THE ROLE OF HEPATOCYTE GROWTH FACTOR IN LIVER DISEASE AND LIVER REGENERATION

by

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TABLE OF CONTENTS

TABLE OF CONTENTS

TABLE OF CONTENTS

TABLE OF CONTENTS

TABLE OF CONTENTS

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

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Declaration

This thesis represents my efforts to investigate the role of HGF in human liver disease and in an animal model of liver regeneration.

I personally performed the laboratory work involved in expression, identification and purification of HGF proteins. Mr. Alan Duncan collaborated with me in the production and sequencing of HGF α266 recombinant HGF protein. Mr. Duncan and I visited Aberdeen for two days to observe and assist Mr. Bryan Dunbar at Marischal College, Aberdeen University, in the sequencing of recombinant HGF proteins. Mr. Duncan included his work in a thesis submitted for the degree of Bachelor of Science in Experimental Pathology at the University of Glasgow.

I personally carried out all of the laboratory work involved in purification of human placental HGF and generation of polyclonal antibodies to this material and to recombinant HGF peptides.

In the attempts to produce monoclonal antibodies to HGF I collaborated with Mr. Pat Ferry. I was personally responsible for the assessment of polyclonal murine antibody response and screening of hybridomas.

I carried out all of the work involved in the development and application of ELISAs for detection of HGF in human serum. I stored and catalogued all of the human sera and frozen liver biopsies. I examined each of the hospital case records relating to these patients and stored the important clinical details using the Paradox 3.5 database program.

I performed the immunocytochemistry for HGF and associated studies using each of the various antibodies, assisted latterly by Mrs. Teri McShane. I was

assisted in the HGF mRNA *in situ* work by Mr. Rod Ferrier. Mr. John Stewart assisted me with immunocytochemistry for c-*met*, the HGF receptor.

I carried out the HGF *in vitro* inhibition studies. All animal experiments were carried out with appropriate Home Office licences. I was assisted in hepatocyte isolation by Dr. Phil Roberts. I performed the animal work involved in HGF *in vivo* inhibition studies assisted by Dr. Kenneth J. Hillan.

Summary

Hepatocyte growth factor (HGF) is a novel protein which is believed to have an important role in liver regeneration. Initially identified as the most potent mitogen for cultured hepatocytes, levels of HGF have since been shown to be elevated in serum and liver in animal models of liver disease. Raised levels have also been reported in serum from patients with liver disease. The receptor for HGF has been identified as the c-*met* proto-oncogene product. Although much has been learned of the structure and function of HGF *in vitro*, the significance of HGF and its receptor in controlling liver growth in health and disease remains to be determined.

The work of this thesis represents my attempts to investigate the significance of HGF in human liver disease and in an animal model of liver regeneration.

The aims of this work were as follows:

(1) Production of recombinant proteins based on human and rat HGF in order to to raise antibodies to native HGF.

(2) Purification of native human HGF in order to produce monoclonal and polyclonal antibodies to HGF.

(3) To establish an Enzyme Linked Immunosorbent Assay (ELISA) to measure serum HGF in patients with various liver diseases and to correlate these levels with disease type and severity.

(4) Immunocytochemical localisation of HGF and its receptor in human liver biopsies.

(5) Examination of the expression of HGF mRNA in human liver biopsies by *in situ* hybridisation techniques.

(6) Modification of the hepatic regenerative response to experimental liver injury by administration of neutralising anti-HGF antibody.

Specific cDNA fragments were prepared from mRNA extracted from rat liver and human placenta and cloned into suitable expression vectors for protein production. The recombinant HGF proteins were expressed in *Escherichia coli*, purified and identification confirmed by SDS-PAGE and N-terminal amino acid sequencing.

Native human HGF was purified from fresh human placentas by two stage column chromatography involving heparin agarose and hydroxylapatite. Eluted fractions containing HGF were identified by ELISA. A purified preparation of human placental HGF was produced containing 2 μg/ml HGF.

Rabbit polyclonal antibodies were produced following immunisation with recombinant proteins based on human and rat HGF, and with purified human placental HGF. These antibodies were characterised by SDS-PAGE. Murine antibody response following immunisation with purified human placental HGF was confirmed by ELISA. No suitable monoclonal antibody producing hybridoma clones were produced.

A number of ELISAs were produced and evaluated as a means of measuring HGF. This work culminated in the development of a sensitive, specific ELISA for the measurement of HGF in human serum. The reliability of the assay was determined and stability of HGF in serum assessed. Using the ELISA serum levels of HGF were determined in healthy controls, disease controls and in patients with a variety of liver diseases.

Thirty three healthy controls were assayed for HGF and only two had detectable levels of HGF. A group of patients with a diagnosis of acute myocardial infarction (n = 11) were used as a disease control group and none had detectable levels of HGF. When the healthy control group was compared with groups of patients with various liver diseases significant differences in HGF serum level were found in a number of cases. Serum HGF concentration was statistically significantly higher in patients with acute alcohol induced liver disease, chronic alcohol induced liver disease, acute (non alcoholic) hepatitis and chronic intrahepatic cholestasis. In contrast there was no difference in serum HGF level between healthy controls and patients with chronic hepatitis, extrahepatic biliary obstruction, metastatic liver disease, paracetamol overdose or chronic hepatitis C.

When serum HGF level was compared to a number of clinical indices of liver disease, several of these demonstrated a relationship with elevated levels of HGF. Patients with increased serum levels of HGF were found to have higher serum levels of AST, ALT and alkaline phosphatase than those with undetectable levels. Similarly, raised HGF levels were associated with prolonged prothrombin time, reduced haemoglobin concentration, albumin concentration and platelet count.

A greater proportion of patients with increased serum HGF level were jaundiced, had evidence of encephalopathy, hepatomegaly or ascites than those with an undetectable HGF level.

No relationship was present between HGF level and clinically evident hepatic inflammation (tenderness on examination) serum gamma glutamyl transferase, urea, creatinine or white cell count.

All of the available antibodies to HGF were assessed for use in immunocytochemistry, however, none of the antibodies were suitable for the demonstration of HGF in liver sections.

Using *in situ* hybridisation techniques, attempts were made to examine the expression of HGF mRNA in human and rat liver. A probe, pRBC-1, based on the rat HGF molecule failed to localise HGF mRNA in human liver tissue. However, using rat liver tissue obtained following partial hepatectomy and common bile duct ligation the probe demonstrated HGF mRNA in cells which on morphological grounds resembled Ito cells. A probe based on the human HGF nucleotide sequence was prepared but failed to localise HGF mRNA in normal or diseased human liver tissue.

A monoclonal antibody to the extracellular domain of the HGF receptor, c-*met*, was used to examine the expression of the receptor in human liver biopsies.

Histologically normal liver tissue demonstrated the presence of fine hepatocyte membranous staining consistent with the presence of membrane bound HGF receptor on hepatocytes. In diseased liver of various aetiologies, the level of HGF receptor on hepatocyte membrane appears to be reduced.

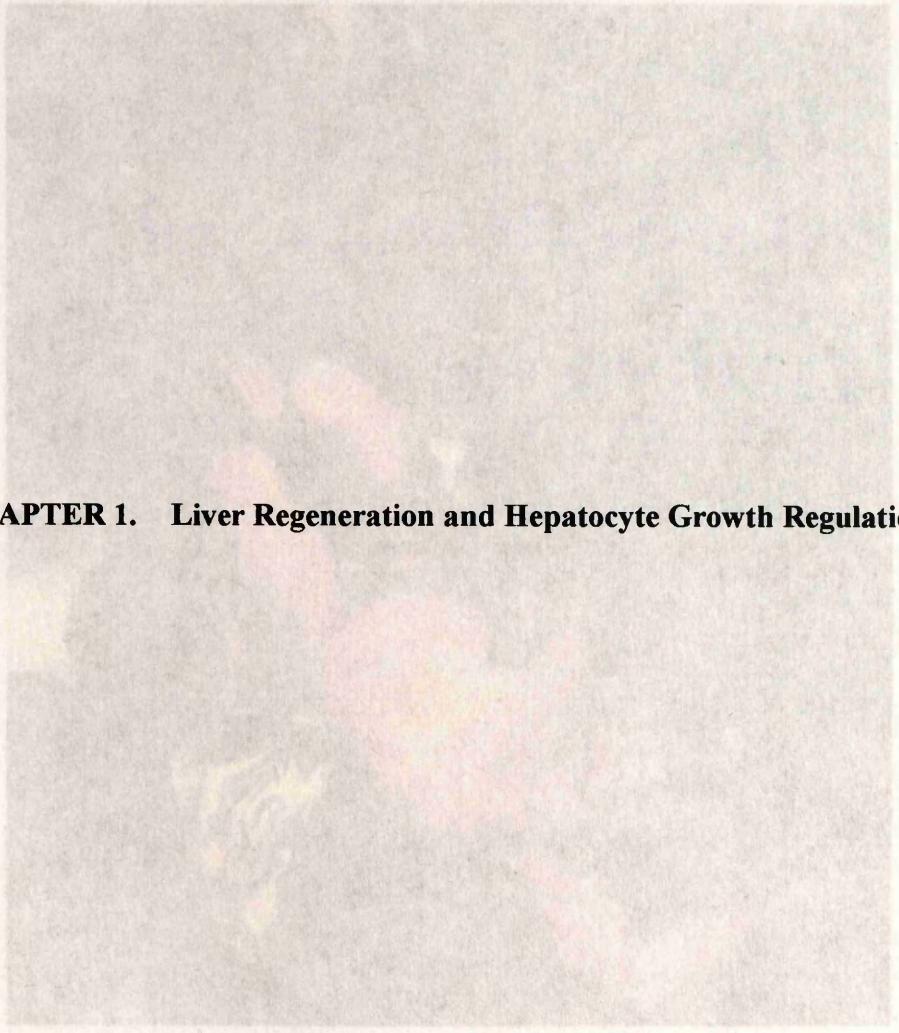
To investigate the contribution made by HGF to the hepatic regenerative response, *in vivo* inhibition of HGF was attempted in rats subjected to partial hepatectomy. Groups of rats were injected with monoclonal antibody to HGF, polyclonal antibody to HGF or appropriate control antibody and subjected to two thirds partial hepatectomy. Further antibody doses were administered post-operatively. The rats were killed at either 24 or 72 hours post-operatively and the regenerative response examined by histology and rate of DNA synthesis

(Bromodeoxyuridine uptake) identified using a standard immunocytochemical technique. Liver tissue from all animals showed considerable DNA synthesis. No difference was found between groups given anti-HGF and those given control antibodies.

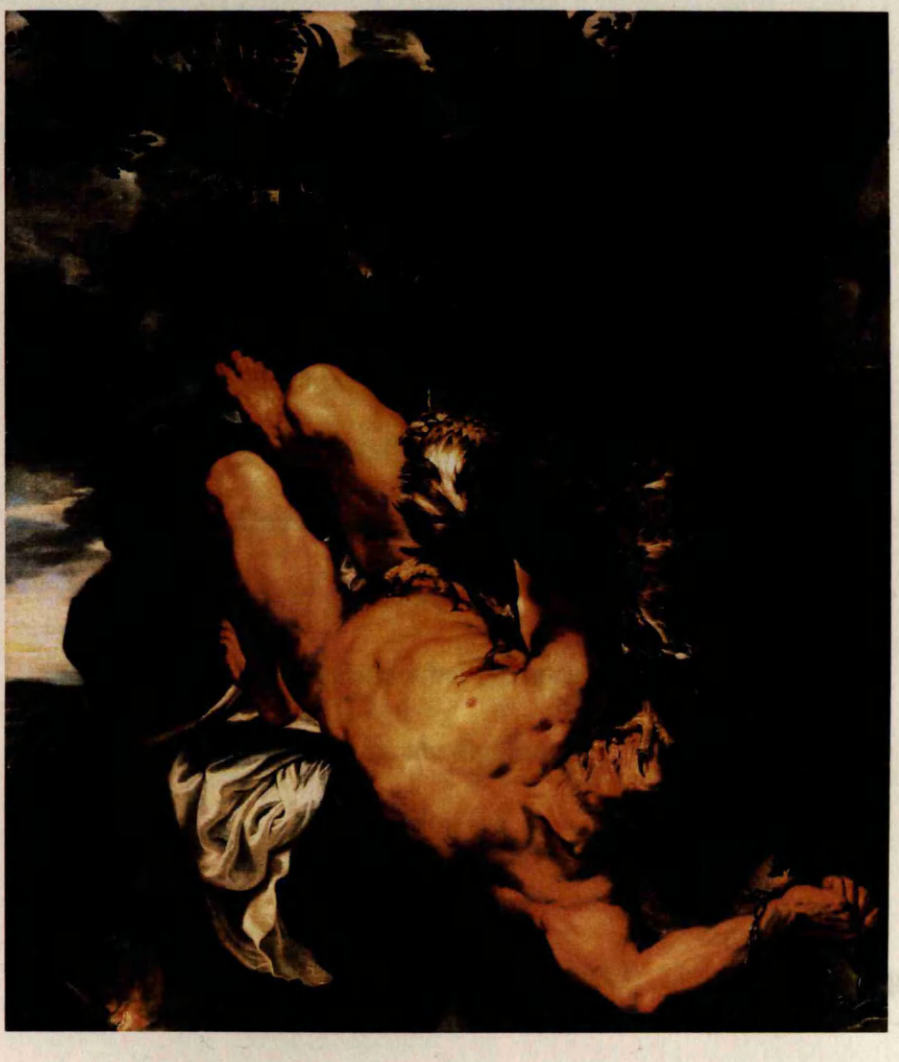
Abbreviations

ALT	alanine aminotransferase
ANIT	α-naphthylisothiocyanate
APES	3-aminopropyl tri-ethoxysilane
AST	aspartate aminotransferase
ATZ	anilinothiozolinone
bp	base pairs
BrdU	bromodeoxyuridine
BSA	bovine serum albumin
CAPS	3-[cyclohexylamino]-1-propane sulphonic acid
cos-1 cells	monkey kidney cells
CSM	cell surface modulator
Cys	cysteine
DEPC	diethylpyrocarbonate
dH_2O	distilled water
E. coli	*Escherichia coli*
EDTA	ethylene diamine tetra-acetic acid
EGF	epidermal growth factor
ELISA	enzyme linked immunosorbent assay
αFGF	acidic fibroblast growth factor
F-TCF	fibroblast derived tumour cytotoxic factor
GGT	gamma glutamyl transferase
GST	glutathione-s-transferase
HGF	hepatocyte growth factor
HGF/SF	hepatocyte growth factor/scatter factor
hpHGF	human placental hepatocyte growth factor
HPI	hepatocyte proliferation inhibitor
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
HSS	hepatic stimulatory substance
Ig	immunoglobulin
IL	interleukin
IMR-90 cells	human embryonic lung diploid fibroblasts
IPTG	isopropyl-β-D-thiogalactopyranoside
kb	kilobase
KB cells	human epidermoid carcinoma cells
kDa	kilo Dalton
M	Molar concentration

μCi	micro Curie
MDCK	Madin Darby canine kidney cells
Mr	relative molecular mass
MRC-S cells	human embryonic lung fibroblasts
mRNA	messenger
MW	molecular weight
N	normole concentration
NBT/BCIP	nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate
NSS	normal swine serum
$O.D._{280}$	optical density at 280nm
OPD	orthophenylene diamine
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
polymorph	polymorphonuclear leucocyte
PTH	phenylthiohydantoin
PVDF	polyvinylidenedifluoride
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TCA	trichloroacetic acid
TGFα	transforming growth factor α
TGFβ	transforming growth factor β
Tris	tris (hydroxymethyl) methylamine
Tyr	tyrosine



CHAPTER 1. Liver Regeneration and Hepatocyte Growth Regulation



PETER PAUL RUBENS
Flemish, 1577–1640

Prometheus Bound, 1612–18. Purchased for the W. P. Wilstach Collection

And in ineluctable, painful bonds

he fastened Prometheus

of the subtle mind, for he drove a stanchion
through his middle. Also

He let loose on him the wing-spread eagle

And it was feeding

On his imperishable liver, which by night

would grow back

To size from what the spread-winged bird

Had eaten in the day-time

Hesiod (c 750-700BC) Theogeny

1. INTRODUCTION

1.1 Liver Regeneration

The concept of liver regeneration has long been accepted as scientific fact, but original thought on the subject first arose in the ancient Greek myth of Prometheus. The benevolent titan, in a bid to improve the lot of mortal man, stole fire from the gods in a stalk of fennel and carried it down to earth. Zeus, enraged by this act ordered Prometheus to be lashed to a rocky crag in the Caucasus mountains. The unfortunate Prometheus' torment was multiplied by an eagle sent by Zeus which daily tore on his liver - the organ regrowing by night. The myth relates that only after Heracles intervened some 30,000 years later was Prometheus freed (1).

The scientific world first considered the idea of hepatic regeneration in 1833 when Cruveilhier suggested the possibility (2). The earliest experimental work on the subject was conducted in Italy in the 1880's but von Podwyssozki is generally credited with the first valuable scientific studies. In 1886 he described evidence of regeneration in hepatic cells adjacent to the site of excision of small wedges of liver in rats, cats, guinea pigs and rabbits (3).

Further studies confirming the phenomenon of hepatic regeneration followed and in 1931 Higgins and Anderson published a quantitative assessment of liver regeneration in the white rat, demonstrating that following 70% partial hepatectomy, restoration of hepatic mass was complete by 14 days (4).

1.2 Organisation of Liver Structure

The liver is composed mainly of epithelial cells, of which parenchymal cells constitute 90-95% of cell mass but only 60-65% of cell population. Non-parenchymal cells, including the littoral cell population of endothelial cells, Kupffer cells, fat storing (Ito) cells, pit cells (in the rat), fibroblasts and bile duct cells account for the bulk of the remaining cell population (5). This mass of epithelial cells is permeated by wide vascular channels known as sinusoids through which blood flows from terminal branches of the hepatic arteries and portal vein

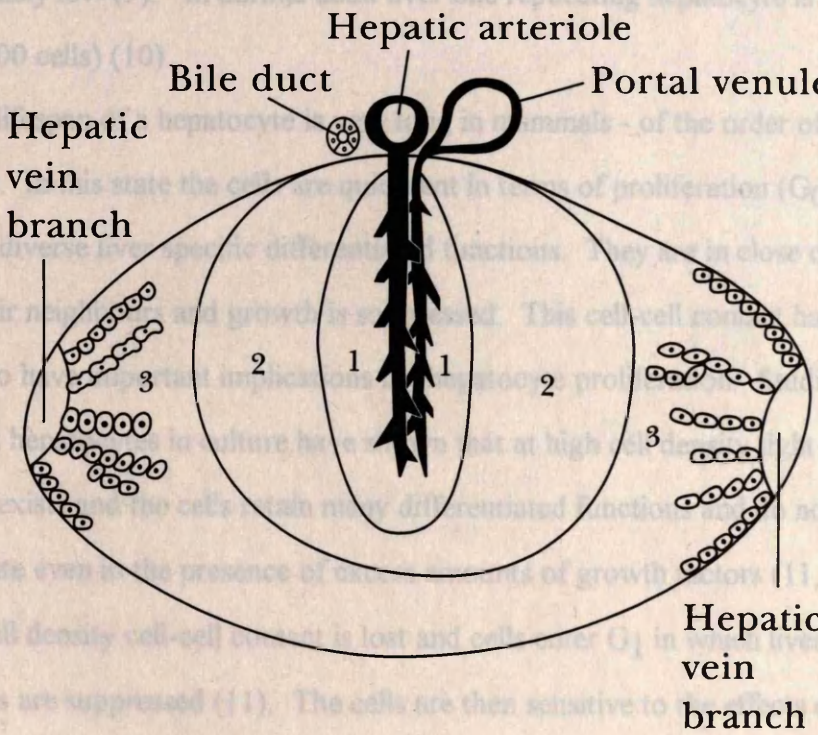
to reach terminal hepatic venules. The channels are separated by a system of interconnected walls or plates of hepatocytes which in rats and humans comprise only a single cell layer (5). The sinusoids are lined by a population referred to as littoral cells comprising Kupffer cells and endothelial cells. Kupffer cells are members of the mononuclear phagocytic (reticulo-endothelial) system. The main function of these cells is the removal of particulate material and effete red cells from portal blood. Endothelial cells form a thin discontinuous cytoplasmic sheet, the endothelial defects referred to as fenestrae. The function of these cells is believed to include that of a filtration barrier while allowing plasma to come into direct contact with hepatocytes. Endothelial cells are also capable of endocytosis and may be involved in sequestration of blood borne matter including proteins (6,7). Bile secreted by hepatocytes passes into a system of minute canaliculi which consist of fine channels passing between adjacent hepatocytes. The walls of the canaliculi are formed by the plasma membranes of adjacent hepatocytes. Bile flows towards bile collecting vessels in the portal tracts then from the intra-hepatic bile collecting system to the right and left hepatic ducts which combine to form the common hepatic duct. The cystic duct joins the common hepatic duct to form the common bile duct through which bile enters the duodenum.

