during zymography, inactive progelatinase A can revert to an activated 72 kDa form. The SDS in the gel will denature progelatinase A by removing the cysteine of the propeptide. During the washing stage of zymography the propeptide cannot bind to the inhibitory cysteine as the active site becomes complexed with water. This therefore leaves the progelatinase in a 72 kDa active form.

Media were collected from HSC cultured in the presence or absence of various mediators for 24 hours in serum- free conditions. The media were clarified by centrifugation. Media, combined with an equivalent volume of non- reducing sample buffer, were loaded onto 8% polyacrylamide gels containing 20 μg/ml gelatin* corresponding to equal DNA content. Positive control HSC treated with concanavalin A (con A), a stimulus for MT1-MMP activation that in turn is an activator of gelatinase A. The gel was run at 110 V for approximately 90 minutes in electrode buffer* in gel apparatus (Biorad, Hemel Hempstead, Hertfordshire, UK).

Once run, gels were washed twice for 30 minutes in 2.5% Triton X-100* buffer to remove the SDS in the gel, followed by 2 washes for 5 minutes in gelatinase incubation buffer*. The gels were incubated overnight at 37 °C in gelatinase incubation buffer. After incubation, gels were stained with Coomassie blue stain* for 30 minutes. The dye stains proteins blue therefore all areas of the gel that still contained gelatin would stain blue. Gels were destained twice for 30 minutes using a destain buffer* to reveal clear bands indicative of gelatinase activity.

2.2.18. DETECTION OF MAPK ACTIVITY IN HSC STIMULATED BY PAR-1 AND PAR-2 AGONISTS

MAPKs are a family of serine/ threonine- specific protein kinases believed to be involved in a number of cellular responses including cell proliferation. In smooth muscle cells and enterocytes, PAR-2 agonists activate MAPK ERK1/ERK2 and weakly stimulate the MAPK homologue p38 (Belham *et al,* 1996), whilst thrombin can activate ERK1/ERK2 in platelets and CCL-39 fibroblasts (Molloy *et al,* 1996).The activation or ERK1/ ERK2 was investigated in HSC stimulated with serum, 0.4 mU/ml thrombin, 30 μM PAR-1 agonist

(SFFLRN) and 0.6 mU/ml tryptase or 30 μM PAR-2 agonist SLIGRL in the presence or absence of a specific ERK1/ ERK2 inhibitor PD 98059 (2' amino- 3'-methoxyflavone) (Calbiochem, Nottingham, UK).

HSC were cultured on 12 well plates until they became activated and 90% confluent (approximately 7 days). The cells were then washed three times in DMEM and then cultured for 48 hours in serum-free conditions. Growth- arrested HSC were stimulated with the PAR-2 agonist for the times indicated and rinsed in ice-cold PBS. Cells were lysed with sample buffer, loaded equally for DNA onto and resolved by SDS- PAGE (10% gel) and transferred to PVDF membranes. Immunoblotting was performed as described in method 2.2.8.4, using a polyclonal anti-MAPK antibody. As positive control for MAPK induction, one well of cells per plate was treated with 16% FCS for 15 mins. To confirm that PAR-2 activated ERK1/ ERK2, the specific inhibitor PD98059 at various concentrations, was added to the growth- arrested HSC 30 minutes before treatment with agonist.

To support the MAPK protein detection by immunoblotting, parallel proliferation assays were performed with growth- arrested HSC treated with the PAR-2 agonist in the presence or absence of the ERK1/ ERK2 inhibitor PD98059. After stimulation, cells were left for 24 hours and were pulsed with 1 μCi ml^{-1} ^{3}H- thymidine (Amersham International, Amersham, Buckinghamshire, UK) overnight (16 hours). Cell proliferation was assessed as previously described in section 2.2.14. Proliferation was assessed by calculating the resulting counts per minute, averaged for each PAR-2 agonist or PD98025 concentration added to HSC. The results were presented as mean ± SEM of percentage proliferation of treated HSC in relation to untreated HSC.

2.2.19. STIMULATION OF COLLAGEN SYNTHESIS IN HSC BY TRYPTASE AND PAR-2 PEPTIDE (SLIGRL)

Collagen assays were set up to determine if tryptase and the PAR-2 peptide could induce *de novo* HSC collagen synthesis. Collagen synthesis was measured by assessing the incorporation of ^{3}H- proline into newly synthesised hepatic collagen. The total amount of synthesised collagen was determined by digesting the newly synthesised collagen in the cellular supernatants by collagenase and comparing quantities of incorporated ^{3}H- proline in respective cellular supernatants which are not treated with collagenase, as described by Peterkofsky and Diegelman, 1970. The total collagen synthesis is equivalent to ^{3}H- proline incorporation in non-collagenase treated supernatants minus ^{3}H- proline

incorporation in collagenase treated supernatants as determined by scintillation counting. All samples were normalised for DNA content

HSC were passaged into 12 well plates and cultured until 90% confluent. HSC were washed 3 times in serum- free D-MEM and treated with 0.6 mU/ml tryptase or 30 μM SLIGRL for 24 hours in D-MEM containing 25 μg/ml ascorbic acid and 0.01% BSA (D-MEM + AA). As a negative control HSC were cultured with D-MEM + AA only and as a positive control HSC were treated with 10 μg/ml TGFβ. After 24 hours incubation at 37 °C the cells were washed 3 times in serum free D-MEM and then treated with the same stimulus in D-MEM+ AA in the presence of 1 μCi ml^{-1} ^{3}H- proline (Amersham International, Amersham, Buckinghamshire, UK) for 24 hours at 37 °C. For each treatment supernatants were removed and were split into two aliquots, one for treatment with collagenase and the other for no collagenase treatment. 100 μl of each supernatant were added to a MultiScreeen 96 well filtration plate (Millipore (UK) Limited, Watford, Hertfordshire) in duplicate. 50μl of collagenase(125 U) (Lorne Laboratories, Reading, Berkshire) plus buffer* were added to each collagenase treated supernatant for 90 minutes at 37 °C . Non- treated supernatants were treated with 50μl of collagenase buffer* only. Collagen protein was then precipitated onto the membrane by the addition of μl of 50% tri-chloroacetic acid (TCA) on ice for 1 hour. The membranes were vacuum dried following 3 washes in 10% TCA. Incorporation of ^{3}H- proline was determined by scintillation counting. Total collagen synthesis was calculated as the amount of ^{3}H- proline incorporation in non-collagenase treated supernatants minus ^{3}H- proline incorporation in collagenase treated supernatants and normalised for DNA.

2.2.20. STATISTICIAL ANALYSIS

Where applicable results were presented as mean ± standard error of the mean (SEM). Adhesion and ^{3}H- thymidine and proline incorporation assays were calculated and plotted as a percentage change of treated HSC relative to untreated HSC. Parametric Student's paired *t*-tests were performed as *n* values varied from 2 to 6. To confirm and supplement significance, where possible, non-parametric Wilcoxon signed rank tests were applied. $p < 0.05$ were considered statistically significant.

CHAPTER THREE
HEPATIC STEM CELL FACTOR SYNTHESIS: A POSSIBLE MECHANISM OF MAST CELL RECRUITMENT IN LIVER FIBROSIS

3.1 INTRODUCTION

It has been found in various studies that MC numbers dramatically increase in liver fibrosis (Miller *et al*, 1994; Peng *et al*, 1994; Ramos *et al*, 1994; Farrell *et al*, 1995; Xu *et al*, 1994; Rioux *et al*, 1996; Ambrust *et al*, 1997; Nakamura *et al,* 1997). In liver fibrosis there is a marked increase in the hepatic deposition of ECM proteins, especially collagen I (Rojkind *et al,* 1979), which can be attributed to activated HSC (McGee and Patrick, 1972; Maher *et al,* 1988; Milani *et al,* 1990; Rockey *et al*, 1992). Activated HSC take on many of the characteristics of fibroblasts in other tissues. The work in this chapter aimed to investigate whether activated HSC might contribute to MC hyperplasia observed in liver fibrosis. SCF may recruit MC to the liver following injury as it is a potent MC chemoattractant (Meininger *et al*, 1992; Nilsson *et al*, 1994), survival factor (Tsai *et al*, 1991a; Takagi *et al*, 1992) and influences MC differentiation from resident tissue precursors (Nocka *et al,* 1990; Irani *et al,* 1992a). I have investigated hepatic SCF production by activated HSC *in vitro* using several techniques as a possible mechanism for MC recruitment to liver during injury and fibrosis.

3.2. RESULTS

3.2.1. SCF mRNA EXPRESSION IN RAT LIVER FIBROSIS BY RT-PCR

Initially, it was necessary to investigate the expression of SCF in liver fibrosis before investigating the cellular source of the cytokine. RT-PCR detects minute quantities of nucleic acids using relatively little amounts of starting biological material, therefore it was an ideal tool to use to primarily detect the presence of SCF mRNA (section 2.2.6).

Previously in our laboratory SCF mRNA and protein expression were examined in total RNA extracted from human liver by Dr R C Benyon. The liver samples used were five normal livers and six livers from PSC and PBC explanted livers from patients undergoing transplantation. By RT-PCR, SCF mRNA expression appeared to be upregulated in human diseased liver compared to normals (figure 3.1i). SCF mRNA was virtually undetectable in two out of five normal livers. However in cirrhotic livers, all six PSC and PBC livers, SCF mRNA was consistently expressed. To complement these studies of mRNA expression, SCF protein expression was quantified by use of a commercially available ELISA (R & D Systems, Abingdon, Oxon, UK).This showed that 96 $\pm$20 ng/mg of immunoreactive SCF was present in normal livers (n=5), whilst in PSC and PBC livers (n=6) SCF protein expression was significantly upregulated 2-3 fold, 317 $\pm$87 ng/mg ($p< 0.05$) and 231 $\pm$35 ng/mg ($p< 0.05$) respectively as assessed by Mann-Whitney non-parametric testing (figure 3.1ii).

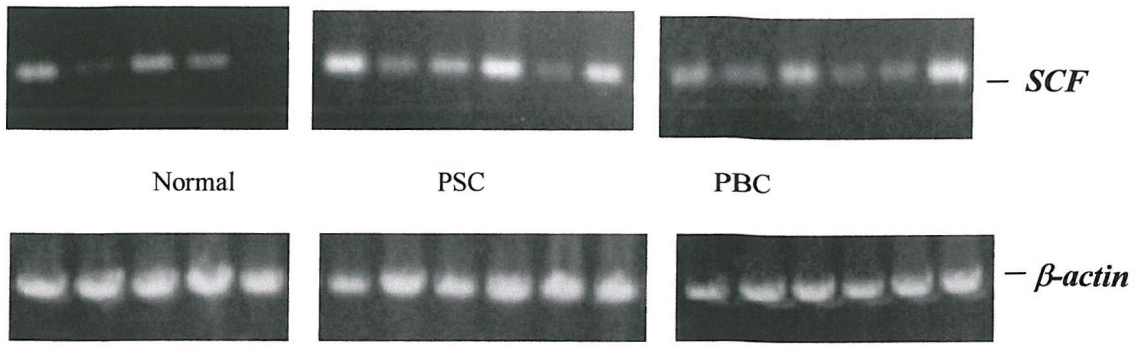


Figure 3.1i. RT-PCR analysis of SCF mRNA expression in normal human livers and in cirrhotic livers from patients with primary sclerosing cholangitis (PSC) and primary biliary cirrhosis (PBC), previously performed by Dr R C Benyon. β–actin confirmed the integrity of the cDNA samples.

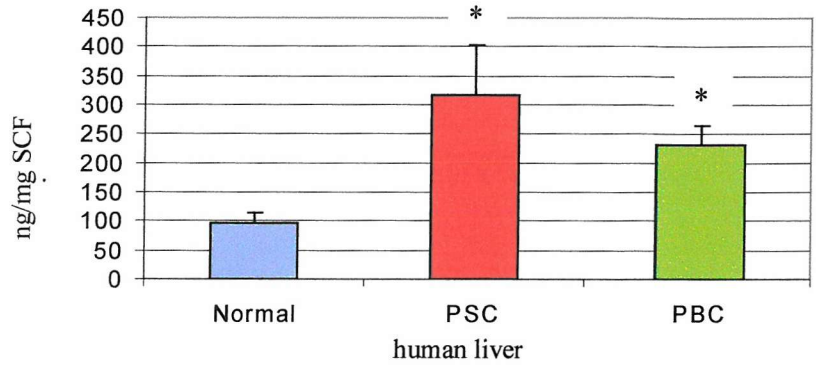


Figure 3.1ii. SCF protein expression in normal human livers (n=5) and in cirrhotic livers from patients with primary sclerosing cholangitis (PSC) (n=6) and primary biliary cirrhosis (PBC) (n=6) as detected by ELISA, previously performed by Dr R C Benyon . *= $p<0.05$, Mann- Whitney U-test.

To examine the time course of SCF expression during progressive liver fibrogenesis, two different rat models of liver fibrosis, CCl_4 and BDL were used. SCF mRNA expression was investigating using conventional RT-PCR. Total RNA was extracted from rat liver homogenates, reverse transcribed and using specific primers to SCF (table 2.2.1), target cDNA was amplified and analysed on a 1.5% agarose/TBE gel (section 2.2.6.3). This resulted in the detection of a 259 bp PCR product, as confirmed in positive control rat skin fibroblasts (RSF). SCF mRNA expression was quantified by densitometric analysis using Scion Image program by Scion Corporation, USA. Results were plotted as the mean ±SEM percentage change in SCF mRNA expression in treated liver compared to untreated liver at the time points indicated.

3.2.1.1. CCl_4 model of rat liver fibrosis

As described in section 2.2.1.1, male Sprague-Dawley rats were injected intra-peritoneally with 0.2 ml/100 g of sterile CCl_4 in a 1:1 ratio with olive oil, twice weekly for 4 weeks. Control rats (c) were treated with olive oil only at the same time. Control and treated rats

were sacrificed 6 hours, 24 hours, 1 week and 4 weeks after the initial treatment of CCl_4. Two rats were used for treated time points (i and ii).

A strong band corresponding to 259 bp was detected in RSF positive control cells (figure 3.2). In the negative control samples (cDNA template replaced by distilled water), no signal was detected suggesting that the signal in RSF was specific for SCF mRNA. In the early treated CCl_4 rat livers (6 and 24 hours) a 259 bp PCR product was clearly detected in the treated livers (i and ii) compared to control untreated livers (c). β–actin, a house-keeping gene, PCR confirmed the integrity of the cDNA samples used in these PCRs and confirmed equal loading and efficiency of amplification between different samples.

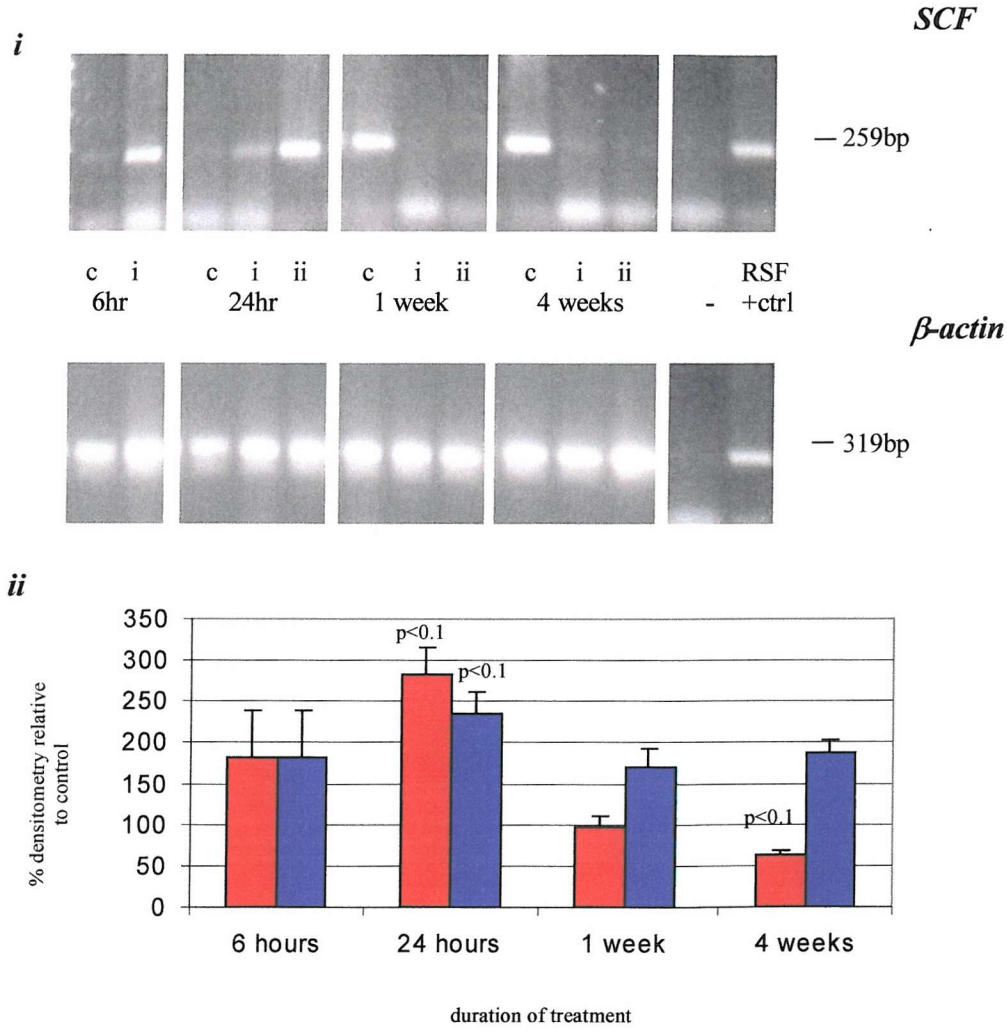


Figure 3.2i. Representative one of four different experiments detecting SCF mRNA using RT-PCR in CCl_4 development model of rat liver fibrosis. SCF mRNA was detected in positive control RSF and in the early stages of fibrosis in treated livers (i, ii) compared to untreated control livers (c). β-actin PCR confirmed the integrity of cDNA samples. **ii.** Densitometric analysis of SCF mRNA expression in CCl_4 development model of rat liver fibrosis, n=4. Red bars indicate SCF mRNA expression in treated livers compared to same time point control. SCF mRNA expression appeared to increase in early fibrosis (24 hours, 283%±32) but decreased as fibrosis developed (4 weeks, 63%± 4.9). Blue bars indicate SCF mRNA expression in treated livers all compared to control at 6 hours. At all time points SCF mRNA expression was greater then control (100%). $p<0.1$ Student's *t*-test.

Densitometric analysis of SCF mRNA expression in treated livers compared to corresponding control time points (red bars, figure 3.2ii) indicated in early treated livers, 6 and 24 hours, SCF mRNA was expressed at higher levels compared to later treated livers, 81.3%±58 and 183.3%±32 ($p<0.1$, Student's *t*-test) respectively. However the apparent falls in SCF mRNA at 1 and 4 weeks may be due to a rising baseline in control livers. This was shown with further densitometric analysis of SCF mRNA expression in treated livers compared to control at 6 hours (blue bars, figure 3.2ii). SCF mRNA expression at all time points was greater then control (100%) but still maximal at 24 hours, 163% ±56 ($p<0.1$, Student's *t*-test).

3.2.1.2. BDL model of experimental rat liver fibrosis

As previously described in section 2.2.1.3, male Sprague-Dawley rats were anaesthetised, had their bile ducts double ligated and then divided. Control animals were sham operated. Control and treated rats were sacrificed 6 hours, 24 hours, 3 days and 7 days after BDL.

A SCF mRNA PCR product of 259 bp was strongly detected in rat BDL livers (i, ii and iii) and control livers (c) at all time points (figure 3.3i). β–actin PCR confirmed the integrity of the cDNA samples used in SCF PCR.

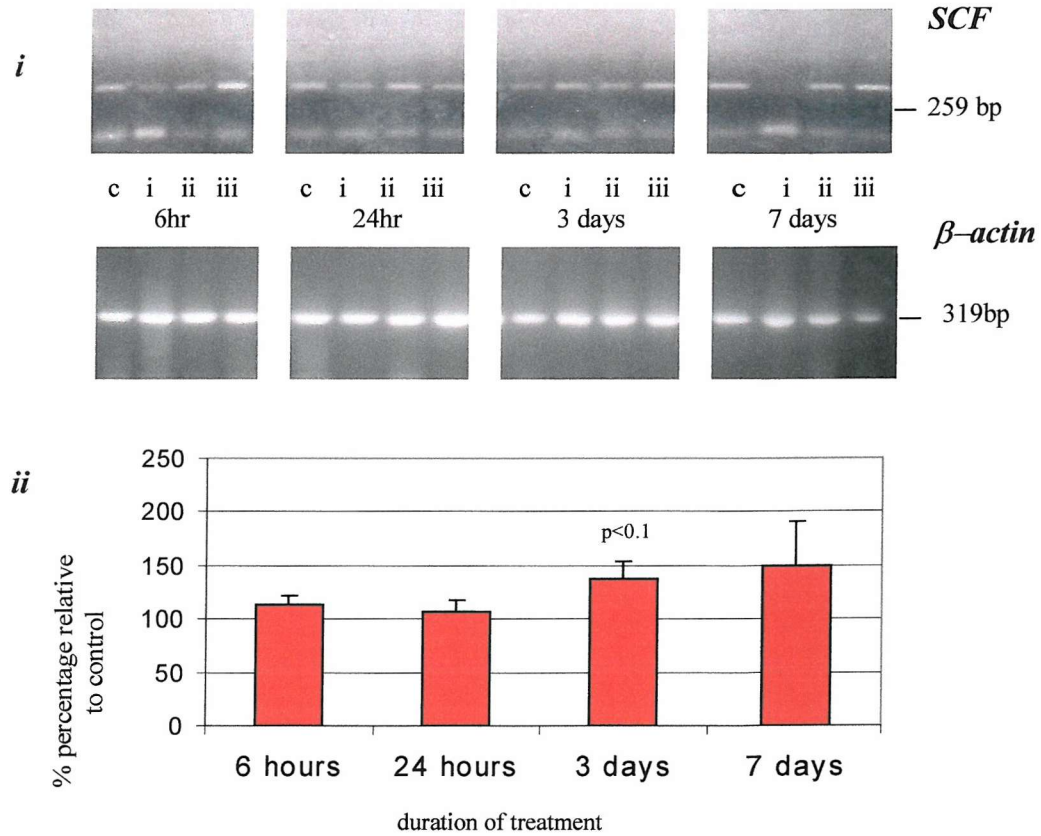


Figure 3.3i. Representative example one of four gels of SCF mRNA detection in BDL rat fibrotic livers using PCR. SCF mRNA was consistently expressed in treated livers (i, ii, iii) and untreated control livers (c). β-actin PCR confirmed integrity of the samples. **ii.** Densitometric analysis of SCF mRNA, n=4, showing a slight increase in SCF mRNA expression in BDL livers compared to untreated livers. $p<0.1$ Student's *t*-test.

Densitometric analysis indicated SCF mRNA expression in BDL increases slightly during course of treatment. At 3 and 7 days, there was a 32.29% $\pm$15.8 ($p<0.1$, Student's *t*-test) and 49.88$\pm$41.2 increase in SCF mRNA expression respectively compared to control untreated livers.

3.2.2. DETECTION OF SCF mRNA EXPRESSION IN PURIFIED HSC BY RT-PCR

SCF mRNA was detected in rat liver homogenates in two different rat models of liver fibrosis by use of RT-PCR. As HSC are the principal cells involved in liver fibrosis, SCF mRNA expression was further examined in purified rat HSC cultures.

Previously in our laboratory by use of RT-PCR, SCF mRNA expression was detected in human HSC (figure 3.4). Purified human HSC were isolated from normal margins of liver resected for hepato- carcinoma, as previously described (Casini *et al,* 1993) and activated by culture on plastic for 14 days. Total RNA were extracted, reverse-transcribed and by PCR, SCF mRNA was detected in human HSC.

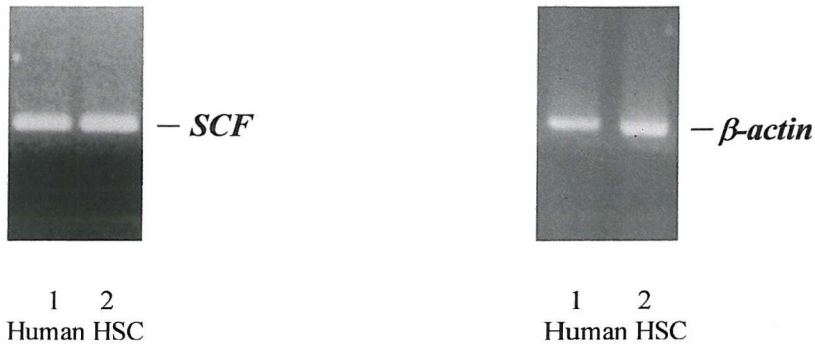


Figure 3.4. RT-PCR analysis of SCF mRNA expression in cultured human HSC. β–actin confirmed the integrity of the cDNA samples. Results shown are from two independent HSC isolates.

SCF mRNA expression was investigated in rat HSC using conventional RT-PCR. Total RNA was extracted from isolated HSC cultured *in vitro* on tissue-culture plastic at various time points (0 and 7 days in culture and HSC passaged twice (P2)), reverse-transcribed and using the same specific primers to SCF as used in rat liver homogenates, target cDNA was amplified and detected on a 1.5% agarose/ TBE gel. This resulted in the detection of a PCR product of 259 bp in RSF positive control samples (figure 3.5). Expression of SCF mRNA was also detected in day 0, 7 and P2 rat HSC, as 259 bp product.

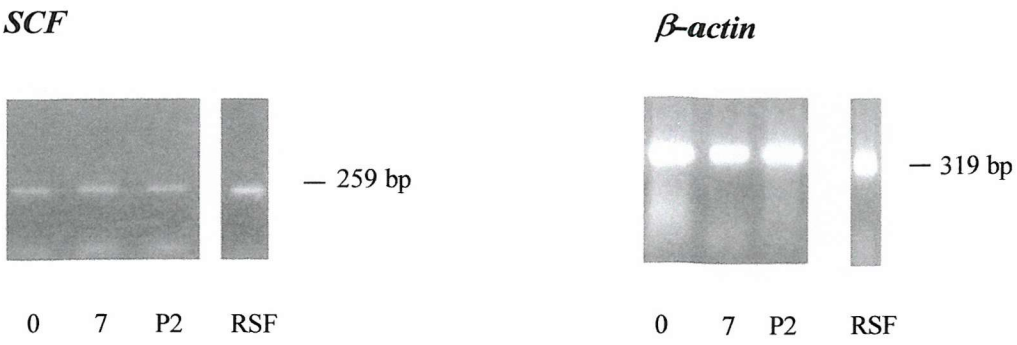


Figure 3.5. Representative example of three independent gels of SCF mRNA analysis in cultured rat HSC samples. By RT-PCR a 259 bp product was clearly detected in 0, 7 day HSC and in P2 HSC which corresponded to RSF positive control. β-actin RT-PCR showed the integrity of the cDNA samples

3.2.3. RIBONUCLEASE PROTECTION ASSAY TO DETECT SCF mRNA EXPRESSION IN CCL_4 RAT MODELS OF FIBROSIS

RPA was carried out on RNA isolated from rat CCl_4 development model of fibrosis to complement RT-PCR studies. A radio-labelled antisense riboprobe synthesised from SCF PCR products was unsuccessful in detecting SCF mRNA expression in any of the CCl_4 rat liver RNA samples and in RSF positive control cells (figure 3.6).