The functional unit of the liver is the acinus which consists of a region of hepatic tissue with its own blood supply (a branch of the portal vein and a hepatic artery) and a bile duct (8). Afferent blood streams through sinusoids, between the single cell thick hepatocyte plates, to a hepatic vein branch, draining several acini (Fig. 1.1).

This leads to the concept of acinar zones 1, 2 or 3 described in relation to the afferent supply. These zones surround the axial structures of the acinus like layers of a bulb. Zone 1 is closest to the supplying vessels and receives blood of a similar composition to that in the afferent vessels. Zone 3 is the most distant from the supply vessels of that acinus as well as from those feeding the neighbouring acini. In this way, zone 3 cells are situated at the micro-circulatory periphery and

Figure 1.1 Schematic illustration of a liver acinus

The acinus consists of a region of hepatic tissue with its own blood supply (a branch of the portal vein and a hepatic artery) and a bile duct. Acinar zones 1,2 and 3 are shown. Zone 1 receives blood similar in composition to that in the afferent vessels. Zone 3 is situated at the microcirculatory periphery and receives blood which has already supplied zones 1 and 2. Hepatocytes are arranged in single cell thick plates.


Hepatic arteriole
Bile duct
Portal venule
Hepatic vein branch
1
2
3
Hepatic vein branch

receive blood which has already supplied zones 1 and 2. These cells are therefore more sensitive to injury by ischaemia, congestion and nutritional deficiency. Zone 2 forms a transitional region the boundaries of which are not fixed. Zones 1 or 3 may encroach on zone 2 in different microcirculatory conditions, depending on fluctuations in arteriolar smooth muscle tone (8) (Fig. 1.2).

1.3 Normal Growth

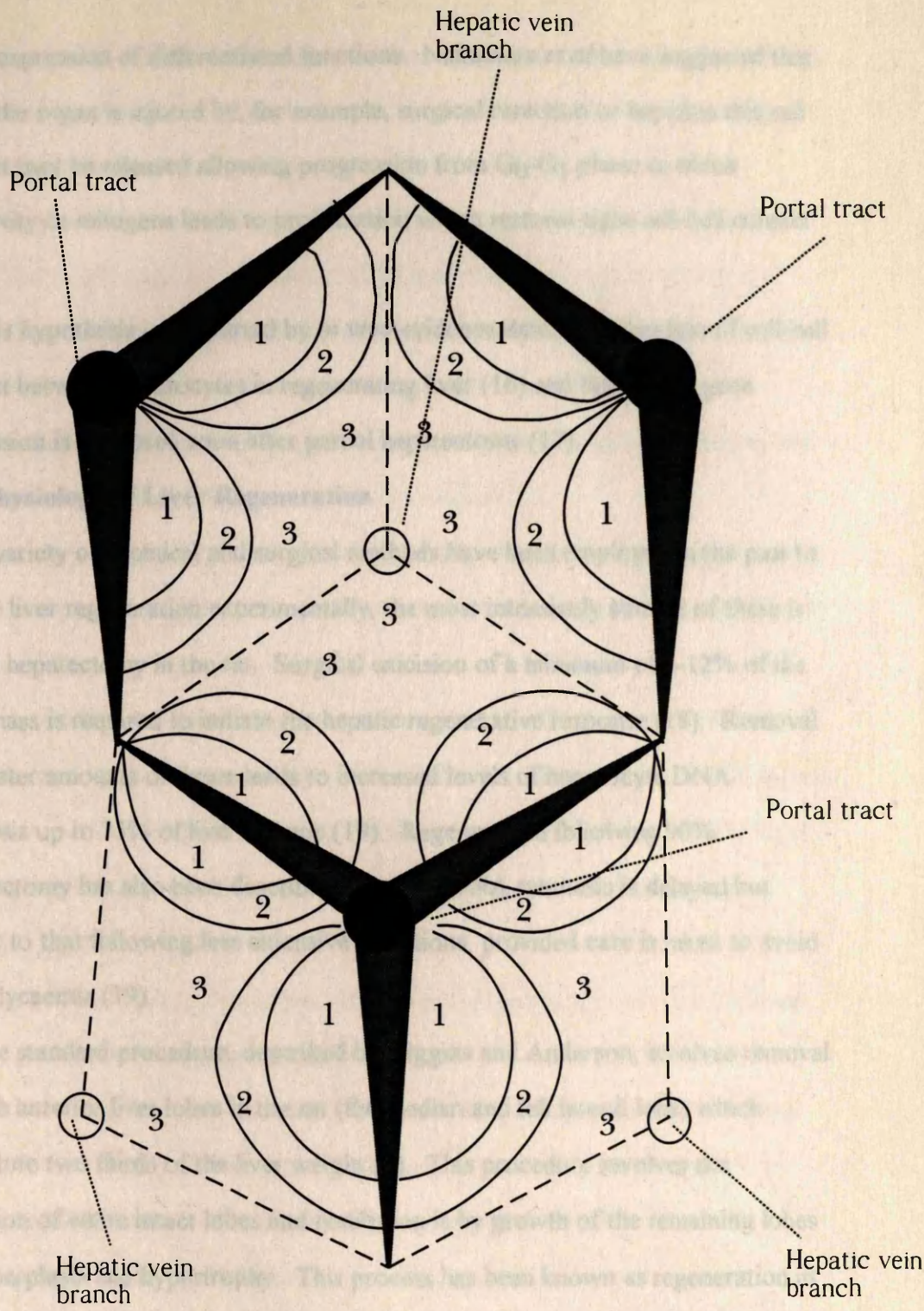
Following fetal and postnatal liver growth, the level of hepatocyte proliferation is extremely low (9). In normal adult liver one replicating hepatocyte is seen per 10-20,000 cells) (10).

The lifespan of a hepatocyte is very long in mammals - of the order of 200-400 days (9). In this state the cells are quiescent in terms of proliferation (G_0), and express diverse liver specific differentiated functions. They are in close contact with their neighbours and growth is suppressed. This cell-cell contact has been shown to have important implications for hepatocyte proliferation. Studies using adult rat hepatocytes in culture have shown that at high cell density tight cell-cell contact exists and the cells retain many differentiated functions and do not proliferate even in the presence of excess amounts of growth factors (11,12). At lower cell density cell-cell contact is lost and cells enter G_1 in which liver specific functions are suppressed (11). The cells are then sensitive to the effects of growth factors, and can be driven from G_1 to S-phase (12). Expression of the proto-oncogene c-*myc*, which is known to be related to cell proliferation, is increased at low cell density and suppressed at high cell density (13).

This G_0-G_1 cell cycle transition in hepatocytes has been shown to be regulated by a plasma membrane protein named "cell surface modulator" (CSM) (14). Addition of this 670kDa protein, extracted from the plasma membrane of liver tissue, inhibits hepatocyte responsiveness to mitogens, when cells are cultured at low density (14). It is thought that cell-cell contact inhibition mediated by CSM may be involved in the regulation of growth and differentiation of hepatocytes in liver regeneration. In intact liver, hepatocyte CSM may suppress proliferation and

Figure 1.2 Schematic illustration of several adjacent liver acini

Acinar zones 1, 2 and 3 surround the axial structures of the acinus like layers of a bulb. Zone 3 is the most distant from the supply vessels and receives blood from adjacent acini. Zone 2 forms a transitional region the boundaries of which are not fixed and which depend on fluctuations in arteriolar smooth muscle tone.


Hepatic vein branch
Portal tract
Portal tract
Portal tract
Hepatic vein branch
Hepatic vein branch

allow expression of differentiated functions. Nakamura *et al* have suggested that when the organ is injured by, for example, surgical resection or hepatitis this cell contact may be released allowing progression from G_0-G_1 phase in which sensitivity to mitogens leads to proliferation which restores tight cell-cell contact (15).

This hypothesis is supported by *in vivo* evidence demonstrating loss of cell-cell contact between hepatocytes in regenerating liver (16) and that c-*myc* gene expression is increased soon after partial hepatectomy (17).

1.4 Physiology of Liver Regeneration

A variety of chemical and surgical methods have been employed in the past to induce liver regeneration experimentally, the most intensively studied of these is partial hepatectomy in the rat. Surgical exicision of a minimum of 9-12% of the liver mass is required to initiate the hepatic regenerative response (18). Removal of greater amounts of tissue leads to increased levels of hepatocyte DNA synthesis up to 75% of liver volume (19). Regeneration following 90% hepatectomy has also been described, in which DNA synthesis is delayed but similar to that following less extensive resections, provided care is taken to avoid hypoglycaemia (19).

The standard procedure, described by Higgins and Anderson, involves removal of both anterior liver lobes in the rat (the median and left lateral lobe) which constitute two thirds of the liver weight (4). This procedure involves the resection of entire intact lobes and restitution is by growth of the remaining lobes by hyperplasia and hypertrophy. This process has been known as regeneration in the scientific literature although the term is not strictly accurate, since the organ responds by compensatory hyperplasia rather than regrowth of excised portions.

1.4.1 Parenchymal Cells

Within thirty minutes of partial hepatectomy morphological changes are evident in cells present in acinar zone 1, including dispersion of cytoplasmic basophilic bodies. When seen by electron microscopy these changes are reflected

in the disruption of the endoplasmic reticulum. This appearance gradually spreads toward acinar zone 3 over the following 8 hours. There is also 'cloudy swelling' of mitochondria (13,20).

After several hours there is an accumulation of lipid, which is maximal by 10 hours, and a fall in glycogen content. Glycogen is almost undetectable at 10 hours, after which there is a slow increase and at 72 hours the value is still only a third of normal.

An increase in the size of cells, nuclei and nucleoli is evident by 12 hours, and is maximal at 12 (or 24) hours when mitosis begins (21).

The endoplasmic reticulum begins to reform by 16-18 hours starting in acinar zone 3 and progressing toward zone 1 with restoration of normal structure by 36-48 hours (20).

DNA synthesis, as determined by radioautography of radioactive nucleotide incorporation, rapidly increases 16-18 hours after partial hepatectomy to a peak at 20-24 hours followed by a sharp fall. Labelled nuclei predominate initially in the periportal areas, then extend toward the terminal hepatic venule and become randomly distributed (22).

Cells in zone 1 of the acinus have been reported to undergo several divisions while a few in zone 3 may not divide at all.

Mitosis follows DNA synthesis by 6-8 hours and exhibits a similar pattern of distribution within the acinus (22). At the time of maximal mitotic activity, mitotic cells represent 3-4% of total hepatocyte number.

1.4.2 Non-Parenchymal Cells

Non-parencymal cells as well as those of peritoneal mesothelium forming the capsular surface, blood vessel walls, bile ducts and connective tissue lag behind hepatocytes in synthesising DNA by about one day. Mitosis is not maximal until the end of the second or early on the third day. However, these cells do not exhibit the same clear peak of mitosis as parenchymal cells (21,22). Kupffer cells, for example, increase gradually, doubling in number over three days (23).

Connective tissue elements are restored very slowly; periportal fibroblasts continue mitotic activity longer than other cell types (collagen formation, beginning at 48 hours, remains low even after six weeks) (21).

1.4.3 Sequential Proto-oncogene Expression

The precisely regulated events of hepatocyte replication have been studied in ever increasing detail in an attempt to determine the initiating events and regulatory mechanisms. Studies of the expression of various cellular proto-oncogenes during liver regeneration have shown that as early as 15 minutes after partial hepatectomy, four fold increases in levels of c-*fos* mRNA can be detected (24). It has also been demonstrated that three main characteristics of such proto-oncogene expression exist such that it is:

A) Specific

B) Sequential

C) Transient

(A) During liver regeneration there is an increase of mRNAs for c-*fos*, c-*myc*, p53 and c-*ras* genes, while c-*abl*, c-*mos* and c-*src* remain unaffected.

(B) A well defined order is evident; *fos*, *myc*, p53 then *ras* h and *ras* k.

(C) Expression of these genes is transient - lasting only a few hours (*fos*, *myc*, p53) or longer (*ras* - 30 hours) during liver regeneration then returning to basal levels (9).

1.5 Control of Hepatic Regeneration

The mechanisms involved in the regulation of this process have been, and remain, the focus of intense investigation. Two main regulatory mechanisms have been proposed involving;

(a) Haemodynamic factors.

(b) Humoral factors.

1.5.1 Haemodynamic Factors

Increased blood flow particularly of portal blood was thought to be of prime importance in initiating regeneration following partial hepatectomy and early

evidence supporting this suggestion was produced by Mann (25,26). However, later work demonstrated that regeneration could occur even in the absence of the portal blood supply (27,28) and that increasing hepatic blood flow several-fold by arterialisation does not lead to a commensurate increase in liver growth (28). Moreover, blood flow remains the same in cases of regeneration following carbon tetrachloride induced necrosis (27). Finally, increased DNA synthesis in heterotopic partial autografts of rat liver following partial hepatectomy confirmed that portal blood and increased blood flow are unnecessary for proliferation of hepatocytes (29).

1.5.2 Humoral Factors

In 1949 Christensen and Jacobsen described work on the hepatic regenerative response in parabiotically united rats (30). Partial hepatectomy was performed on one of each pair and at 52 hours the animals were killed and mitosis counted in the livers of each parabion. It was found that the number of mitoses was increased in both animals of a parabiotic pair. Bucher produced similar results by counting mitoses in livers of non-hepatectomised rats following partial hepatectomy in animals parabiotically united in pairs or triplets (10).

These experiments were refined by Moolten and Bucher (31) and Sakai (32). The later studies, using ^{14}C-thymidine incorporation as an index of DNA synthesis, again confirmed the possibility of a humoral mediator of regeneration.

Further work involving the study of the effect of partial hepatectomy on DNA synthesis and mitosis in heterotopic partial autografts of rat liver (29) and on transplanted hepatocytes (33) showed that DNA synthesis and mitosis is increased in the grafted tissue.

These studies provided strong evidence for a humoral mediator of hepatic regeneration. The nature of the agent, or agents responsible, has been under intense investigation and a number of candidates have been suggested.

1.6 Hormonal Influences

Work on whole animals with removal of endocrine glands or injection of hormones has demonstrated a modifying but not determining role for known endocrine factors (20). Of course, such experiments are fraught with difficulties, given the complex biochemical interactions present within the intact animal. With the advent of isolated primary hepatocyte culture in the 1960s and 1970s the possibility of focussing on precise mechanisms controlling hepatocyte growth became a reality.

In 1976, Richman *et al* (34) described conditions in which primary isolated hepatocytes could be made to synthesise DNA, and suggested a prominent role for epidermal growth factor in concert with insulin and glucagon.

Following this work various manipulations of hepatocyte culture medium were performed and it was found that serum obtained from rats 48 hours after partial hepatectomy was consistently more effective in stimulating DNA synthesis than normal rat serum (35).

1.7 Hepatotropic Agents Hepatocyte Growth Factor (HGF)

The *in vitro* means of studying hepatocyte functions led to publication of a number of reports describing DNA synthesis promoting factors. Morley and Kingdom in 1973 (36) reported liver specific stimulation of DNA synthesis by a heat stable protein of 26kDa isolated from serum of rats 24 hours after partial hepatectomy. This was followed in 1982 by Strain *et al* (37) who described stimulation of DNA synthesis in cultured hepatocytes by a rat platelet associated substance. Paul and Piasecki (38) demonstrated the presence in rat platelets of hepatocyte growth factor(s) distinct from PDGF. In 1984, three laboratories independently reported partial purification of a hepatocyte growth factor or hepatopoietin A from rat platelets (39) or serum (40,41). This factor was subsequently purified to homogeneity from rat platelets (42) and human plasma (43,44), rabbit serum (44), rat liver (45) and human placenta (46). Hepatocyte growth factor (HGF) was shown to be a heat and acid labile heterodimeric protein

which is sensitive to reduction with dithiothreitol, consists of a larger α chain of 54-70kDa mass and a smaller β chain of 29-35kDa, and which is distinct from other growth factors.

1.7.1 Molecular Cloning and Deduced Primary Structure of HGF

In 1989 (47,50) and 1990 (49) cDNA coding for human and rat HGF was cloned and the primary structure of HGF deduced. This led to the demonstration that rat, rabbit and human HGF are highly homologous molecular species. In each case native HGF was purified and the amino acid sequence for parts of the protein determined. Degenerate oligonucleotide primers were synthesised on the basis of these amino acid sequences and used to screen liver or placental cDNA libraries. Northern hybridisation studies demonstrated that the mRNA coding for HGF is ~6kb in size. Following this method the nucleotide sequence of the entire HGF cDNA was determined. It comprises a 5' non-coding region of 134 nucleotides, a single open reading frame of 2,184 nucleotides, and a 3' non-coding region of 3,580 nucleotides. The 3' non-coding region contains an A U-rich sequence, which is known to be a recognition signal for a processing pathway for specific degradation of the mRNA. This A U-rich sequence is commonly associated with mRNAs which are expressed transiently (13).

The deduced protein sequence of HGF showed that a polypeptide of 728 amino acids is encoded by human HGF cDNA in a single open reading frame which includes a signal peptide and both the α- and β-chains.

Calculated relative molecular masses (Mr) of human HGF have been reported as 83.132kDa (47) or 83.126kDa (48) consisting of an α-chain of 440 amino acids (Mr; 50.808kDa (48)) and a β-chain of 234 amino acids (Mr; 25.994) (47) or 26.089 (48)). Four asparagine linked glycosylation sites have been identified (α-chain positions 294, 402; β-chain positions 566, 653). Glycosylation at these sites may account for the differences in mass of HGF subunits as determined by SDS-PAGE and those predicted from the cDNA sequence (in addition to the over-estimation of the molecular weight by relaxation of disulphide bonds).

The protein is produced as a pre-promolecule, the N-terminal of which begins with a 54 amino acid pre-pro sequence. The first 29 amino acids are hydrophobic as is commonly found in signal sequences. This is followed by the pro-sequence of 25 amino acids then the N-terminus of the α-chain. The α and β chains are synthesised together from 55-728, the ß-chain starting at 495. The protein is produced in this pre-pro form, then undergoes proteolytic processing with separation at position 494/495 (Arg-Val). The single chain form of HGF is inactive, biological activity only being acquired following conversion to the two chain form by extracellular serine-protease (50) referred to as HGF-converting enzyme (51). The two chains remain bound, by a disulphide bond between CYS 487 in α-chain and CYS 604 in β-chain (Fig. 1.3).

A second type of cDNA coding for a naturally occurring variant of HGF has been cloned (52). This c-DNA encodes a form of HGF with a 5 amino acid deletion in the first kringle domain. The biological activity of this form of HGF is similar to that of full length HGF.

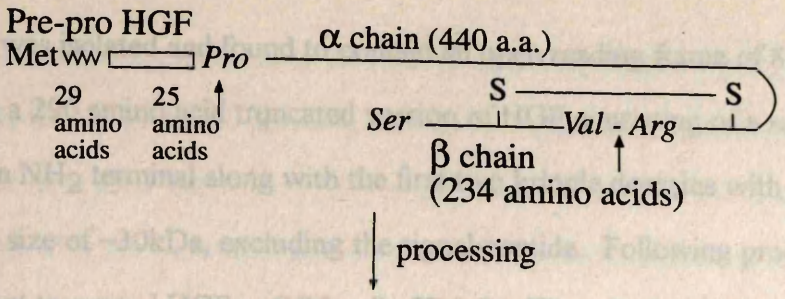
An interesting structural feature of the HGF molecule is the presence of four kringle domains. These looped, disulphide bridged regions were named for their likeness to a particular type of Danish pastry (53) and are thought to be involved in binding regulatory ligands. The α chain also has a N-terminal hairpin loop structure and the β chain a serine protease like domain. The N-terminal hairpin region, together with the first two kringles are essential for biological activity and for binding to the receptor (54,55)

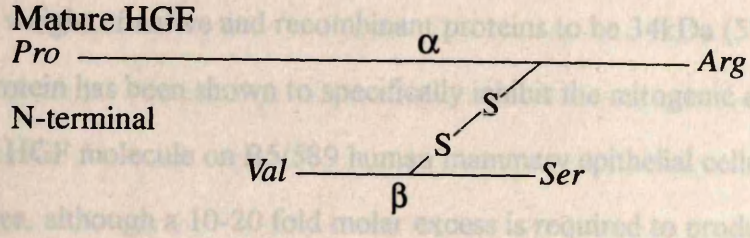
1.7.2 HGF Antagonist

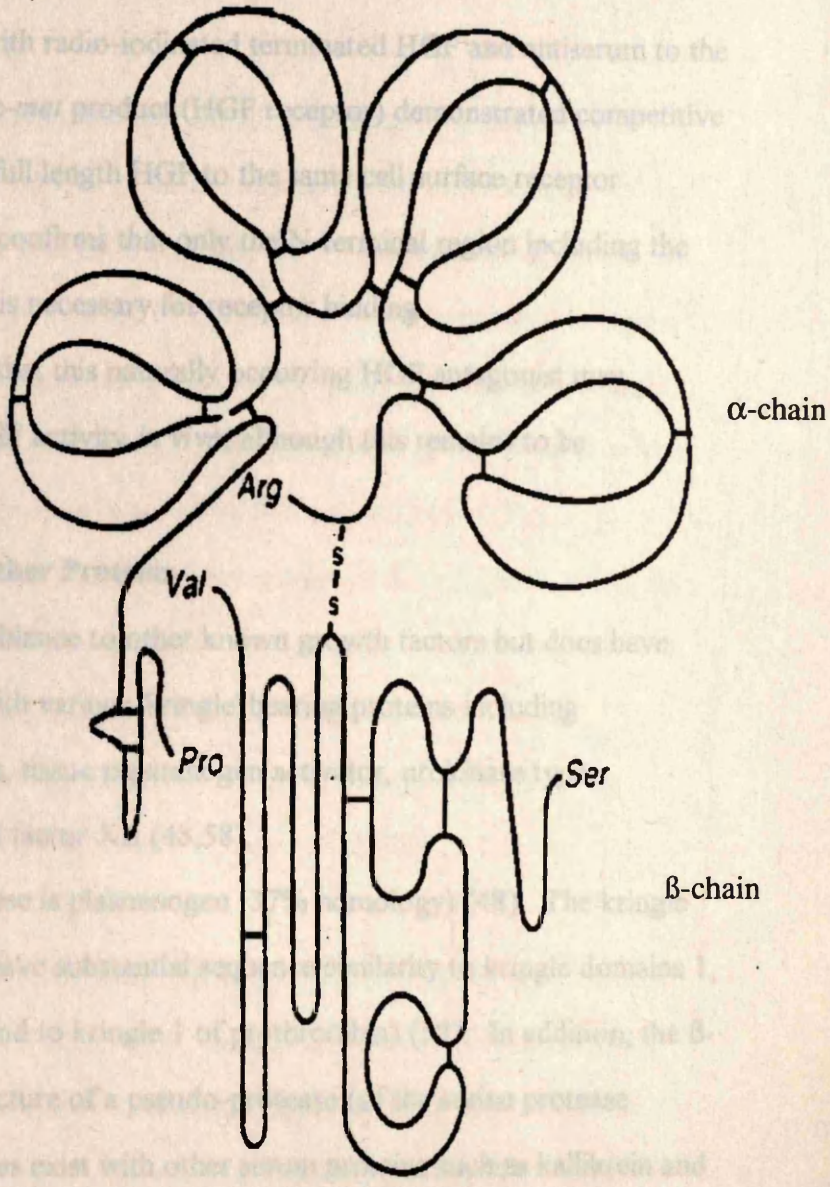
An alternative, truncated form of HGF has also been identified. This naturally occurring peptide is encoded by an alternatively processed mRNA generated from the human HGF gene (56,57). The alternative transcript (1.5kb (56), 1.3kb (57)) is found in human placenta and is identical to the full length form from the 5' end for 865 nucleotides, but diverges at the 3' end. It is generated by alternative RNA processing events involving the second kringle exon. A cDNA sequence for the

Figure 1.3 Schematic representation of HGF protein

HGF is produced as a pre-pro molecule which undergoes proteolytic processing to form an active 2-chain peptide. The α-chain is characterised by 4 kringle domains and is joined to the β-chain by a disulphide bond.


Pre-pro HGF
Met
Pro
α chain (440 a.a.)
29 amino acids
25 amino acids
S
S
Ser
Val - Arg
β chain
(234 amino acids)
processing


Mature HGF
Pro
α
Arg
N-terminal
S
S
Val
Ser
β


α-chain
Arg
S
S
Val
Pro
Ser
ß-chain

transcript was isolated and found to contain an open reading frame of 870bp predicting a 290 amino acid truncated version of HGF consisting of a signal peptide, an NH_2 terminal along with the first two kringle domains with a calculated size of ~30kDa, excluding the signal peptide. Following production of recombinant truncated HGF in COS cells SDS-PAGE analysis confirmed the molecular weight of native and recombinant proteins to be 34kDa (57).

This protein has been shown to specifically inhibit the mitogenic effects of the full length HGF molecule on B5/589 human mammary epithelial cells and human melanocytes, although a 10-20 fold molar excess is required to produce a 50% reduction (57) in DNA synthesis.

Crosslinking studies with radio-iodinated terminated HGF and antiserum to the carboxyl terminus of the c-*met* product (HGF receptor) demonstrated competitive binding of truncated and full length HGF to the same cell surface receptor molecule (52). This also confirms that only the N-terminal region including the first two kringle domains is necessary for receptor binding.

It has been suggested that this naturally occurring HGF antagonist may modulate the effect of HGF activity *in vivo*, although this remains to be demonstrated.

1.7.3 Homology with Other Proteins

HGF bears little resemblance to other known growth factors but does have considerable homology with various 'kringle' bearing proteins including plasminogen, prothrombin, tissue plasminogen activator, urokinase type plasminogen activator and factor XII (48,58).

The most similar of these is plasminogen (37% homology) (48). The kringle domains present in HGF have substantial sequence similarity to kringle domains 1, 4 and 5 of plasminogen (and to kringle 1 of prothrombin) (58). In addition, the ß-chain of HGF has the structure of a pseudo-protease (of the serine protease superfamily) and similarities exist with other serum proteins such as kallikrein and factor XII (58). However, two (histidine, serine) of the three amino acids of the

protease active site are replaced in the HGF β-chain by glutamine and tyrosine respectively and HGF has no protease activity (58) (similarly, neither plasminogen nor plasmin have HGF activity) (13). The presence of such structural similarities is consistent with the view that HGF is of relatively recent phylogenetic derivation (60). The amino acid sequence homology of human and rat HGF is greater than 90% (49). This helps to explain the absence of species specificity in terms of biological activity. The principle differences that do exist include insertions and deletions in the β-subunit sequence such that human HGF β1-chain = 234 and rat HGF β-chain = 233 amino acids, also the C-terminus of human β-chain is serine whereas in rat this is replaced by leucine (49).

1.7.4 Human HGF Gene

In 1991 Seki *et al* (59) characterised the gene coding for human HGF, demonstrating that it is composed of 18 exons interrupted by 17 introns and is about 70kb in length. The first exon contains the 5' untranslated region and the signal peptide. The α-chain is coded by the next 10 exons, with each kringle domain coded by a pair of exons, as is the case for other proteins containing kringle domains. The short spacer region between the α- and β-chains is coded by the 12th exon and the ß-chain is coded by the final six exons (Fig. 1.4).

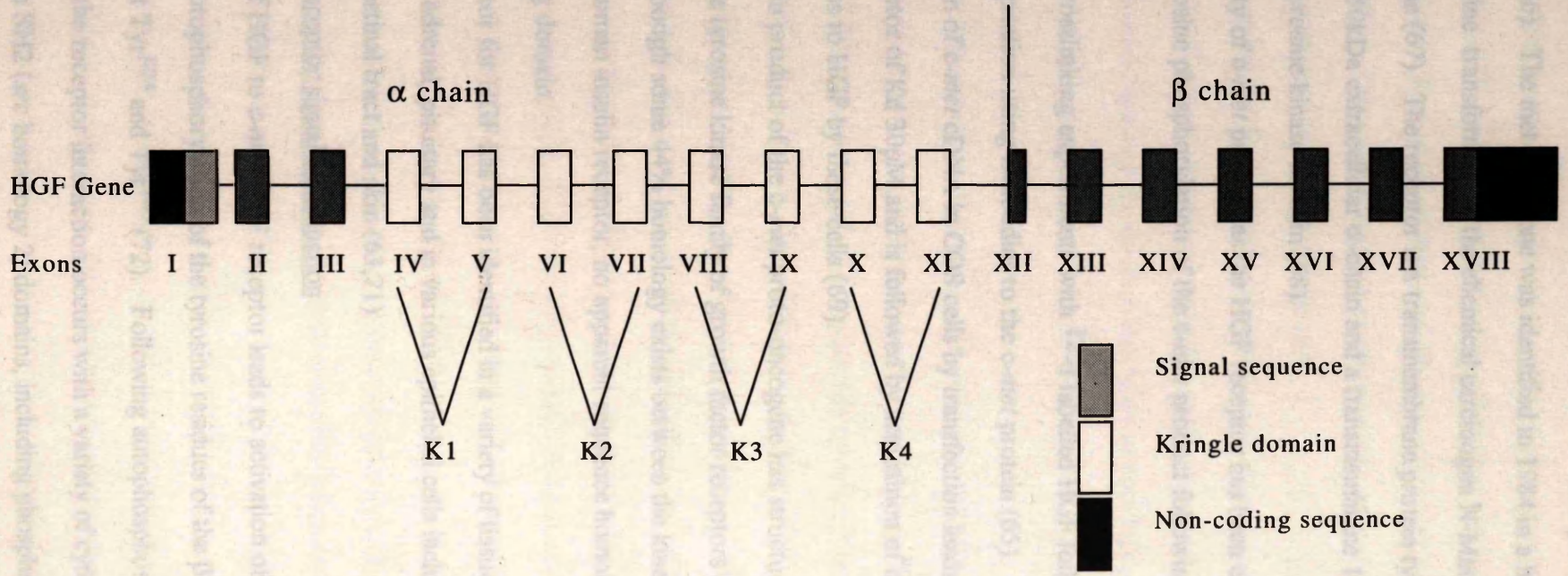
The organisation of the HGF gene is very similar to that of genes coding for plasminogen and other serine proteases involved in blood coagulation and fibrinolysis. This is thought to represent further evidence that HGF is evolutionally related to these proteins. In 1991, the human HGF gene was mapped to the long arm of chromosome 7, band q21.1 (60).

1.7.5 HGF Receptor

Investigation of the means by which HGF exerts its biological effects led to attempts to identify receptor(s) for HGF. A high affinity HGF receptor with a dissociation constant (Kd) of 20-36pM and an apparent molecular weight of 220kDa was identified on primary cultured rat hepatocytes and other epithelial cells (61,62,63,64).

Figure 1.4 HGF Gene Organisation

The gene coding for HGF is approximately 40kb in length and is composed of 18 exons interrupted by 17 introns.


α chain
β chain
HGF Gene
Exons
I
II
III
IV
V
VI
VII
VIII
IX
X
XI
XII
XIII
XIV
XV
XVI
XVII
XVIII
K1
K2
K3
K4
Signal sequence
Kringle domain
Non-coding sequence

In 1991 the receptor for HGF was identified as the c-*met* proto-oncogene product (65,66). The met oncogene was identified in 1984 in a human osteogenic sarcoma cell line transformed by the chemical carcinogen N-Methyl-N-Nitro-N-Nitroguanidine (67). The receptor is a transmembrane protein tyrosine kinase of 2 subunits; a 50kDa extracellular α-chain and a transmembrane 145kDa β-subunit which has a tyrosine kinase domain (68).

The identity of c-*met* product as the HGF receptor has been confirmed by:

(1) Rapid tyrosine phosphorylation of the c-*met* product following HGF treatment (65,66).

(2) Affinity crosslinking experiments with ^{125}I labelled HGF followed by immunoprecipitation using antibodies to the c-*met* protein (65).

(3) Expression of c-*met* cDNA in COS cells by transfection leads to expression of an HGF receptor of Kd 30pM and is followed by acquisition of mitogenic responsiveness to HGF by these cells (69).

The protein product of the c-*met* proto-oncogene has structural and functional features of the tyrosine kinase family of growth factor receptors (68,70). However, although some 44% homology exists between the kinase domain of c-*met* and the human insulin receptor, no apparent sequence homology exists in the ligand binding domain.

The receptor for HGF has been identified in a variety of tissues such as liver, lung, kidney, adrenal, pituitary and in various epithelial cells including the lining of the gastrointestinal tract and skin (63,71).

<u>c-*met*/HGF receptor signal transduction</u>

Binding of HGF to c-*met*/HGF receptor leads to activation of tyrosine kinase resulting in autophosphorylation of the tyrosine residues of the β-subunit (most importantly at Tyr^{1234} and Tyr^{1235} (72). Following autophosphorylation and activation of the receptor interaction occurs with a variety of cytoplasmic proteins which contain SH2 (*src* homology 2) domains, including phosphatidyl(inositol)-3-kinase (PI-3 kinase), phospholipase Cδ (PLC-δ), *ras* GTPase activating protein

(*ras* GAP) and cytoplasmic tyrosine kinases of the c-*src* family (73,74). Activation of phospholipase Cδ occurs as a result of phosphorylation following stimulation with HGF, and induces production of inositol-1,4,5-triphosphate and 1,2-diacylglycerol followed by calcium mobilisation and activation of protein kinase C (75,76). This cascade of phosphorylation and activation ultimately leads to altered gene expression and DNA synthesis.

1.7.6 Evidence for HGF as a Mediator of Hepatic Regeneration

Following its discovery and characterisation as the most potent mitogen known for cultured hepatocytes, evidence has been accumulating in favour of the view that HGF plays a major role in the process of hepatic regeneration following injury.

A number of reports have described increased levels of HGF in both liver and plasma of rats subjected to partial hepatectomy or carbon tetrachloride induced liver injury (77,78). Similarly carbon tetrachloride treatment leads to an elevation of circulating HGF in mice (78). In addition, examination of the expression of HGF mRNA in liver tissue from rats subjected to a variety of insults such as carbon tetrachloride poisoning, D-galactosamine induced hepatitis, partial hepatectomy or common bile duct ligation indicates an increase in the level of HGF gene transcription (79, 80,81). Increased HGF mRNA expression in liver remnant following partial hepatectomy is evident at 12 hours and maximal at 24 hours i.e. at the same time as initial peak of DNA synthesis. Significantly increased plasma HGF levels are however detected as early as 3 hours post resection, suggesting that extrahepatic sources of HGF may provide the regenerative trigger rather than HGF production within the liver (79).

Such a view is supported by the finding that HGF mRNA expression has been shown to be increased in intact kidney, spleen (82) and lung (83) following partial hepatectomy in rats. Furthermore, following hepatic injury in rats, marked HGF receptor down regulation is seen; interestingly, the down regulation only occurs in the organ type injured, i.e. in liver following partial hepatectomy, or in kidney

following unilateral nephrectomy (63). There is also evidence from *in vivo* studies that HGF has a role in liver regeneration and may ameliorate the effects of hepatotoxins. Ishiki *et al* (84) have reported that intravenous administration of recombinant HGF to mice subjected to 30% hepatectomy, carbon tetrachloride or α-naphthylisothiocyanate (αANIT) poisoning results in remarkably enhanced liver regeneration *in vivo*. Moreover, the addition of HGF prevented any marked increase in serum levels of liver enzymes and bilirubin in mice treated with αANIT (84). Similar findings of reduction in αANIT hepatotoxicity when administered with recombinant HGF were reported by Roos *et al* (85).

Studies using transgenic mice which expressed increased levels of HGF under the control of albumin regulatory sequences demonstrated that in such animals the rate of regeneration after partial hepatectomy could be doubled (86). There have, however, been no reports of attempts to inhibit HGF function *in vivo*. Such a study may establish conclusively the significance of the contribution made by HGF to the hepatic regenerative response.

1.7.7 Sites of Origin of HGF

In situ hybridisation studies initially identified the hepatic source of HGF as Kupffer cells and sinusoidal endothelial cells (87). However, convincing evidence has been produced demonstrating that the Ito cell is the main cell type producing HGF in the liver (88,89).

HGF mRNA has also been shown, by Northern blot analysis, to be present in adult rat kidney, lung, thymus and brain (49) in addition to human placenta (47), fetal liver (90) and embryonic lung fibroblast (91).

As described above, HGF activity in the circulation is markedly increased within 3 hours of liver injury, suggesting that extrahepatic sources of HGF may be involved in the initiation of hepatic regeneration. The level of expression of HGF mRNA rapidly increases in the intact lung, spleen and kidney after partial hepatectomy or carbon tetrachloride administration.

In situ hybridisation studies show that the cells expressing HGF mRNA appear to be fenestrated endothelial cells in the kidney and endothelial cells in the lung (87,62). Localisation of HGF protein in tissues has also been reported in rabbits, rats and humans (92,93). Positive staining, using an immunohistochemical technique, was found in a variety of tissues and cell types, including acinar cells of pancreas, neurons in the brain, c-cells of the thyroid, salivary glands and Brunners glands in the rabbit (92). In rat and human most surface epithelia, distal tubules and collecting ducts of the kidney; syncytiotrophoblast, extra-villous trophoblast, large neurons, megakaryocytes and granulocytes were stained positively (93). However, questions arise with regard to the discrepancy found between tissues positive for HGF protein by immunocytochemistry in which HGF mRNA cannot be detected. Also, contradictory immunocytochemistry results have been reported (94) which describe positive staining for HGF in pancreatic islet 'A' cells and an absence of acinar staining.

Currently the balance of evidence is in favour of the view that HGF may exert hepatotropic effects in an endocrine as well as paracrine fashion.