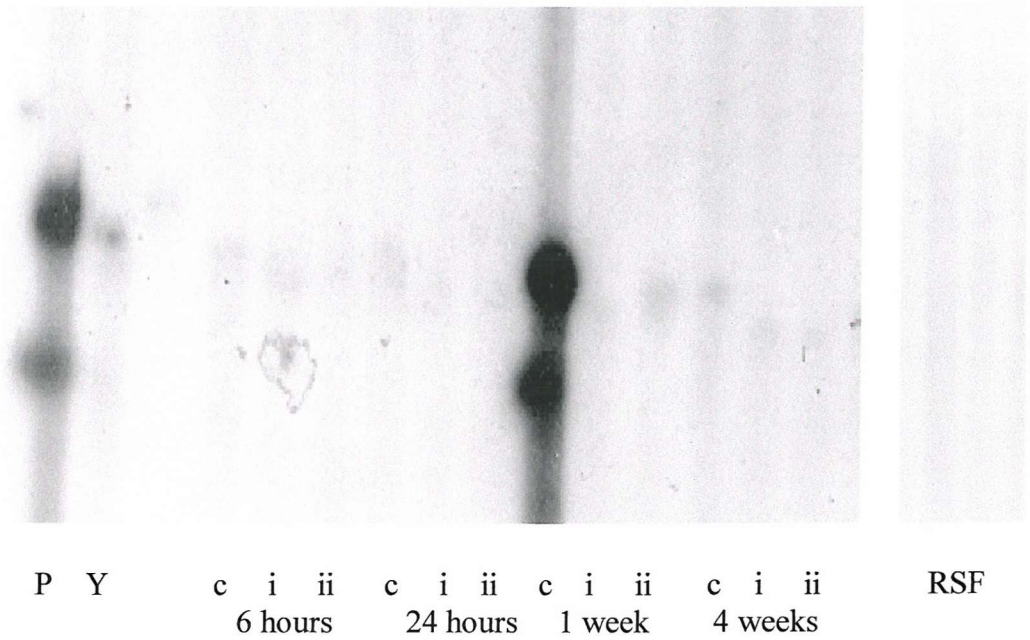


Figure 3.6. Autoradiograph of a RPA investigating SCF mRNA expression in rat liver homogenates of CCl_4 experimental model of fibrosis. Representative one of two independent RPAs. An anti-sense probe made from purified PCR products was unsuccessfully in detecting SCF mRNA expression.

3.2.4. SCF mRNA DETECTION BY NORTHERN BLOTTING ANALYSIS

RNA isolated from rat culture-activated HSC were immobilised on a nylon membrane, and hybridised with a [α- ^{32}P] -radioactive random-primed probe made from purified SCF PCR products. A 6.5 kb product, was detected in RSF positive control samples but not in our HSC samples (figure3.7). This correlated to previous Northern blotting of human HSC within our laboratory and was in accord with the reported molecular weight of SCF (Zhang and Anthony, 1994). As a positive control, membranes were probed with a [α- ^{32}P] - radioactive random-primed β-actin probe made from purified β-actin PCR products. β-actin was clearly detected in rat HSC samples and in RSF.

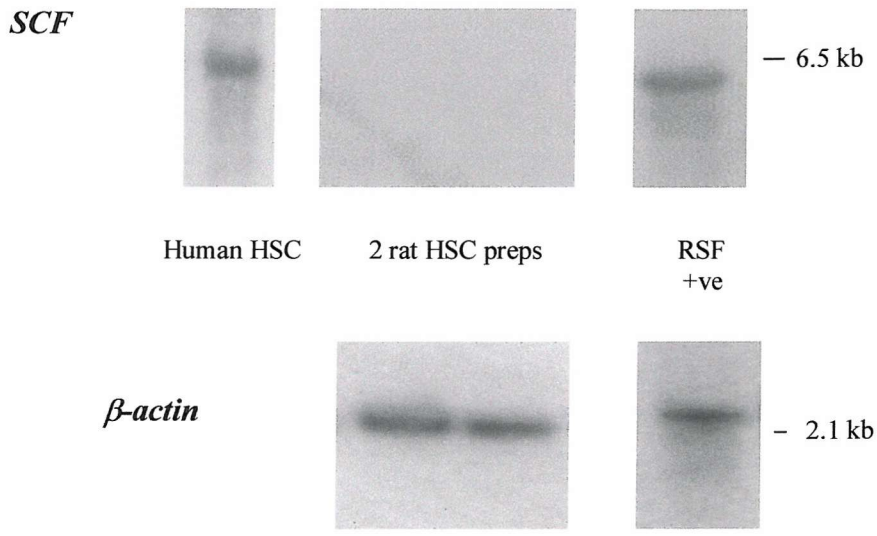


Figure 3.7. Northern blotting analysis to detect SCF mRNA in rat HSC. Radioactive random- primed SCF cDNA probe successfully detected SCF mRNA of reported size (6.5 kb) in RSF positive control samples and in human HSC. However SCF mRNA was undetected in 2 different rat HSC samples. β-actin was used as a control probe.

3.2.5. IMMUNODETECTION OF SCF PROTEIN IN RAT HSC

SCF protein synthesis by HSC was examined by immunodection to complement the above studies of mRNA expression. During HSC transformation from a quiescent to an activated phenotype, there is an increase in cellular protein synthesis. To control for these changes in protein, samples were loaded onto SDS gels according to equal amounts of DNA (section2.2.9.1). Equal amounts of HSC whole cell lysates (3 days, 14 days and P2, and human culture activated HSC) (0.5 μg) were subjected to electrophoresis on a 12 % SDS-polyacrylamide gel and proteins subsequently transferred onto PVDF. Membranes were incubated with 10 μg/ml polyclonal anti-mouse SCF (Genzyme Diagnostic, West Malling, Kent, UK) or negative control antibody (non-immune IgG) followed by incubation with a secondary antibody conjugate to horseradish- peroxidase (1:10,000). By use of chemiluminescence, a band of 30 kDa, corresponding to 3T3 positive control sample was

detected in 2 different human HSC preparations. In rat HSC it can be observed that in progressive activation of HSC in culture there follows an increased expression of SCF protein (figure 3.8i).

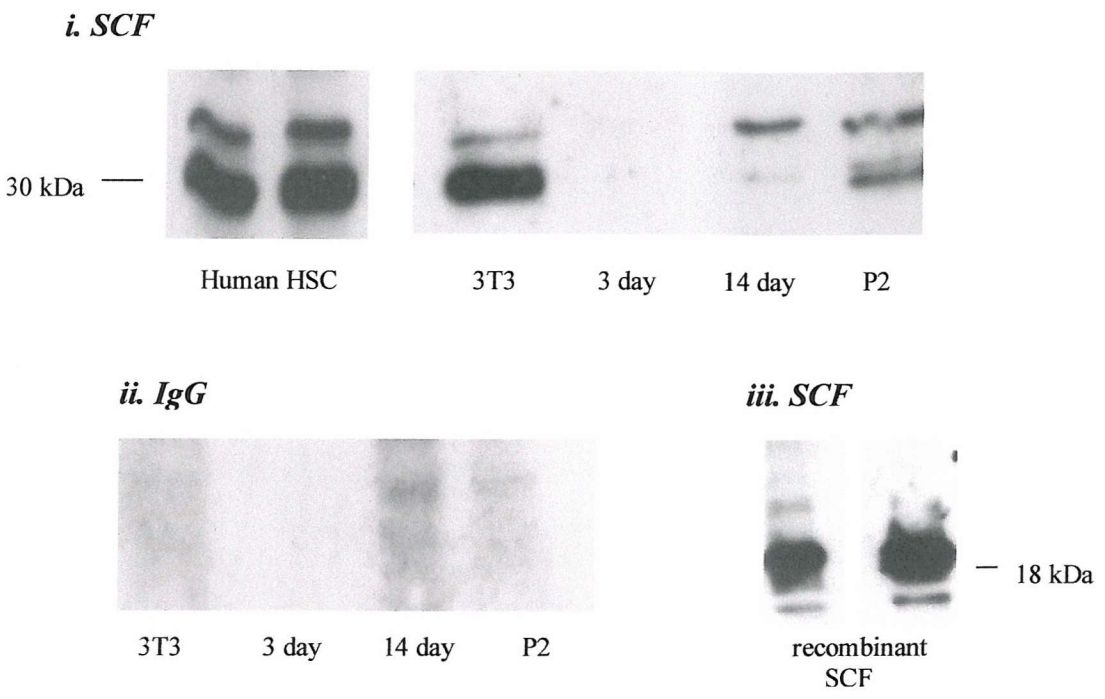


Figure 3.8. **(i)** SCF protein expression in human and rat HSC by Western blotting using a polyclonal SCF antibody. **(ii)** Non-immune IgG immunoblotting. **(iii)** Recombinant SCF protein expression using a polyclonal SCF antibody.

Use of non-immune IgG as a negative control for the primary SCF antibody, identified faint bands on the membrane but these did not correspond to the 30 kDa SCF protein (figure 3.8ii). To verify our findings as a further control, Western blots of recombinant unglycosylated SCF were examined. A signal was detected at the expected molecular weight of 18.5 kDa (figure 3.8iii).

3.2.6. IMMUNOSTAINING OF CULTURED RAT HSC

Third passage rat HSC, cultured on slides were investigated for SCF protein expression using fluorescence immunostaining. Slides were viewed using a Leica fluorescence microscope and by confocal microscopy (section 2.2.10).

Staining of the intermediate filament protein desmin in HSC as a positive control, clearly showed a cytoplasmic fibrillar staining of the whole stellate cell population (figure 3.9i). Staining against SCF within this HSC population showed a diffuse pattern of staining within the cells suggesting that this protein was also expressed homogeneously (figure

3.9ii). The negative control IgG non- immune staining was completely negative with only the HSC nuclei fluorescing red from the propidium iodide counter stain (figure 3.9iii).

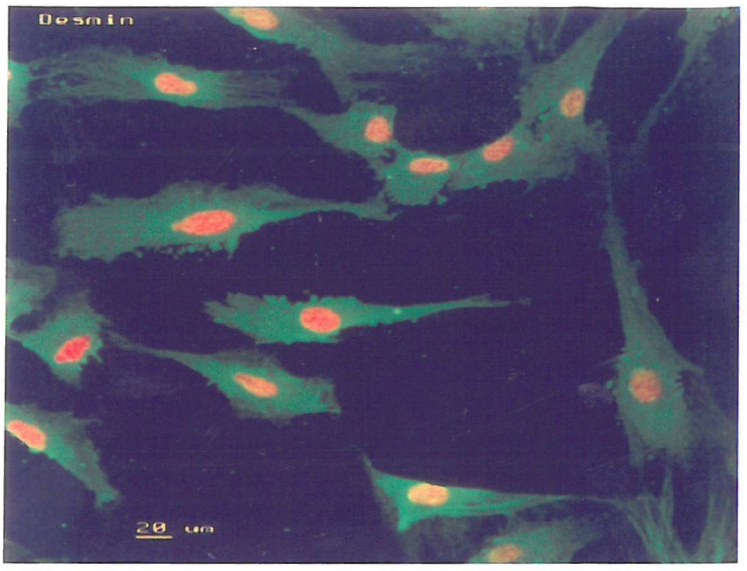
3.2.7. IMMUNOSTAINING OF SCF IN RAT CCL_4 FIBROTIC LIVER SECTIONS

6 μm paraffin- embedded tissue sections from rat normal and 8 week CCl_4 fibrotic livers were investigated for SCF protein expression using strepavidin- biotin horseradish peroxidase immunostaining (section 2.2.12). Control rat normal and fibrotic liver tissues were independently stained with anti-SMA antibody as a positive control and non-immune IgG (5 μg /ml) in place of primary antibody. All the stained sections had slight background staining.

Within the negative control IgG non- immune staining in normal and fibrotic livers there was no clear specific cellular staining. In the normal rat liver, the portal triads (PT), central vein (CV) and hepatic vein (HV) could be clearly detected (figure 3.10 i). However in the fibrotic tissues, abnormal liver architecture was apparent. Fibrotic septae (FB) could be seen distributed throughout the tissue (figure 3.10ii). Excess connective tissue was evident surrounding the PT and HV causing restrictive hepatic blood flow. In normal rat livers, although there is a slight background, the connective tissue surrounding the HV was clearly stained when α-SMA antibody was used as positive control (figure 3.11i). α-SMA staining detected the subendothelial connective tissue and smooth muscle cells found within the vessels. α-SMA was observed within PT in the portal vein (PV), bile ducts (BD) and hepatic artery (HA). In the fibrotic livers however abnormal liver tissue morphology and structure was clearly evident, due to excess connective tissue deposition. α-SMA antibody clearly detected the FS around the liver lobules, formed due to the excess synthesis and deposition of connective tissue (figure 3.11ii). Under high power (x40) magnification, HSC were apparent within the FS and identified accumulating within the necrotic hepatocyte parencyhma (figure 3.11iii).

Staining of the sections with SCF gave contrasting results between the normal and fibrotic rat liver sections. In normal liver sections specific SCF staining was observed within the columnar epithelial cells lining the BD lumen (figure 3.12i), and this could be clearly seen at higher magnification (figure 3.12ii). This observation was previously reported by Fujio *et al,* 1996 and Tsuneyama *et al,* 1999. In the fibrotic rat liver however, SCF staining was visibly stronger within the BD and appeared within the FS (figure 12.iii). At higher magnification SCF staining was evident in the HSC found within the FS and in the necrotic parenchyma (figure 3.12iv). This finding suggests that hepatic SCF synthesis during injury maybe as a result of HSC activation and also from the BD epithelium.

i



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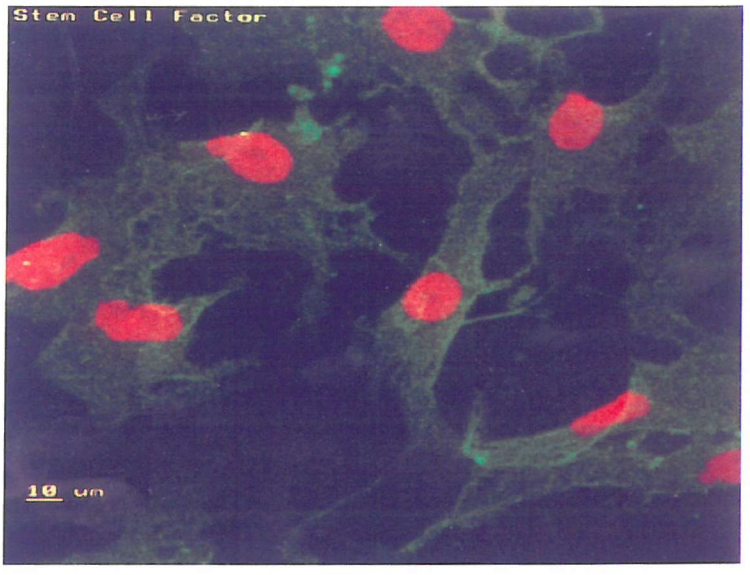

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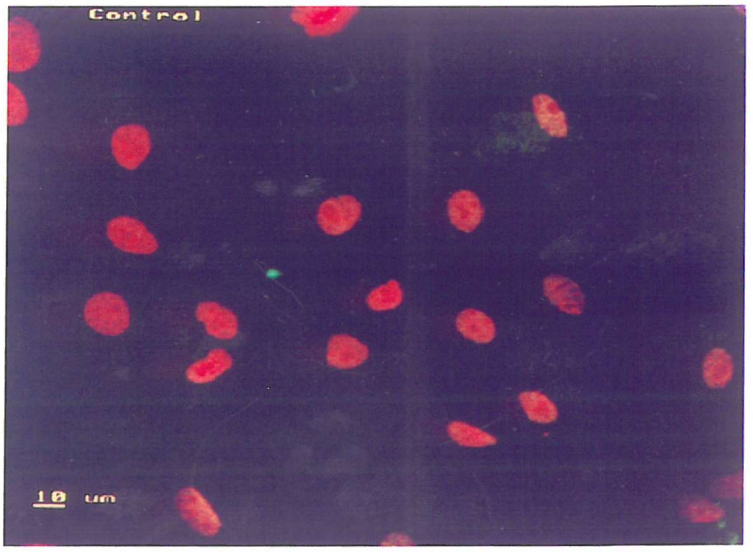
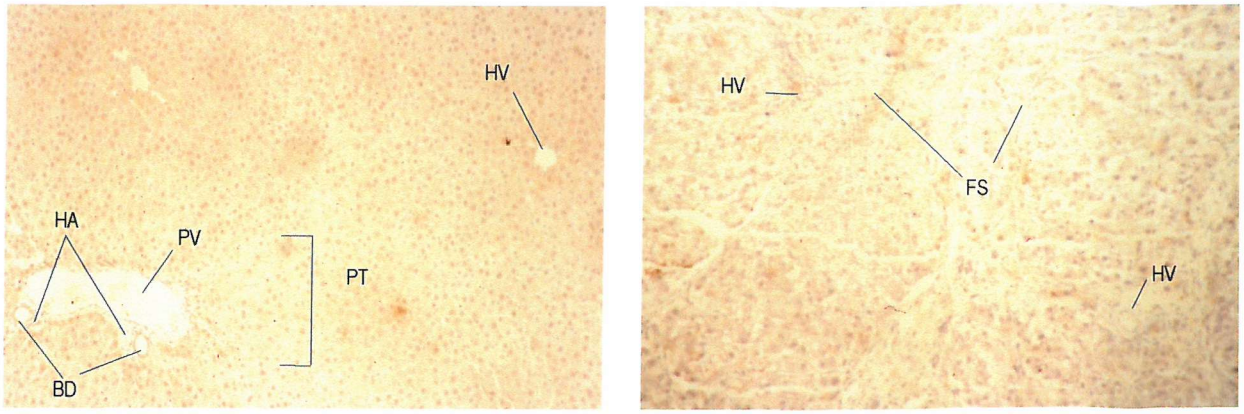
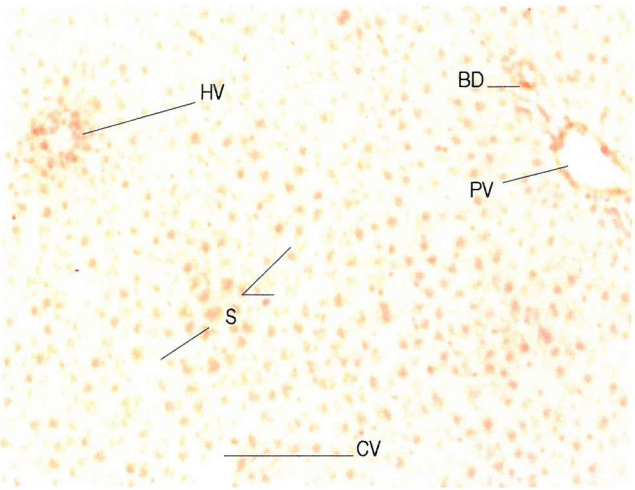


Figure 3.9. Fluorescent immunocytochemistry analysis of SCF production in passaged rat HSC cultured on glass slides. **(i)** Positive control desmin immunostaining. **(ii)** SCF immunostaining using a polyclonal antibody. **(iii)** Non- immune IgG staining of rat HSC as negative control.



i *ii*

Figure 3.10. Strepavidin- biotin horseradish peroxidase complex non- immune IgG immunostaining in rat normal and CCl_4 fibrotic liver sections. **(i)** IgG negative control staining in normal rat livers, x100 magnification. The portal triad (PT) can be clearly seem, consisting of bile ducts (BD), Hepatic artery (HA) and portal vein (PV). The hepatic vein (HV) is the main vessel in which the blood leaves the liver. **(ii)** IgG negative control staining in fibrotic rat livers, x100 magnification. The normal architecture of the liver has been destroyed by the deposition of ECM forming fibrotic septae (FS).



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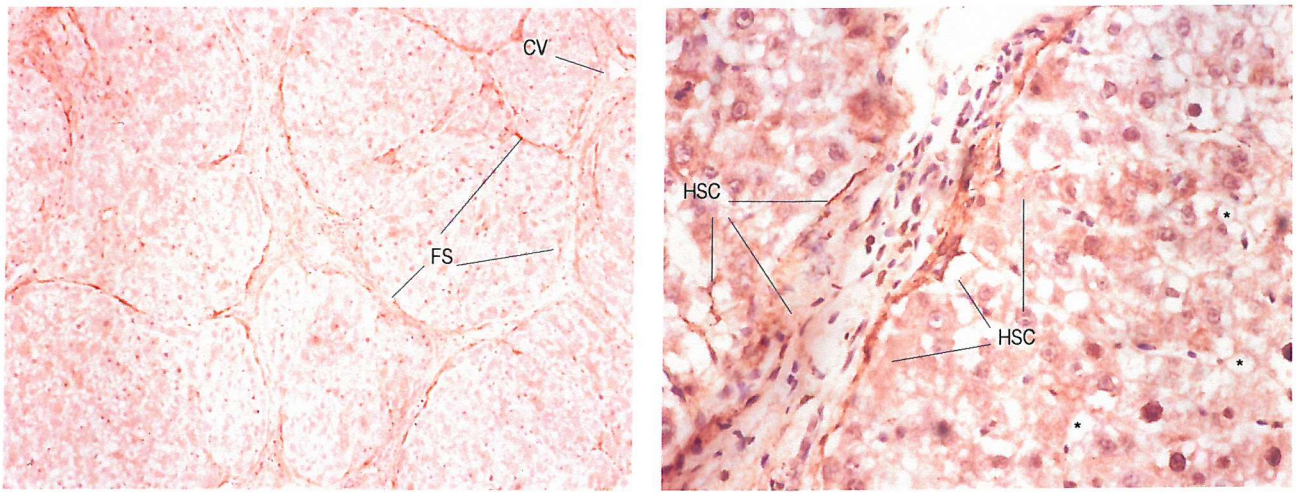


ii *iii*

Figure 3.11. Strepavidin- biotin horseradish peroxidase complex α-SMA immunostaining in rat normal and CCl_4 fibrotic liver sections. **(i)** α-SMA positive control staining in normal rat livers, x100 magnification. The connective tissue around the hepatic vein (HV), and bile duct (BD) and portal vein (PV) showed positive for α-SMA. Sinusoids (S) appear as light areas between the cords of hepatocytes. **(ii)** α-SMA positive staining in fibrotic rat livers, x100 magnification. The fibrotic septae (FS) can be clearly seen stained positive for α-SMA. **(iii)** α-SMA staining in fibrotic rat livers, x400 magnification. HSC can be clearly seen within the connective tissue in the FS and in the necrotic liver parenchyma (*).

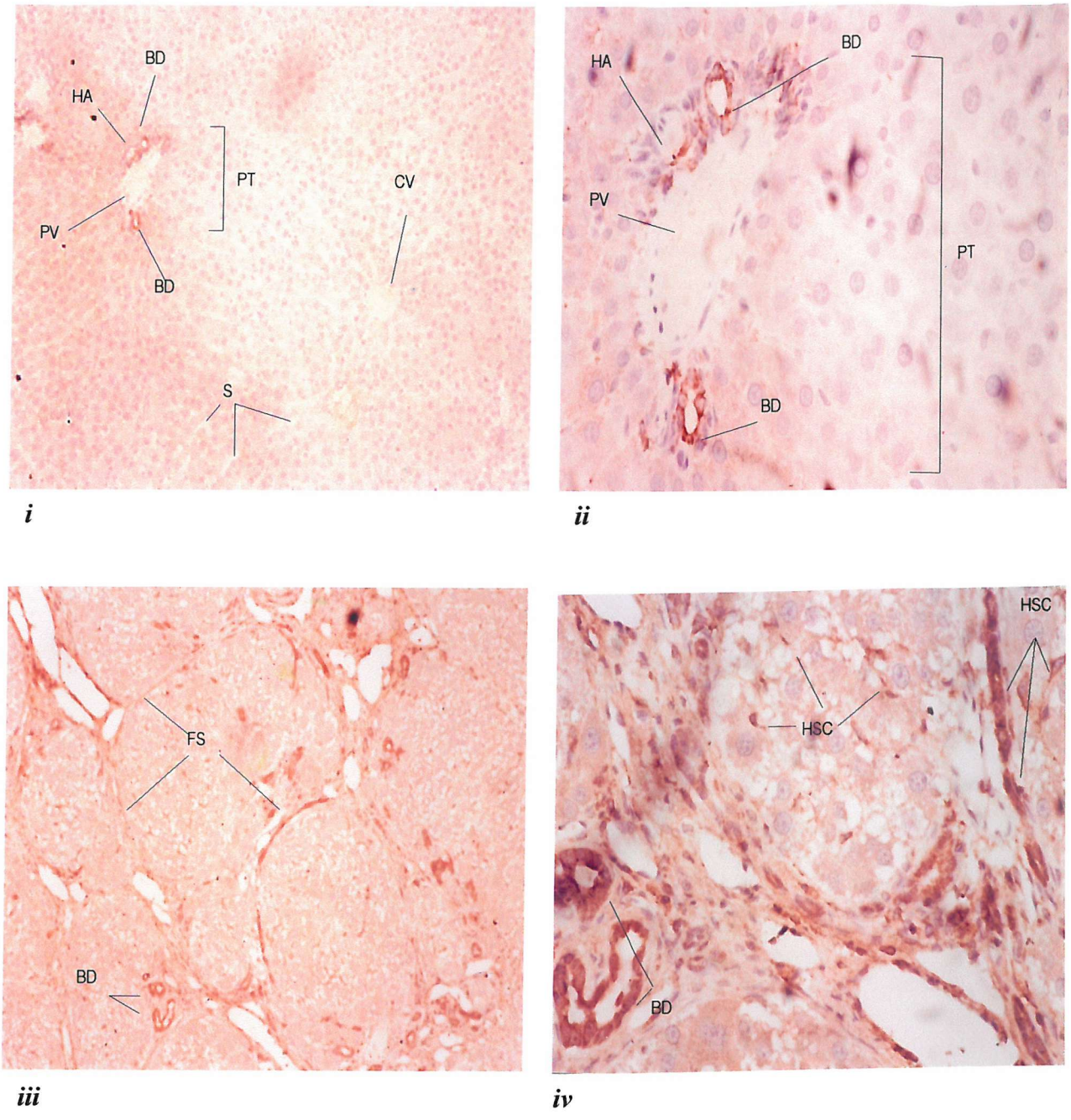


Figure 3.12. Strepavidin- biotin horseradish peroxidase complex SCF immunostaining in rat normal and CCl_4 fibrotic liver sections. **(i).** SCF staining in normal rat liver, x100 magnification. SCF was clearly observed within the epithelium of the bile ducts (BD) of the portal triads (PT). The central vein (CV) have sinusoids (S) penetrating the wall of the vein and lack surrounding connective tissue. **(ii).** SCF staining in normal rat liver, x400 magnification. At this magnification, SCF is evident within the biliary epithelium. **(iii).** SCF staining in fibrotic rat liver, x100 magnification. SCF staining was apparent within the fibrotic septae (FS) of the liver and much stronger staining within the BD epithelium. **(iv).** SCF staining in fibrotic rat liver, x400 magnification. At a higher magnification, SCF staining was located in the HSC within the FS and the liver parenchyma, within the sinusoids. BD showed intense SCF staining.

3.2.8. *IN- VITRO* CO-CULTURE OF HSC AND MC- ADHESION STUDIES

Purified human MC obtained as described (section 2.2.4) were co-cultured for 1 hour at 37 °C in 5% CO_2 in a humidifying incubator on monolayers of passaged rat HSC in the presence or absence of a neutralising SCF antibody (ranging from 0- 10 μg/ml) . After incubation, non-adherent cells were washed off in DMEM. Adherent MC were stained using toluidine blue, which binds to heparin in MC granules and counted. Darkly stained MC were clearly observed adhering to the flattened large HSC monolayer (figure 3.13i). Numbers of MC adherent to HSC were assessed in triplicate wells for each treatment by counting nine low power fields.

In the co-culture studies, it was observed that at high concentrations of SCF neutralising antibody there was an inhibition of MC adherence to HSC. This observation was consistent in 5 different co-culture experiments and resulted in an inhibition of MC adherence from 14.3±9.7 % ($p< 0.05$) to 55.6 ±9.3 % ($p<0.05$) over a SCF neutralising antibody range from 0.039 μg/ml to 10 μg/ml (red bars, figure 3.13ii).

As a negative control, non-immune IgG at concentrations of 0.625 μg/ml and 2.5 μg/ml (which corresponded to the concentration of SCF neutralising antibody that gave inhibition of MC adherence) was used in place of the neutralising SCF antibody. It was found that in three separate experiments there was a 22.5 %±4.73 and 28 %±5.29 inhibition of MC adhesion respectively (blue bars, figure 3.13ii).

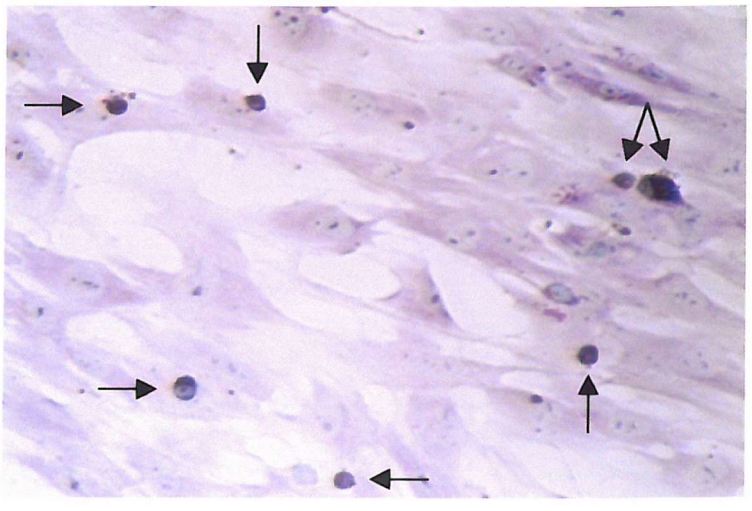


Figure 3.13i. Detection of human MC adherent to cultured monolayer of passaged rat HSC. Purified MC were left for 1 hour to adhere to HSC monolayers. Non-adherent cells were removed by washing. Adherent MC were stained with toluidine blue and counted using a Leica light microscope (x 200 magnification).

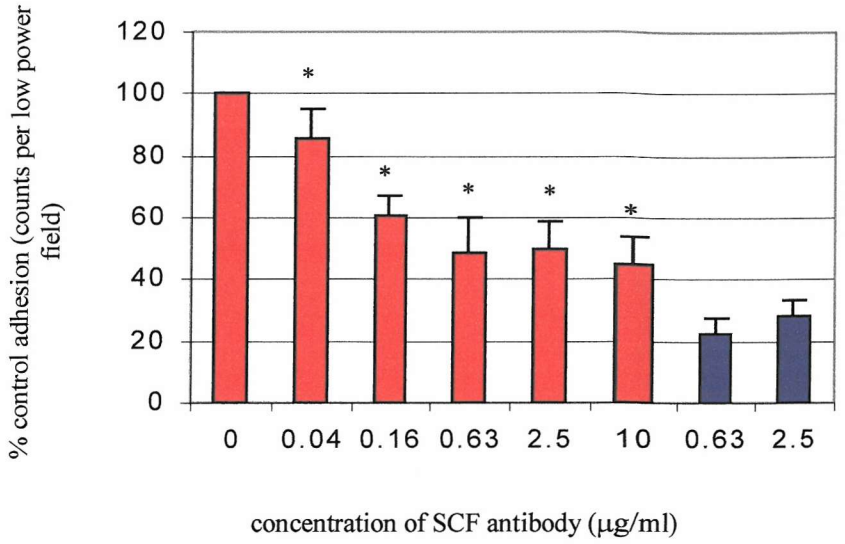


Figure 3.13ii. Graph showing inhibition of MC adherence to HSC monolayer by neutralising SCF antibody (red bars). Non- immune IgG negative control added to co-cultures blocked adhesion by 28%±5.29 (blue bars). Results are shown as mean ± SEM of nine low power field counts in 3 to 5 independent co- cultures. *=$p<0.05$, paired *t*-test and non-parametric Wilcoxon signed rank test.

3.3. DISCUSSION

A number of groups have recently shown that MC accumulate in the human and rat liver during injury (Miller *et al*, 1994; Peng *et al*, 1994; Ramos *et al*, 1994; Farrell *et al*, 1995; Xu *et al*, 1994; Rioux *et al*, 1996; Ambrust *et al*, 1997; Nakamura *et al,* 1997). MC are distributed periportally in the human and rat liver within the portal tracts and sinusoids. MC have been shown to be present in the fibrotic liver in areas of connective tissue deposition (Farrell *et al,* 1995), also known as the fibrotic septae. As activated HSC play a vital role in liver fibrosis by synthesising and secreting ECM proteins (Friedman *et al,* 1985), I have examined the potential role of these cells in recruiting MC to the liver during injury by producing SCF. SCF is fundamental in MC biology having multifunctional effects; promotes MC maturation from tissue resident precursors and MC progenitors (Nocka *et al*, 1990; Irani *et al*, 1992a; Rennick *et al,* 1995), prolongs MC survival in culture (Tsai *et al*, 1991a; Takagi *et al*, 1992), induces MC mediator release (Bischoff *et al*, 1992; Columbo *et al*, 1992; Coleman *et al,* 1993) and most importantly acts as a potent MC chemoattractant in fibrotic tissues (Meininger *et al*, 1992; Nilsson *et al*, 1994; Hiragun *et al,* 1998 Fireman *et al,* 1999).

Primarily it was necessary to verify that SCF was synthesised in liver fibrosis before investigating the hepatic cellular source of the cytokine. Initially the expression of SCF mRNA in rat liver fibrosis was examined using two established models of fibrosis, CCl_4 induced fibrosis and bile-duct ligation. A number of approaches were considered to measure SCF expression. ELISA used for human livers could not be used as the reagents used do not cross react with rat, therefore RPA and RT-PCR were employed. RPA was not successful in detecting SCF mRNA in rat liver homogenates, however by RT-PCR analysis using SCF- specific primers, SCF mRNA expression was clearly detected in rat liver homogenates although expression did not correlate significantly with the development of fibrosis in both models. This maybe as SCF protein was found to be consistently expressed in the bile duct by use of immunohistochemistry, and RT-PCR may detect this expression as a constant background. In contrast, previously in our laboratory SCF mRNA expression as detected by RT-PCR, appeared to be upregulated in human diseased liver compared to normals. In cirrhotic human livers, PSC and PBC, SCF mRNA was consistently detectable as compared to normal livers. Due to RT-PCR limitations, the increase in SCF expression in human fibrotic livers was quantified by the use of a commercially available human SCF ELISA. ELISA confirmed the human RT-PCR

findings and indicated SCF protein expression was significantly upregulated 2-3 fold in cirrhotic PSC and PBC livers compared to normal.

Fibroblasts are a potential source of SCF, which has been demonstrated by numerous MC- fibroblast co-culture systems (Irani *et al,* 1992; Levi- Schaffer *et al,*1995;Levi-Schaffer *et al,*1996). As activated HSC have many similarities to fibroblasts, these cells were investigated as a potential source of hepatic SCF. By RT-PCR SCF mRNA expression was investigated in rat HSC, as SCF was previously detected in human HSC by RT-PCR in our laboratory. SCF mRNA was detected in cultured activated rat HSC and in RSF positive cells expressing SCF. Northern blotting however was not successful in detecting SCF mRNA expression in rat HSC, although by this technique SCF mRNA was previously detected in human HSC. RSF positive SCF- synthesising cells also clearly expressed SCF mRNA, a 6.5 kb mRNA product, which corresponded to the previously reported size of SCF mRNA (Zhang and Anthony, 1994). This suggests that SCF mRNA is expressed at low levels in the rat compared to the human, as rat SCF mRNA can only be detected when the sensitive RT-PCR technique is employed. This difference in SCF mRNA expression suggests that there may be manifestation differences between the species.

To complement the mRNA SCF studies, SCF protein synthesis was investigated. Western blotting detected SCF protein expression in human and rat HSC lysates. Using a polyclonal antibody to mouse SCF, SCF protein at 30 kDa was detected in two different cultures of human HSC. SCF protein was only detected in culture- activated rat HSC (day 14) and passaged HSC (P2) cultures and not in freshly isolated rat HSC. This lower level of SCF expression by activated rat HSC relative to human may account for the higher abundance of MC found in the human liver compared to that in the rat liver during fibrosis; an 8 fold increase in MC numbers in human cirrhotic liver (Farrell *et al,* 1995) compared to 2 fold increase in MC in rat cholestatic disease (Rioux *et al,* 1996). This suggests that greater MC numbers are recruited to the human liver during injury than in the rat. Immunofluorescent staining of SCF protein in rat HSC confirmed the Western blotting studies. Third passage rat HSC uniformly within the culture expressed SCF protein pericellularly and clearly expressed a fibrillar distribution of positive desmin staining which confirmed activation status of the stained rat HSC. Immunohistochemical studies confirmed the presence of SCF within rat liver tissues. In normal rat livers the epithelial cells lining the bile duct lumen stained strongly for SCF, as previously reported by Fujio *et al,* 1996. In the rat fibrotic livers (8 weeks of CCl_4 treatment) SCF staining was apparent again in the bile ducts but also within the fibrotic septae and necrotic parencyhma. Upon

closer examination HSC were shown to be positive for SCF within the fibrotic rat liver. These studies in the rat liver therefore confirm the findings *in vitro* that activated HSC produce SCF.

Many studies of MC and fibroblast co-cultures provide further evidence that MC and fibroblasts interact to stimulate development, maturation and activation of each other aided by the synthesis of SCF. These studies therefore suggest that there is a bi-directional communication between MC and fibroblast (Levi-Schaffer *et al,* 1985; Levi-Schaffer *et al,* 1987; Levi-Schaffer *et al,* 1995). Within this chapter I have addressed the possibility that MC and HSC may too have an association within the liver and interact with each other via SCF and its ligand *c-kit* on the MC surface. Co-cultures of MC and HSC clearly indicate adhesion between MC and HSC *in vitro*. By blocking membrane- expressed SCF in activated rat HSC using a neutralising SCF antibody, MC adherence to HSC was inhibited up to 55 % $\pm$ 9.3 ($p<0.05$). As this adhesion of MC to HSC was not completely blocked, this suggests that factors other than SCF/*c-kit* are involved in MC/ HSC intercellular adhesion. Our co-culture studies clearly confirm a dynamic relationship between HSC and MC which is already reported between fibroblasts and MC (Levi *et al,* 1985).

In summary these results suggest that during liver disease, HSC become activated and synthesise SCF protein and mRNA. As a consequence of this increased hepatic production of SCF, MC are potentially recruited into the injured liver. It has been recently reported that co-culture of MC and HSC resulted in the induction of SCF in the presence of TNFα (Brito *et al,* 1997). This study further confirms that there is an interaction between liver connective tissue stroma cells and MC in hepatic fibrosis.

In conclusion, activated HSC may contribute to MC recruitment and hyperplasia during liver fibrosis by the production of SCF.

CHAPTER FOUR
MAST CELLS AND THEIR MEDIATORS IN FIBROTIC LIVER AND THEIR EFFECT ON HEPATIC STELLATE CELL ACTIVATION

4.1. INTRODUCTION

During fibrosis MC are reported to aggregate in areas of tissue damage. It has been found in various studies that MC numbers dramatically increase in human and rat liver fibrosis, especially within the sinusoids and the portal tracts (Miller *et al*, 1994; Peng *et al*, 1994; Ramos *et al*, 1994; Farrell *et al*, 1995; Xu *et al*, 1994; Rioux *et al*, 1996; Ambrust *et al*, 1997; Nakamura *et al*, 1997). The participation of MC in fibrosis has been directed at the variety of profibrogenic mediators secreted by MC upon subsequent activation. For example, serine proteases tryptase and chymase, and cytokines IL-4 and TNF-α have known fibrogenic effects on fibroblasts and can upregulate collagen synthesis (Gruber *et al*, 1987; Gruber *et al*, 1989; Brito *et al*, 1991; Rous *et al*, 1991; Sempowski *et al*, 1994;Cairns and Walls, 1996; Cairns and Walls, 1997). Whilst the last chapter examined a mechanism which HSC might recruit MC to the injured liver, in this chapter the potential role of the mast cell in activating HSC was investigated. Initially the presence of MC proteases was examined in the injured liver. The mitogenic effects of various MC mediators on *in-vitro* activated rat HSC were studied, especially the MC protease tryptase.

4.2. RESULTS

4.2.1 MC PROTEASE mRNA EXPRESSION IN HUMAN LIVER DISEASE BY RT-PCR

In human liver fibrosis MC accumulation has been detected and confirmed by immuno-histochemistry using antibodies targeted to MC proteases tryptase and chymase (Farrell *et al*, 1995; Ambrust *et al*, 1997). However the expression of MC protease mRNA has never been investigated. To confirm previous findings and to further investigate the role of MC in liver fibrosis I have analysed MC protease mRNA expression by RT-PCR. Published primers were used to detect MC protease mRNA expression in reverse- transcribed liver homogenates from liver cirrhosis (Xia *et al*, 1995). Tryptase and chymase mRNA expression were examined in human liver diseases such as PBC and PSC and in normal livers (N), see figure 4.1. Tryptase mRNA and chymase mRNA were clearly detected as 151 bp and 277 bp cDNA products, as previously reported (Xia *et al*, 1995). Tryptase mRNA was constitutively expressed in all cirrhotic and normal liver samples. The expression of chymase mRNA was similar to that of tryptase as it was found expressed in all three PBC livers, in three of four PSC livers and in two of three normal livers. β–actin PCR clearly indicated integrity of the cDNA samples used, figure 4.1.

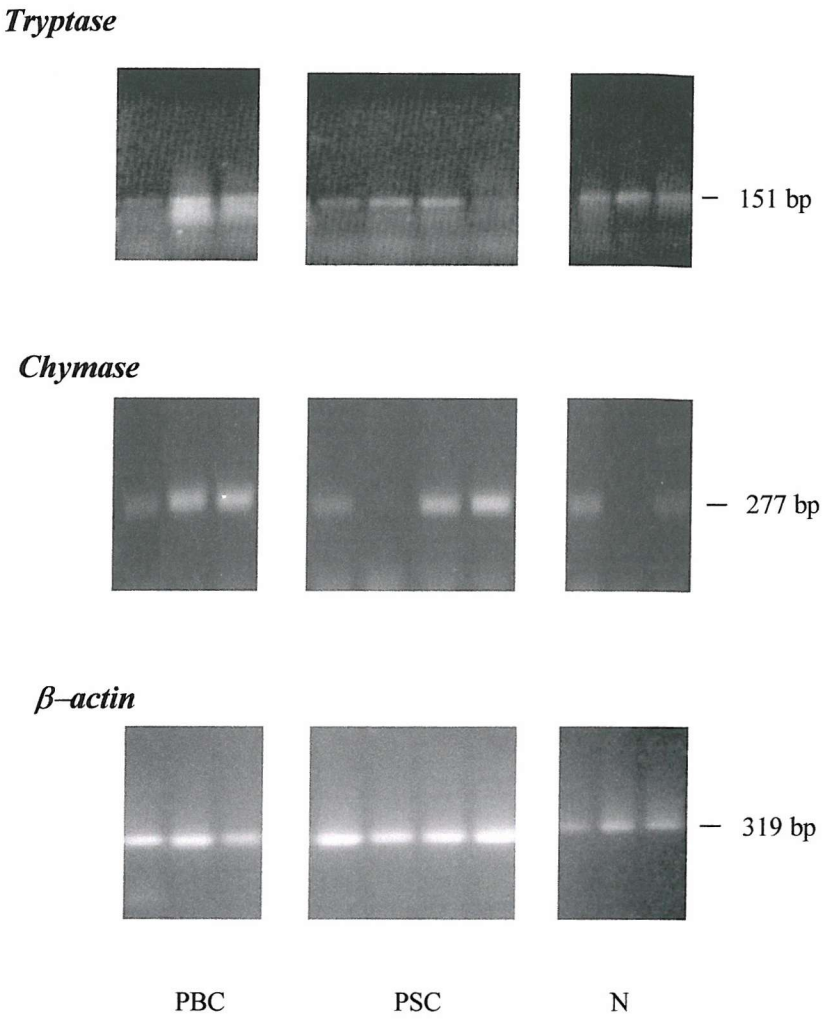


Figure 4.1. RT-PCR analysis of tryptase and chymase mRNA expression in normal human liver and human cirrhotic liver homogenates. PBC= primary biliary cirrhosis; PSC= primary sclerosing cholangitis; N= normal livers. β-actin RT- PCR was performed to confirm integrity of cDNA samples.

4.2.2. RAT MC PROTEASE mRNA AND PROTEIN EXPRESSION IN EXPERIMENTAL RAT MODELS OF LIVER FIBROSIS BY RT-PCR AND IMMUNOSTANING

The distribution of human MC in liver fibrosis has been extensively investigated by the use of immunostaining (Farrell *et al*, 1995; Ambrust *et al*, 1997; Nakamura *et al*, 1997). In BDL and CCl_4 rat models of liver fibrosis MC accumulation has also been detected and confirmed by histochemical analysis (alcian blue/ safranin or toluidine blue staining) and by RMCPII ELISA (Rioux *et al*, 1996; Ambrust *et al*, 1997). It was found that MMC predominated in the fibrotic rat livers (Xu *et al*, 1994; Rioux *et al*, 1996). Miller *et al* (1994), also found that in rodents infected with *Schistosoma mansoni* parasites

MMC were recruited into the liver. To try and confirm these findings, by the use of RT-PCR and immunostaining I have investigated rat MC protease mRNA and protein expression in two rat models of liver fibrosis.

4.2.2.1. RAT MC PROTEASE mRNA EXPRESSION IN EXPERIMENTAL RAT MODELS OF LIVER FIBROSIS BY RT-PCR

Published primers (Rouleau *et al,* 1994) were used to detect RMCP I and RMCP II mRNA expression in reverse- transcribed liver homogenates from CCl_4 and BDL models of rat liver fibrosis.

4.2.2.1.1. CCl_4 model of rat liver fibrosis

As previously described in section 2.2.1.1, male Sprague-Dawley rats were injected intra-peritoneally with 0.2 ml/100 g of sterile CCl_4 in a 1:1 ratio with olive oil, twice weekly for 4 weeks. Control rats (c) were treated with olive oil only at the same time. Control and treated rats were sacrificed 6 hours, 24 hours, 1 week and 4 weeks after the initial treatment of CCl_4. Two rats were used for treated time points (i and ii).

By RT-PCR, a PCR product of 423 bp which correlated to RMCPI mRNA, was weakly and inconsistently detected in fibrotic livers (i, ii) and in control livers (c), figure 4.2. RMCPII mRNA expression was detected in all treated (i, ii) and control (c) livers at all time points as a 436 bp product, figure 4.2. β–actin PCR confirmed the integrity of the cDNA samples used in these PCRs and confirmed equal loading and efficiency of amplification between different samples.

4.2.2.1.2. BDL model of rat liver fibrosis

As previously described in section 2.2.1.3, male Sprague-Dawley rats were anaesthetised, had their bile ducts double ligated and then divided. Control animals were sham operated. Control and treated rats were sacrificed 6 hours, 24 hours, 3 days and 7 days after BDL.

RMCPI mRNA expression was weakly detected as PCR product of 423 bp in rat BDL livers (i, ii and iii) and control livers (c) at all time points, figure 4.3. This expression was clearly upregulated in 7 day BDL rat livers. RMCP II mRNA expression however was observed as a 436 bp product only in 7 day BDL treated rat livers (i, ii, iii), which suggests that RMCP II mRNA expression is upregulated in BDL livers. β–actin PCR confirmed the integrity of the cDNA samples used in RT-PCR.

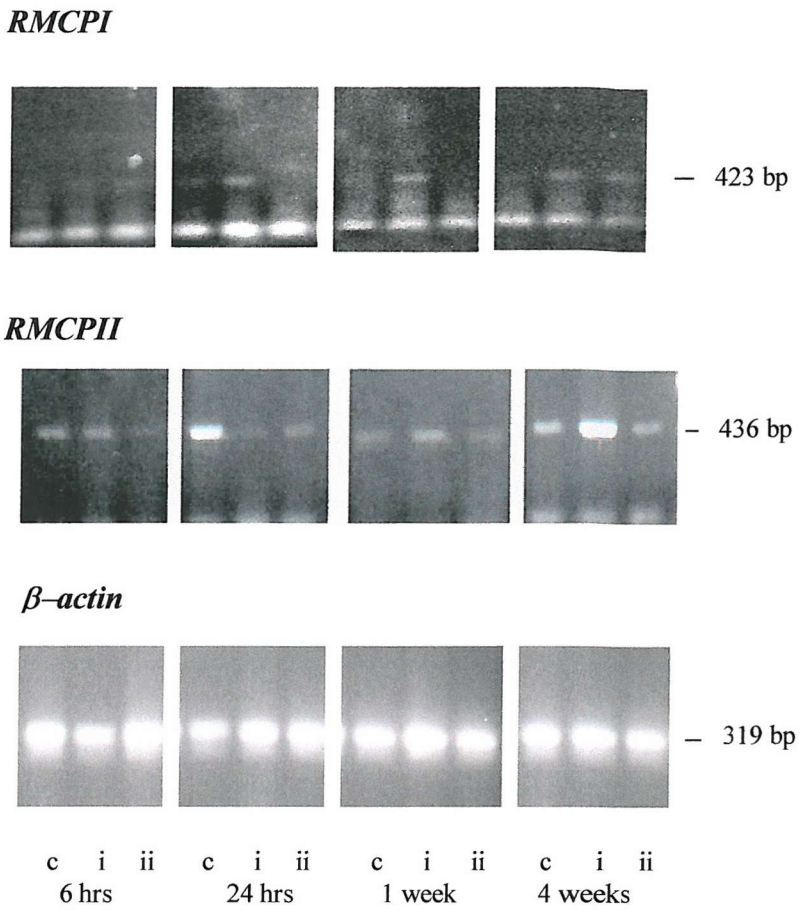


Figure 4.2. Representative example of three RT-PCRs detecting RMCPI and RMCPII mRNA expression in CCl_4 development model of rat liver fibrosis. β-actin RT-PCR was used to confirm cDNA integrity.

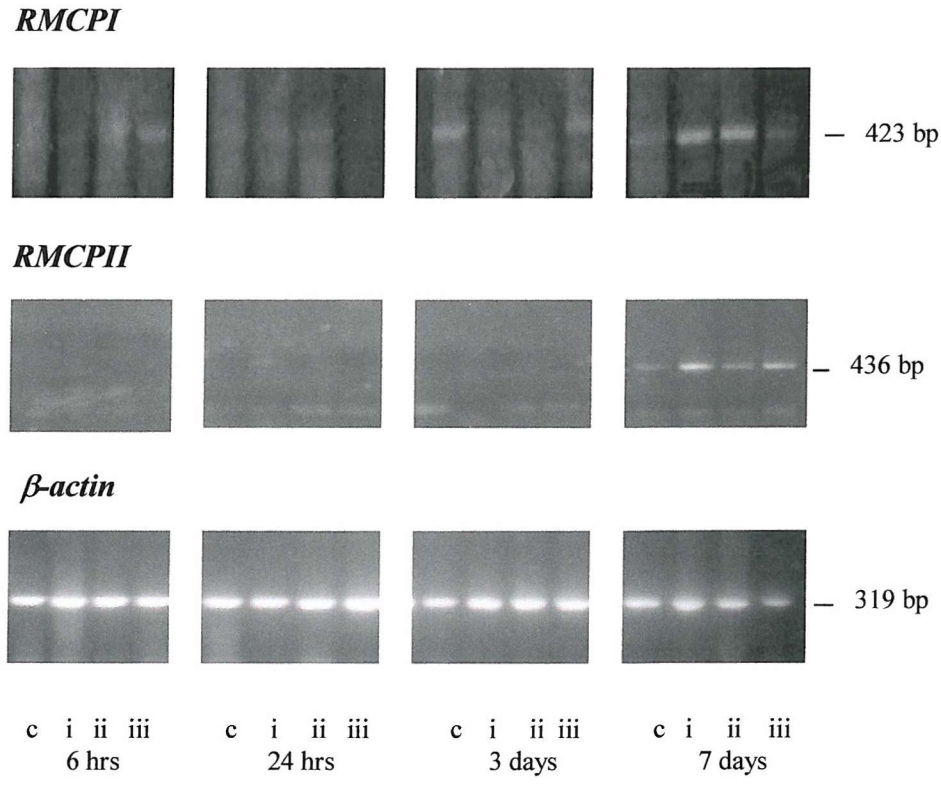


Figure 4.3. Representative example of three RT-PCRs detecting RMCPI and RMCPII mRNA expression in BDL model of rat liver fibrosis. β-actin RT-PCR was used to confirm cDNA integrity.

4.2.2.2. IMMUNOSTAINING OF RMCPI AND RMCPII PROTEIN IN RAT CCL_4 FIBROTIC LIVER SECTIONS

6 μm paraffin- embedded tissue sections from rat normal and CCl_4 fibrotic livers were analysed for RCMPI and RMCPII protein expression using antibodies that were gifts from Drs. G Newlands and H Miller, Moredun Research Institute, Edinburgh, UK. Immunostaining was carried out as in method 2.2.11. Control rat normal and fibrotic liver tissues were independently stained with anti-SMA antibody (1:400 dilution) as a positive control and non- immune IgG (5 μg /ml) in place of primary antibody (section 3.2.6).

In normal rat liver RMCPI staining identified the presence of a few CTMC associated within the periportal connective tissue of the portal triads (PT) (figure 4.4i), which could be clearly seen at higher magnification (figure 4.4ii). Within the fibrotic rat livers (figure 4.4iii), although there was high background staining, CTMC accumulation was evident within the fibrotic septae running through the injured liver. Again CTMC were observed concentrated within the connective tissue surrounding the portal tracts, which could be clearly seen at higher magnification (figure 4.4iv). A few CTMC were found within the necrotic hepatic parenchyma.