1.7.8 Injurin

Although HGF appears to be a major effector molecule in liver and kidney regeneration, much has yet to be learned of the mechanisms which control HGF production. Matsumoto *et al* (95) have described a protein factor which has the capacity to induce expression of the gene for HGF. Rat serum from animals subjected to partial hepatectomy or ischaemic injury injected intra-peritoneally into intact rats led to a marked increase of HGF mRNA expression in the lung of the recipient. The effect was also shown *in vitro* with MRC-5 cells which responded by increased expression of HGF mRNA and increased production of HGF. Preliminary purification studies suggest that the agent responsible for these effects is a protein of molecular weight 10-20kDa and that its effects are not duplicated by known cytokines. Using MRC-5 cells in a functional assay, levels of this factor, named "injurin" are seen to rise within 3 hours (i.e. preceding the

increase in expression of HGF mRNA) of hepatic or renal injury including unilateral nephrectomy, mercury chloride (HgCl) or kanamycin administration.

Injurin may therefore represent another link in the chain of events triggered by liver (and renal) injury which results in growth and repair in the affected system.

1.7.9 Other Biological Activities of HGF

In addition to its well documented ability to stimulate DNA synthesis in isolated cultured rat hepatocytes, HGF has been found to promote DNA synthesis in a variety of epithelial cells including rabbit renal tubular epithelial cells (97), human epidermal melanocytes (91,97), keratinocytes (98) and other epithelial cells (99). However, no growth stimulation of fibroblast/mesenchyme derived cells has been described. Another function of HGF is stimulation of cell motility. Workers investigating a fibroblast secreted protein which could disperse epithelial colonies of a variety of cell types in culture named the protein scatter factor and only when sequence data became available was scatter factor found to be identical to HGF (100). Examples of cell types which can be made to disperse in culture under the influence of HGF/SF include human keratinocytes (98), Madin Darby Canine Kidney (MDCK) cells (101), Hep G2 cells, human epidermoid carcinoma A431 and mouse keratinocytes PAM 212 (13).

In addition to enhancing cell dispersion, HGF also acts as a 'morphogen'; MDCK cells grown in collagen cells can be induced to form branching tubular structures under the influence of HGF (101). Similar effects have been demonstrated using cell lines derived from hepatic non-parenchymal epithelial cells (102). In view of the potent stimulatory effect on cell growth HGF was considered as a promoter of hepatocellular carcinoma. However, it is now known that HGF actually inhibits a variety of hepatocellular carcinoma cell lines (103). Experiments involving such neoplastic cells stably transfected with an albumin-HGF expression vector have demonstrated *in vitro* growth inhibition and when these cells are transplanted into mice, the resultant tumours are found to be smaller (~10% of expected size) than control lesions (103). Growth inhibition has

also been seen with B6/F_1 mouse melanoma cells and human squamous carcinoma cell lines (104).

Interestingly, as in the case of scatter factor/HGF, workers in the field of tumour cytotoxic agents identified a tumour cytotoxic factor derived from human embryonic lung fibroblast IMR-90 cells which was then shown to be identical to HGF (105). Fibroblast derived tumour cytotoxic factor (F-TCF)/HGF has cytotoxic activity against sarcoma 180 and cytostatic activity against KB cells (105).

Additional processes in which a role for HGF has been proposed include embryogenesis (106,107), angiogenesis (108), wound healing (91) and inflammation (via neutrophil priming) (109).

1.7.10 Renal Regeneration

HGF has also been implicated in renal regeneration. As described previously, HGF is a potent mitogen for a variety of epithelial cells including rabbit renal tubular epithelial cells (96). Moreover, HGF mRNA has been detected in a number of organs including liver, kidney, lung, heart and brain of rat (49). It is known that following unilateral nephrectomy renal compensation occurs by hyperplasia and hypertrophy, mainly of proximal tubular epithelial cells in the remaining kidney (110). This, together with *in vitro* and *in vivo* evidence supporting the concept of a humoral mediator in compensatory renal hyperplasia (111,112) led to the investigation of HGF as a candidate for such a role. Nagaike *et al* (62) have reported that following unilateral nephrectomy, expression of HGF mRNA in the intact kidney increased markedly to a maximum at 6 hours and was followed by an increase in HGF activity at 12 hours. Similar findings were noted in rats treated with carbon tetrachloride. A reduction in HGF receptor number was also seen, without change in receptor affinity. Receptor numbers fell as early as 2 hours after surgery and between 24 and 48 hours were undetectable. This may result from HGF binding to its receptor *in vivo* followed by internalisation of the complex. Since HGF appears to be produced in endothelial cells of the kidney

(62,112), a paracrine mechanism may exist whereby HGF acts to stimulate compensatory growth following injury. Recent work involving administration of HGF in a model of acute ischaemic renal injury in rats suggests that HGF accelerates recovery from such injury. Acute ischaemic renal injury was induced by bilateral renal artery occlusion for 75 minutes and 30 minutes after reperfusion a single subcutanous dose of recombinant HGF was administered. Compared with controls, rats which had received HGF had improved renal function, reduced mortality, greater renal cortex mitogenic activity and reduced histological evidence of injury 7 days following reperfusion (113).

Thus HGF has been implicated in stimulation of normal cell growth, inhibition of neoplastic cell growth, cell motility and morphogenesis. Multiplicity of function has been described in relation to other growth factors, e.g. transforming growth factor β (TGFß), which is capable inhibition of epithelial cell growth and stimulation of mesenchymal cell growth (114). This growth factor has been associated with various functions including wound healing and stimulation of angiogenesis (9).

1.7.11 HGF and Human Liver Disease

A considerable volume of evidence has been gathered to support the hypothesis that HGF has a role in hepatic regeneration. In human liver disease regeneration is often a feature, prompting investigation of a possible role for HGF. HGF has been purified from the plasma of patients with fulminant hepatic failure (43,115) and levels of HGF as determined by bioassay have been found to be related to coma grade (116,117). HGF has also been identified in plasma and ascites of patients with liver cirrhosis (118).

In 1991 the first report describing a highly specific ELISA protocol for HGF in human serum was published (119). Using a sandwich technique ELISA, HGF serum levels in patients with fulminant hepatic failure, acute hepatitis, chronic hepatitis, cirrhosis and normal controls were determined. Statistically significant elevations were seen in patients with fulminant hepatic failure and acute

hepatitis but not in chronic hepatitis/cirrhosis. The same ELISA system has been used to show that levels of HGF in fulminant hepatic failure can be correlated to clinical outcome (120) and to demonstrate increased levels of HGF in serum following partial hepatectomy for neoplasm (121). Another report using this ELISA describes increased serum levels of HGF in both acute and chronic liver disease (122). It also suggests that HGF level may serve as an index of severity of liver dysfunction in acute and chronic liver disease.

However, the precise contribution made by HGF in liver disease remains to be determined. Further measurement of HGF serum levels in different liver diseases would be of particular interest (a) in an attempt to clarify whether HGF levels are or are not increased in chronic liver disease, (b) in order to explore the possibility that HGF measurement may be useful as an index of hepatic dysfunction/ regeneration in human liver disease.

1.8 Other Regulators of Hepatic Growth

A number of other substances are known to affect hepatocyte proliferation. These may be classified as complete and incomplete hepatic mitogens and growth inhibitors.

1.8.1 Complete Hepatocyte Mitogens

These are factors independently capable of stimulating DNA synthesis and mitosis in quiescent hepatocyte populations in defined medium, without serum.

The following is a summary of substances known to have such properties:

Epidermal Growth Factor (EGF)

Epidermal growth factor was the first substance shown to be able to stimulate DNA synthesis in hepatocytes (34). Insulin is not required but its addition maximises the response to EGF (123). The amino acid proline is very strongly implicated in EGF growth promotion, in its absence the labelling index of cultured hepatocytes falls to less than 5% (114).

Two receptors for EGF have been defined: 'high' and 'low' affinity. Following culture of hepatocytes *in vitro* the 'high' affinity receptors disappear but 'low'

affinity receptors persist in sufficient numbers (124) and are believed to be the more important group.

EGF is also known to have effects on amino acid transport and protein synthesis - TGFß inhibits EGF stimulated mitogenesis but not protein synthesis stimulation (125). The role of EGF in partial hepatectomy is unclear; reduced numbers of EGF receptors are found by 8 hours with a trough at 40 hours following partial hepatectomy. There is also a decline in the EGF dependent tyrosine kinase activity of the receptor. However, a number of explanations including TGFα (which shares the receptor for EGF) secretion in regenerating liver may account for this. In addition, noradrenaline has been shown to cause heterologous down regulation of EGF receptors *in vitro* (114). EGF is not produced in the liver (126) and its plasma concentration remains unchanged during liver regeneration (126,127).

Transforming Growth Factor Alpha (TGFα)

This protein shares the same receptor as EGF (128) and has ~33% sequence homology with EGF (129). However, TGFα is a more potent hepatocyte mitogen than EGF (130). It is produced by regenerating hepatocytes, a number of neoplastic cells (132), in the early fetus and in normal epidermis (132). It is also implicated in wound healing (130).

Expression of TGFα mRNA in normal liver is undetectable, but following partial hepatectomy an increase is noted at 18 hours with a peak at 24 hours (130). This coincides with the decrease in the number of EGF receptors which is maximal at 36 hours (133). Serum levels of TGFα are increased within 8 hours of partial hepatectomy with a peak of 24 hours (and a later peak at 72 hours) which is parallel with DNA synthesis (114).

Acidic Fibroblast Growth Factor (acidic FGF)

Acidic FGF is a heparin binding 16kDa protein capable of stimulating hepatocyte DNA synthesis. However, heparin is required for this function, and aFGF is less potent than EGF (e.g. 1/3 or 1/5 as effective) (114,13). Acidic FGF

is secreted by hepatocytes and non parenchymal cells and its mRNA expression increased 4 hours after partial hepatectomy reaching a peak at 24 hours (13).

Hepatopoeitin B

This little known factor was identified by Michalopoulos *et al* at the time of purification of hepatoprotein A/HGF (41). Although a complete mitogen, this small (500Da) glycolipid factor is less potent than EGF or HGF and its origin remains unknown.

Hepatic Stimulatory Substance (HSS)

Purified from neonatal and regenerating livers HSS is a heparin binding protein of 16kDa (114) which has been reported to stimulate hepatocyte mitogenesis *in vivo* (134). HSS is not a complete mitogen for hepatocytes in culture, but does augment EGF/insulin (114).

1.8.2 Incomplete Hepatocyte Mitogens

A number of substances have been identified as incomplete hepatocyte mitogens. This important group includes factors which, although unable to directly stimulate DNA synthesis alone in serum free cultures, can significantly enhance the growth promoting properties of other agents.

Noradrenaline

Three important characteristics of this group have been demonstrated *in vitro* for noradrenaline. (a) Inability to independently stimulate DNA synthesis by hepatocytes (139). (b) Promotion of the mitogenic effect of HGF (135). (c) Inhibition of TGFβ inhibitory effects on hepatocyte DNA synthesis (136,137). This feature has been dramatically illustrated by culture of hepatocytes isolated from regenerating liver at 12-16 hours after partial hepatectomy in the presence of EGF and TGFβ. Merely by adding adrenaline the labelling index can be increased from less than 4% to greater than 70% (114).

Reduction of α_1-adrenergic effect by surgical hepatic denervation or administration of Prazosin, an α_1-adrenergic receptor antagonist, led to a reduction in hepatic DNA synthesis during the first 24 hours after partial

hepatectomy. However, chronic guanethidine injections (3-6 weeks) reduced liver catecholamine levels but did not alter the ability of the liver to regenerate (138).

Vasopressin, Angiotensin II, Angiotensin III

These hormones also promote EGF induced mitogenesis but to a lesser degree than noradrenaline (114). Similarly, they can antagonise the inhibitory effect of TGFß, although this effect is less potent than in the case of noradrenaline (137).

Rat strains which are congenitally deficient in vasopressin production (Brattleboro strain) demonstrate impaired liver regeneration (120).

Oestrogens

A number of reports implicate oestrogens in liver regeneration (140,141). Serum levels of oestrogens increase after partial hepatectomy peaking at 24-48 hours in man (141) and at 72 hours in rats (140). There is a concomitant increase in total hepatic content and nuclear retention of oestrogen receptors (140).

In contrast, following partial hepatectomy there is a fall in serum testosterone levels, hepatic androgen receptor activity and in activity of androgen responsive proteins (140). Injection of rats with Tamoxifen, an oestrogen receptor antagonist, soon after partial hepatectomy has been shown to significantly inhibit hepatic DNA synthesis in the regenerating liver (142).

Insulin and Glucagon

Insulin is a necessary constituent of hepatocyte culture media, since in its absence the cells degenerate and die (114). Rats subjected to resection of the gastrointestinal tract, pancreas, spleen and 70% partial hepatectomy demonstrate greatly delayed and attenuated hepatic regeneration. However, when insulin and glucagon are administered together the regenerative rate is dramatically restored to normal (143). The effects of glucagon appear to be mediated by cAMP since they may be duplicated by substitution of cAMP or agents which increase cAMP levels (144). In spite of these findings insulin does not stimulate hepatocyte DNA synthesis in culture alone or with glucagon (144). In addition, injection of insulin

and glucagon to either normal or eviscerated animals whose livers are intact fails to initiate hepatocyte proliferation (143).

1.8.3 Inhibitors of Hepatic DNA Synthesis

The complex processes involved in the phenomenon of controlled cell growth as illustrated by hepatic regeneration cannot, of course, be entirely regulated by promoters of cell growth. A number of factors have been studied which can inhibit mitogenesis. This property has been defined in the context of EGF mitogenic effects on primary cultured hepatocytes (114).

Transforming Growth Factor β (TGFβ)

TGFβ is a 25kDa disulphide linked homodimer which has been identified as a promoter of mesenchymal cell growth and a potent inhibitor of epithelial cell growth (145). This factor is known to exist in two main forms, $TGF\beta_1$ and $TGF\beta_2$. $TGF\beta_1$ is the more abundant, constituting 80-85% of total recovered TGFβ in both bovine bone and porcine platelets. In most assays $TGF\beta_1$ and $TGF\beta_2$ are functionally indistinguishable and although it has been suggested that $TGF\beta_2$ may have a unique function this has not yet been demonstrated (145). TGFβ has been associated with embryogenesis, wound healing and bone formation (145) it is known to increase the expression of extracellular proteins such as collagen and fibronectin (146) and their cellular receptors. It also reduces secretion of protease activators and increases secretion of protease inhibitors (147).

$TGF\beta_1$ is a potent inhibitor of primary cultured hepatocyte DNA synthesis (148,149). The early phase of regeneration following partial hepatectomy can be inhibited by intravenous administration of $TGF\beta_1$ or $TGF\beta_2$ at the time of, or shortly after, hepatic resection (150). However, it should be noted that the inhibitory effect of $TGF\beta_1$ and $TGF\beta_2$ in such studies was released eight days after partial hepatectomy. Moreover, complete restoration of liver cell mass was achieved by five days even if $TGF\beta_1$ or $TGF\beta_2$ was administered repeatedly (150). Further evidence that this protein is implicated in the control of hepatic

regeneration includes the finding that TGFβ mRNA expression is increased in regenerating liver following partial hepatectomy (151).

$TGF\beta_1$ mRNA expression in non parenchymal cells, especially sinusoidal endothelial cells, first becomes detectable by 4-8 hours, remains low until 24 hours then rises steadily, peaking at 72 hours (48-120 hours). Significant levels of expression persist for more than 96 hours (151,152). Thus, the timing of the peak of TGFβ mRNA expression, occurring after the 24 hour peak of DNA synthesis in hepatocytes is appropriate. However, in addition to the delay in peak production of the protein, the effect of TGFβ may be delayed because of its production in a latent form. This latent form is composed of a complex of $TGF\beta_1$ with a high molecular weight binding protein, also referred to as 'masking protein', 'TGFB-latency associated protein' or 'TGFβ binding protein' (153). This binding protein is a 180-220 kDa glycoprotein consisting of 2 x 39kDa and a single large subunit of 105-120kDa linked by disulphide bonds. The 39kDa protein is identical to the N-terminal part of the TGFβ precursor (154).

This $TGF\beta_1$/binding protein complex, linked by non covalent bonds, may be dissociated *in vitro* by treatment with 1M acetic acid, 6M urea or heat. However, it is not yet known how this activation of the complex takes place *in vivo* (154).

Interleukins IL-1b and IL-6

IL-1b is also a potent inhibitor of hepatocyte proliferation although less effective than TGFβ. Similarly, a weak inhibitory effect is seen with IL-6 (155).

Hepatocyte Proliferation Inhibitor (HPI)

This factor, of MW 17-19kDa, purified from adult rat liver, is an inhibitor of proliferation of normal rat liver cells (hepatocytes and other epithelial liver cells). Although similarities exist between $TGF\beta_1$ and HPI, differences include dose needed for inhibition and failure of antibodies to TGFß to inhibit HPI activity (156).

Clearly liver growth and regeneration are complex phenomena and many different mechanisms are likely to play a part. The recent discovery of HGF, the

most potent mitogen for hepatocytes known, has been seen as an exciting event in the history of the study of these processes. However, although much has been learned of the structure and function of HGF *in vitro*, the significance of HGF and its receptor in continuing liver growth in health and disease remains to be determined. It is hoped that a fuller understanding of these processes may ultimately help to provide a basis for the development of new approaches to the treatment of liver disease, in which inadequate or disordered regeneration frequently plays a part.

1.9 Aims

The work of this thesis represents my attempts to investigate the significance of HGF in human liver disease and in an animal model of liver regeneration.

1. To establish an ELISA to measure serum HGF in patients with various liver diseases, and to correlate these levels with disease type and severity.
2. Production of recombinant proteins based on human and rat HGF to raise antibodies to native HGF.
3. Purification of native human HGF in order to produce monoclonal and polyclonal antibodies to HGF.
4. Immunocytochemical localisation of HGF and its receptor in human liver biopsies.
5. Examination of expression of HGF mRNA in human liver biopsies by *in situ* hybridisation techniques.
6. Modification of the hepatic regenerative response to experimental liver injury by administration of neutralising anti-HGF antibody.

CHAPTER 2. Preparation of Recombinant Bacterial Proteins Based on Rat and Human HGF

2. INTRODUCTION

2.1 Recombinant HGF Proteins

A significant problem encountered in the course of this project has been the limited availability of useful antibodies to HGF. A number of antibodies have been kindly provided by collaborating research groups, however, at the time of this study there were no commercial sources. In addition, none of the antibodies provided were able to localise HGF immunocytochemically. Consequently, part of this project has involved the production of antibodies intended for use in immunocytochemistry as well as ELISA and *in vivo* HGF inhibition studies.

Although HGF has been purified from human plasma (43,115), ascitic fluid (118) and placenta (46), the processes involved are demanding and yield only very small quantities of pure protein. Therefore, in addition to purifying native placental HGF, synthetic HGF polypeptides have been produced. Using recombinant DNA techniques large quantities of pure protein may be manufactured. The fundamental steps involved in the generation of such proteins include the cloning and amplification of an appropriate gene fragment followed by insertion of such clones into cultured prokaryotic or eukaryotic cells. Following expression, the protein is purified from the host cell proteins.

2.2 Cloning of Polymerase Chain Reaction (PCR) Products into Vector

The essence of recombinant DNA technology involves two stages - (1) joining a DNA segment of interest to a DNA molecule which is able to replicate and (2) providing a milieu which allows propagation of the joined unit. In this study plasmid vectors were used to introduce cDNA sequences and to drive the expression of these sequences in *Escherichia coli*.

2.3 Transfected Cells

Recombinant proteins may be produced in both prokaryotic (e.g. bacteria) and eukaryotic cells (e.g. mammalian, insect). The use of bacteria for gene expression and protein synthesis has long been established. The ease with which bacteria may be transfected, cultured and selected has been a major part of the reason for

the enduring popularity of these organisms. Eukaryotic (mammalian and insect) cell culture is technically more demanding but significant differences exist between the two methods. The cells of the most commonly used bacteria, *Escherichia coli* are unable to carry out N- or O-linked glycosylation, to completely remove the N-terminal methionine residue from a number of proteins or to acetylate, phosphorylate or palmitate proteins. In addition, *E. coli* has an internal reducing environment, which interferes with disulphydryl bridges such that proteins may not be folded properly. Thus the proteins produced may differ immunologically and functionally from native proteins. However, notwithstanding these potential difficulties, antibodies have previously been successfully produced following immunisation with recombinant proteins from *E. coli*. In this study *E. coli* were used to express recombinant proteins based on rat and human HGF.

2.4 Plasmid Vectors

Plasmids are naturally occurring extrachromosomal circular DNA molecules found in most bacterial species and in some eukaryotic species. They have been adapted and modified by molecular biologists as tools for DNA manipulation. Such vectors contain transcriptional promoters which may be used to control expression of cloned cDNA and multiple restriction enzyme sites which allow the insertion of DNA sequences in frame. Vectors also frequently contain genes which code for resistance to certain antibiotics e.g. ampicillin, tetracycline. These provide a simple means of identifying host cells which contain either plasmid or plasmid with cDNA insert.

The transcriptional promoter region of the vector may be controlled by another gene product of the vector in such a way that transcription can be manipulated as required. pTAC is an example of this system; normally repressed by the Lac I^q gene product, it is induced by isopropyl-ß-D-thiogalactopyranoside (IPTG) which binds to and inactivates the repressor protein. In this study two plasmid vector types were used; the pET and pGEX families.

2.4.1 pET Protein Expression Vectors

The pET family of plasmids (plasmid for expression by T7 RNA polymerase) express target DNA in bacteria under the control of bacteriophage T7 RNA polymerase (Figure 2.1). T7 RNA polymerase is not expressed by the plasmid and requires to be supplied, usually be transfection of the cell with another vector such as bacteriophage CE_6 which expresses the enzyme or by use of bacteria which contain an integrated copy of the T7 polymerase gene. The plasmids in this family all confer antibiotic resistance and possess translation initiation signals (Shine Delgarno sequence) for the strongly expressed T7 s10 protein (a T7 capsid protein). Foreign DNA may be cloned into the BamH I site on the 3' end leading to a fusion peptide consisting of the 11 amino-acid N-terminus of the s10 protein with the target protein sequence. In a similar fashion the target protein alone may be produced by inserting the target DNA into an NcoI site behind the translation initiation codon. This system allows high level expression of target RNA and protein in bacterial hosts. In this project the pET plasmid pET-8_c was used with BL21 (DE3) bacteria which contain an integrated copy of the T7 polymerase gene controlled by the *lac* UV5 promoter. The addition of Isopropyl-ß-D-thiogalactopyranoside (IPTG) induces the expression of T7 polymerase. The plasmid pET-8_c contains the promoter recognised by T7 polymerase (157,158).

2.4.2 pGEX Protein Expression Vectors

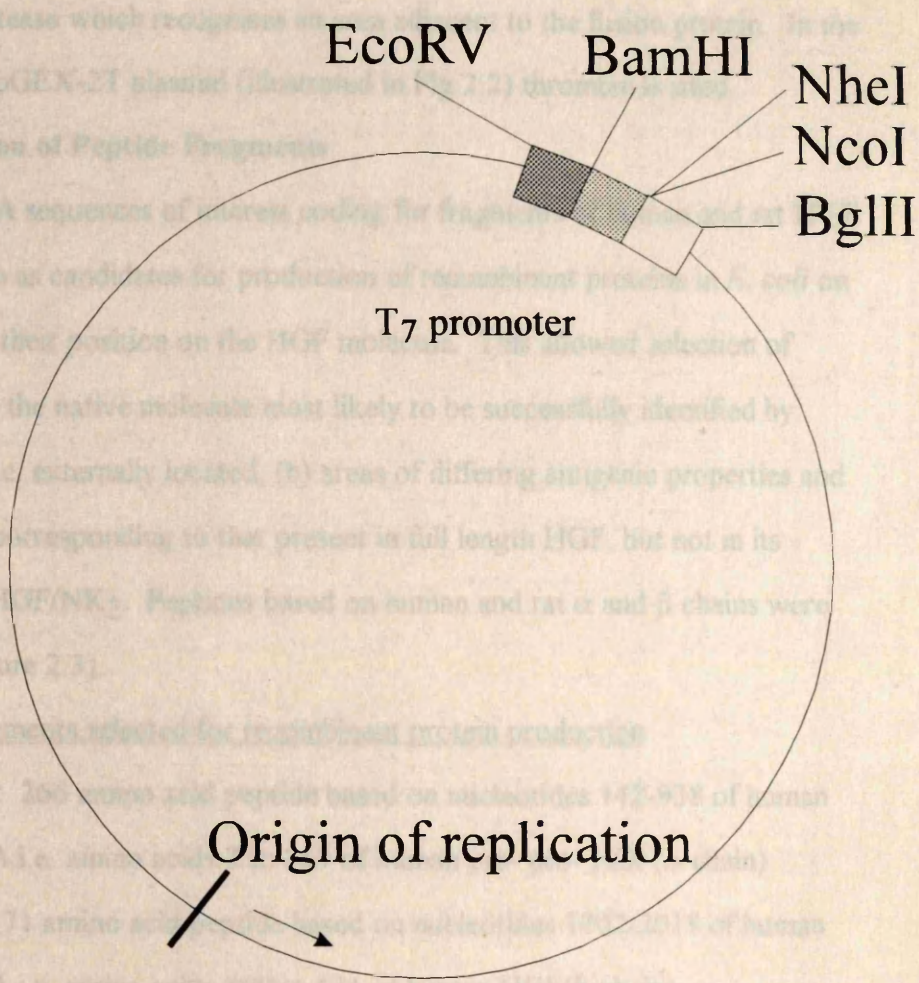
The pGEX protein expression vectors (Promega Ltd, Southampton, UK) are designed for high level expression of proteins as fusions with *Schistosoma japonicum* glutathione-S-transferase (159,160,161). These plasmids possess the Amp gene which confers resistance to 50µg/ml ampicillin and each has a cloning site which allows the cDNA to be inserted in frame with the promoter and stop codons. They contain a *tac* promoter which is normally repressed by the *Lac* I^q gene product but which is induced in the presence of IPTG (isopropyl-ß-D-thiogalactopyranoside).

Figure 2.1 Schematic representation of plasmid pET-8c

The plasmid includes a T_7 phage promoter that is recognised by the phage T_7 RNA polymerase but not by host *E. coli* polymerase. In this case T_7 RNA polymerase is provided by use of BL21 (DE3) bacteria which contain an integrated copy of the gene, controlled by the lac UV5 promoter.

Foreign DNA may be inserted into the Bam HI or Nco I sites leading to either a fusion peptide consisting of the 11 amino acid N-terminus of the s10 protein and target protein or target protein alone.

The plasmid also carries a gene for ampicillin resistance.



Represents the T_7 terminator

Represents the insertion site

Represents the T_7 promoter

Such fusion proteins help to simplify purification of target proteins - in this case fusion proteins are removed from bacterial lysates by affinity chromatography using glutathione sepharose 4B. Cleavage of the 26kDa glutathione S-transferase domain from the fusion protein is by use of a site specific protease which recognises an area adjacent to the fusion protein. In the case of the pGEX-2T plasmid (illustrated in Fig 2.2) thrombin is used.

2.5 Selection of Peptide Fragments

The DNA sequences of interest coding for fragments of human and rat HGF were chosen as candidates for production of recombinant proteins in *E. coli* on the basis of their position on the HGF molecule. This allowed selection of (a) areas on the native molecule most likely to be successfully identified by antibodies i.e. externally located, (b) areas of differing antigenic properties and (c) an area corresponding to that present in full length HGF, but not in its antagonist HGF/NK_2. Peptides based on human and rat α and β chains were chosen (Figure 2.3).

Peptide fragments selected for recombinant protein production

hHGFα266: 266 amino acid peptide based on nucleotides 142-938 of human HGF mRNA i.e. amino acids 3 to 269 of human pre- pro- HGF(α chain).

hHGFβ71: 71 amino acid peptide based on nucleotides 1802-2018 of human HGF mRNA i.e. amino acids 560 to 631 of human HGF(β chain).

rHGFα94: 94 amino acid peptide based on nucleotides 215-499 of rat HGF mRNA i.e. amino acids 25 to 119 of rat HGF(α chain).

rHGFβ121: 121 amino acid peptide based on nucleotides 1658-2023 of rat HGF mRNA i.e. amino acids 506 to 627 of rat HGF (β chain) (Figs. 2.3, 2.4).

Appropriate specific oligonucleotide primers complementary to the cDNA sequence of interest were chosen and synthesised by the β-cyanoethyl phosporamidate method on a DNA synthesiser. The fragment was amplified by a reverse polymerase chain reaction (PCR). The antisense primer was hybridised to a sample of RNA containing HGF mRNA. Addition of reverse transcriptase to

Figure 2.2 Schematic Representation of the pGEX-2T Vector

This plasmid allows insertion of foreign DNA in frame with the promoter and stop codons. The target DNA is expressed as a fusion protein with *Schistosoma japonicum* glutathione-S-transferase which may be cleaved by the proteolytic action of thrombin.

The *tac* promoter and *lac* I^q gene allow chemically inducible high level expression.

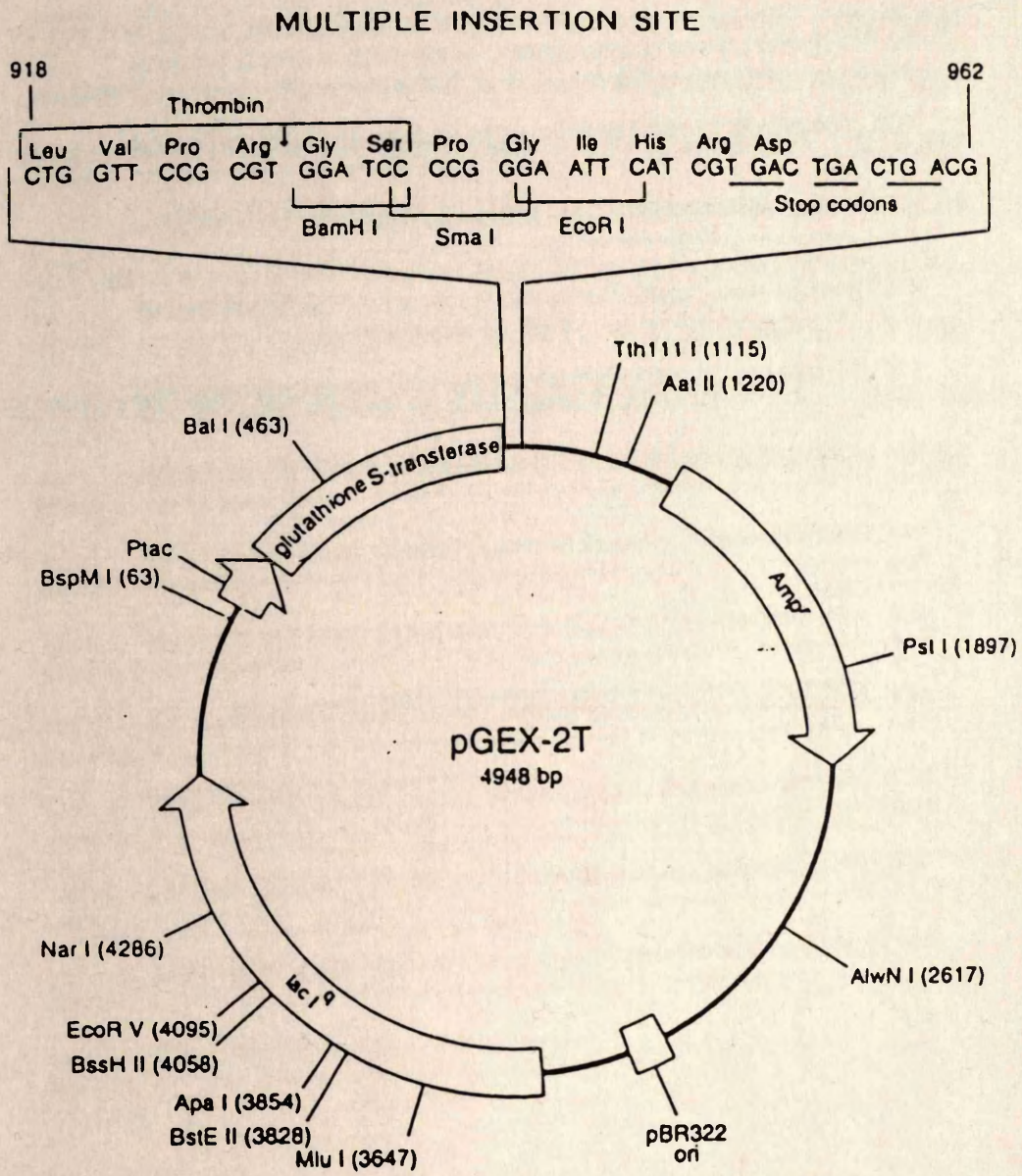
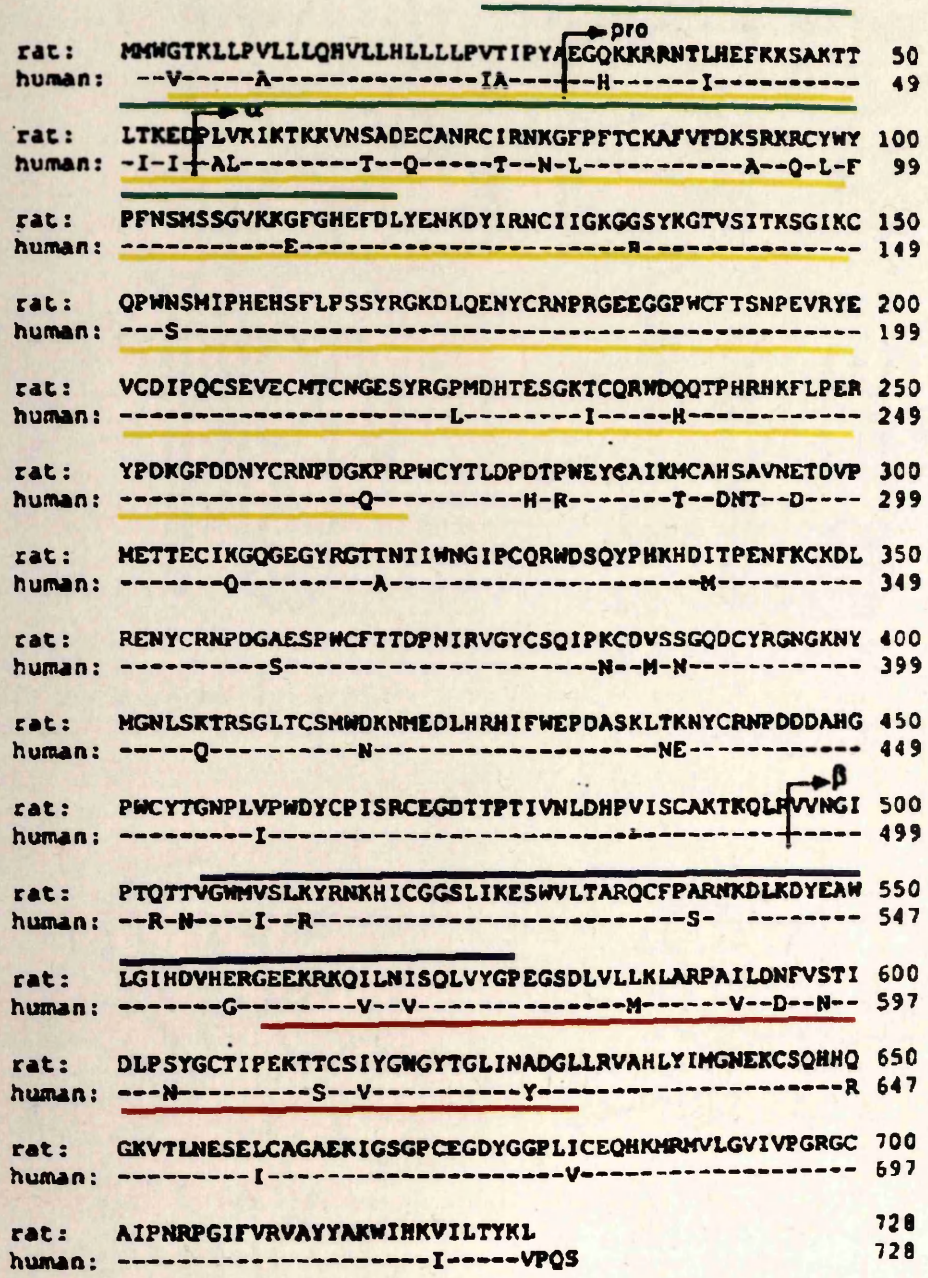

MULTIPLE INSERTION SITE
918
962
Thrombin
Leu Val Pro Arg Gly Ser Pro Gly Ile His Arg Asp
CTG GTT CCG CGT GGA TCC CCG GGA ATT CAT CGT GAC TGA CTG ACG
BamH I
Sma I
EcoR I
Stop codons
Tth111 I (1115)
Aat II (1220)
Bal I (463)
glutathione S-translerase
Ptac
BspM I (63)
Amp
Pst I (1897)
pGEX-2T
4948 bp
Nar I (4286)
lac I q
AlwN I (2617)
EcoR V (4095)
BssH II (4058)
Apa I (3854)
BstE II (3828)
Mlu I (3647)
pBR322
ori

Figure 2.3 Amino Acid Sequence of Recombinant HGF Proteins

Amino acid sequences of rat and human HGF and location of recombinant HGF protein sequences. Amino acid numbers are given on the right. Identical amino acids are indicated by dashes in the sequence of human HGF.

hHGFα266: 266 amino acid peptide based on nucleotides 142-938 of human HGF mRNA i.e. amino acids 3 to 269 of human pre- pro- HGF(α chain).
hHGFβ71: 71 amino acid peptide based on nucleotides 1802-2018 of human HGF mRNA i.e. amino acids 560 to 631 of human HGF(β chain).
rHGFα94: 94 amino acid peptide based on nucleotides 215-499 of rat HGF mRNA i.e. amino acids 25 to 119 of rat HGF(α chain).
rHGFβ121: 121 amino acid peptide based on nucleotides 1658-2023 of rat HGF mRNA i.e. amino acids 506 to 627 of rat HGF (β chain).