RMCPII gave a similar pattern of staining as RMCPI.. In normal rat liver, although there was background staining, a few MMC were associated within the connective tissue of the portal triads (PT) (figure 4.5i), which could be clearly seen at higher magnification (figure 4.5ii). Within the fibrotic rat livers (figure 4.5iii), although not as evident as CTMC, MMC accumulation was apparent within the fibrotic septae in the liver. At higher magnification, MMC were observed concentrated within the connective tissue surrounding the portal tracts figure (4.5iv).

These results suggest that within the normal rat liver very few MC are present. However in the fibrotic livers both CTMC and MMC accumulate and are present, mainly within the fibrotic septae and the portal tracts.

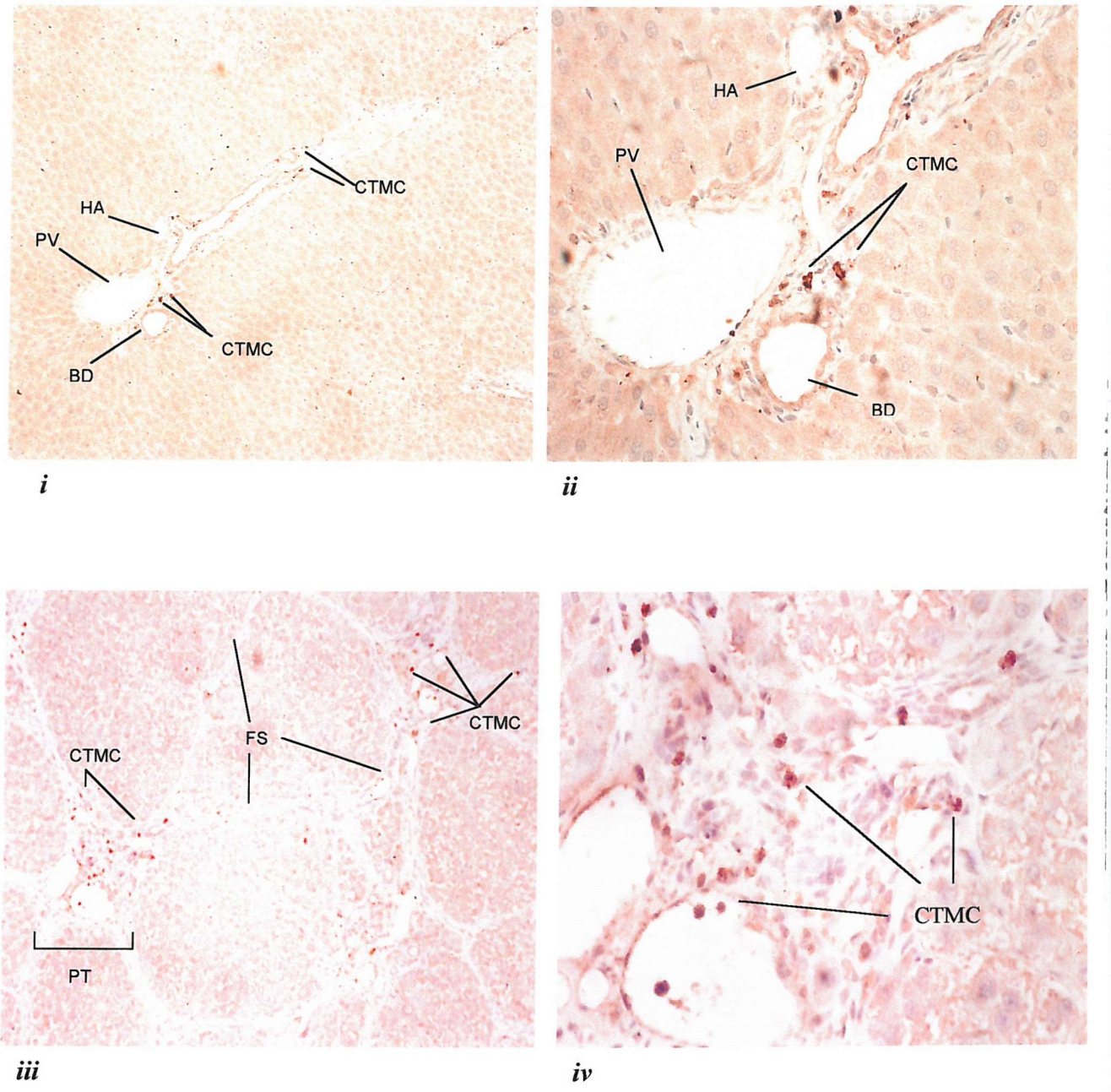


Figure 4.4. Strepavidin-biotin horseradish peroxidase complex RMCPI immunostaining in rat normal and CCl_4 fibrotic liver sections. **(i).** RMCPI staining in normal rat liver, x100 magnification. A few MC (CTMC) were observed within the surrounding periportal connective tissue. **(ii).** RMCPI staining in normal rat liver, x400 magnification. **(iii).** RMCPI staining in fibrotic rat liver, x100 magnification. MC were apparent within the fibrotic septae (FS) running through the fibrotic liver mainly associated within the fibrotic PT. A few MC were observed within the fibrotic liver parenchyma. **(iv).** RMCPI staining in fibrotic rat liver, x400 magnification. At this magnification MC could be clearly seen accumulating within the fibrotic PT.

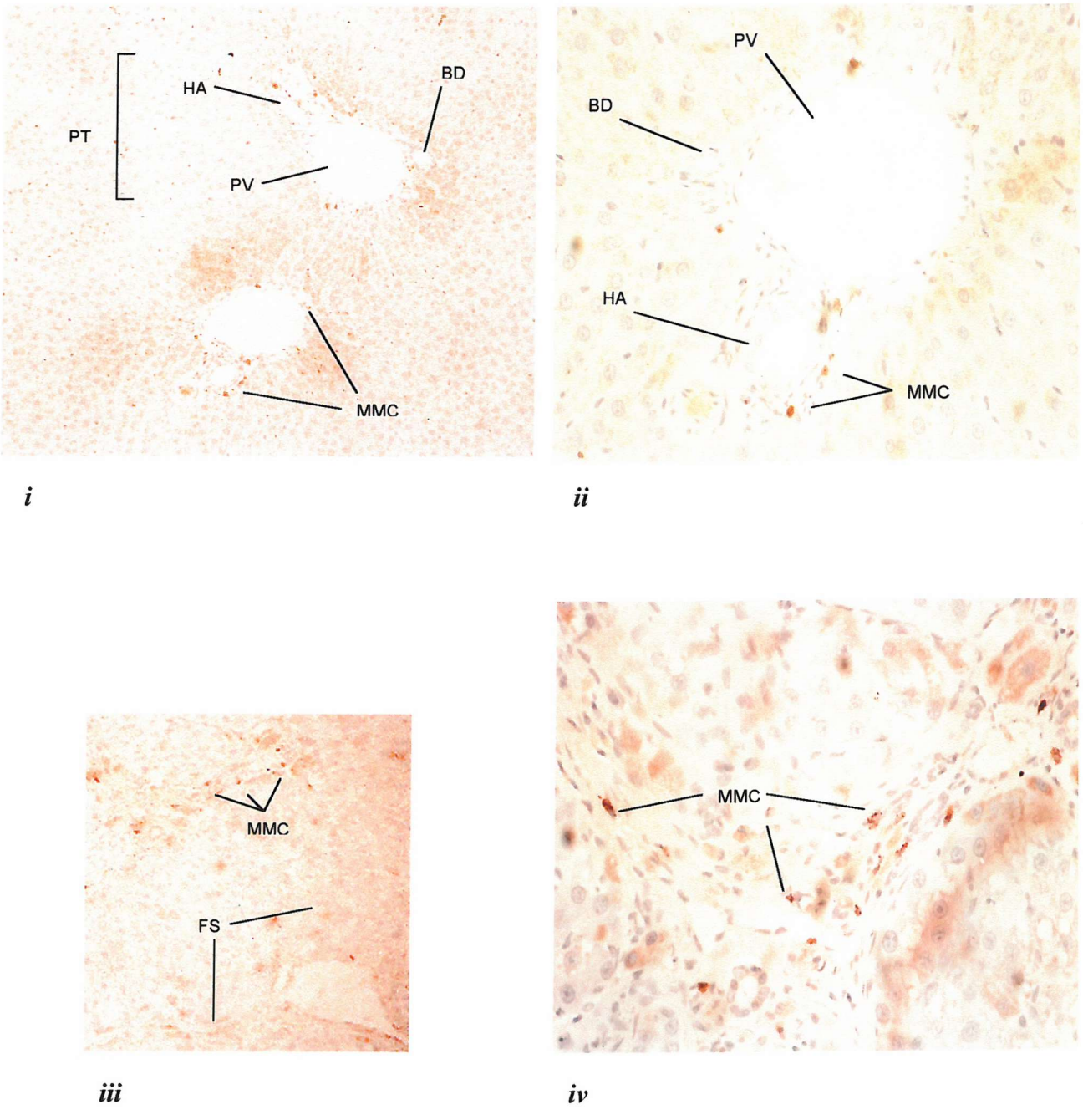


Figure 4.5. Strepavidin-biotin horseradish peroxidase complex RMCPII immunostaining in rat normal and CCl_4 fibrotic liver sections. **(i).** RMCPII staining in normal rat liver, x100 magnification. A few MC (MC) were observed within the connective tissue surrounding the portal triads (PT) of the liver. **(ii).** RMCPII staining in normal rat liver, x400 magnification. **(iii).** RMCPII staining in fibrotic rat liver, x100 magnification. MC were apparent within the fibrotic septae (FS) and within the fibrotic PT. **(iv).** RMCPII staining in fibrotic rat liver, x400 magnification. At this magnification MC could be clearly seen accumulating within the fibrotic PT.

4.2.3. THE MITOGENICITY OF MC MEDIATORS ON HSC IN CULTURE AS DETERMINED BY PROLIFERATION ASSAYS

MC mediators in fibrosis have been extensively studied and reported to have fibrogenic effects. Histamine released from MC has been shown to dose- dependently stimulate the proliferation of human skin and lung fibroblasts (Russell *et al,* 1977; Jordana *et al,* 1988). Tryptase has been shown to be a potent mitogen for several cell types including human lung fibroblasts, Chinese hamster lung and rat fibroblasts (Ruoss *et al,* 1991; Cairns and Walls, 1997; Gruber *et al,* 1997), smooth muscle cells, and epithelial cells (Cairns and Walls, 1996). However heparin has been reported to have anti-proliferative effects on fibroblasts (San Antonio *et al,* 1992). Initially the effect of various MC mediators on HSC proliferation was studied to examine the possibility that MC mediators might have pro-fibrotic effects in liver by increasing HSC number. Primary HSC were cultured on tissue plastic for 7 days to adopt a myofibroblastic phenotype. HSC were quiesced in D-MEM containing 0.5% FCS for 24 hours after which they were treated with various MC mediators for 24 hours, in triplicate. Proliferation was assessed by the incorporation of ^{3}H-thymidine. Due to different degrees of absolute amounts of ^{3}H- thymidine incorporation between separate experiments, data were expressed as a percentage of HSC proliferation in relation to control (0.5% FCS) treated HSC. The results were presented as mean $\pm$ SEM percentage proliferation of treated HSC relative to untreated HSC. Where applicable paired *t*-tests were performed and further confirmed using non-parametric Wilcoxon signed rank test, $p < 0.05$ was considered statistically significant.

4.2.3.1. Proliferative effect of tryptase on cultured HSC

Previously within our laboratories it was shown that purified human lung mast cell tryptase induced a 145% increase in human HSC proliferation at 3mU/ml compared to control treated HSC and 247% increase at 10 mU/ml, figure 4.6. (Benyon et al, 1997).

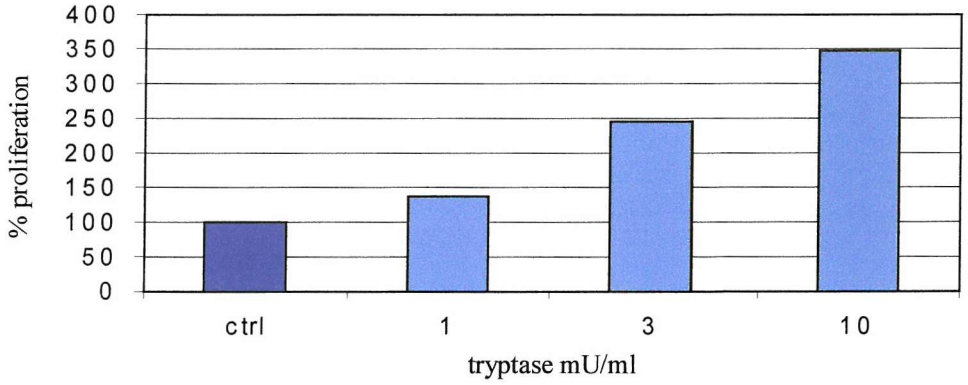
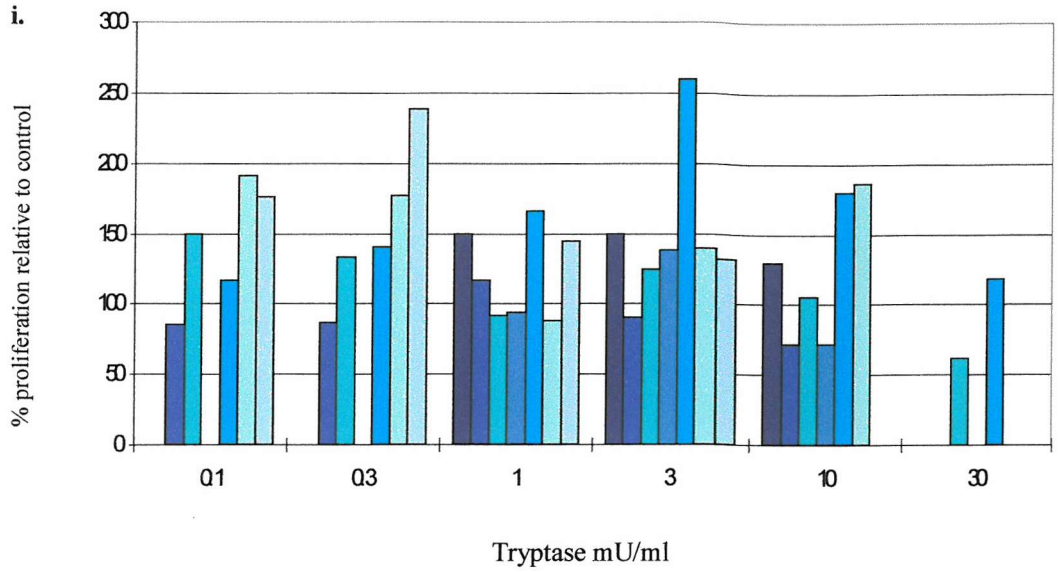


Figure 4.6. Proliferative effect of purified tryptase on cultured human HSC as assessed by ^{3}H- thymidine incorporation (n=2).

i.



ii.

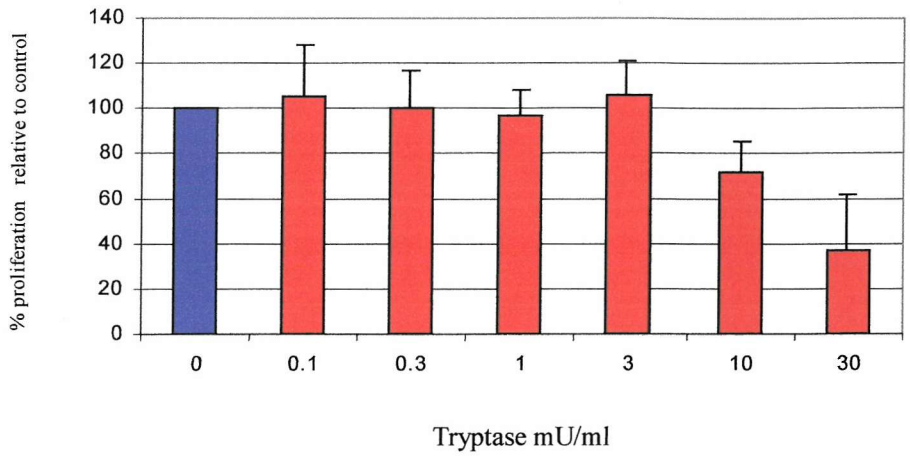


Figure 4.7. Proliferative effect of purified tryptase on cultured rat HSC. Results were plotted as percentage of HSC proliferation in presence of tryptase compared to untreated HSC. **i).** Each bar represents a different experiment (n=2 to 7), and clearly highlights the variability in response of HSC to treatment with this tryptase. **ii).** The results were averaged and plotted as mean ± SEM and this second graph shows that tryptase had no proliferative effect on HSC.

Therefore rat HSC were examined for proliferative response to purified tryptase. Donated purified human mast cell tryptase (Immunopharmacology Group, University Medicine, Southampton General Hospital) was used in proliferation studies at concentrations ranging from 0.1 mU/ml to 30 mU/ml . However as can be seen from figure 4.7, there was a great deal of variability of HSC response to this mediator. When the results were averaged and plotted as mean ± SEM, it was found that this donated tryptase had no proliferative effect on primary rat HSC, indeed at higher concentrations of the enzyme there was a trend to inhibition of HSC proliferation. This might be due to the high salt (2 M NaCl) content within the tryptase solution causing a dramatic decrease in HSC proliferation at 30 mU/ml concentration and could be resolved by subsequent dialysis of the enzyme in a low- salt buffer.

4.2.3.1.1. Purity of tryptase as assessed by silver staining

To assess the purity of the donated tryptase it was further examined by means of SDS-PAGE and silver staining, in parallel with purified tryptase (Biogenesis) which became commercially available after the above studies were done.

Tryptase samples were separated on 10% SDS-PAGE and protein was detected by silver staining. As can be seen from figure 4.8, there was a clear difference in purity between the two tryptase samples. The commercially available tryptase gave only a single band of approximately 33 kDa, the reported molecular weight of monomeric tryptase. However the donated tryptase appeared to have a variety of bands suggesting that there were contaminants within this preparation which might have caused the variability of HSC response to this tryptase when used in proliferation studies. Biogenesis tryptase was therefore used to reassess tryptase mitogenicity on HSC by means of further proliferation assays.

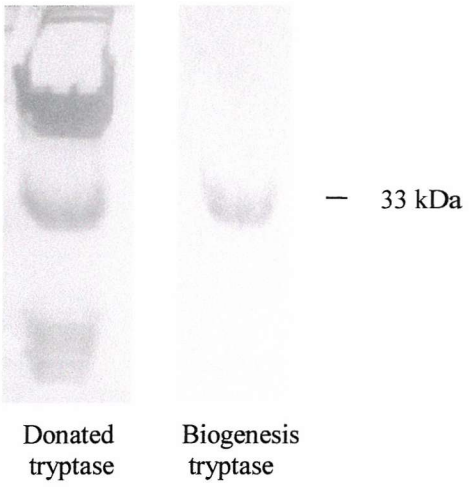


Figure 4.8. Tryptase from donated sources and Biogenesis tryptase were subjected to SDS-PAGE. By silver staining it can be clearly seen that in the donated tryptase sample contaminating bands are present, however in the Biogenesis tryptase only one clear band can be seen at 33 kDa representing the monomeric form of tryptase.

4.2.3.2. Proliferative effect of Biogenesis tryptase on cultured HSC

Enzymatic activity of Biogenesis tryptase was assessed by BAPNA hydrolysis as described in method 2.2.14., and was added to quiesced 7 day cultured HSC at concentrations ranging from 0.2 mU/ml to 6 mU/ml. As shown in figure 4.9, tryptase at concentrations ranging from0.2 mU/ml to 2 mU/ml gave significant increases in HSC proliferation as calculated using parametric Student's t-test and further confirmed using non-parametric Wilcoxon signed rank test. Results are shown as mean $\pm$ SEM of three to five independent

experiments carried out in triplicate. Maximal proliferation was observed at tryptase concentration of 0.6mU/ml giving 104% ± 26.23 ($p<0.05$) increase in HSC proliferation compared to control treated HSC.

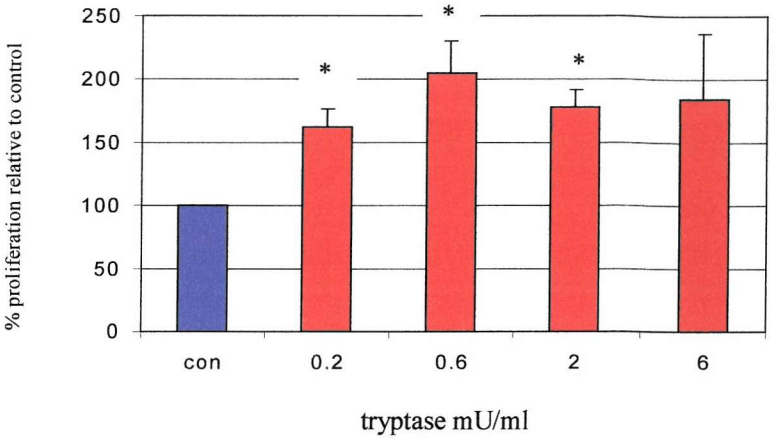


Figure 4.9. Graph showing proliferative effect of MC mediator tryptase (Biogenesis) on primary HSC (n= 3 to 5). *= $p<0.05$, paired *t*-test and non-parametric Wilcoxon signed rank test.

4.2.3.3. Proliferative effect of heparin and Biogenesis tryptase on cultured HSC

Heparin is found within the granules in MC. The main function of heparin in MC is to stabilise the tryptase tetrameric complex. Heparin alone has anti-proliferative effects on fibroblasts (San Antonio *et al,* 1992), whilst in conjunction with tryptase, may enhance the mitogenic effects of tryptase.

Heparin was added to tryptase (1 μg/ml to every 1mU/ml of tryptase), to see if this changed tryptase mitogenicity on HSC as previously assessed by ^{3}H- thymidine incorporation. As shown in figure 4.10i, at corresponding tryptase concentrations, addition of heparin did slightly increase the proliferative effect of tryptase (from 2 to 64% increase), however the effect was not statistically significant when compared to control untreated HSC and corresponding tryptase treated HSC. In parallel, heparin alone (0.1- 1000 μg/ml) was added to HSC to assess effects on HSC proliferation. As shown in figure 4.10ii, when heparin was added alone to HSC there was a dose- dependent inhibition of HSC proliferation, which became significant at 10 and 100 μg/ml, 63.8% ±9.4 ($p<0.05$) and 72.6% ±8.6 ($p<0.05$) respectively using Student's paired t-test. Confirmation of statistical significance was confirmed using non-parametric Wilcoxon signed rank test and confirmed the significant decrease of HSC proliferation at 10 μg/ml ($p<0.05$) (not enough data for

100 μg/ml heparin as n=3). At the lowest heparin concentration (0.01μg/ml) there was an overall 9% increase in proliferation when compared to 100% control treated HSC.

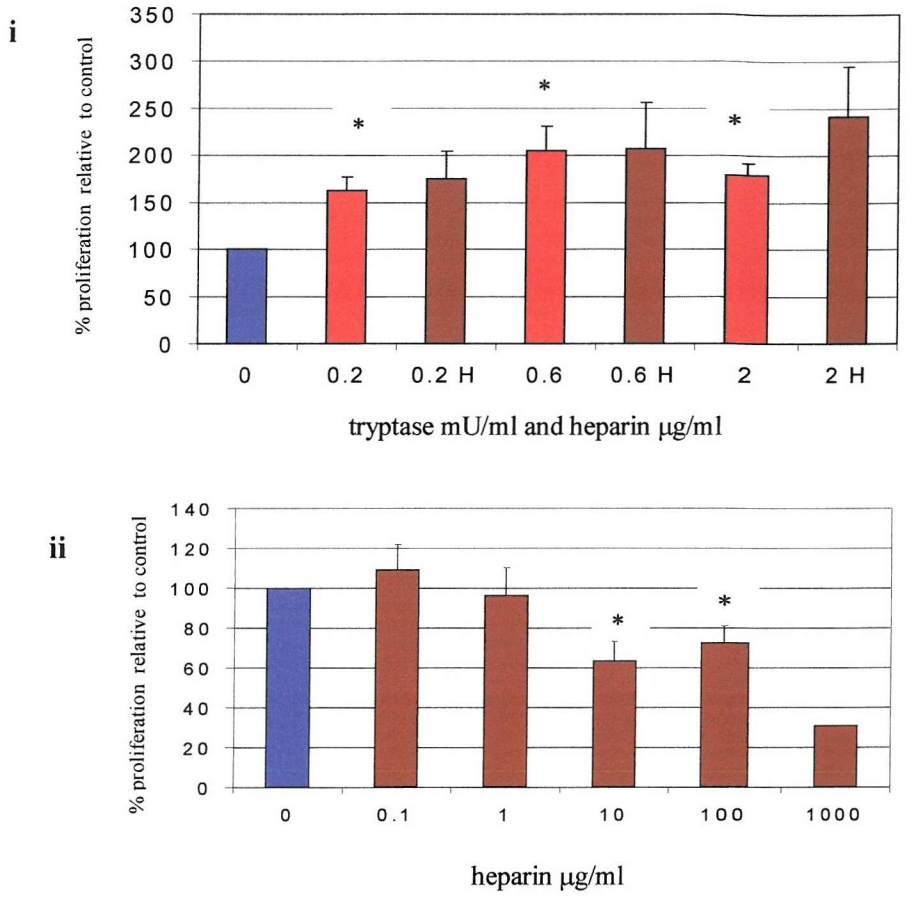


Figure 4.10. i). Graph showing the effect of tryptase and heparin on primary rat HSC (n= 3 to 5). 1 μg/ml of heparin was added to every mU/ml of tryptase. **ii).** Heparin was added alone to HSC, (n= 2 to 5). *=$p<0.05$, Student's paired *t*-test and Wilcoxon signed rank test.

4.2.3.4. Proliferative effect of histamine, IL-4 and TNF-α on HSC

Further MC mediators, histamine, IL-4 and TNF-α were added individually to HSC to see if they could induce proliferative effects on HSC as previously reported in fibroblasts (Russell *et al,* 1977; Jordana *et al,* 1988). Although histamine from MC has been shown to dose- dependently stimulate the proliferation of human skin and lung fibroblasts (Russell *et al,* 1977; Hatmochi *et al,* 1985; Jordana *et al,* 1988), it had little or no proliferative effects on HSC. Histamine (figure 4.11i), induced a dose- dependent inhibition of HSC proliferation, although this was not statistically significant. Maximal HSC proliferation was observed at the lowest histamine concentration of 0.1μg/ml and was 8% greater than that of control. The treatment of HSC with IL-4 also resulted in inhibition of HSC proliferation in a dose- dependent manner (figure 4.11ii). The lowest concentration of IL-4

(0.01 μg/ml) induced a significant 120% ±20 ($p<0.1$) increase in HSC proliferation when compared to control untreated HSC. However as the concentration of IL-4 increased, the proliferative response was reduced until it became 56% less then control at 100 μg/ml of IL-4. TNF-α however, figure 4.11iii, at all concentrations increased HSC proliferation, in a dose- dependent manner. Proliferation became significant ($p<0.1$, Student's *t*-test) at 10 ng/ml and 100 ng/ml having 36% ±16 and 73% ±34 increase in proliferation, respectively, when compared to 100% untreated control.