Represents hHGFα 266

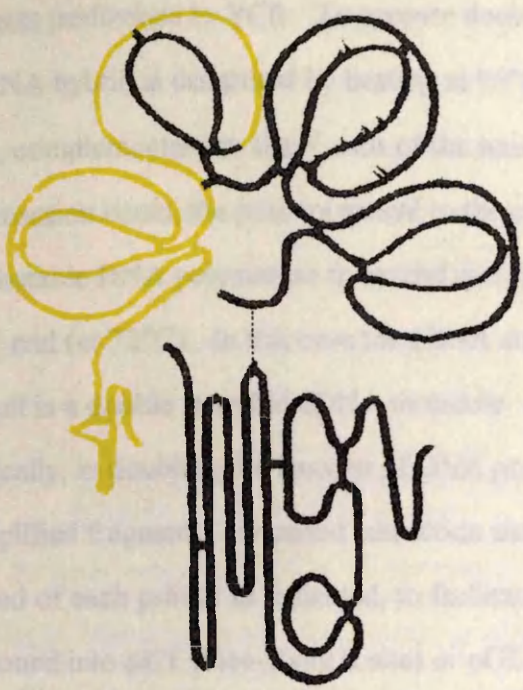
Represents rHGFα 94

Represents hHGFβ 71

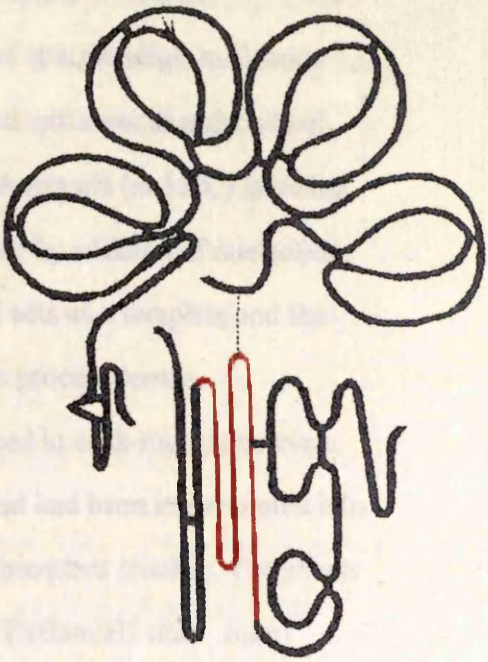
Represents rHGFβ 121

Figure 2.4 Size and Position of Recombinant HGF Proteins

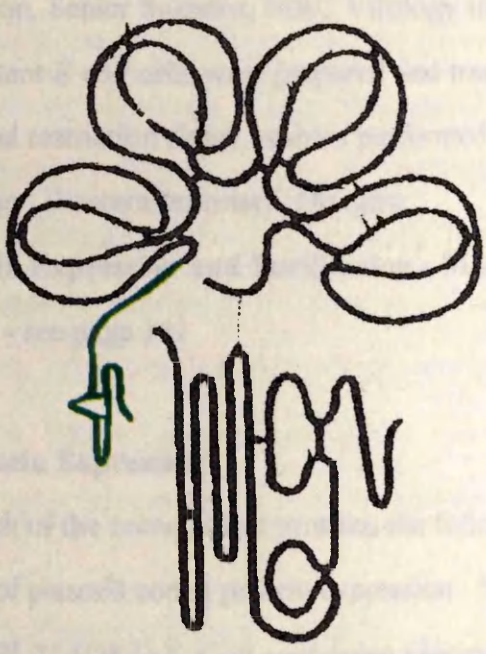
Illustration of the size and position of recombinant HGF proteins in relation to full length native human and rat HGF.



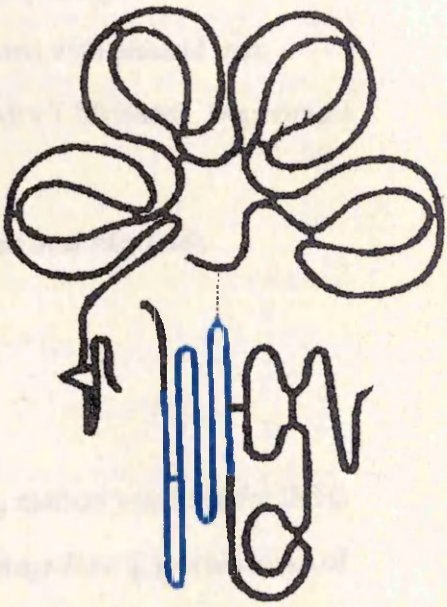
hHGFα266



hHGFβ71



rHGFα94



rHGFβ121

the primed mRNA results in synthesis of a DNA/RNA hybrid. Amplification of cDNA was performed by PCR. To prepare double stranded DNA, the DNA/RNA hybrid is denatured by heating at 95°C and specific oligo-nucleotide primers, complementary to the 5' ends of the sense and antisense strands, added. As the reaction cools, the primers anneal to the cDNA strands (at 55°C) allowing a thermostable DNA polymerase to extend each primer by addition of nucleotides at the 3' end (at 72°C). In this case the cDNA strand acts as a template and the end result is a double stranded cDNA molecule. This process results, theoretically, in doubling the amount of DNA produced in each successive cycle. The amplified fragments contained restriction sites that had been incorporated into the 5' end of each primer as indicated, to facilitate subsequent cloning. Fragments were cloned into pET (Nco-BamHI site) or pGEX-2T (Bam HI site). Insert orientation in pGEX-2T was checked by restriction enzyme digestion or by DNA sequencing.

Cloning of cDNA fragments was carried out by Dr K Hillan in association with Dr C Preston, Senior Scientist, MRC Virology Institute, Glasgow.

Competent *E coli* cells were prepared and transfected with plasmid then selected and restriction digest analysis performed by Mrs T McShane, Department of Pathology, Western Infirmary, Glasgow.

2.6 Protein Expression and Purification - Materials and Methods

Materials - see page 141

Methods

2.6.1 Protein Expression

For each of the recombinant proteins the following method was used for IPTG induction of plasmid coded protein expression. Scrapings from glycerol stocks of DH5 and BL21 (DE3) *E. Coli* containing plasmids pGEX-2T266 and pET-121 (stored at -70°C) were mixed with 0.5ml of YT broth and the solution used to inoculate culture plates containing medium (with ampicillin 50μg/ml). Following incubation at 37°C overnight, a single colony was picked and used to inoculate

2ml YT broth containing ampicillin 50µg/ml and incubation continued at 37°C for 2 hours. IPTG was added (100µl of 100mM IPTG) and culture continued for a further 2 hours. Control cultures not treated with IPTG were included. Proteins were harvested by repeated cycles of freeze thawing (liquid nitrogen/37°C incubation) then centrifuge separation at 6000rpm for 10 minutes using a "Microcentaur" microfuge (Fisons Scientific Equipment, Leicestershire, UK) from culture medium. Lysates from the bacterial cell cultures were analysed by SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) (162).

2.6.1.1 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) Analysis of Proteins

Proteins were resolved by electrophoresis on an SDS-polyacrylamide gel using "Biorad Miniprotean II" electrophoresis equipment (BioRad Laboratories Ltd, Hertfordshire, UK). Resolving and stacking gels were prepared as outlined in Appendix 1. After pouring, the resolving gels were overlaid with water saturated isobutanol and allowed to set. The isobutanol was poured off and the top of the gel rinsed with water. The stacking gel was poured, a toothed comb inserted, and allowed to set. Following removal of the comb, the stacking gel and its walls were rinsed with water. The gel was placed in a buffer tank with electrode buffer (Appendix 2). Protein samples for electrophoresis were mixed with an equal volume of loading buffer (Appendix 2). Samples to be reduced were mixed with loading buffer containing 5% mercaptoethanol and placed in boiling water for 4 minutes. Molecular weight markers were prepared for loading in the same fashion. Fifteen microlitres of each sample were loaded per well and electrophoresis performed at 25mA until the dye front reached the bottom of the gel.

2.6.1.2 Coomassie Blue Staining

Gels to be stained were removed from electrophoresis equipment, placed in 200ml of Coomassie blue stain (Appendix 3) and incubated overnight at room

temperature. Gels were destained by immersion in destain solution (Appendix 3) which was changed several times.

2.6.2 Purification of Protein from SDS-PAGE Gels

Large scale SDS-polyacrylamide gels (20cm x 20cm electrophoresis equipment, Scotlab, Coatbridge, UK) were prepared for protein purification. All samples, buffers and reagents were as for minigels. Each gel included the following samples; molecular weight markers (14,300-71,500 Da (BDH) or 29,000-205,000 Da (Sigma)), whole bacterial cell lysate of *E. coli* transfected with plasmid but not induced by IPTG and *E. coli* transfected with plasmid and induced by IPTG.

Gels were run at 25mA until dye front reached bottom of gel. The gel was removed and vertical strips containing the outer two wells with their corresponding resolving gel areas excised. These strips were stained with Coomassie blue overnight and the central portion wrapped in polythene film ('saran' barrier food wrap, Dow Chemical Company) and stored at 4°C. After destaining, the side strips were aligned with the central unstained portion of the gel and the position of the protein band of interest determined (Figure 2.5). This area was then excised from the gel and cut into small fragments ($1mm^3$ or less). These fragments were placed in 10ml of 0.1% (weight/volume) SDS in water and the protein allowed to elute overnight at room temperature. The solution was removed and concentrated by placing in dialysis tubing and covering the tubing with sucrose to remove water by osmosis. The final solution was decanted and protein content estimated by measuring absorbance of ultraviolet light at 280nm (using OD_{280} = mg protein/ml) (Beckman DH-64 spectrophotometer, Beckman RIIC Ltd, Glenrothes, UK). Purity was determined by SDS-PAGE analysis.

2.6.3 Purification of hHGFα266 using Glutathione-Sepharose 4B

Recombinant protein hHGFα266 was produced as a fusion protein with glutathione-S-transferase (GST) of *Schistosoma japonicum*. Bacteria containing the plasmid coding for hHGFα266 were lysed by repeated freeze/thaw cycles and

Figure 2.5 Purification of recombinant bacterial protein by SDS-PAGE of bacterial cell lysate.

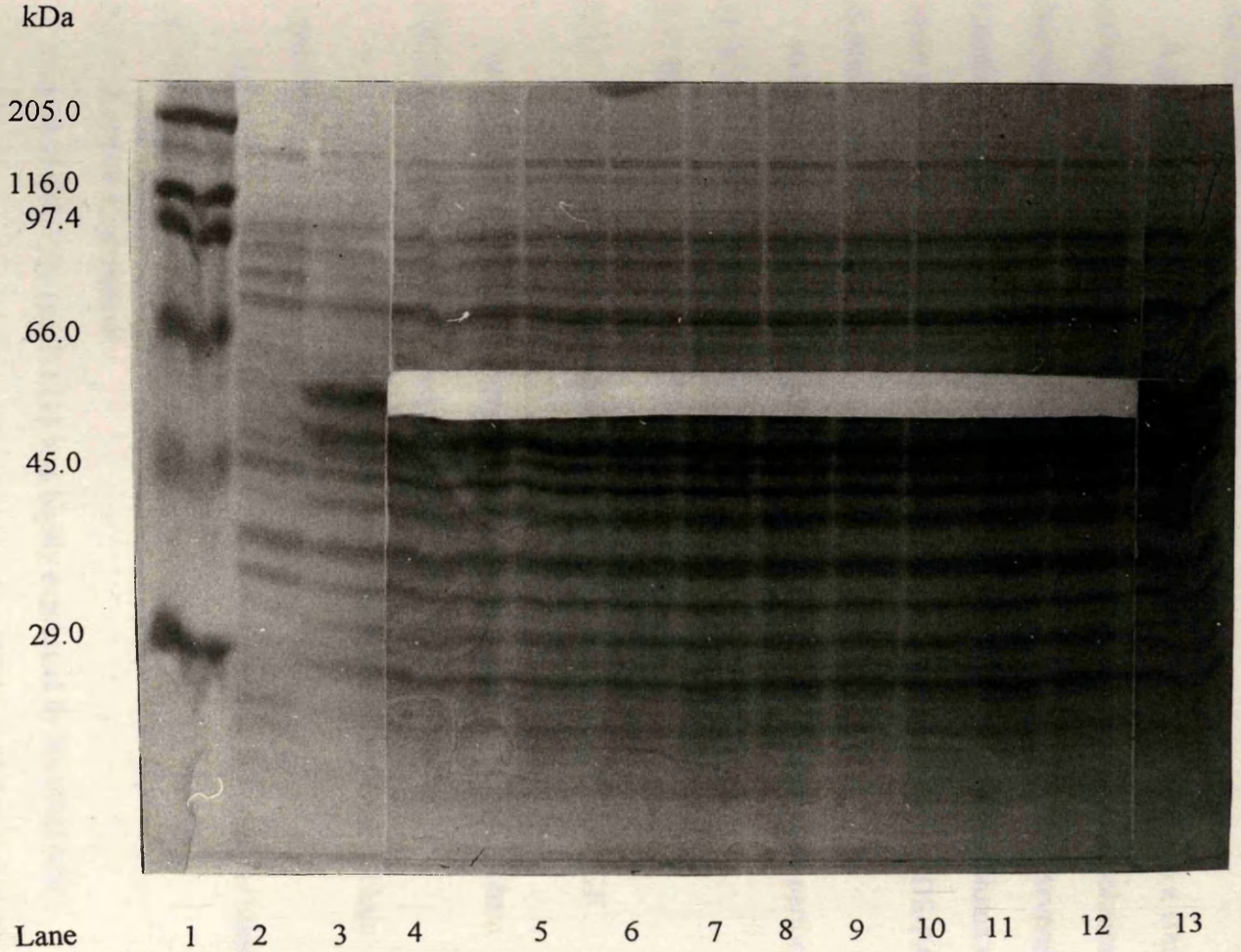
Coomassie Blue stain and excision of band containing separated protein of interest (12% polyacrylamide gel).

Lane 1 Molecular weight markers (kDa)

2 Cell lysate of *E. coli* transfected with plasmid pGEX2T which does not contain cDNA coding for hHGFα266.

Lanes 3-13 Cell lysate of *E. coli* transfected with plasmid pGEX2T which contains cDNA coding for hHGFα266.

Protein hHGFα266 can be seen as a band of approximately 56 kDa in lanes 3 and 13 of the Coomassie Blue stained gel. The central portion of gel (lanes 1-12) has been stained after excision of the area corresponding to protein hHGFα266.


kDa
205.0
116.0
97.4
66.0
45.0
29.0
Lane
1
2
3
4
5
6
7
8
9
10
11
12
13

centrifuged at 6000rpm ('Microcentaur' microfuge) for 10 minutes. The supernatant and pellet of cell debris and insoluble proteins were analysed by SDS-PAGE. Methods to solubilise the recombinant proteins included sonication and the use of the following solutions: PBS, PBS containing 1% (volume/volume) Tween 20, PBS containing 2% (volume/volume) Triton X-100, 2M urea, 4M urea and 8M urea.

A glutathione-sepharose 4B gel was washed in PBS and poured into a 10ml syringe to form a chromatography column. Bacterial cell lysate in the solutions described was poured through the column. After washing the gel with several column volumes of PBS, bound proteins were eluted by washing the column with 5mM glutathione, pH 8.0. Gel and eluted fractions were analysed by SDS-PAGE as described.

N-terminus amino acid sequencing of the recombinant proteins was performed as described in chapter 3.

2.7 Results

2.7.1 Cloning of cDNA Fragments Coding for Human and Rat HGF Peptides

An 800bp cDNA fragment coding for a peptide based on human α-chain (hHGFα266) was produced and cloned into the PGEX 2T vector.

A 365bp cDNA fragment coding for a peptide based on rat HGF ß-chain (rHGFβ121) was produced and cloned into pET vector.

Attempts to produce additional cDNA fragments coding for peptides based on rat HGFα-chain and human HGF ß-chain were unsuccessful.

2.7.2 Protein Expression

Protein rHGFβ121 (pET-121) was highly expressed by bacterial cells transfected with pET-121 plasmid and induced by IPTG, as shown in Figure 2.6. The band corresponding to rHGFβ121 is 14kDa in size, consistent with a protein comprising 121 amino acids.

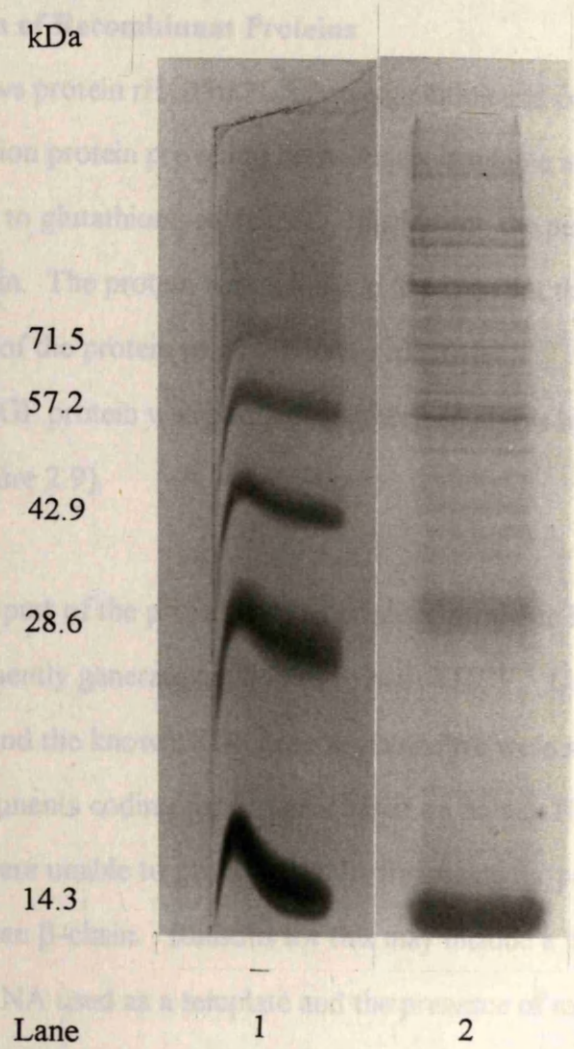
Figure 2.6 SDS-PAGE Analysis of lysate of *E. coli* transfected with pET-121

Expression of protein rHGFβ121 (pET-121) by bacterial cells transfected with pET-121 plasmid, induced by IPTG SDS-PAGE of bacterial cell lysate and molecular weight markers, 12% Polyacrylamide gel, Coomassie Blue stain.

Lane 1 Molecular weight markers (kDa)

2 Cell lysate of *E. coli* expressing rHGFβ121.

Protein rHGFβ121 can be seen as a band of approximately 14 kDa in lane 2. No such band was seen when a lysate of *E. coli* containing plasmid without the cDNA fragment was subjected to SDS-PAGE and stained.


kDa
71.5
57.2
42.9
28.6
14.3
Lane
1
2

Similarly, cells transfected with the plasmid coding for hHGFα266 expressed a 56kDa protein when induced with IPTG (Figure 2.7). This mass is consistent with a peptide of 266 amino acids and a fusion protein of 26.5kDa.

2.7.3 Purification of Recombinant Proteins

Figure 2.8 shows protein rHGFß121 following elution and concentration.

hHGFα266 fusion protein proved to be relatively insoluble and could not be purified by affinity to glutathione-sepharose. In addition, the peptide could not be cleaved by thrombin. The protein was soluble in 8M urea but this concentration prevented binding of the protein to glutathione-sepharose.

The insoluble HGF protein was purified as described above by SDS-PAGE and band excision (Figure 2.9).

2.8 Discussion

The aim of this part of the project was to produce synthetic HGF polypeptides in order to subsequently generate antibodies to native HGF. Using recombinant DNA technology and the known HGF gene sequence we were successful in cloning cDNA fragments coding for proteins based on human HGF α-chain and rat β-chain. We were unable to produce cDNA fragments for proteins based on rat α-chain or human β-chain. Reasons for this may include a problem of integrity of the mRNA used as a template and the presence of mRNA secondary structure which interferes with reverse PCR (163).

In the case of both of the cDNA fragments produced protein expression by bacterial cells transfected with plasmids containing the cDNA fragment was sufficient to assist in identification of the protein and to facilitate purification. Had the expression of the recombinant protein by the bacterial cells been low, even greater difficulty would have been experienced in identifying and purifying the target proteins, since both of these processes depended on visible differences in protein quantity on SDS-PAGE.

Protein rHGFβ121 which was produced without fusion to a carrier protein proved to be more easily purified than protein hHGFα266 which was formed as a

Figure 2.7 SDS-PAGE analysis of lysate of *E. coli* transfected with pGEX-2T containing cDNA coding for hHGFα266

Expression of protein by bacterial cells transfected with plasmid pGEX-2T containing cDNA coding for hHGFα266. SDS-PAGE of bacterial cell lysate and molecular weight markers, 12% Polyacrylamide gel, Coomassie Blue stain.

Lane 1 Molecular weight markers (kDa)

2 Cell lysate of *E. coli* transfected with plasmid pGEX2T which does not contain cDNA coding for hHGFα266

3 Cell lysate of *E. coli* transfected with plasmid pGEX2T which contains cDNA coding for hHGFα266

Protein hHGFα266 can be seen as a band of approximately 56 kDa in lane 3. This corresponds to hHGFα266 formed as a fusion protein with glutathione-S-transferase of *Schistosoma japonicum*. The protein is not present in lane 2.

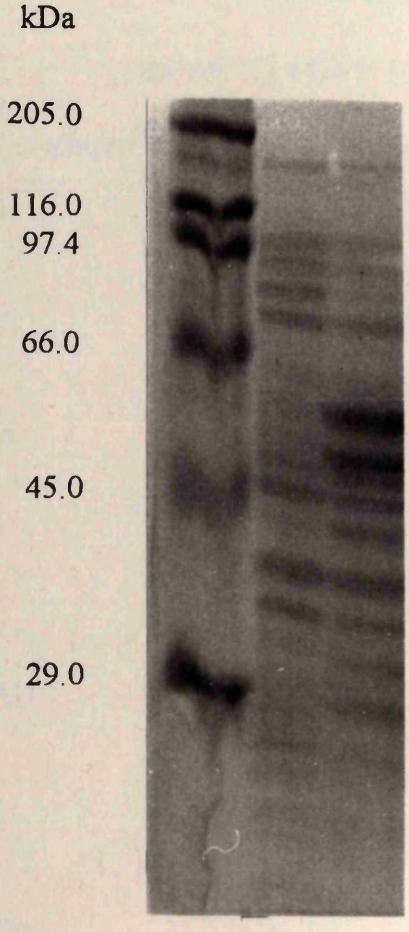

kDa
205.0
116.0
97.4
66.0
45.0
29.0
Lane
1
2
3

Figure 2.8 SDS-PAGE analysis of purified rHGFα121 protein

SDS-PAGE analysis of purified recombinant protein rHGFα121 and molecular weight markers, 10% polyacrylamide gel, Coomassie Blue stain.

Lane 1 Molecular weight markers (kDa)

2 Purified, concentrated rHGFα121 protein

Lane 2 illustrates the purity of the isolated protein, with respect to other bacterial proteins of different molecular weight.

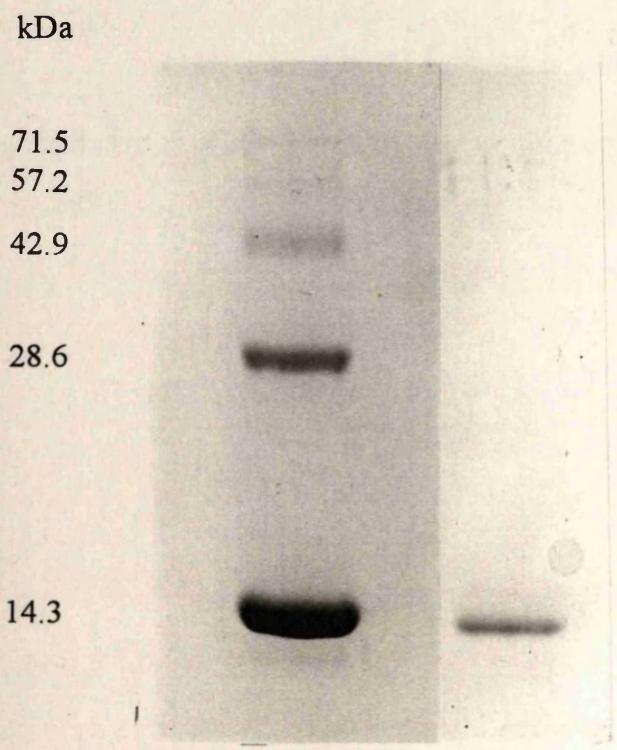

kDa
71.5
57.2
42.9
28.6
14.3
Lane
1
2

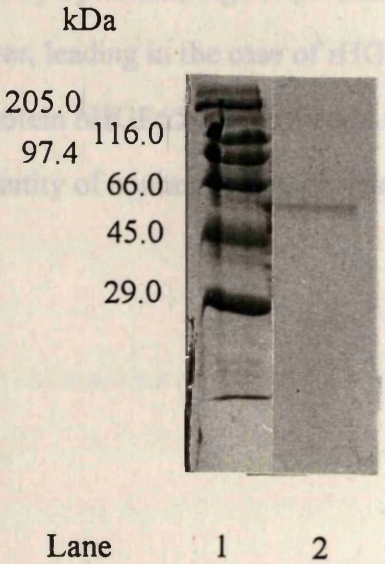
Figure 2.9 SDS-PAGE analysis of purified hHGFα266 protein

Purification of recombinant protein hHGFα266. SDS-PAGE analysis of purified recombinant protein hHGFα266 and molecular weight markers, 10% polyacrylamide gel, Coomassie Blue stain.

Lane 1 Molecular weight markers (kDa)

2 Purified concentrated hHGFα266 protein

Lane 2 demonstrates isolation of the protein of 56kDa corresponding to hHGFα266.


kDa
205.0
97.4
116.0
66.0
45.0
29.0
Lane
1
2

fusion protein with Glutathione-S-transferase (GST) of *Schistosoma japonicum*. The relative instability of hHGFα266 and the failure of thrombin to enzymatically cleave the GST protein led to difficulty in purification of the target protein. We were unable to use the manufacturer's recommended technique of affinity chromatography using Glutathione Sepharose 4B as the concentration of urea required to solubilise the protein prevented binding of the protein to Glutathione-Sepharose, possibly by denaturing the protein. Satisfactory purification was achieved however, leading in the case of rHGFβ121 to a single band on SDS-PAGE. The protein hHGFα266, purified in a similar fashion, was associated with a small quantity of contaminating proteins on SDS-PAGE.

CHAPTER 3. Recombinant Protein Sequencing

3. INTRODUCTION

To confirm the identity of the recombinant proteins hHGFα266 and rHGFβ121 N-terminal amino acid sequencing was performed. This work was carried out at the Science and Engineering Research Council Protein Sequencing Facility, Marischal College, University of Aberdeen, with the assistance of Mr Bryan Dunbar and Professor John Fothergill.

3.1 Protein Sequencing

Proteins are sequenced by degradation from their N-terminus by the Edman reaction using phenylisothiocyanate (PITC) (164). The reaction is divided into three steps which result in removal of the N-terminal residue leaving the protein with a free N-terminus which can undergo another cycle of the reaction. The three steps are:

Coupling: in which PITC modifies the N-terminal residue

Cleavage: under anhydrous acidic conditions, removing the N-terminal residue as an unstable anilinothiozolinone (ATZ) derivative.

Conversion: of the ATZ derivative to a stable phenylthiohydantoin (PTH)-amino acid.

Identification of Amino Acids

The PTH-amino acid is injected onto an HPLC and the elution time of a peak (absorbance at 269nm) identifies the amino acid. A major problem in protein sequencing is "blockage" of the N-terminus commonly by contaminants such as cyanate and aldehyde agents. Care is necessary in sample preparation to preserve the N-terminus and to avoid chemicals which may interfere with other steps in the Edman reaction, these may include buffers and non-ionic detergents.

To separate samples for sequencing, bacterial cell lysate proteins were subjected to SDS-PAGE, transferred to "ProBlott" membrane, stained and the appropriate band excised. The method of protein purification described in chapter 2 is unsuitable for the preparation of proteins for sequencing. Polyacrylamide gel electrophoresis prior to sequencing requires particular conditions including "pre-

running" the gel and the addition of free-radical scavengers such as glutathione and sodium thioglycolate. Proteins were sequenced using an Applied Biosystems 477A pulsed liquid protein sequencer with a 120A on-line PTH analyser. PTH-amino acids are determined on the 120A analyser using reverse phase HPLC at an absorbance of 269nm with computer generated chromatographs and results. Amino acids are identified by comparing the chromatograph of each cycle (1 residue) with a reference chromatograph.

3.2 Materials and Methods

Materials - see page 141.

Methods

3.2.1 SDS-PAGE Sequencing: A 12% resolving gel was prepared as described in Chapter 2, section 2.6.1.1. The 5% stacking gel was prepared as described in Appendix 1 with pH 8.8 buffer. Electrode buffer (Appendix 2) containing 10mM glutathione was poured into the top reservoir and an aliquot of loading buffer placed in a lane. Electrophoresis at 10mA was performed until the dye front had reached the resolving gel. At this stage, the upper buffer was changed to pH 6.8 electrode buffer (see Appendix 2) containing 0.1mM sodium thioglycolate and reduced samples of bacterial cell lysate hHGFα266 and rHGFβ121, as described in chapter 2, loaded. Electrophoresis was carried out at 10mA for 45 minutes after which the gels were removed and equilibrated with CAPS (3-[cyclohexylamino]-1-propane sulfonic acid) transfer buffer (Appendix 2). Separated proteins were then transferred from gel onto 'ProBlott' membrane using "Biorad Mini-Protean II" blotting equipment (BioRad Laboratories, Hertfordshire, UK) and CAPS transfer buffer with a current of 250mA for 1 hour an 45 minutes.

After transfer, membranes were stained with amido black (Appendix 3) for 5 minutes then destained (Appendix 3) by 3 washes of 10 minutes and the band of interest excised.

The excised band was sequenced on the Applied Biosystems 477A automated protein sequencer, using the 'Blott' reaction cell.

3.3 Results

3.3.1 Recombinant Bacterial Protein rHGFß121, based on rat HGF, β Chain.

The cDNA fragment coding for rHGFβ121 codes for a polypeptide of 121 amino acids. The predicted N-terminal amino acid sequence is as illustrated in Figure 3.1. A methionine 'Cap' at the N-terminus of recombinant proteins is commonly found, thus the sequence confirms the identity of rHGFβ121.

3.3.2 Recombinant Bacterial Protein hHGFα266, based on Human HGF, α Chain

The N-terminus of hHGFα266 consists of the fusion protein glutathione-S-transferase followed by the HGF peptide. Sequencing of this protein proved difficult and it was not possible to identify a precise sequence (several amino acids were suggested for each residue). However, for almost every residue the predicted amino acid was present among the suggested amino acids.

Figure 3.2

Although the identity of HGF could not be proved with this method, the results suggest the presence of GST fusion protein.

3.4 'Vestec' Laser Desorption Mass Spectrometry

The precise molecular weight of rHGFß121 was determined with the assistance of Mr Ian Davidson, Marischal College, University of Aberdeen. The laser desorption massspectrometer, which may be used to investigate subtle differences in protein size and structure, can determine the molecular weight of a sample to within 0.1%. In this case the molecular weight helped to characterise the protein rHGFβ121. The sample of lysed cells, diluted in distilled water was placed in the ion chamber of the spectrometer and the proteins ionised by nitrogen laser pulses. The ionised particles were accelerated by 25,000 volt plates and passed through a 0.7m flight tube. Mass is determined by the time of flight (from

Figure 3.1 N-terminus Amino Acid Sequence of rHGFβ-121

The amino acid sequence is illustrated using the single letter code for amino acids, the N-terminus on the left. A methionine residue has been added to the N-terminus of rHGFβ-121, a common event when recombinant proteins are expressed in bacteria. Following this residue the next twenty amino acids correspond exactly with the expected sequence of rat HGF, confirming the identity of rHGFβ121.

N-terminus amino acid sequence of rHGFβ-121

Predicted {V-G-W-M-V-S-L-K-Y-R-N-K-H-I-C-G-G-S-L-I

Determined {M-V-G-W-M-V-S-L-K-Y-R-N-K-M-I-C-G-G-S-L-I

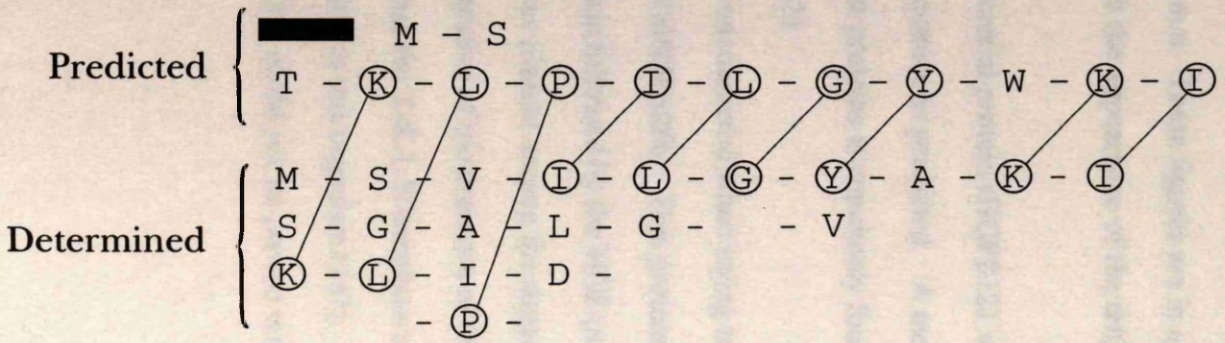
Figure 3.2 N-terminus amino acid sequence of glutathione-s-transferase fusion peptide, hHGFα266

The amino acid sequence is illustrated using the single letter code for amino acids, the N-terminus on the left. Where predicted amino acids are present in the sequence report, these residues are circled.

Amino acid sequencing for this protein was less satisfactory, and several amino acids were suggested for each residue. However, in all but one, the predicted amino acid was present among the suggested amino acids.

Note The T-K-L N-terminus may be replaced in *E. coli* due to cDNA instability

N-terminus amino acid sequence of GST-fusion peptide



laser pulse to protein ion detection set at 3.4kV at the end of the flight tube) with reference to standard proteins.

Protein rHGFβ121 was found to exist in two forms, with molecular weights of 13984 m.u. and 14190 m.u. These figures are in agreement with the predicted mass of 14kDa and with the appearance of the rHGFβ121 band on SDS-PAGE.

3.5 Discussion

The recombinant bacterial protein rHGFβ121 was found to have the same N-terminus amino acid sequence as predicted. A methionine 'cap' at the N-terminus of recombinant proteins is commonly found, thus the sequence confirms the identity of rHGFβ121.

More difficulty was encountered when trying to determine the N-terminus amino acid sequence of hHGFα266. This protein consists of the fusion protein Glutathione-S-transferase followed by the HGF peptide. The fact that the predicted amino acid was present among the suggested amino acids for almost all residues is at least suggestive of the presence of GST fusion protein. In addition, it has been suggested that the T-K-L N-terminus may be replaced in *E. coli* by M-S due to cDNA instability in this organism (161). One must, however, accept that the identity of hHGFα266 could not be proved with this method.

CHAPTER 4. Purification of Native Human Placental HGF (hpHGF)

4. INTRODUCTION

4.1. Placenta: A Source of Native HGF

As described in chapter 2 none of the available antibodies to HGF localised HGF immunocytochemically. One possible explanation for this might be antigenic differences between native and recombinant HGF, to which all of the available antibodies had been raised. It is known that significant differences in protein folding and glycosylation may exist between native and recombinant proteins. These differences may alter the antigenicity of a molecule (165) or affect its recognition by antibody. In an attempt to overcome these difficulties part of this project involved purification of native human HGF and the production of monoclonal and polyclonal antibodies to this material. Although HGF and its mRNA have been localised in a variety of tissues, suitable sources of HGF are restricted to plasma (43) and placenta. Since HGF is present in very small quantities (ng/ml serum) (119,122) a large volume of plasma would be required to isolate sufficient protein for the purposes of antibody production. However, human placenta has been identified as a source of significant quantities of HGF (46). This source was selected as supplies of fresh placenta were easily secured.

Human placental HGF (hpHGF) can be purified on the basis of its affinity for heparin and its hydroxylapatite binding properties, by adapting the methods of Hernandez *et al* (46) and Gohda *et al* (166). These properties were utilised in column chromatography steps with the eluted fractions containing hpHGF, identified by ELISA.

4.2 Materials and Methods

Materials - see page 141

Methods

4.2.1 Heparin Agarose/Hydroxylapatite Chromatography

Freshly obtained normal human term placenta was cut into $2cm^3$ cubes and homogenised in one litre of cold 1M NaCl (100g wet tissue to one litre 1M NaCl) using an 'ATOMIX' homogeniser. The homogenate was diluted to 0.5M NaCl

with water and clarified by centrifugation at 15000g for 30 minutes at 4°C. The supernatant was applied, at a flow rate of 60ml/hr, to a heparin-agarose column (bed volume 200ml) equilibrated with PBS containing 0.5M NaCl, pH 7.2. The column was washed with 4 litres of PBS containing 0.5M NaCl until the protein content of the efferent wash buffer returned to the baseline (protein content was estimated by measuring absorbance of ultraviolet light at 280nm OD_{280} = mg protein/ml). The material bound to the immobilised heparin was eluted by 1 litre of PBS containing 1.3M NaCl and fractions of 10ml collected. Every third fraction from the column was analysed for conductivity (NaCl concentration), optical density at 280nm (protein content), pH and HGF content (ELISA (d) as described in Chapter 6, section 6.2.5).

Those fractions containing peak amounts of hpHGF were pooled and dialysed against 5 litres of PBS. Triton X-100 solution was added to the dialysed preparation at a final concentration of 0.013% (volume/volume) and applied to a hydroxylapatite column (bed volume 40ml) equilibrated with PBS containing 0.013% Triton X-100, at a flow rate of 60ml/hr. The column was washed with 200ml of the same buffer, then with 200ml of 0.1M sodium phosphate buffer (pH 7.1) containing 0.15M sodium chloride and 0.013% Triton X-100. Bound hpHGF was then eluted with 160ml of 0.45M sodium phosphate buffer (pH 7.1) containing 0.15M sodium chloride and 0.013% Triton X-100 at room temperature (all other stages carried out at 4°C). Fractions of 2.5ml were collected and every third fraction assessed for pH, protein content (O.D. at 280nm), phosphate concentration (conductivity) and hpHGF content (ELISA (d)). Those fractions containing the greatest amounts of hpHGF with lowest total protein content were pooled, dialysed and concentrated by removal of water with sucrose as described in Chapter 2, section 2.6.2.

4.2.2 Analysis

Purified hpHGF was analysed by SDS-PAGE (method described in Chapter 2, section 2.6.1.1) using Coomassie blue (method described in Chapter 2, section 2.6.1.2) and silver staining techniques and by Western blotting.

Silver stain: Following SDS-PAGE, gels were incubated for 30 minutes in 50% methanol/10% acetic acid, followed by 30 minutes in 5% methanol/7.5% acetic acid then 30 minutes in 10% glutaraldehyde, using a shaking water bath. The gels were next resuspended in 2 litres of distilled water overnight. The water was changed several times to remove glutaraldehyde and gels incubated for 30 minutes in 150mls dithiothreitol solution (5µg/ml) followed by 30 minutes in 150mls 0.1% (weight/volume) silver nitrate. After rinsing with distilled water gels were developed with 3% (weight/volume) sodium carbonate containing 500µl filtered formaldehyde per litre. Sodium carbonate solution changed after a few minutes to prevent excessive precipitation. Reaction stopped by addition of 3M citric acid to reduce pH to 7. The gels were finally resuspended in water and viewed using a light box.

Western Blotting

Proteins present in samples of fractions collected from hydroxylapatite column and samples of concentrated purified hpHGF together with molecular weight markers were separated by SDS-PAGE as described in Chapter 2.

The separated proteins were transferred from gel onto PVDF (polyvinylidenedifluoride) membrane using ice box cooled "BioRad Mini-Protean II" blotting equipment (Bio Rad Laboratories, Hertfordshire, UK) and a carbonate buffer (Appendix 2) with a current of 400mA for 1 hour.