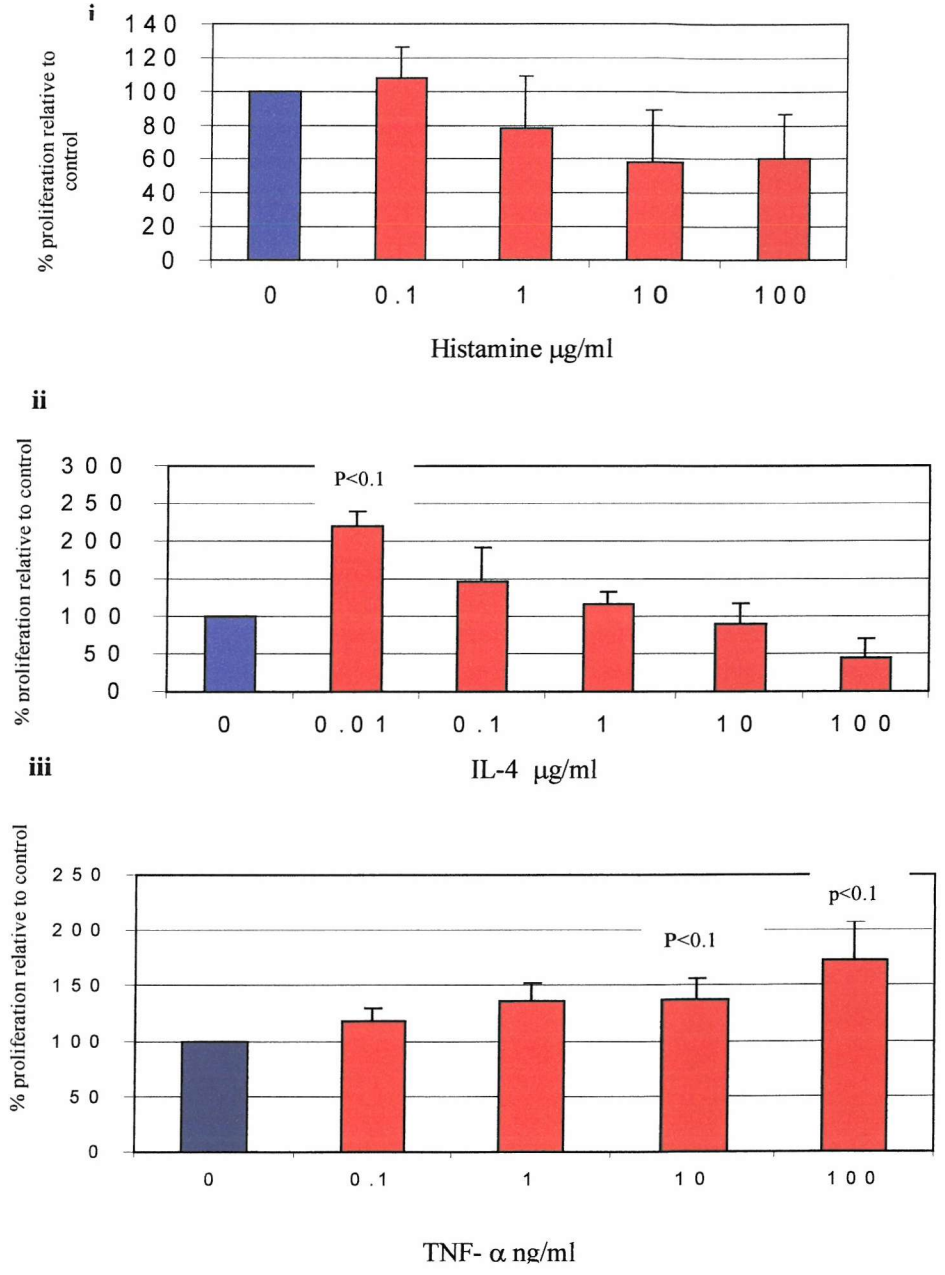


Figure 4.11. Graphs showing the proliferative effects of various MC mediators on rat HSC. **i).** The effect of histamine on HSC proliferation (n=3). **ii).** Effect of IL-4 on HSC proliferation (n=3). Maximal and significant proliferation was seen at 0.01μg/ml which gave 120% ±20 increase in proliferation compared to control. **iii).** Effect of TNF-α on HSC proliferation (n= 4 to 6). p=<0.1 Student's *t*-test.

4.2.4. DETECTION OF GELATINASE IN HSC CULTURE MEDIA BY ZYMOGRAPHY

During HSC activation, there is an increase in the protein and mRNA synthesis of MMPs, especially gelatinase A (Arthur *et al*, 1992). It has previously been reported that MC mediators such as tryptase and chymase may modulate the activation of MMPs such as collagenase (Gruber *et al,* 1988;Gruber *et al,* 1989;Lees *et al,* 1994), urokinase (Stack *et al,*1994), stromelysin (Lees *et al,* 1994) and gelatinase (Lohi *et al,* 1992; Fang *et al,* 1996 ; Fang *et al,* 1999). By the use of zymography, I have investigated whether various MC mediators such as heparin, histamine and MC tryptase can induce the activation of gelatinase A in cultured HSC.

Media were collected from HSC cultured in the presence of histamine, heparin and tryptase at different concentrations, for 24 hours in serum- free media and subjected to SDS-PAGE in 8% polyacrylamide gels containing 20 μg/ml gelatin as described in section 2.2.16. As a positive control, HSC were treated with 10 μg/ml of con A, an activator of pro-gelatinase A.

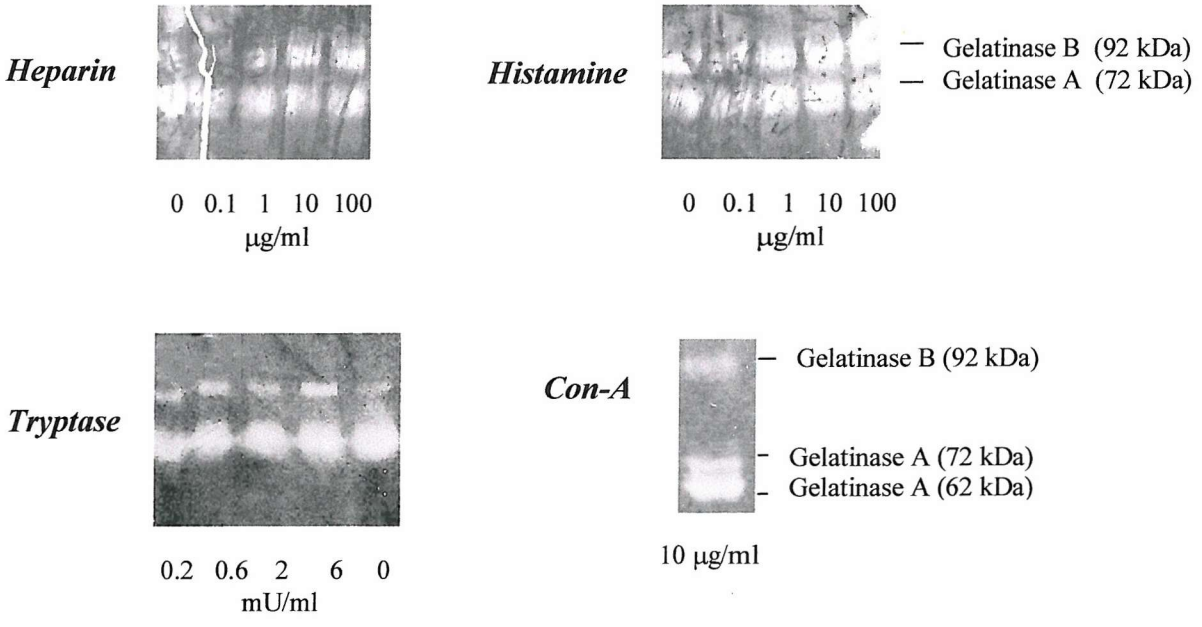


Figure 4.12. The detection of gelatinase activity in HSC cultured with various MC mediators by zymography.

From figure 4.12, it can be clearly seen that incubation of HSC with histamine, heparin or tryptase at varying concentrations did not have any effect on the activation of pro-gelatinase A (72 kDa). Only pro-gelatinase A was detected upon zymography. However, treatment of HSC with 10 μg/ml of con A clearly resulted in the activation of pro-gelatinase A (62 kDa).

4.2.5. STIMULATION OF COLLAGEN SYNTHESIS IN HSC BY TRYPTASE

Collagen synthesis in HSC was determined by the incorporation of ^{3}H- proline in HSC supernatants following incubation with 0.6 mU/ml tryptase or 10 ng/ml TGF-β as a positive control for HSC collagen synthesis (section 2.2.19). Tryptase increased collagen synthesis by 78% ± 68 in two independent experiments. TGF-β however induced a 130%± 49 ($p<0.05$, Student's *t*-test and Wilcoxon signed rank test) increase in HSC collagen synthesis in five independent experiments.

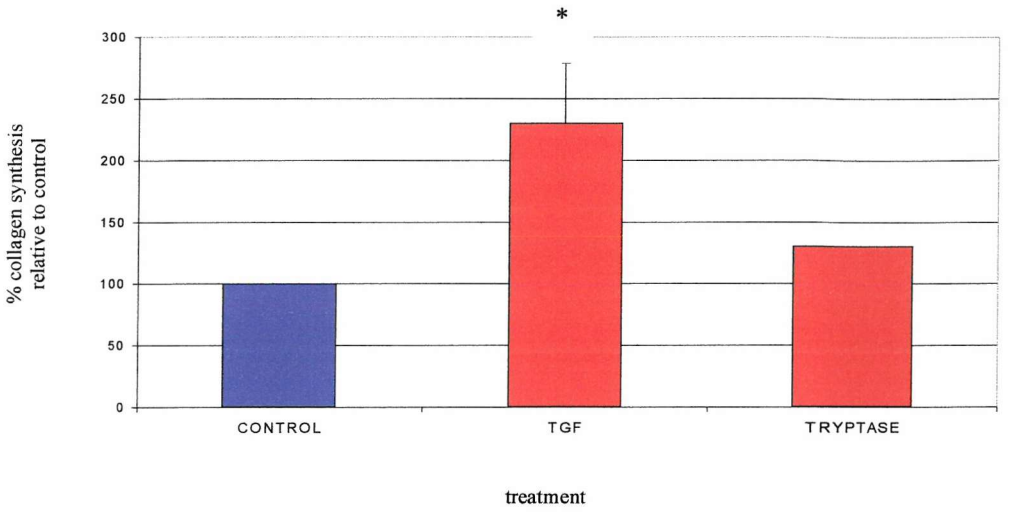


Figure 4.13. Collagen syntheisis in HSC stimulated with 0.6 mU/ml tryptase n=2 and TGF-β, n=5. *= $p<0.05$ Student's paired *t*-test and Wilcoxon signed rank test.

4.3. DISCUSSION

In fibrotic disease of many tissues MC hyperplasia is prevalent (Kawanami *et al,* 1979; Claman *et al,* 1990; Hunt *et al,* 1992; Irani *et al,* 1992; ; Li *et al,* 1992; Pesci *et al,* 1993; Peng *et al,* 1994; Xu *et al,* 1994; Farrell *et al,* 1995; Rioux *et al,* 1996; Ambrust *et al,* 1997; Nakamura *et al,* 1997; Ramos *et al,* 1997). However the actual role of MC in fibrosis has not yet been established.

In human cholestatic disease (primary biliary cirrhosis,) an eight- fold increase in MC numbers compared to normal livers has been observed. This MC hyperplasia has been correlated with collagen deposition in portal tracts (Farrell *et al,* 1995). Increased amounts of MC mediators have been detected in the circulation during liver disease implying MC activation in liver fibrosis. For example, in chronic cholestatic liver disease, plasma histamine levels are increased almost two-fold compared to normal levels (Gittlen *et al,* 1994). In fibrotic livers from *S. mansoni* infected rats, there is an increase in MC numbers and corresponding increase in TNF-α, which subsequently contributes to liver fibrosis (Brito *et al,* 1998). In cholestatic rats treated for 21 days a two fold increase in MC was reported which correlated with collagen deposition within the portal tracts assessed by Sirius Red staining (Rioux et al, 1996).

To complement previous studies examining the existence of MC in human liver disease by use of immunohistochemistry, I have examined the mRNA expression of MC proteases, tryptase and chymase, in human fibrotic and normal livers by RT-PCR. Expression of these proteases can be useful markers in assessing the type of MC recruited during liver injury as chymase is a biological marker for human MC heterogeneity. Tryptase mRNA, the major human MC protease found in both MC_T and MC_{TC} subpopulations, was detected in all fibrotic (primary biliary cirrhosis and primary sclerosing cholangitis) and normal liver samples suggesting that it is constitutively expressed in the liver. Chymase, the serine protease only present in MC_{TC} subpopulation, mRNA expression was also investigated by RT-PCR. Surprisingly chymase mRNA was detected in all but one of the fibrotic livers and in all but one of the normal livers. This suggests that MC_{TC} subpopulation of MC is present at all times within the liver, that is in normal and in fibrotic livers. However previous findings suggest that MC_{TC} are found in the liver during fibrosis (Ambrust *et al,* 1997). Further quantification of these proteases needs to be undertaken by quantitative PCR or Northern blotting to verify these findings.

As a continuation, the expression of rat MC protease mRNA, RMCPI and RMCPII, was investigated in the CCl_4 and BDL models of rat liver fibrosis by RT-PCR. As these

proteases are synthesised by different MC, that is RMCPI by CTMC, and RMCPII by MMC, expression of these proteases can be useful markers in assessing the type of MC recruited during liver injury. Previously it has been shown by immunohistochemistry and ELISA that in the bile duct ligation rat model of liver fibrosis, MMC predominate (Rioux *et al,* 1996), whilst in diethylnitrosamine (DEN) induced hepatocarcinogenesis CTMC predominate (Ruan *et al,* 1992). By RT-PCR RMCPI mRNA was weakly detected in all treated and control livers at all time points in CCl_4 and in BDL livers. However in CCl_4 and BDL livers at the latest time-points, RMCPI mRNA expression appeared to be upregulated. RMCPII mRNA however was detected in all CCl_4 treated and control livers but only in day 7 of BDL livers. These results suggest that in CCl_4 livers both MMC and CTMC sub-types are present. Moreover RMCPII mRNA was easily detected in normal and fibrotic livers suggesting that MMC are constitutively present in the liver. In BDL livers it appeared that RMCPI mRNA was weakly expressed in normal and fibrotic liver, suggesting a constant CTMC presence within the liver. RMCPII mRNA was found to be expressed only in 7 day livers that indicate MMC are upregulated in rat cholestatic liver disease. Nevertheless, further examination of these protease mRNAs by quantitative PCR or Northern blotting are needed to verify these findings.

Immunohistochemistry of CCl_4 rat livers using antibodies to RMCPI and RMCPII showed CTMC and MMC within normal and CCl_4- induced fibrotic rat liver and is in agreement with previous findings (Rioux *et al*, 1996; Ambrust *et al*, 1997) In the normal rat liver, a small number of both CTMC and MMC were found distributed around the hepatic portal tracts within the connective tissues. In CCl_4- induced fibrotic rat livers, both CTMC and MMC were apparent in greater numbers within the fibrotic septae and within the connective tissue of the portal tracts. Therefore these results imply that MC are present and accumulate at the site of increased connective tissue formation during liver fibrosis. MC mediators are mitogenic for many cells including fibroblasts. Histamine induces human skin and lung fibroblast proliferation (Russell *et al,* 1977; Jordana *et al,* 1988) whilst tryptase has been reported to be a potent mitogen for human lung fibroblasts, Chinese hamster lung and rat fibroblasts (Rouss *et al,* 1991; Cairns and Walls, 1997; Gruber *et al,* 1997. As activated HSC adopt many features of fibroblasts, the effects of MC mediators on HSC were examined by means of proliferation assays. Previous data from our laboratory showed that tryptase at concentrations of 3 mU/ml and 10 mU/ml induced 145% and 247% increases in human HSC proliferation, respectively (Benyon et al, 1997). Consequently, the effect of tryptase on rat HSC was initially studied. Donated tryptase appeared to give variable responses in HSC proliferation. This may have been a result of

contaminants within the tryptase preparation causing a dramatic inhibition of HSC proliferation at high concentrations, 30 mU/ml. Further analysis of this tryptase by SDS-PAGE and silver staining proved that this tryptase contained several contaminants which also could have influenced HSC responses to tryptase. However use of commercially available tryptase (Biogenesis) was seen to induce a significant and maximal 104% ±26.23 ($p<0.05$) increase in HSC proliferation at 0.6 mU/ml when compared to untreated HSC control. The effect of heparin in combination with tryptase and alone was also investigated. With tryptase, heparin enhanced HSC proliferation, however was not statistically significant when compared to untreated HSC or HSC treated with tryptase. Heparin alone appeared not to induce any HSC proliferation, indeed it induced a dose-dependent inhibition of HSC proliferation. At low concentrations of heparin, 0.01 μg/ml, an overall 9% increase in proliferation was observed whilst at higher concentrations proliferation was reduced until proliferation was less than half of untreated HSC control (1000 μg/ml). Histamine and IL-4 induced a maximal 8% and 120% ±20 ($p<0.1$)increase in HSC proliferation respectively at 0.1 μg/ml and 0.01 μg/ml, but at all other concentration induced a dose- dependent decrease in HSC proliferation. The fibrogenic cytokine TNF-α induced proliferation at all concentrations, 0.1 to 100 ng/ml, that resulted in maximal proliferation 73% ± 34 ($p<0.1$) compared to untreated HSC control. These results suggest that not all MC mediators play a role in HSC proliferation. However these mediators were added to HSC cultures individually and collectively may have different effects.

Using ^{3}H- proline- incorporation assays followed by collagenase digestion, tryptase was found to stimulate a 78% increase in collagen synthesis in HSC relative to control untreated HSC in two independent experiments. This increase in HSC collagen synthesis was not as great as TGFβ stimulated HSC, used as a positive control, that gave significant increase of 112.8%±48 ($p<0.05$) in collagen synthesis. These results suggest that tryptase released from activated MC may also upregulate collagen synthesis in activated HSC during liver disease.

The ability of these MC mediators to induce activation of gelatinase A, an MMP expressed in activated HSC (Benyon *et al,* 1999) was investigated by zymography. However various concentrations of these mediators incubated with HSC did not cause the subsequent activation of pro-gelatinase A, as confirmed by con A treated HSC.

Previous studies have detected the increased expression of MC and MC mediators in liver disease. In this chapter I have confirmed these findings as I have shown by RT-PCR and immunocytochemistry of rat livers, that MC are present within normal and fibrotic livers. This study has shown that the MC mediators TNF-α and tryptase influence HSC

proliferation. Tryptase has been detected in liver fibrosis and can induce mitogenic effects on HSC as previously reported (Benyon *et al,* 1997).

In conclusion, MC are present in the liver during fibrosis in areas of increased connective tissue deposition. The true role of MC in fibrosis, especially liver fibrosis has yet to be identified. However the increase in HSC proliferation and collagen synthesis as a direct result of tryptase stimulation, may be an important mechanism in the activation of HSC and the development of liver fibrosis.

CHAPTER FIVE
PROTEINASE ACTIVATED RECEPTOR-2 EXPRESSION IN HEPATIC STELLATE CELLS AND LIVER DISEASE

5.1. INTRODUCTION

Preceding this chapter, I have shown that activated HSC synthesise SCF which may contribute to the recruitment of MC to the liver during injury. By various methods I have examined the expression of MC proteases in liver injury and investigated the proliferative effect of various MC mediators on quiescent HSC. The MC mediator tryptase was shown to induce proliferation of quiescent HSC and increase collagen synthesis. These actions of tryptase on HSC might contribute to liver fibrogenesis. It has recently been suggested that tryptase mediates its effect via a 'novel' receptor, namely proteinase activated receptor-2 (PAR-2) (Corvera *et al,* 1994; Nystedt *et al,* 1994; Molino *et al,* 1997). In this chapter I have examined the possible mechanism of HSC proliferation and collagen synthesis by tryptase by examining the expression of PAR-2 as an intermediary for tryptase action on HSC. PAR-1, the first member of the family of proteolytic activated receptors has previously been discovered in the liver and its expression during human cholestatic liver disease is upregulated (Marra *et al,* 1998). By use of *in- situ* hybridisation and immunostaining, PAR-1 was localised to liver endothelial cells, infiltrating monocytes and most importantly activated HSC. I have investigated the expression of PAR-2 by various techniques and examined a possible signal transduction pathway associated with activation of PAR-2 using the rat PAR-2 activating peptide (SLIGRL) on HSC in culture. In parallel to these studies I have examined the expression of PAR-1 and the effect of the PAR-1 activating peptide (SFFLRN) on cultured HSC.

5.2. RESULTS

5.2.1 PAR-2 mRNA EXPRESSION IN RAT EXPERIMENTAL MODELS OF FIBROSIS BY RT-PCR

Initially it was necessary to investigate the mRNA expression of PAR-2 in rat normal and fibrotic liver homogenates. By use of published rat PAR-2 primers targeted to new amino-terminal domain (Saifeddine *et al,* 1996), that is from the protease cleavage site to the second extracellular loop of the transmembrane spanning region, expression of this serine protease receptor in rat CCl_4 development model and bile duct ligation model of liver was examined by RT-PCR. As can be seen in figure 5.1, PAR-2 mRNA was detected in early and late stages of both fibrosis models. However in the later stages of CCl_4 development (4

weeks) the expression PAR-2 mRNA appeared to be upregulated, as can be compared with β-actin expression.

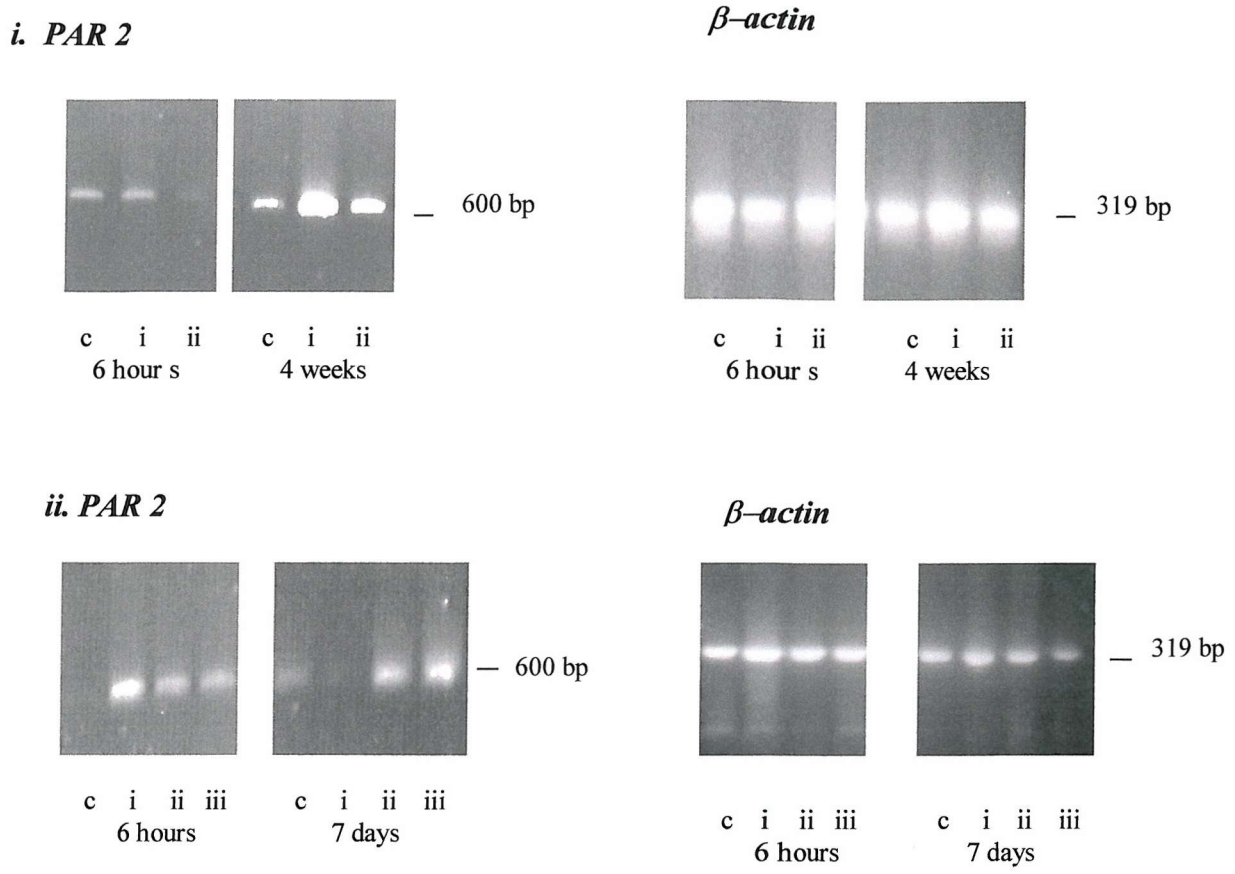


Figure 5.1 Representative one of three RT-PCRs detecting PAR-2 mRNA in CCl_4 development model of fibrosis (**i**) and bile duct ligation model of fibrosis (**ii**). β–actin PCR confirmed the integrity of the cDNA samples.

5.2.2. PAR-1 AND PAR-2 mRNA EXPRESSION IN RAT HSC BY RT-PCR

Following the successful detection of PAR-2 mRNA expression in rat livers, PAR-2 mRNA expression was further investigated in HSC by RT-PCR. Using the same PAR-2 primers in section 5.2.1, PAR-2 mRNA expression was examined in culture- activated rat HSC at day 0, 7, 14 and passaged HSC (P1). 3T3 and RSF cells were used as positive control cells, expressing PAR-2. As shown in figure 5.2, a 600 bp product, as reported in the literature, was clearly detected in the positive control 3T3 and RSF cells. This 600 bp product was also detected in all the cultured HSC samples. Upon progressive activation of HSC in culture (day 0 to day 14) it appears that the signal for PAR-2 mRNA increased. PAR-1 mRNA expression was also examined in parallel in culture-activated HSC, by use of published rat PAR-1 primers (Saifeddine *et al,* 1996). Expression of PAR-1 mRNA was similar to that of PAR-2. A reported PCR product of 400 bp was detected in quiescent and culture-activated HSC as well as positive control RSF and 3T3 cells.

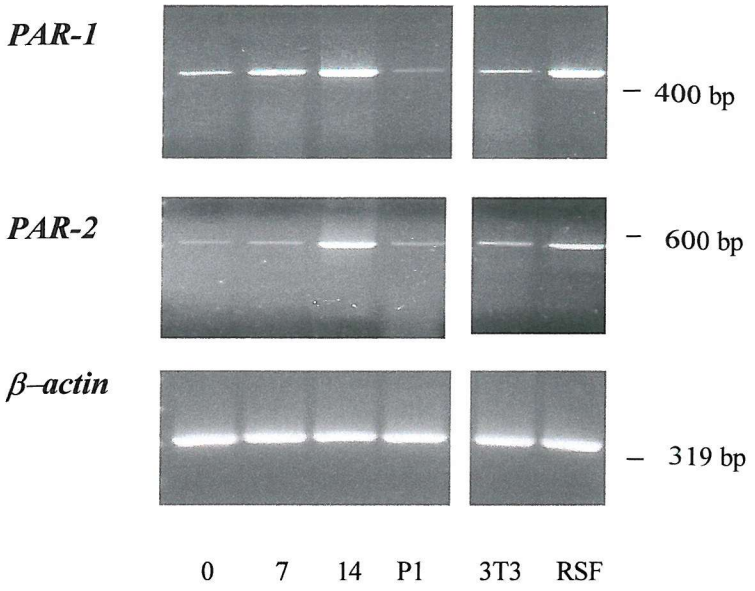


Figure 5.2. Representative of three RT-PCRs detecting PAR-1 and PAR-2 mRNA in cultured rat HSC. β-actin PCR indicates the integrity of the cDNA samples used.

5.2.3.DETECTION OF PAR-1 AND PAR-2 mRNA IN RAT HSC BY NORTHERN BLOTTING

RNA isolated from rat culture-activated HSC (0, 3, 7 and 14 days) was immobilised on a nylon membrane, and hybridised with a [α- ^{32}P] -radioactive random-primed probe made from purified PAR-1 and PAR-2 PCR products. A 3.5 kb product , was detected in all HSC samples and in positive control RSF and was in accord with the reported molecular weight of PAR-1 (McNamara *et al,* 1993). Expression of PAR-1 mRNA was upregulated with activation of HSC on culture (figure 5.3). PAR-2 mRNA however was not detected in RSF or HSC time course samples. As a positive control, membranes were probed with a [α- ^{32}P] -radioactive random-primed β-actin probe made from purified β-actin PCR products. β-actin was clearly detected in all the rat HSC samples and RSF.

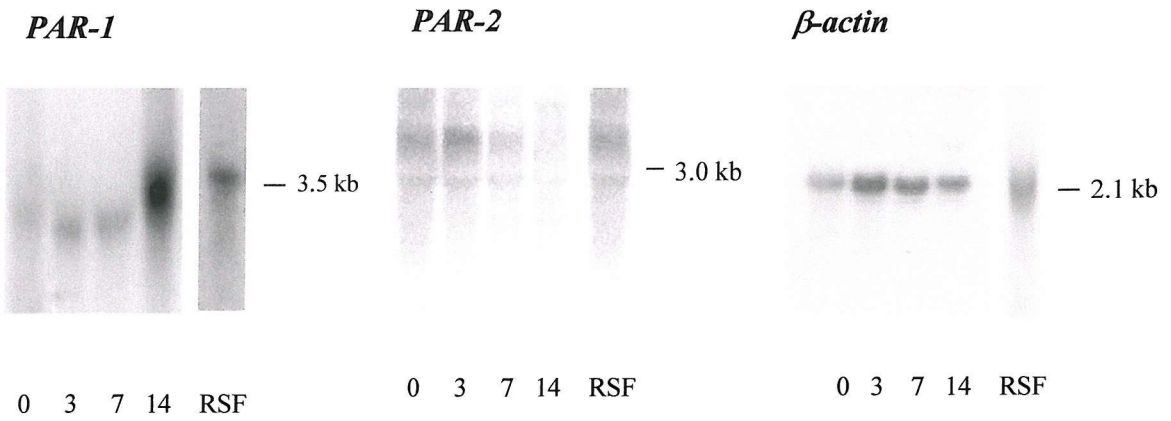


Figure 5.3. Northern blotting analysis to detect PAR-1 and PAR-2 mRNA in rat HSC. PAR-1 mRNA expression was upregulated in culture-activated HSC. PAR-2 mRNA was undetected in rat HSC samples. β-actin, a control probe, was however detected in all HSC samples. RSF were used as positive controls for PAR-1 and PAR-2.

5.2.4.DETECTION OF PAR-2 PROTEIN IN RAT HSC BY WESTERN BLOTTING

PAR-2 protein expression was examined in cultured rat HSC by immunodetection. Equal amounts of HSC whole cell lysates (day 21 and passage 2 (P2)) and positive control cell lysates (3T3 and RSF) were subjected to electrophoresis on a 12% SDS- polyacrylamide gel and subsequent transfer of proteins to PVDF membranes by Western blotting. Membranes were probed using an in-house synthesised rabbit polyclonal PAR-2 antibody raised to synthetic peptides corresponding to the activation site of human PAR-2 (a gift from Dr S. Compton, University Medicine, Southampton General Hospital, UK) at a dilution of 1:100 and by use of chemiluminescence PAR-2 was detected. 2 bands were detected at 45 kDa and 42 kDa (figure 5.4), in HSC samples and in positive control samples. These bands were consistent with the reported molecular weight of the two forms of PAR-2, the higher band the inactivated receptor and the lower band the activated and cleaved PAR-2 on the surface of the cell. To test that these bands were not artefacts, the same blot was probed with a non- immune IgG as a negative control. As expected for a negative control, no apparent bands were seen corresponding to those obtained with the polyclonal PAR-2 antibody.

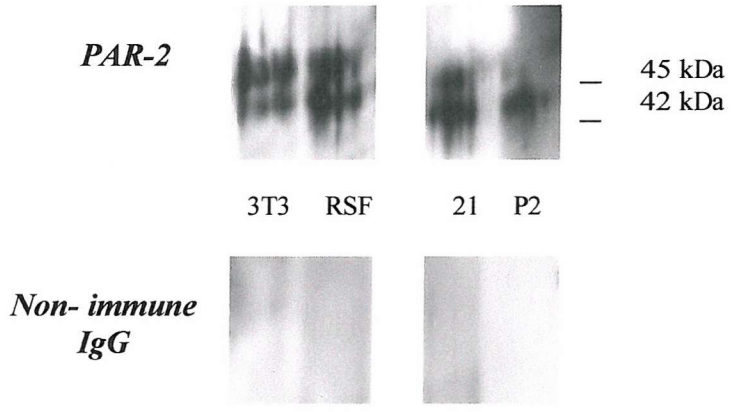


Figure 5.4 PAR-2 protein expression in rat HSC detected by Western blotting using a polyclonal PAR-2 antibody. A double band correlating to the reported mw of inactivated and activated receptor was seen in HSC samples and in positive control samples.

5.2.5. IMMUNOSTAINING OF PAR-2 PROTEIN IN CULTURED HSC

Three stage immunocytochemistry was employed to determine the presence of PAR-2 protein in culture- activated rat HSC. As described above in section 2.2.9, passaged HSC were stained for PAR-2 using a monoclonal anti- human PAR-2 antibody. Slides were viewed using a Leica light microscope. Cells were independently stained with anti-SMA antibody as a positive control and mouse non- immune IgG as a negative control. Staining of rat HSC against α-SMA as a positive control resulted in a cytoplasmic fibrillar pattern of staining within the whole HSC population (figure 5.5i). PAR-2 staining however was

more diffuse than the α-SMA staining but was present in rat HSC (figure 5.5iii). This could be clearly seen at higher x400 magnification (figure 5.5iv). The negative control non- immune IgG staining resulted in only the HSC nuclei faintly staining blue from the Harris haematoxylin counter-stain (figure 5.5ii).

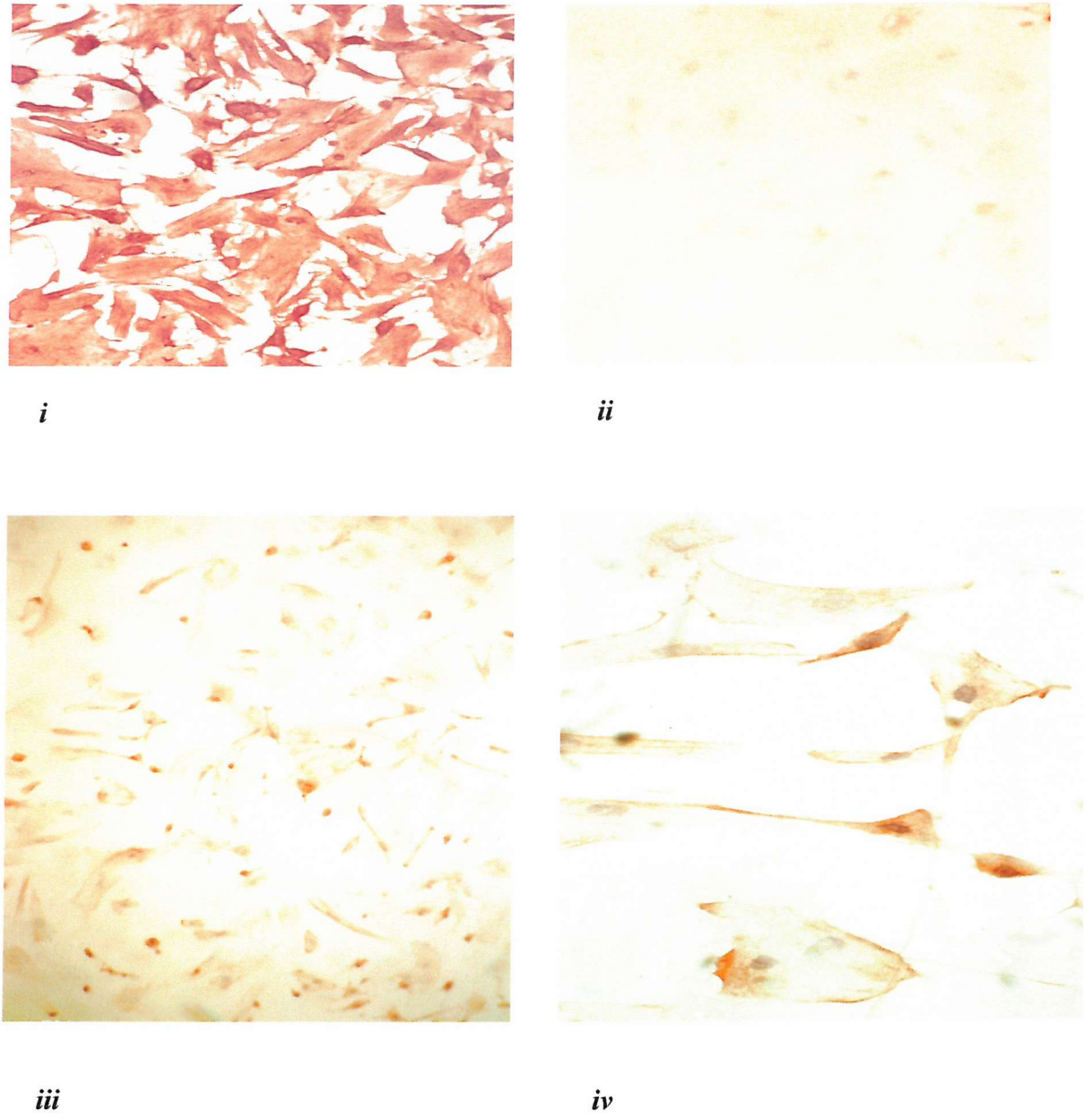


Figure 5.5. Strepavidin- biotin horseradish peroxidase complex immunostaining of PAR-2 protein in cultured HSC. **(i)** Positive control α-SMA immunostaining, x100 magnification. **(ii)** Non- immune IgG staining of rat HSC as negative control, x100 magnification. **(iii)** PAR-2 immunostaining using a monoclonal anti- human PAR-2 antibody, x100 magnification. **(iv)** PAR-2 immunostaining using a monoclonal anti-human PAR-2 antibody, x400 magnification.

5.2.6. PROLIFERATIVE EFFECT OF PAR-1 AND PAR-2 AGONISTS ON HSC IN CULTURE

The proliferative effect of trypsin on HSC, a natural agonist of PAR-2 (Nydstedt *et al,* 1994) and the rat PAR-2 activating peptide (SLIGRL), thrombin the natural agonist of PAR-1 and the rat PAR-1 activating peptide (SFFLRN) were examined as described in section 2.2.16. Briefly, HSC were cultured on tissue plastic for 7 days to develop a myofibroblastic phenotype. HSC were quiesced in D-MEM containing 0.5% FCS for 24 hours after which they were treated with trypsin, SLIGRL, thrombin and SFFLRN at various concentrations for 24 hours in triplicate. Proliferation was assessed by the incorporation of ^{3}H- thymidine. Due to absolute amounts of ^{3}H- thymidine incorporation between separate experiments, data was expressed as a percentage of HSC proliferation in relation to control (0.5% FCS) treated HSC. The results were presented as mean ± SEM of percentage proliferation of treated HSC in relation to untreated HSC. Where applicable Student's paired t-tests with $p=< 0.05$ were considered statistically significant.

It was found that trypsin at doses from 1μM to 1000 μM, induced concentration related proliferation of HSC which was significant ($p<0.1$) at 1 μM (17% ±7.3 increase in proliferation) and which became maximal at 1000 μM inducing 45%±29 increase in proliferation (figure 5.6).

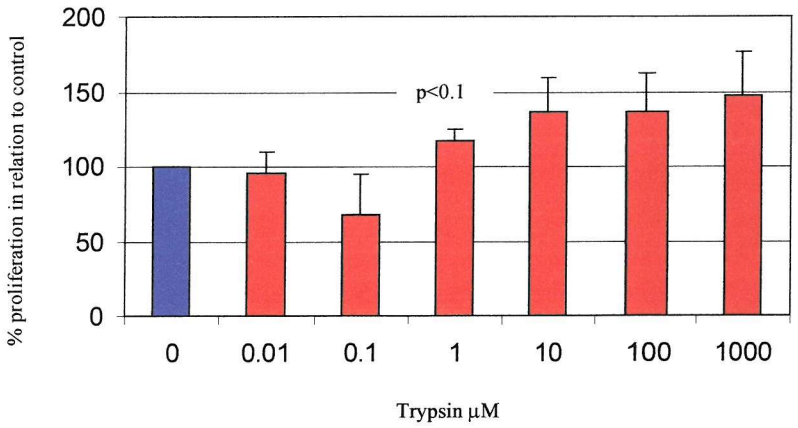


Figure 5.6. Graph showing proliferative effect of trypsin on HSC as detected by thymidine incorporation. Concentrations of trypsin 1μM and above induced HSC proliferation. Results plotted as mean ± SEM of n=4. *= $p<0.1$, Student's *t*-test.

The rat PAR-2 activating peptide, SLIGRL, corresponding to the first six amino acids of the new amino- terminal of PAR-2, (section 1.9.2) was added at various concentrations ranging from 1 μM to 100 μM to quiescent HSC. SLIGRL induced an increase in HSC proliferation at all concentrations ranging from a 19.5%±20.6 to 126%±40.7 increase.

SLIGRL concentration of 30 μM gave the maximal significant increase in proliferation 126%±40.7 ($p<0.05$) by Student's *t*-test and Wilcoxon signed rank test. Results are shown as mean ± SEM of 4 to 6 independent experiments carried out in triplicate (figure 5.7).

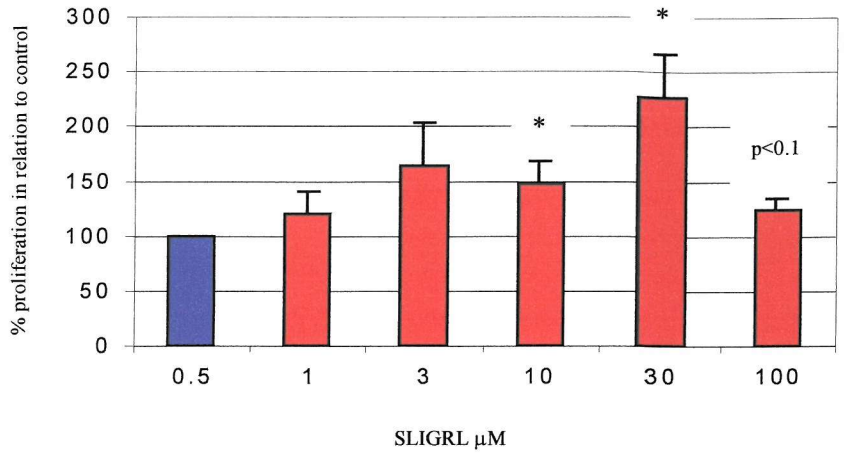


Figure 5.7. Graph showing proliferative effect of PAR-2 activating peptide on primary HSC as detected by thymidine incorporation. All concentrations of PAR-2 activating peptide induced a proliferative response. 30 μM gave a 220% significant increase in HSC proliferation, n=4 to 6. *= $p<0.05$, Student's t-test and Wilcoxon signed rank test.

In parallel to these studies the proliferative effect of the natural and synthetic agonist, thrombin and the hexapeptide SFFLRN respectively, were examined on cultured HSC. Thrombin has previously been shown to induce up to a fourfold increase in human HSC proliferation (Marra *et al,* 1995). In rat HSC, thrombin at concentrations from 0.2 mU/ml to 4 mU/ml induced concentration related HSC proliferation (figure 5.8). Maximal proliferation was observed at 0.4 mU/ml in which there was a 96% ±26 increase in proliferation compared to untreated control HSC.

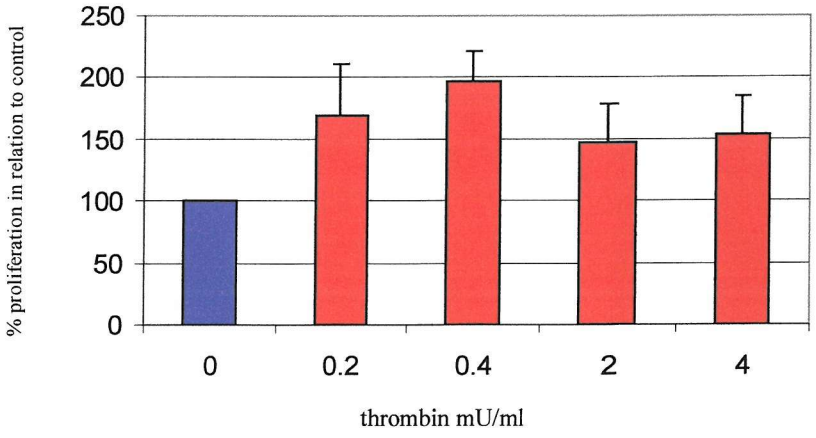


Figure 5.8. Graph showing proliferative effect of thrombin on primary HSC as detected by thymidine incorporation. All concentrations of thrombin induced HSC proliferation, n=3.

The rat PAR-1 activating peptide, SFFLRN, corresponding to the first six amino acids of the new amino- terminal of PAR-1, (section 1.9.2) was added at various concentrations ranging from 1 μM to 100 μM to quiescent HSC. SFFLRN induced significant increases in HSC proliferation at all concentrations ranging from a 45%±11 ($p<0.05$) to 120%±10 ($p<0.05$)increase (figure 5.9). SFFLRN concentration of 30 μM gave the maximal significant increase in proliferation 123%±21 ($p<0.05$). Results are shown as mean ± SEM of 4 to 6 independent experiments carried out in triplicate and analysed by Student's *t*-test and confirmed using non-paramentric Wilcoxon signed rank test..

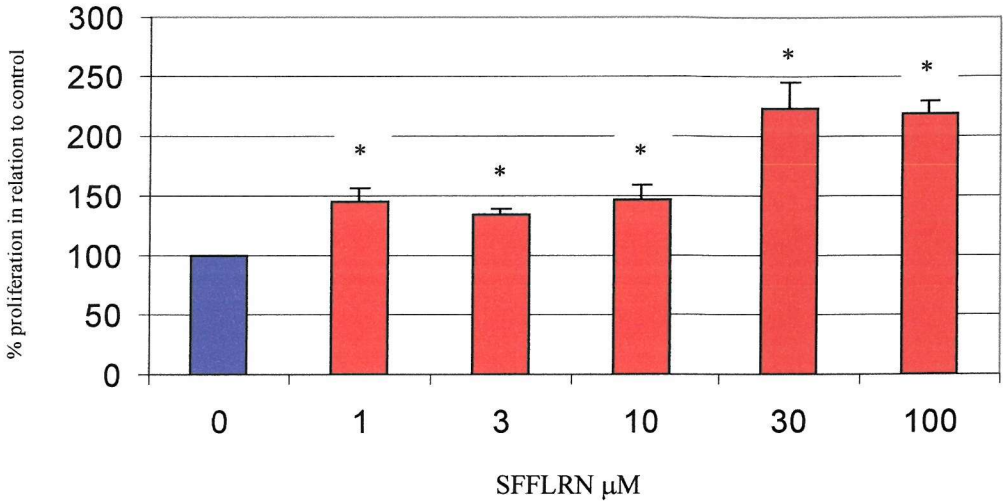


Figure 5.9. Graph showing proliferative effect of PAR-1 activating peptide, SFFLRN on primary HSC as detected by thymidine incorporation. All concentrations of SFFLRN induced HSC proliferation, n=4 to 6. *=$p<0.05$, Student's *t*-test and Wilcoxon signed rank test.

5.2.7. IMMUNODETECTION OF MAPK ACTIVITY IN HSC STIMULATED BY TRYPTASE, THROMBIN, SFFLRN AND SLIGRL

MAPKs are a family of serine/ threonine- specific protein kinases believed to be involved in a number of cellular responses including cell proliferation. Thrombin, PAR-1 and PAR-2 peptide agonists are known to activate MAPK ERK1/ERK2 in various cell types (Belham *et al,* 1996; Molloy *et al,* 1996). Using a specific antibody for the activated phosphorylated forms ERK1/ERK2, MAPK protein expression was examined in stimulated HSC by immunoblotting. MAPK protein expression was quantified by densitometric analysis using Scion Image program by Scion Corporation, USA. Results were plotted as the mean ±SEM percentage change in MAPK protein expression in treated HSC compared to untreated control HSC.

Briefly, HSC were cultured on 12 well plates until they became activated, quiesced for 48 hours in serum- free media and then stimulated with 16% FCS, tryptase, thrombin, SFFLRN or SLIGRL. Equal cell equivalents of stimulated HSC (0.5 μg DNA) were resolved by SDS- PAGE (10% gel) and transferred to PVDF membranes. Immunoblotting was performed as described in section 2.2.9 and 2.2.18, using a polyclonal anti-MAPK antibody at 1:10, 000 dilution. PD98059, a specific inhibitor of ERK1/ERK2 was added to quiesced cells, 30 minutes prior to stimulation, to confirm activation of MAPK by PAR-2 peptide. PD98059 works by binding to the inactive form of mitogen activated protein kinase kinase (MEK) preventing its activation by *Raf* and other upstream activators which subsequently prevents the activation and phosphorylation of ERK1/ERK2. PD98059 however does not inhibit MEK once this molecule is already activated (Alessi *et al,* 1995).HSC stimulated with 16% FCS in identical conditions as the PAR-2 peptide, were used as positive controls for MAPK induction.

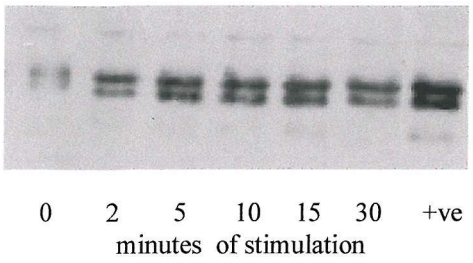
To test the induction of MAPK in HSC, as a positive controlled experiment, HSC were initially stimulated with 16% FCS. By use of chemiluminescence, 2 bands were detected at 44 and 42 kDa and were consistent with the reported molecular weight of the forms of activated ERK1/ERK2 (figure 5.10). HSC treated with 16% FCS stimulated ERK1/ERK2 in a time- dependent manner. By immunoblotting, ERK1/ERK2 were maximally stimulated after 15 minutes of HSC exposure to 16% FCS. At 0 and 10 minutes, activation of the MAPK increases. However at 20 and 30 minutes of FCS stimulation, this activation decreases slightly. These results were quantified and illustrated by densitometric analysis.

Figure 5.10. Activation of ERK1/ERK2 by 16% FCS on HSC as detected by Western blotting and quantified by densitometry, n=1.

HSC treated with the PAR-2 activating peptide, SLIGRL had a similar pattern of MAPK activation, figure 5.11i. At 0 and 2 minutes activation of ERK1/ERK2 gradually increases. Between 5 and 15 minutes of stimulation, ERK1/ERK2 activation reaches a maximum and decreases at 30 minutes. However PAR-2 agonist stimulation did not activate ERK1/

ERK2 as much as the positive control 16% FCS. Densitometric analysis of three independent experiments confirmed this pattern of MAPK activation, figure 5.11.ii. MAPK expression reached a maximum at 15 minutes of SLIGRL stimulation, 169.6%±64 increase compared to untreated HSC control.

i



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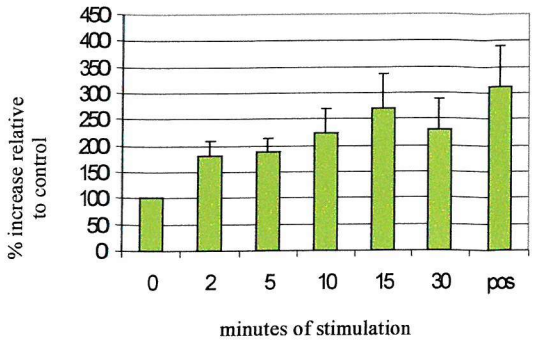

Figure 5.11. i. Representative one of three independent Western blots examining the upregulation of ERK1/ERK2 by 30 μM PAR-2 peptide (SLIGRL) on HSC. HSC were stimulated with 30 μM SLIGRL for the times indicated and MAPK protein expression was examined by Western blotting. +ve= HSC stimulated for 15 minutes with 16% FCS. **ii.** Graph illustrating activation of MAPK by SLIGRL as analysed by densitometry, n=3.

From the last two experiments it was evident that MAPK activation was maximal at 15 minutes of stimulation. Therefore quiescent HSC were stimulated for 15 minutes with optimum concentrations of mediators as previously assessed by proliferation assays; 0.6 mU/ml tryptase, 30 μM SLIGRL, 0.4 mU/ml thrombin and 30 μM SFFRLN. 16% FCS was used as a positive stimulus whilst untreated HSC as a negative control. From figure 5.12i, tryptase, the rat PAR-2 activating peptide, thrombin and the rat PAR-1 activating peptide induced ERK1/ERK2 activation. However this activation was not as great as stimulus with 16% FCS. This pattern of expression could be clearly seen illustrated when analysed by densitometry, figure 5.12.ii.

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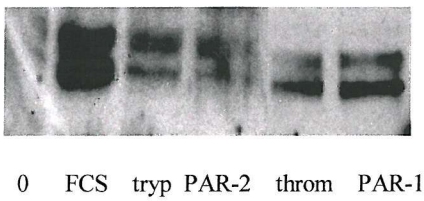

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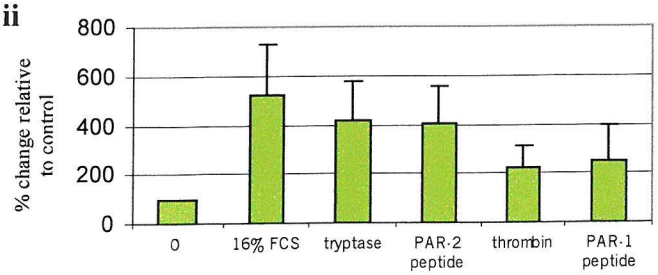

Figure 5.12. i. Representative one of three Western blots examining the activation of ERK1/ERK2 by various mediators, **ii.** Graph illustrating activation of MAPK by various mediators as analysed by densitometry, n=3.

PD98059, a specific inhibitor of ERK1/ERK2 was added to HSC to confirm activation of MAPK. SLIGRL, the rat PAR-2 activating peptide was used, as it was in greater abundance than tryptase. Treatment of HSC with various concentrations of PD98059 with SLIGRL (lanes 1, 2 and 3) slightly reduced the activation of ERK1/ERK2 when compared to treatment of HSC with SLIGRL alone (lane 4), figure 5.13i. PD98059 did not reduce all MAPK activation by SLIGRL. Perhaps the inhibitor needed to be added for longer to act on the HSC before addition of MAPK stimulants. Densitometric analysis, figure 5.13.ii illustrates the inhibition of ERK1/ERK2 in two to three independent experiments.

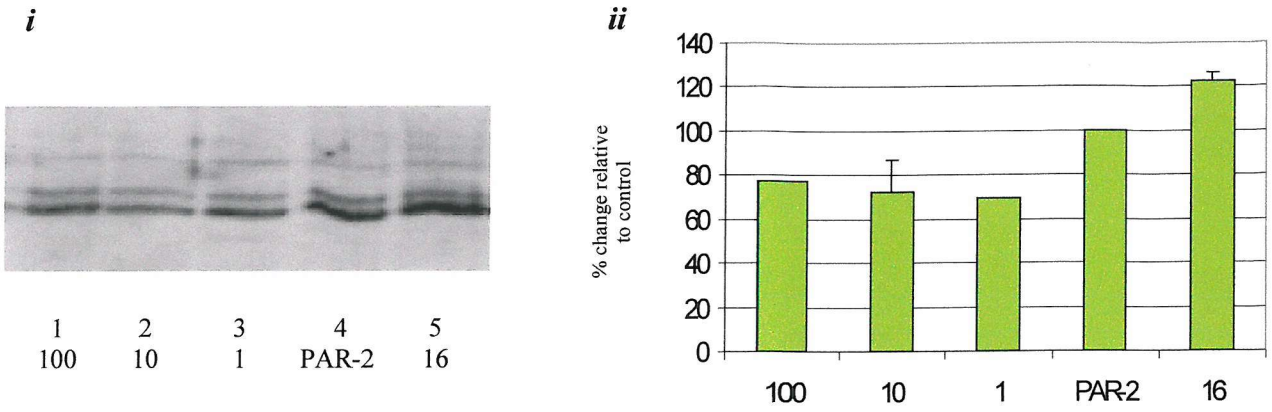

Figure 5.13. i. Representative one of three Western blots examining the activation of ERK1/ERK2 by 30 μM PAR-2 peptide in the presence or absence of the ERK1/ER2 inhibitor PD98059 on HSC. PD98059 was added to HSC 30 minutes prior to stimulation with 30 μM PAR-2 for 15 minutes. 1= 100 μM PD98059; 2= 10 μM PD98059; 3= 1μM PD98059; 4= 30 μM SLIGRL; 5= 16% FCS. **ii.** Graph illustrating the inhibition of MAPK activation by SLIGRL using PD98059 as analysed by densitometry, n=2 to 3.

5.2.8. MAPK ACTIVATION DURING HSC PROLIFERATION

To support the MAPK protein detection by immunoblotting, parallel proliferation assays were performed with growth- arrested HSC treated with the PAR-2 agonist in the presence or absence of the ERK1/ ERK2 inhibitor PD98059. After stimulation, cells were left for 24 hours and during the last 4 hours of incubation the cells were pulsed with 1 μCi ml^{-1} ^{3}H-thymidine. Cell proliferation was assessed as previously described in section 2.2.18. Proliferation was assessed by calculating the resulting counts per minute, averaged for each PAR-2 agonist or PD98025 concentration added to HSC. The results were presented as mean ± SEM of percentage proliferation of treated HSC in relation to untreated HSC. Where applicable Student's paired *t*-tests with $p=< 0.05$ were considered statistically significant and conformed using non-parametric Wilcoxon signed rank test. 16% FCS was added to each proliferation assay as a positive control marker to ensure HSC were responding to treatment.

Initially, the ERK1/ER2 inhibitor PD98059 was added to HSC before stimulation with 16% FCS, as a positive control. Relative to untreated HSC control, 16% FCS gave 701% ±48.5 increase in proliferation. Addition of 10 μM PD98059 significantly reduced FCS- induced proliferation to 258.95% ±57.5 ($p< 0.05$ versus (vs) FCS stimulated HSC), an approximate 2- fold reduction in 16% FCS- induced proliferation, (figure 5.14).

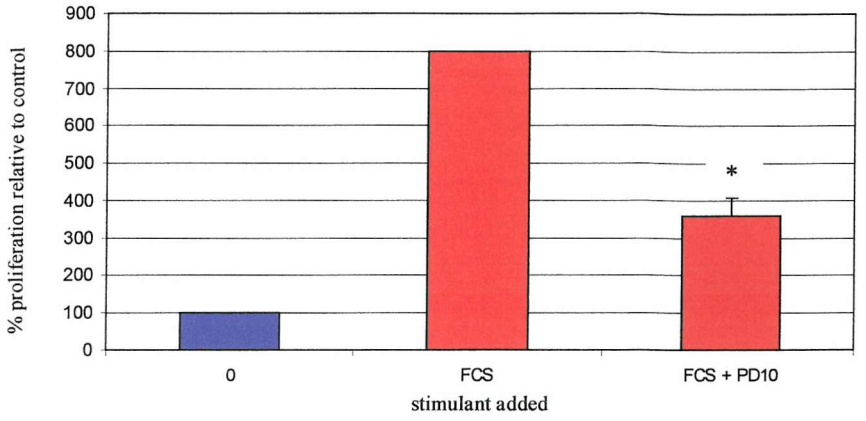

Figure 5.14. Effect of MAPK inhibition on FCS- induced HSC proliferation, n= 4 to 6. Serum starved HSC were pre-incubated with 10 μM PD98059 for 30 minutes before addition of 16% FCS. *= $p<0.05$ vs. FCS stimulated HSC, Student's t-test and Wilcoxon signed rank test.

The effect of MAPK inhibition on SLIGRL- induced HSC proliferation was then investigated, figure 5.15. HSC were pre-incubated with various concentrations of PD98059 (1 to 100 μM) before stimulation with 30 μM SLIGRL. As previously, SLIGRL induced a significant 236% ±28.8 increase in HSC proliferation. Pre-treatment of HSC with PD98059 at 1, 10 and 100 μM gave significant dose- dependent reduction in SLIGRL-induced HSC proliferation, 2.6, 3.7 and 10 fold reduction in proliferation.

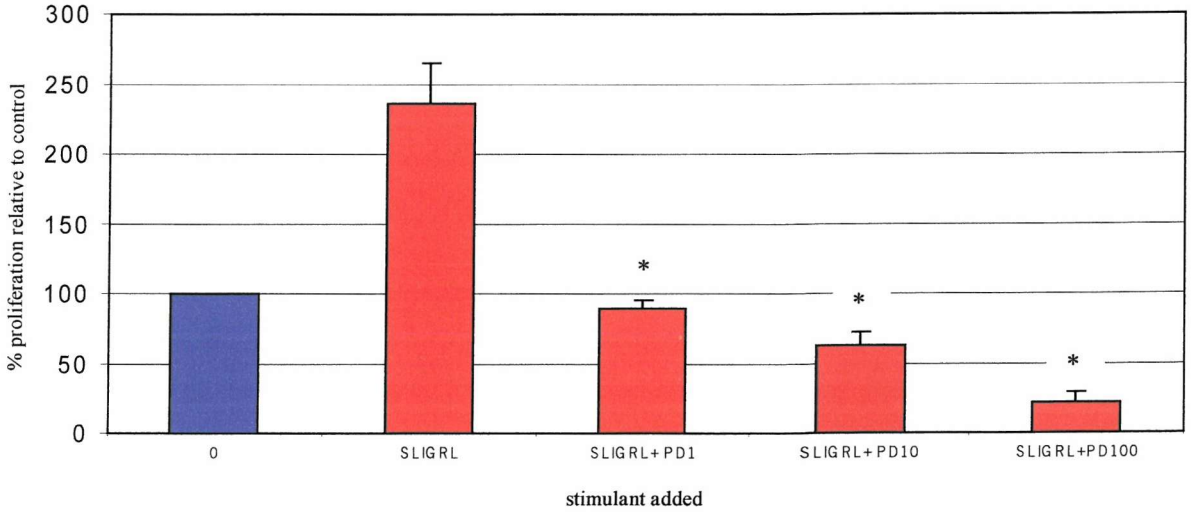

Figure 5.15. Effect of MAPK inhibition on PAR-2 peptide (SLIGRL)- induced HSC proliferation, n= 4 to 6. Serum starved HSC were pre-incubated with 1 to 100 μM PD98059 for 30 minutes before addition of 30 μM SLIGRL. *= $p<0.05$ vs. SLIGRL stimulated HSC, Student's t-test and Wilcoxon signed rank test.

The 10-fold reduction in HSC proliferation by 100 μM PD98059, upon examination of HSC in culture during the proliferation assay, proved to be cytotoxic. HSC under these conditions lost their myofibroblast phenotype and became spherical. However HSC treated with 1 and 10 μM PD98059 maintained their myofibroblastic phenotype.

As a further control, the proliferative effect of PD98059 at concentrations of 1, 10 and 100 μM and DMSO, the diluent of PD98059, at amounts found within 100 and 10 μM of PD98059 (0.05% and 0.025% respectively) on HSC were examined, figure 5.16. It was found that PD98059 alone gave a dose-dependent inhibition of HSC proliferation, however not as large as that observed in the presence of SLIGRL. The effect of 100 μM PD98059 on HSC was cytotoxic as light microscopy examination of these HSC showed the activated myofibroblastic phenotype of these cells was lost. However addition of DMSO at 0.05% and 0.025% induced little or no proliferation of HSC which suggests that the inhibition of MAPK activity was as a result of PD98059.

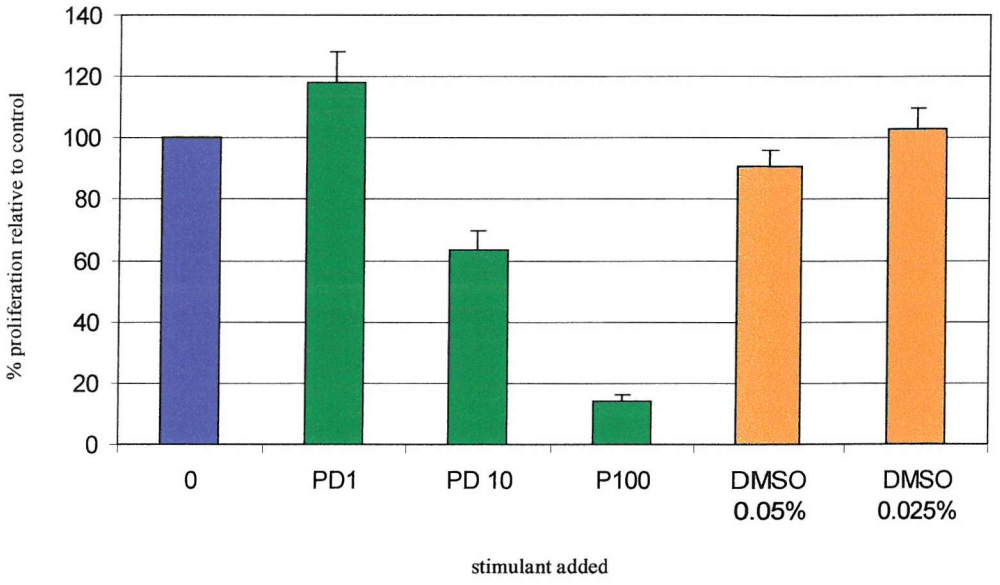

Figure 5.16. Various concentrations of PD98059 and DMSO (inhibitor diluent) on HSC proliferation, n= 4 to 6.

5.2.9. STIMULATION OF COLLAGEN SYNTHESIS IN HSC BY SLIGRL

Previously I have shown that pre-treatment of HSC can induce collagen synthesis (section 4.2.5) . Using the rat PAR-2 activating peptide SLIGRL, I have examined if this stimulation of collagen synthesis can be mediated via the PAR-2 receptor. As in section 4.2.5, collagen synthesis in HSC was determined by the incorporation of ^{3}H- proline in HSC supernatants following incubation with 30 μM SLIGRL or 10 ng/ml TGF-β as a positive control for HSC collagen synthesis (section 2.2.19). SLIGRL increased collagen

synthesis by 95% ±50 ($p<0.05$), figure 5.17. TGF-β induced a 130%±49($p<0.05$) increase in HSC collagen synthesis as analysed by Student's *t*-test and confirmed using non-parametric Wilcoxon signed rank test.

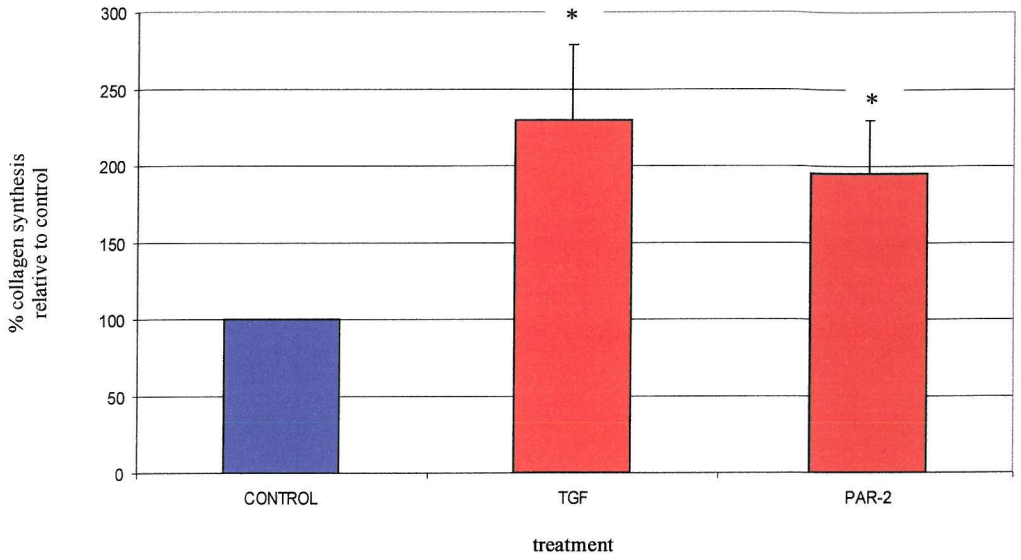

Figure 5.17. Collagen synthesis in HSC stimulated with SLIGRL and TGF-β, n=6. *= $p<0.05$, Student's t-test and Wilcoxon signed rank test.

5.3. DISCUSSION

PARs are a novel family of G-protein- coupled serine protease receptors that are activated by proteolysis (Vu *et al,*1991; Nystedt *et al,* 1994; Ishihara *et al,*1997) . To date there are four members of this family, PAR-1 and PAR-3 that are activated by thrombin (Vu *et al,*1991; Ishihara *et al,*1997), PAR-2 that is activated by pancreatic trypsin and MC tryptase (Corvera *et al,* 1994; Nystedt *et al,* 1994; Molino *et al,* 1997), and PAR-4 which is activated by thrombin or trypsin (Xu *et al,* 1998). The receptor agonist activates the receptor by cleaving the extracellular NH_2- terminus at a recognised putative cleavage site exposing a new NH_2- terminus, or 'tethered ligand'. Specific residues within the tethered ligand interact with extracellular domains of the receptor resulting in its activation (Vu *et al,*1991). Synthetic peptides corresponding to the first six amino acids of the tethered ligand have been shown to activate respective PARs by non- proteolytic mechanisms (Nystedt *et al,* 1994).

Previous studies have identified the presence of PAR-2 within the liver (Nystedt *et al,*1994; Böhm *et al,* 1996). MC tryptase has been shown to cleave and activate PAR-2 in many cell types such as endothelial cells, enterocytes and colonic myocytes (Corvera *et al,* 1994; Molino *et al,* 1997). Previously, in chapter 4 I have shown that tryptase can induce HSC proliferation and collagen synthesis. As PAR-2 is the suggested receptor for tryptase and has been previously detected in the liver and MC hyperplasia is observed in the fibrotic liver (Miller *et al,* 1994; Peng *et al,* 1994; Ramos *et al,* 1994; Farrell *et al,* 1995; Xu *et al,* 1994; Rioux *et al,* 1996; Ambrust *et al,* 1997; Nakamura *et al,* 1997), I have investigated the potential expression of PAR-2 in liver tissue and in cultured HSC by various techniques.

By use of RT-PCR I have examined PAR-2 mRNA expression in rat experimental models of liver fibrosis. In early and in late stages of both CCl_4 and BDL fibrotic livers PAR-2 mRNA expression was detected. Therefore the receptor is present in normal and fibrotic rat livers. By RT-PCR, PAR-2 mRNA expression was further examined in culture- activated rat HSC, at day 0, 7 and 14 and in passaged HSC. Upon activation of HSC in culture it appears that PAR-2 mRNA increases, from day 0 to day 14 when compared to β–actin controls. This increased expression needs to be confirmed by other quantitative methods such as use of quantitative PCR ('Taq-man').Northern blotting however was less successful in detecting PAR-2 mRNA expression in HSC. This suggests that PAR-2 mRNA is weakly expressed in HSC and can only be detected when using sensitive techniques such as PCR. In parallel to these studies, PAR-1 mRNA expression

was investigated. It was found that in culture-activated rat HSC, PAR-1 mRNA expression was similar to that of PAR-2 in that expression was upregulated during activation of HSC (day 0 to day 14) and this was confirmed by RT-PCR and Northern blotting.

To complement the mRNA studies, PAR-2 protein synthesis was investigated. Western blotting detected PAR-2 protein expression in rat HSC lysates. Using a polyclonal antibody to human PAR-2 (a gift from Dr S. Compton, University Medicine, Southampton General Hospital, UK), PAR-2 protein at 45 and 42 kDa was detected in day 21 HSC and passaged HSC. PAR-2 was also detected in the positive control 3T3 and RSF samples. Immunostaining of PAR-2 protein using a monoclonal antibody to human PAR-2 (a gift from Professor L Brass, University of Pennsylvania, USA) in rat HSC confirmed the Western blotting studies. Culture-activated rat HSC expressed PAR-2 protein pericellularly that appeared slightly fibrillar in distribution. Positive α-SMA staining confirmed the activation status of the stained rat HSC. Further co-localisation of PAR-2 to receptors expressed on activated HSC (such as PDGF) will enable the confirmation of the distribution of PAR-2 within the HSC.

PAR-2 mRNA and protein have been successfully detected in the rat liver and in culture-activated HSC. In chapter 4, I have shown that tryptase, an agonist of PAR-2 can induce HSC proliferation. To further confirm the presence of PAR-2 I have examined the proliferative effect of two other PAR-2 agonists, trypsin and the PAR-2 peptide agonist SLIGRL. Trypsin at concentrations of 1 to 1000 μM gave 17% ±7.3 to 45% ±29 increase in HSC proliferation. SLIGRL, at concentrations ranging from 1 to 100 μM also gave increases in HSC proliferation. This became maximal and significant at 30 μM having a 126% ±40.7 increase in proliferation. Therefore all three PAR-2 agonists, tryptase, trypsin and SLIGRL, can induce HSC proliferation. The results of these findings suggest the possibility that the action of tryptase released from activated MC on HSC may be mediated by PAR-2.

In analogous experiments, as PAR-1 has been previously identified on HSC (Marra *et al,* 1998) and has been confirmed by my RT-PCR and Northern blotting studies, I have examined the proliferative effect of PAR-1 agonists, thrombin and PAR-1 activating peptide (SFFLRN) on quiescent HSC. Previous studies have shown that thrombin can stimulate a fourfold increase human HSC proliferation (Marra *et al,* 1995). Rat HSC treated with thrombin at concentrations from 0.2 mU/ml to 4 mU/ml induced HSC proliferation. Maximal proliferation was observed at 0.4 mU/ml in which there was a 96% ±25.69 increase in proliferation compared to untreated control HSC. The rat PAR-1 activating peptide SFFLRN at concentrations from 1 to 100 μM induced significant

increases in HSC proliferation from a 45%±11.8 ($p<0.05$) to 120%±10.6 ($p<0.05$) increase. SLIGRL concentration of 30 μM gave the maximal significant increase in proliferation 123%±21.35 ($p<0.05$). Hence two PAR-1 agonists, thrombin and SRRLRN can induce HSC proliferation suggesting that their proliferative effect on HSC may be mediated by PAR-1.

The intracellular consequences of PAR activation on cell membranes are not well characterised. However the MAPK cascade signal transduction pathway has been implicated in the mitogenic effects of trypsin on PAR-2 in rat aortic smooth muscle cells (Belham *et al,* 1996) and of thrombin on platelets and CCL-39 fibroblasts (Molloy *et al,* 1996). MAPK are a family of serine/threonine –specific protein kinases involved in regulating cellular growth and proliferation . The MAPK signalling pathway is a multi-step phosphorylation cascade that transmits signals from the surface of the cell to cytosolic and nuclear targets. HSC proliferation in response to stimulation by PDGF, is reported to be modulated by ERK1/ERK2 (Marra *et al,* 1999). Therefore by use of a specific ERK1/ERK2 antibody and inhibitor (PD98059), I have examined the expression of the MAPK signal transduction pathway in HSC stimulated by PAR-1 and PAR-2 agonists.

By Western blotting I have shown that in quiescent HSC, FCS and SLIGRL can activate ERK1/ERK2 expression in a time dependent manner. Maximal ERK1/ERK2 activity was observed at 15 minutes of FCS. MAPK expression reached a maximum at 15 minutes of SLIGRL stimulation, 169.6%±64 increase compared to untreated HSC control when analysed by densitometry. As 15 minutes was the optimal time for MAPK activation, quiescent HSC were stimulated with various agonists of PAR-1 and PAR-2 to see if they could induce MAPK activation. Tryptase and SLIGRL, PAR-2 agonists, thrombin and SFFLRN, PAR-1 agonists, induced ERK1/ERK2 activation in quiescent HSC. To confirm that MAPK activation was due to addition of these mediators, a specific inhibitor of ERK1/ERK2, PD98059 was used. In these inhibitor studies, the PAR-2 agonist, SLIGRL was used as was in greatest abundance. Addition of the specific ERK1/ERK2 inhibitor PD98059 slightly decreased the expression of ERK1/ERK2 when stimulated with SLIGRL. This was not anticipated, as it was expected this inhibitor would knock out all activation of ERK1/ERK2. If the inhibitor was left on the cells for a longer time, one hour instead of 30 minutes, then possibly all ERK1/ERK2 activation would have been blocked. To support the MAPK immunoblotting, parallel proliferation assays were performed on stimulated HSC. It was found that PD98059 significantly reduced FCS- induced HSC proliferation from 701%±48.4 increase in HSC proliferation to 259%±57.5 ($p<0.05$ vs FCS stimulated HSC), that is an overall 2-fold reduction in HSC proliferation. PD98059

had similar effects on HSC stimulated with SLIGRL. Pre- treatment of HSC with PD98059 at 1, 10 and 100 μM resulted in a significant dose- dependent 2.6, 3.7 and 10 fold reduction in SLIGRL- induced proliferation respectively, however treatment of 100 μM PD98059 was cytotoxic.

In chapter 4 I have shown that tryptase can mediate collagen synthesis in HSC. I have examined if the PAR-2 activating peptide, SLIGRL, can also induce HSC collagen synthesis. ^{3}H- proline- incorporation assays followed by collagenase digestion demonstrated a significant 94.75% $\pm$50 ($p<0.05$) increase in HSC collagen synthesis by SLIGRL relative to control untreated HSC. These results suggest that collagen synthesis stimulated by SLIGRL and tryptase may be mediated by PAR-2.

In summary PAR-1 and PAR-2 are expressed in the liver by HSC. Natural and synthetic agonists to these receptors can induce HSC proliferation and may be modulated by ERK1/ERK2. Collagen synthesis can be stimulated by tryptase and the PAR-2 activating peptide suggesting that this may be mediated by the PAR-2 receptor.

In conclusion, liver fibrogenesis potentiated by the mitogenic effects of thrombin and MC tryptase may be mediated via PAR-1 and PAR-2 respectively.

CHAPTER SIX
FINAL DISCUSSION
AND
FUTURE DIRECTIONS

6.1. DISCUSSION

During fibrosis MC accumulate in areas of tissue damage. Direct analysis of these tissues by immunocytochemistry using antibodies targeted to MC proteases confirm MC hyperplasia in fibrotic tissues. Analysis of MC mediators in the circulating plasma and BAL have detected increased levels of histamine and tryptase released from the recruited and activated MC in these injured tissues. (Kawanami *et al,* 1979; Claman *et al,* 1990; Hunt *et al,* 1992; Irani *et al,* 1992; ; Li *et al*, 1992; Pesci *et al*, 1993; Peng *et al*, 1994; Xu *et al,* 1994; Farrell *et al*, 1995; Rioux *et al*, 1996; Ambrust *et al*, 1997; Nakamura *et al*, 1997; Ramos *et al*, 1997).

The phenomenon of MC hyperplasia has been mainly attributed to its relationship with fibroblasts within the injured tissue, although other recruited and circulating inflammatory cells such as eosinophils and T lymphocytes may play a role. Fibroblasts have been shown to contribute to MC accumulation by their production of SCF (Nocka *et al,* 1990) which becomes upregulated in areas of tissue injury. SCF is an important growth factor and cytokine central to the development of MC from progenitors in the bone marrow to mature MC delivered to injured connective tissues. SCF synthesised by fibroblasts binds to the *c-kit* receptor on the surface of the MC (Lev *et al*, 1992; Blecham *et al,* 1993). The activation of this tyrosine kinase receptor initiates many signal transduction pathways causing the mobilisation of intracellular Ca^{2+} stores which results in *de novo* cytokine synthesis and release and the degranulation of cytoplasmic granules containing mediators such as histamine and serine proteases. As well as exerting fibrogenic effects on the surrounding tissue- environment, the released mediators such as IL-3, IL-4, tryptase and TGFβ, may also act directly on the MC causing an autocrine mechanism of MC activation, or act on the fibroblast (Bradding *et al,* 1992; Gruber *et al,* 1994; He *et al,* 1998).

Tryptase is a known mitogen for fibroblasts and also stimulates their production of collagens (Gruber et al, 1987; Gruber et al, 1989; Rous et al, 1991; Cairns and Walls, 1996; Cairns and Walls, 1997). Therefore tryptase may augment fibrosis within the tissues to which MC are recruited. Hence MC and fibroblasts have an important bi-directional relationship in the tissue environment and together may contribute to the development and maintenance of fibrosis. MC have been shown to increase in numbers in liver fibrosis and are greatest in the fibrotic septa observed within the connective tissues in the portal tracts (Farrell *et al,* 1995; Rioux *et al*, 1996; Ambrust *et al,* 1997).

During liver fibrosis and injury, there is an imbalance in the fibrogenesis, fibrolysis and deposition of hepatic ECM which eventually disturbs the hepatocellular function and liver architecture (Clement *et al,* 1993). Central in maintaining this balance is the HSC, found within the space of Disse in the hepatic sinusoids (Friedman *et al*, 1993; Gressner *et al*, 1994). In normal liver, HSC have a 'quiescent' phenotype, are spherical in shape, show minimal proliferation, and contain abundant cytoplasmic droplets of vitamin A. However, following liver injury HSC transform to enlarged myofibroblastic cells (Friedman,1993; Gressner, 1995) which lose their lipid droplets and have enhanced proliferation. These activated HSC express intracellular α-smooth muscle actin (Ramadori *et al,* 1990) and synthesise a wide variety of ECM proteins including laminin, heparan sulphate proteoglycans and type I and III collagens (Maher *et al,* 1988; Milani *et al,* 1990; McGee and Patrick, 1992; Rockey *et al*, 1992) which are deposited in the space of Disse. The molecular and cellular events underlying the pathogenesis of liver fibrosis and activation of HSC are not fully understood, however my results indicate MC hyperplasia may play a role in HSC activation.

In this thesis I have examined the hypothesis that HSC and MC have a bi- directional relationship in liver fibrosis. To test this hypothesis it was necessary to establish a possible mechanism of MC recruitment within the fibrotic liver, to examine the fibrogenic effects of MC mediators and their subsequent mechanism in HSC activation. As activated HSC adopt many features of fibroblasts, the potential role of these cells in recruiting MC to the liver by producing SCF was initially investigated.

By a variety of techniques, SCF protein and mRNA expression were detected in rat experimental models of fibrosis and in culture activated rat HSC. These results confirm previous findings within our laboratory of SCF production by human HSC (Benyon *et al,* 1996). However, as shown by Western blotting, SCF protein was easier to detect in human HSC than rat HSC. This lower level of SCF production by rat HSC compared to human HSC may explain the relatively modest 1-2 fold increases in MC numbers in injured rat livers previously reported (Rioux *et al,* 1996) compared to the more impressive 8- fold increase in MC in human cirrhotic livers (Farrell *et al,* 1995). Immunohistochemical analysis of rat liver tissues confirmed the presence of SCF. In normal rat livers SCF was found expressed within the columnar epithelial cells lining the bile duct lumen, and this confirmed previous reports by Fujio *et al,* 1996. In the rat fibrotic livers (8 weeks of CCl_4 treatment) SCF staining was apparent again in the bile ducts but also within the fibrotic septae and necrotic parencyhma. Upon closer examination HSC appeared to be staining

Studies of MC and fibroblast co-cultures provide further evidence of MC and fibroblast interaction mediated by SCF (Levi-Schaffer *et al,* 1985; Levi-Schaffer *et al,* 1987; Levi-Schaffer *et al,* 1995). Within this thesis I have addressed the possibility that MC and HSC may also bind to each other via SCF using a co- culture system. This interaction between MC and HSC via SCF/*c-kit* has also been reported in *S. mansoni* infected rat livers (Brito *et al,*1997).The co-culture studies clearly indicate adhesion between MC and HSC that could be inhibited up to 55 % $\pm$ 9.3 ($p<0.05$) with the use of neutralising antibodies to SCF. This interaction was not completely blocked suggesting that other factors besides SCF/*c-kit* may be involved in MC/ HSC intercellular adhesion, such as integrins. MC have been reported to express a variety of integrins including $\alpha4\beta1$, $\alpha v\beta1$, $\alpha5\beta3$ which are receptors for collagen and fibronectin (Metcalfe *et al,*1997). During liver fibrosis, HSC synthesise ECM components including collagens and fibronectin. The ECM made by the HSC may contribute to MC recruitment mediated by MC integrin expression. This may explain why MC hyperplasia is often observed relatively late in the development of liver fibrosis.

Accumulation of MC in liver fibrosis has been studied both in humans and in rat models of fibrosis. Animal models of liver fibrosis are essential in the study of early and the development of fibrosis as it is difficult to study the progression of liver fibrosis in humans as the disease usually presents itself in the patient only when it is highly developed. Serial biopsies also have ethical problems. It is important to realise the limitations of the studies of MC in rat models of liver fibrosis as rat MC differ from humans in protease and proteoglycan content. Nevertheless these models are useful as they allow the closer examination of the disease process and help to contribute to the general knowledge in the development of liver disease. It has been previously reported that in human liver disease MC_{TC} is the prevalent MC subtype (Ambrust *et al,* 1997) whilst in rat liver fibrosis it is the MMC subtype (Rioux *et al,* 1996). By PCR I have observed that in the human liver, both tryptase and chymase mRNA were expressed in normal and fibrotic livers suggesting that MC_{TC} are indeed present in the liver. In normal and rat fibrotic livers RT-PCR for the MMC marker RMCP-II and the CTMC marker RMCP-I indicated that both types of MC are present. Immunocytochemical studies using antibodies targeted to RMCPI and II in rat CCl_4 induced fibrosis, confirmed the PCR studies. In normal livers MMC and CTMC were found sparsely distributed within the connective tissues of the hepatic portal tracts. However in fibrotic livers there were clear increases in MMC and CTMC found within the periportal fibrotic septae and within the portal tracts. These results suggest that in the normal rat liver MC are present however during liver fibrosis MC

accumulate at the site of increased matrix deposition and HSC activation. However it is important to further examine the expression of the different MC subtypes in liver disease to fully understand the pathology of the disease and to help in the possible treatment and development of potential therapeutics. Numerous studies have recognised that MC sub-types differ in activation stimuli; MMC are only activated by IgE dependent mechanisms whilst CTMC are also stimulated by a wide range of IgE- independent stimuli including neuropeptides (Benyon *et al,*1989). The subtypes differ in response to treatment with MC stabilising drugs such as sodium cromoglycate (SCG) and nedocromil sodium. Subtype differences are also seen in human MC. MC dispersed from human skin are insensitive to the inhibitory effects of SCG (Lowman *et al,* 1988), dispersed lung MC are poorly responsive to SCG, needing high concentrations (1000 μM) to have a 30% inhibition of histamine release (Church and Hiroi, 1987) , whilst activated MC from BAL and the intestine are effectively inhibited with SCG (Leung *et al,* 1988). Human MC subtypes also differ in protease and proteoglycan content (Irani *et al,*1986; Schechter, 1990).

MC may potentiate fibrosis by the subsequent release of their mediators upon activation. In humans, MC may contribute to a variety of fibrotic disorders. For example, in idiopathic dilated and ischaemic cardiomyopathies, two conditions characterised by the increase in fibrous components, MC hyperplasia and increases in tissue histamine and tryptase content have been observed (Patella *et al,* 1997); in scleroderma patients MC hyperplasia and degranulation preceded dermal fibrosis (Siebold *et al,* 1990); in fibrotic lung disorders increases in tryptase and histamine concentrations are found in BAL (Crimi *et al,* 1991); in chronic human cholestatic liver disease, plasma histamine levels increase to 275 ±117 μg/ml, compared to normal livers 114 ±72 μg/ml, suggesting MC hyperplasia and MC activation (Gittlen *et al,* 1990). In systemic mastocytosis, a disease in which there is abnormal accumulation of MC in one or more organs, patients are known to develop portal hypertension and liver cirrhosis further suggesting a role for MC in fibrosis (Kyriakou *et al,* 1998; Fonga-Djimi *et al,* 2000). Studies using MC degranulation inhibitors and MC deficient animals also implicate MC in fibrosis. Administration of MC degranulation inhibitor (tranilast) has been shown to reduce collagen production in scleroderma and mastocytoma patients (Taniguchi *et al,*1994; Katoh *et al,* 1996). In a mouse model of human scleroderma, the tight-skin (TSK) mouse develops cutaneous fibrosis having increased MC numbers and degranulation. Use of MC degranulating inhibitors such as SCG and ketotifen reduces dermal fibrosis in TSK mice (Walker *et al,* 1987; Walker *et al,* 1990;), whilst MC deficient mice carrying the TSK locus indicated that dermal fibrosis develops with the recruitment and activation of MC (Everett *et al,* 1995).

Recently, the development of tissue fibrosis has been studied in MC deficient mice. Studies inducing lung and dermal fibrosis using bleomycin in MC deficient mice demonstrated that wild type mice had increased fibrosis that correlated to MC histamine release and hydroxyproline content, however the MC deficient mice had minimal fibrosis with hydroxyproline levels similar to that of untreated controls (O'Brien-Ladner *et al,* 1993; Yamamoto *et al,* 1999). However different results were obtained in studies of liver fibrosis in MC deficient mice and rats. It was found that there were no significant decreases in liver fibrosis induced by CCl_4, BDL and pig-serum in these animals compared to normal controls (Okazaki *et al,* 1998; Sugihara *et al,* 1999). However these studies are open to question. Only end point fibrosis and not the progression of liver fibrosis was studied in all animal models which does not rule out the possibility that MC may play a role in accelerating the formation of liver fibrosis. These results only suggest that MC are not required for inducing liver fibrosis. Another factor not taken into consideration is that anaemia (*W* mutant rats and mice exhibit anaemia as a 'side-effect') increases the sensitivity of hepatocytes to detrimental stimuli and it is conceivable that liver fibrosis is induced more strongly in MC mutant mice and rats than in the wild-type.

Several reports have documented the fibrogenic effects of MC serine proteases and cytokines, such as tryptase, IL-4 and TNF-α on fibroblasts (Gruber *et al*, 1987; Gruber *et al*, 1989; Brito *et al,* 1991; Rous *et al,* 1991; Sempowski *et al,* 1994; Cairns and Walls, 1996; Cairns and Walls, 1997). In this thesis I have addressed the potential effects of MC mediators on rat HSC and where possible, human HSC. Human and rat HSC are phenotypically similar in that they both adopt a myofibroblastic-like phenotype during activation and express ECM components. Rat HSC were mainly used as they are more readily available and could be cultured from quiescence as human HSC were cultured from human explants and were predominately activated. Histamine, heparin, and IL-4, when incubated with cultured HSC at low concentrations (0.1, 0.01 and 0.01 μg/ml respectively) induced HSC proliferation, but at higher concentrations, proliferation was reduced in a dose-dependent manner. However TNF-α and commercially available tryptase induced HSC proliferation. Tryptase was found to induce a maximal proliferation of 104% $\pm$26.23 ($p<0.05$) at 0.6 mU/ml in rat HSC. This proliferative effect was previously reported in our laboratory in human HSC. Tryptase at concentrations of 3 and 10 mU/ml induced a 145% and 247% increase in human HSC proliferation (Benyon *et al,*1997). The concentrations of tryptase used with human HSC are similar to those used in previous studies using human lung fibroblasts (2.3 to 36 mU/ml tryptase) (Cairns *et al,* 1997). Tryptase at 0.6 mU/ml, also induced collagen synthesis in HSC by 78% in two independent experiments.

Recent findings have shown that human lung fibroblasts stimulated with tryptase predominately synthesise type I collagen (Cairns *et al,* 1997). Tryptase therefore, can act as a growth factor for HSC and can stimulate the synthesis of collagen. These findings further indicate that MC may have a role in liver fibrogenesis.

It has recently been suggested that tryptase mediates its effects via a receptor, namely PAR-2 (Corvera *et al,* 1994; Nydstedt *et al,* 1994; Molino *et al,* 1997). PARs are a novel family of G-protein coupled serine protease receptors that are activated by proteolysis (Vu *et al,* 1991; Nydstedt *et al,* 1994; Ishihara *et al,* 1997). At present 4 members of this family have been discovered, PAR-1 and PAR-3 are activated by thrombin (Vu *et al,* 1991; Ishihara *et al,* 1997), PAR-2 is activated by pancreatic trypsin and MC tryptase (Corvera *et al,* 1994; Nystedt *et al,* 1994; Molino *et al,* 1997) and PAR-4 is activated by thrombin or trypsin (Xu *et al,*1998). PAR-1 has previously been found in the liver and its expression during human cholestatic liver disease is upregulated (Marra *et al,* 1998). By use of *in- situ* hybridisation and immunostaining, PAR-1 was localised to liver endothelial cells, infiltrating monocytes and most importantly activated HSC. PAR-2 has been found distributed in many tissues but most importantly it has been found in the liver (Böhm *et al,*1996; Nydstedt *et al,* 1996). To further examine the bi-directional relationship in existence between HSC and MC, I examined the role of PAR-2 as an intermediary for tryptase actions on HSC.

Initially expression of PAR-2 was investigated in models of rat liver fibrosis. By RT-PCR PAR-2 mRNA was detected in normal and fibrotic rat livers. Further RT-PCR detected PAR-2 mRNA expressed on progressively activated HSC. PAR-2 protein was also detected in activated HSC by Western blotting and immunostaining. Further studies indicated PAR-2 is functionally linked to changes in HSC proliferation and activation as PAR-2 agonists trypsin and the rat PAR-2 activating peptide SLIGRL significantly increased HSC proliferation and collagen synthesis relative to control untreated HSC. The intracellular effects of PAR-2 activation were studied by examining the expression of MAPK signal transduction pathway in SLIGRL stimulated HSC. The two MAPK isoforms ERK1/ERK2 have previously been shown to be activated by PAR-2 agonists (Belham *et al,*1996). By Western blotting it was found that SLIGRL activated ERK1/ERK2 in a time dependent manner in HSC and this was decreased by PD98059, a specific ERK1/ERK2 inhibitor. Parallel proliferation assays on HSC treated with SLIRGL in the presence of PD98059 showed a significant reduction in SLIGRL- induced HSC proliferation.

As a supplement to the PAR-2 studies, the expression of PAR-1 in HSC was also investigated. RT-PCR and Northern blotting detected PAR-1 mRNA increasing in

expression in culture-activated HSC and confirmed previous studies (Marra *et al,* 1998). PAR-1 agonists, thrombin and the rat PAR-1 activating peptide (SFFLRN) induced HSC proliferation suggesting their proliferative effect may be mediated by PAR-1.

In conclusion, my findings provide compelling evidence for an important association between MC and HSC in liver fibrogenesis and support the hypothesis that there is a bi-directional relationship occurring between MC and HSC to promote liver fibrosis. A model is suggested in figure 6.1.During liver disease, HSC become activated and synthesise SCF. As a consequence of this, MC are recruited into the injured liver. Once recruited to the developing fibrotic septae of the liver, MC become activated, support and contribute to hepatic fibrogenesis by specific mediators such as tryptase which may potentiate HSC activation by inducing HSC proliferation and collagen synthesis. The induction of HSC proliferation and collagen synthesis by tryptase is suggested to be mediated by PAR-2 via the MAPK signal transduction pathway. Tryptase may also stimulate the conversion of pro-thrombin to thrombin and the activation of pro-MMPs, which in turn will activate HSC.

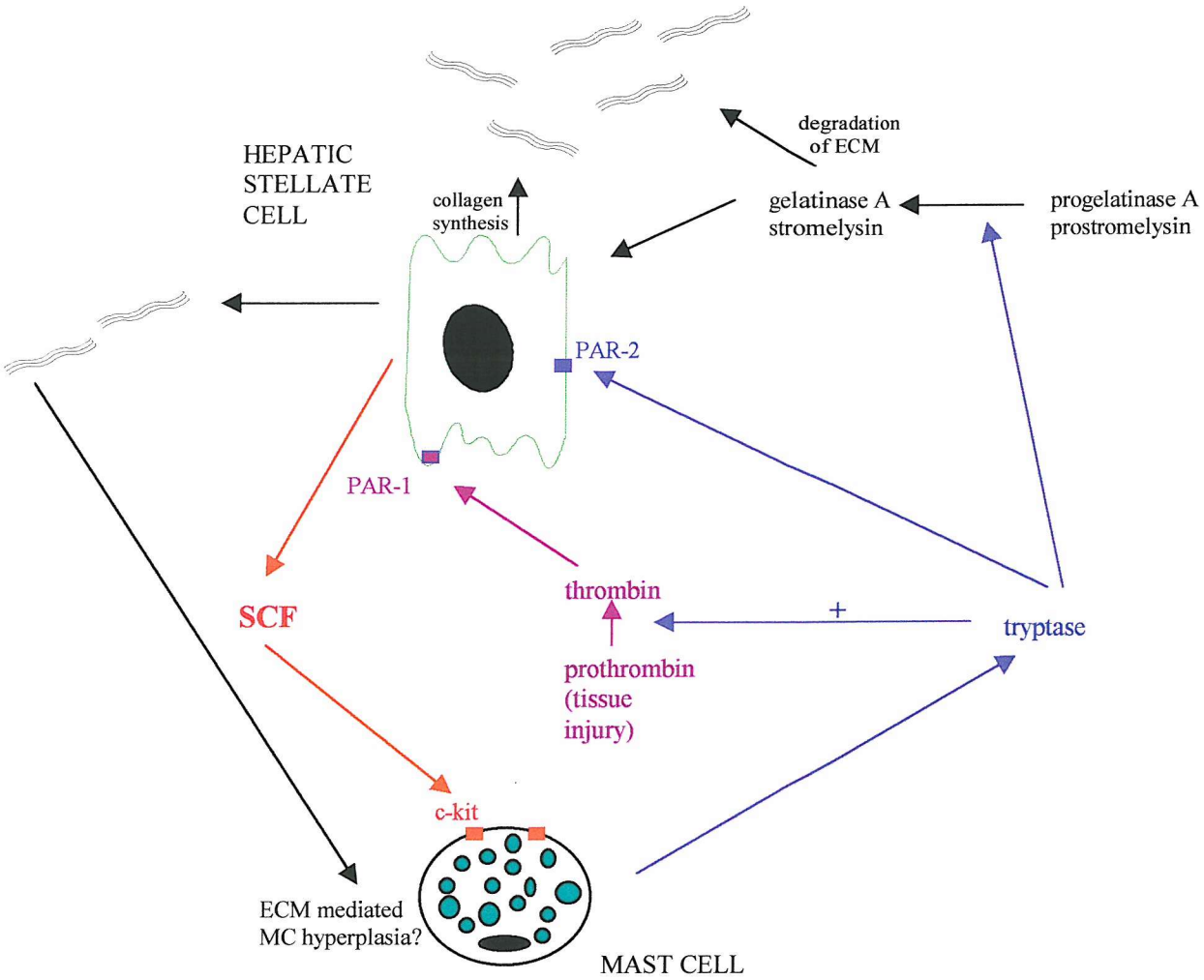

Figure 6.1. A schematic diagram proposing the relationship between mast cells and hepatic stellate cells in liver fibrosis.

6.2. FUTURE DIRECTIONS

Further investigation is necessary to examine the potential role of MC in liver fibrosis. The importance of SCF in the recruitment of MC during liver fibrosis should be further examined in rat models of fibrosis (CCl_4 and BDL). During the development of fibrosis, neutralising SCF antibodies could be administered to the liver to further verify the chemoattractant role of SCF during liver fibrosis. In normal rats, SCF antibodies may be administered to validate MC recruitment to the liver.

To fully understand the relationship between MC and HSC it is necessary to examine other potential adhesion factors involved within the MC/HSC co-culture studies. Use of neutralising antibodies to integrins such as β1 and β2 and disintegrins can be easily adopted to the MC/HSC co-culture system and may highlight possible adhesion factors mediating MC/HSC interactions. Addition of Matrigel and various ECM components such as collagen 1 to MC/HSC cultures may identify the importance and potential role of ECM in the MC/ HSC relationship during the development of liver fibrosis.

Further work is needed to determine the precise subtype of MC involved in liver fibrosis and perhaps the employment of dual immunostaining techniques targeted towards MC proteases may aid this study. Further evidence is required to ensure that MC tryptase has fibrogenic effects on HSC so the synthesis and upregulation of collagens and HSC activation needs to be further examined by the use of tryptase inhibitors (leupeptin). As type I and III collagens are upregulated in liver disease, the type of collagen synthesised by tryptase stimulated HSC should be investigated. In rat models of liver fibrosis the administration of MC degranulation inhibitors (SCG, tranilast) and tryptase inhibitors (leupeptin, APC 366) during progression of liver fibrosis may suggest the importance of activated MC in the development of liver fibrosis.

The role of PAR-2 protein within human livers and on HSC needs to be further investigating by the possible use of PAR-2 knockout mice and immunohistochemistry. It is necessary to understand the function and the signalling transduction pathways mediating PAR-2 HSC activation. This may be achieved by cloning techniques and formation of HSC over-expressing PAR-2 using various transfection mechanisms. These techniques will help further understand the mechanism of PAR-2 activation in HSC with the use of synthetic peptides and reverse peptides.

CHAPTER SEVEN

APPENDIX

7.1 SOLUTIONS AND REAGENTS

Cell culture reagents

PSG:	penicillin 10 mU/ml streptomycin 10 μg/ml gentamycin 32 μg/ml (in sterile water)

Mast cell isolation reagents

Erythrocyte lysis buffer:	1.7 mM ammonium chloride pH 7.4
Kimura stain:	0.8 ml 0.03% light green 5 ml 0.06M phosphate buffer pH 6.4 0.5ml saturated saponin 11ml 0.05% toluidine blue

RNA isolation reagents

DEPC treated water:	1ml DEPC 1 litre distilled water leave overnight then autoclave for 1 hour
4 M GIT:	DEPC water 4M GIT 26 mM sodium acetate to adjust pH to 5

RNA electrophoresis reagents

10X MOPS:	DEPC water 10 mM EDTA 0.2 M MOPS 50 mM sodium acetate adjust to pH 7
RNA loading buffer:	0.5% bromophenol blue DEPC water 7% formaldehyde 50% formamide 7% glycerol 1% MOPS

RT-PCR reagents

5X RT buffer:	50 mM dithiothretol (DTT) 250 mM KCl 20 mM $MgCl_2$ 250 mM Tris-HCl, pH 8.3
10X *Taq* buffer:	100 mM $(NH_4)_2SO_4$ 1% Triton X100 100 mM Tris-HCl, pH 8.8

RPA reagents

6% PAGE solution:	6% acrylamide/bis-acrylamide (19:1) 1 mM EDTA 100 mM Tris- borate pH 8.3 7 M urea
RPA hybridisation buffer:	DEPC water 1mM EDTA 80% formamide 40 mM PIPES 0.2 mM Na Acetate
Indicator plates:	15 g agar 5 g NaCl 10 g tryptone 5 g yeast extract, pH 7 and autoclave cool, add 100 μg/ml ampicillin and pour plates and cool until hard, spread plate with 20 μl X-Gal (50mg/ml) and 100 μl of IPTG (100 mM).
IPTG stock (100mM):	1.2 g IPTG in 50 ml water
LB broth:	15 g agar 5 g NaCl 10 g tryptone 5 g yeast extract pH 7 and autoclave
rNTP mix:	2.5 μl CTP, GTP and ATP 0.2 μl UTP 2.3 μl DEPC water
RPA loading buffer:	0.1 % bromophenol blue 2 mM EDTA 80 % formamide 0.1 % xylene cyanol
5x transcription buffer(Promega):	30 mM $MgCl_2$ 50 mM NaCl 10 mM spermidine 200 mM Tris-HCl (pH 7.9)
stop solution:	DEPC water 1 % SDS 1 mg/ml tRNA
X-Gal stock (50 mg/ml)	100mg 5-bromo-4 chloro-3 indolyl- β-D-galactosidase in 2 ml di-methylformamide

DNA electrophoresis reagents

DNA loading buffer:	0.25% bromophenol blue distilled water 30% glycerol

10X TBE:	0.9M boric acid 20 mM EDTA 0.9M Trizma base adjust to pH 8.4
Northern blotting reagents	
Pre/Hybridization buffer:	0.5% blocking reagent (Boehringer-Mannhein) 5 ml 50X Denhardt's reagent 25 ml formamide 1 ml 10mg/ml herring sperm DNA 2.5 ml 20X sodium orthophosphate -EDTA 0.5g SDS 12.5 ml 20X SSC make up to 50 ml with DEPC water
50X Denhardt's solution:	DEPC water 1% Ficoll 1% PVP
10X SSC	DEPC water 1.5M sodium chloride 150 mM sodium citrate adjust to pH7
20X SSC:	DEPC water 3M sodium chloride 300 mM sodium citrate adjust to pH7
20X sodium orthophosphate –EDTA:	DEPC water 10 mM EDTA 500 mM sodium orthophosphate
0.2% SDS/ 0.2X SSC:	2 g SDS 10 ml 20X SSC make up to 1 litre with DEPC water
SDS- PAGE reagents	
10% resolving gel:	5 ml 40% acrylamide 100 µl 10% APS 8 ml distilled water 0.2 ml 10% SDS 10 µl TEMED 5 ml 1.5M Tris pH 8.8
12% resolving gel:	as 10% but use 6 ml 40% acrylamide and 9 ml distilled water
5X running buffer:	0.2 M glycine 1 mM SDS 0.25 mM Trizma base

sample buffer:	0.2mls 0.5% bromophenol blue 4 ml distilled water 0.8 ml glycerol 1.5 ml 10% SDS 0.5 M tris-HCl pH 6.8
Western blotting reagent	
Transfer buffer:	27 mM glycine 20 % methanol 50 mM tris
Immunodetection reagent	
TTBS:	500 mM sodium chloride 100 mM Trizma base pH 7.5 0.1% Tween-20
Immunostaining reagent	
TBS:	**9** mM HEPES 150 mM sodium chloride 25 mM Trizma base pH 7.6
DAB (instructions as in kit):	2 drops chromogen (DAB) 1 drop hydrogen peroxide 82 µl 15% sodium azide 411 µl substrate buffer 3.6 ml distilled water
Immunohistochemistry reagents:	
Endogenous peroxidase inhibitor:	0.2 ml 30% hydrogen peroxide 11.8 ml methanol
Citric buffer:	2.1 g citric acid 1 litre distilled water pH 6.0
Tryptase assay reagents	
BAPNA:	20 mM BAPNA in DMSO
Tryptase assay buffer:	1 M glycerol 100 mM Trizma base pH 8
Zymography reagents	
8% resolving gel:	4 ml 40% acrylamide 100 µl 10% APS 9 ml distilled water 1 ml 20 µg/ml gelatin 0.2 ml 10% SDS 10 µl TEMED 5 ml 1.5M Tris pH 8.8

2.5 % Triton X-100:	500 mls distilled water 12.5 mls Triton X-100
Gelatinase incubation buffer:	5 mM calcium chloride 50 mM Tris pH8
Coomassie blue stain:	10% acetic acid (v/v) 0.5% Coomassie blue (w/v) 45% distilled water (v/v) 45% methanol (v/v)
Destain:	10% acetic acid (v/v) 80% distilled water (v/v) 10% methanol (v/v)

Collagen assay reagents

Collagenase buffer:	780 µl NEM solution 1820 µl Tris/ $CaCl_2$ buffer
Collagenase plus buffer:	980 µl collagenase (2450 U) 780 µl NEM 980 µl Tris/ $CaCl_2$ buffer
N-Ethylmaleride (NEM) solution:	50 mM NEM in water
Tris/ $CaCl_2$ buffer:	0.3M $CaCl_2$ 0.2M Tris pH 7.5

CHAPTER EIGHT

LIST OF REFERENCES

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