Following transfer, PVDF membranes were soaked in 'blocking solution' of 5% (weight/volume) powdered milk containing 0.1% (volume/volume) Tween 20 in PBS for 1 hour. Primary antibodies were diluted in PBS containing 0.1% (volume/volume) Tween 20 (PBS-Tween) and incubated with membranes overnight at room temperature (polyclonal antiserum to HGF Lot No 201; diluted

1/4000, monoclonal antibody to HGF D9 diluted 1/4, monoclonal antibodies to HGF B25, B43 and A3.1.2-2. all diluted 1/5000). Membranes were then washed in PBS-Tween (3 x 10 minutes) and incubated with secondary alkaline phosphatase conjugated antibodies diluted 1/1000 in PBS-Tween for one hour at room temperature. Membranes were washed in PBS-Tween (2 x 10 minutes) and in 100mM Tris buffer pH 9.5 (1 x 10 minutes). Nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Appendix 3) was used as the substrate for alkaline phosphatase. The reaction was stopped by washing membranes in distilled water.

4.3 Results

4.3.1 Heparin Agarose/Hydroxylapatite Chromatography

The hpHGF (as determined by ELISA (d)) bound to the heparin agarose column was eluted by increasing the sodium chloride concentration to 1.3M NaCl. The peak of hpHGF concentration occurred after the peak of protein concentration eluted from the column, in fractions 25-43 (see Figure 4.1). The fractions containing maximal protein quantities (Nos 13-25) are separate. In this way HGF can be separated from the bulk of other placental proteins. Similarly, hpHGF bound to the hydroxylapatite column was eluted by increasing phosphate concentration to 0.45M. Fractions 31-40 contained the greatest amounts of HGF and fractions 16-31 contained the greatest quantities of total protein (see Figure 4.2).

4.3.2 Analysis

SDS-PAGE (12% gel) of the final concentrated preparation demonstrated the presence of several contaminating bands of unknown identity of molecular weight 10-20kDa (Figure 4.3(a)). Human placental HGF was not visible with either Coomassie blue or silver stain techniques (Figure 4.3(b)).

ELISA (c) (using monoclonal antibody D9, see Chapter 6) was used in preliminary studies to identify fractions containing peak hpHGF concentration. Figure 4.4 illustrates Western blotting of such fractions, eluted from a

Figure 4.1 Elution profile of human placental HGF from a heparin agarose column

Content of hpHGF is indicated by optical density of HGF ELISA reaction (right Y axis). Total protein content is indicated by absorbance of ultraviolet light at 280 nm, (left Y axis). Sodium chloride concentration, rising from 0.5M to 1.3M is indicated by conductivity measurement, (left Y axis X10). Fractions containing maximal hpHGF concentration (Nos. 25-43) are separate from fractions containing maximal protein concentration (Nos. 13-25).

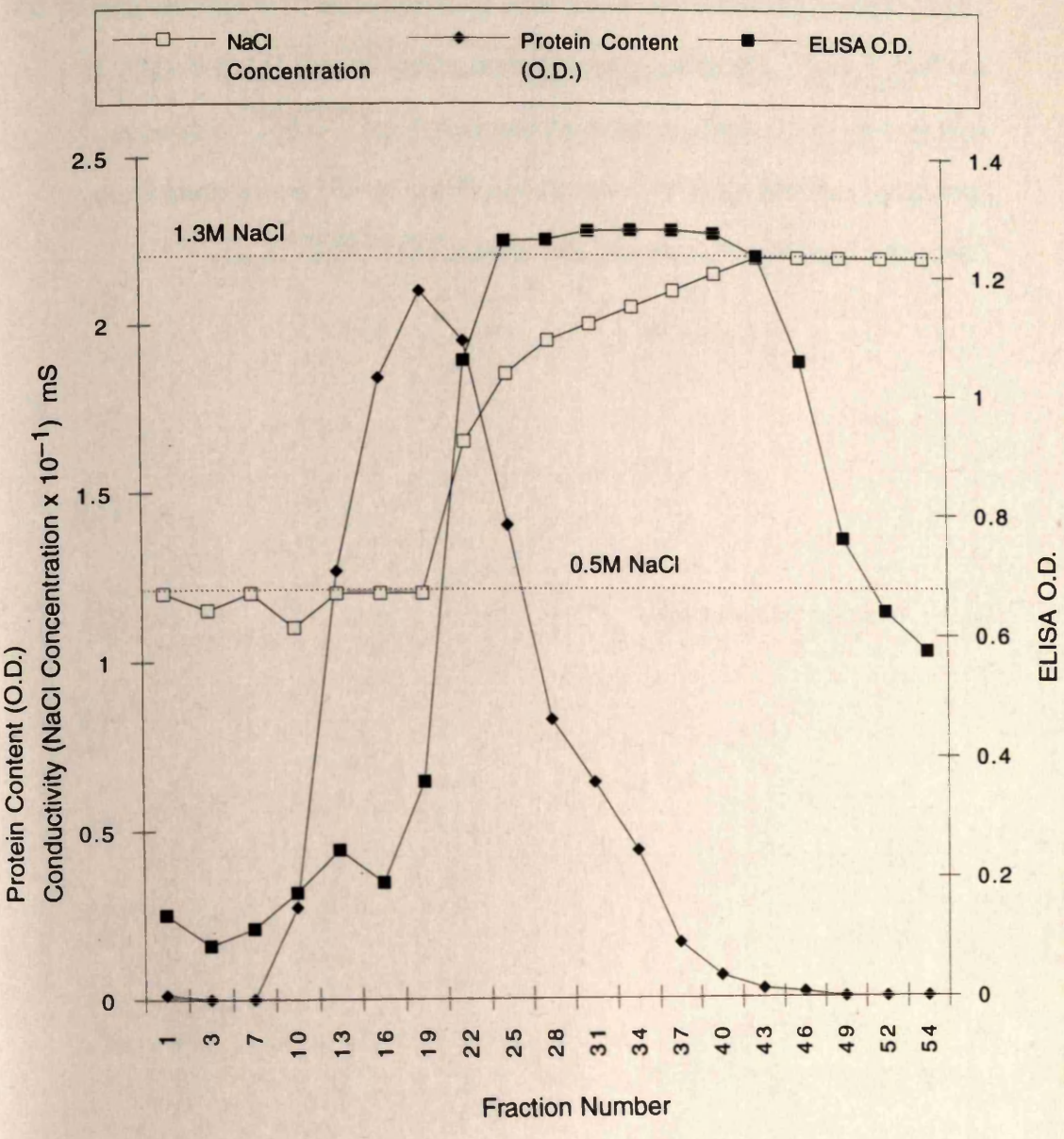

NaCl Concentration
Protein Content (O.D.)
ELISA O.D.
1.3M NaCl
0.5M NaCl
Protein Content (O.D.)
Conductivity (NaCl Concentration x 10^{-1}) mS
ELISA O.D.
Fraction Number
2.5
2
1.5
1
0.5
0
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1 3 7 10 13 16 19 22 25 28 31 34 37 40 43 46 49 52 54

Figure 4.2 Elution profile of human placental HGF from a hydroxylapatite column

Content of hpHGF is indicated by optical density of HGF ELISA reaction (right Y axis). Total protein content is indicated by absorbance of ultraviolet light at 280 nm (left Y axis). Phosphate concentration, rising from 0.1M to 0.45M, is indicated by conductivity in measurement (left Y axis X100). Fractions containing maximal hpHGF concentration (Nos. 31-40) are separate from fractions containing maximal protein concentration (Nos. 16-31).

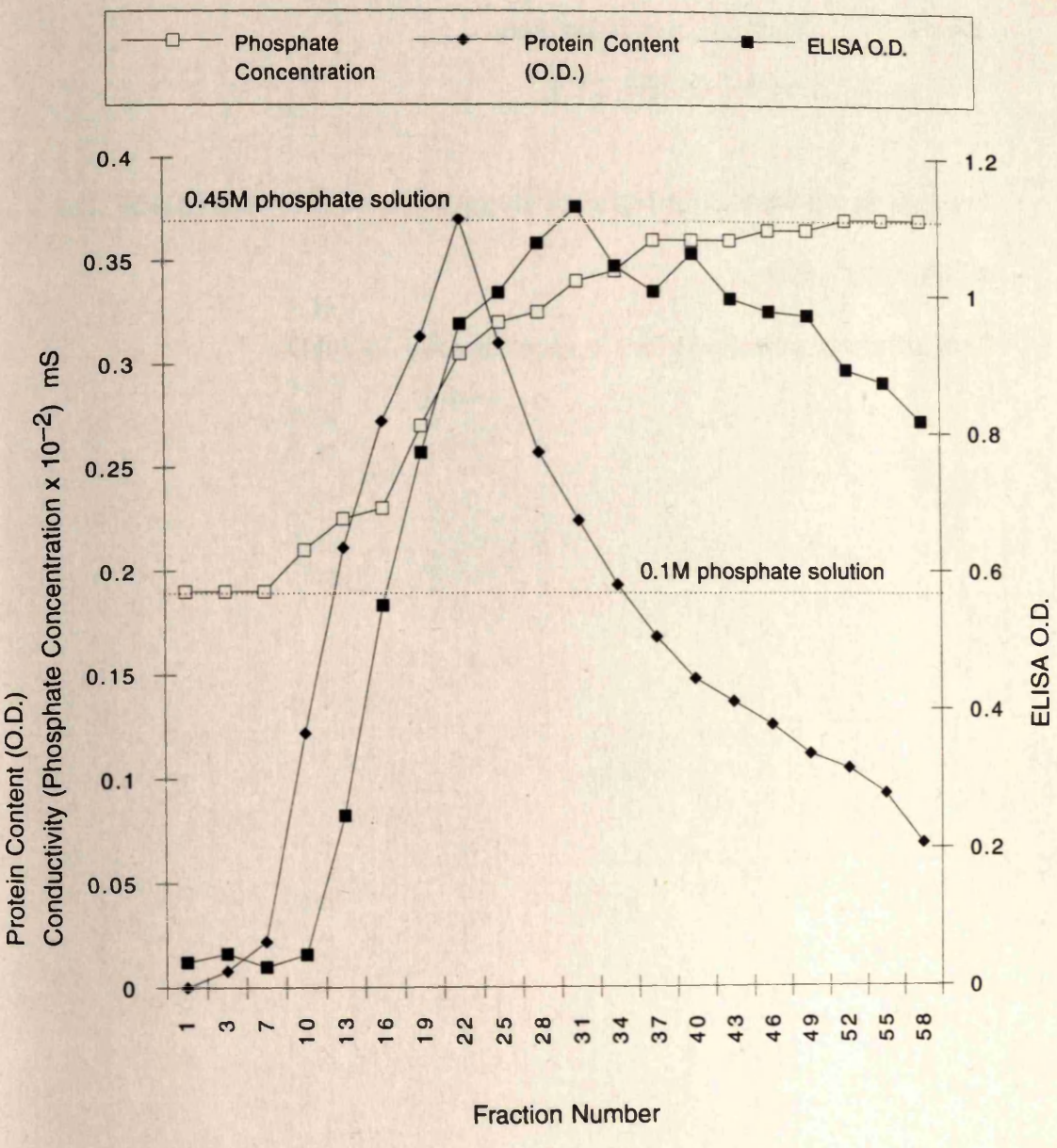

Phosphate Concentration
Protein Content (O.D.)
ELISA O.D.
0.45M phosphate solution
0.1M phosphate solution
Protein Content (O.D.)
Conductivity (Phosphate Concentration x 10⁻²) mS
ELISA O.D.
Fraction Number
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
1.2
1
0.8
0.6
0.4
0.2
0
1 3 7 10 13 16 19 22 25 28 31 34 37 40 43 46 49 52 55 58

Figure 4.3(a) SDS-PAGE analysis of purified human placental HGF (hpHGF)

Coomassie blue stain using 12% resolving gel and non-reduced samples.

Lane 1 Molecular weight markers

2 Purified hpHGF, 15 ng

No staining is evident in the region of the gel expected to contain hpHGF, i.e. adjacent to 80.4kDa.

Contaminating proteins are seen at approximately 10-20kDa.

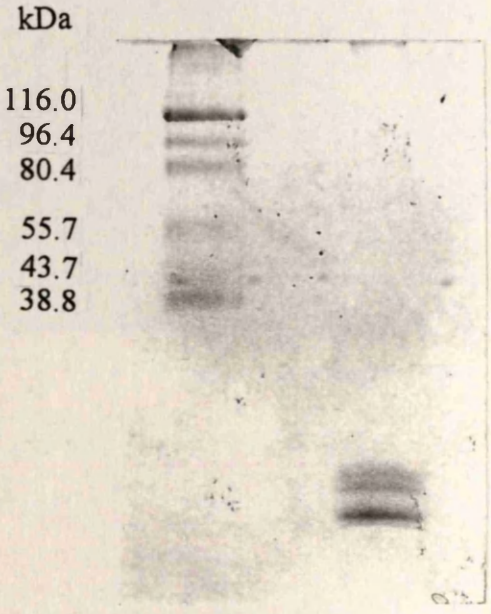

kDa
116.0
96.4
80.4
55.7
43.7
38.8
Lane
1
2

Figure 4.3(b) SDS-PAGE analysis of eluted fractions from a hydroxylapatite column containing maximal hpHGF content as determined by ELISA (c)

Silver stain using a 10% resolving gel and non-reduced samples

Lane 1 Fraction 31 ELISA O.D. i.e. hpHGF content = 0.497

2 Fraction 28 ELISA O.D. = 0.592

3 Fraction 25 ELISA O.D. = 0.617

4 Fraction 22 ELISA O.D. = 0.601

5 Fraction 19 ELISA O.D. = 0.373

6 Molecular weight markers

No staining is evident in the region of the gel expected to contain hpHGF, i.e. adjacent to 80.4kDa.

Contaminating proteins are seen at approximately 40-66 kDa.

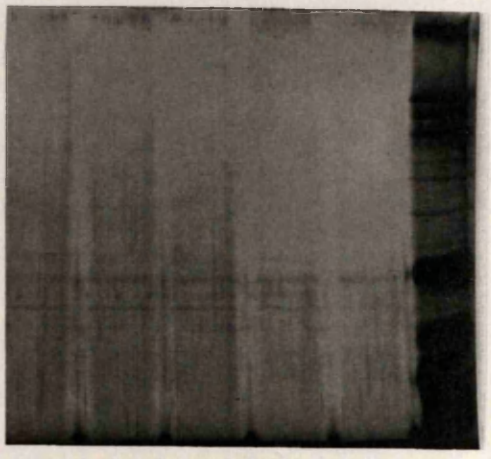

kDa
205.0
116.0
97.4
66.0
45.0
29.0
Lane 1 2 3 4 5 6

Figure 4.4 Western blotting, using polyclonal anti-HGF serum (Lot No. 201), of eluted fractions from hydroxylapatite column containing maximal human placental HGF content as determined by ELISA (c)

SDS-PAGE using a 7.5% resolving gel and non reduced samples probed with rabbit polyclonal anti-HGF serum (Lot No. 201, 1/4000)

Lane 1 Molecular weight markers

2 Fraction 19 ELISA O.D. (i.e. hpHGF content) = 0.373

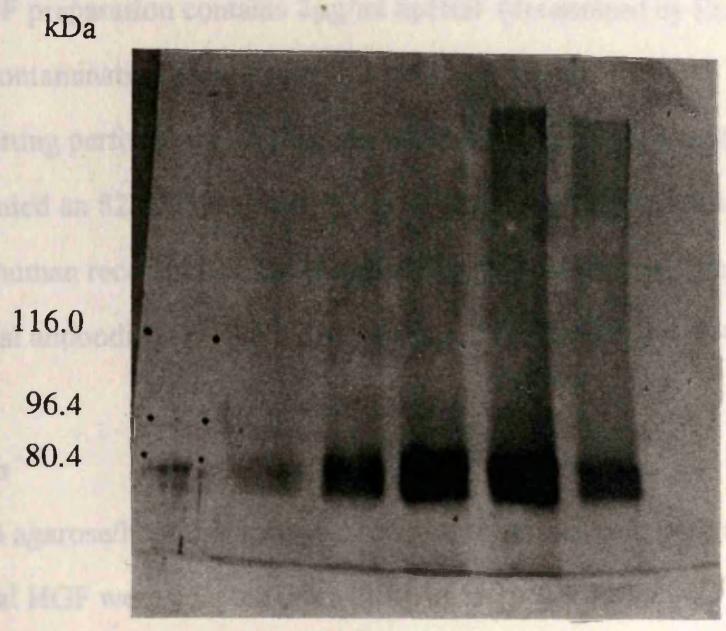
3 Fraction 22 ELISA O.D. = 0.601

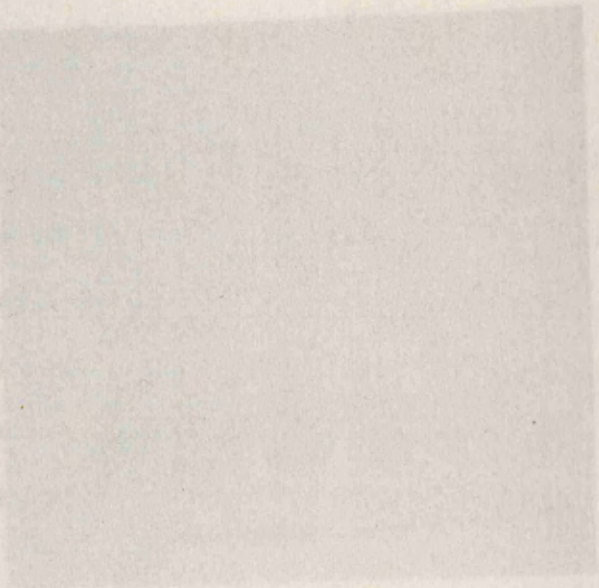
4 Fraction 25 ELISA O.D. = 0.617

5 Fraction 28 ELISA O.D. = 0.592

6 Fraction 31 ELISA O.D. = 0.497

A band is visible adjacent to the molecular weight marker 80.4 kDa, corresponding to human placental HGF.


kDa
116.0
96.4
80.4
Lane 1 2 3 4 5 6



hydroxylapatite column, using polyclonal antiserum to human recombinant HGF (Lot No 201). A band of ~82kDa corresponding to HGF is seen in those fractions which were found to contain peak HGF concentration using ELISA (c). The final hpHGF preparation contains 2μg/ml hpHGF (determined by ELISA) together with contaminating proteins making up to 3.0 mg/ml.

Western blotting performed using this material and the following antibodies to HGF demonstrated an 82kDa band in non-reduced samples with identical appearance to human recombinant full length HGF; polyclonal antiserum Lot No 201, monoclonal antibodies D9, B25, B43, A3.1.2-2. (Figures 4.5, 4.6, 4.7, 4.8, 4.9).

4.4 Discussion

The heparin agarose/hydroxylapatite chromatography methods used to purify human placental HGF were adapted from those of Hernandez *et al* (46) and Gohda *et al* (166). These methods were selected because they had already been shown to be useful in the purification of native HGF and because they could be adapted according to available resources.

In each of the chromatography steps, the fraction containing the peak concentration of protein were separate from those containing greatest concentration of hpHGF. Clearly, however, these methods can only provide partial purification of the target protein. Human placental HGF was not present in sufficient concentration to be demonstrable in SDS-PAGE, although several contaminating proteins were clearly shown. The presence of hpHGF in the eluted fractions and final concentrated preparation was confirmed by Western blotting in which a number of antibodies to HGF, monoclonal and polyclonal, produced similar images of a single band at 82 kDa under non-reducing conditions. This form of HGF does not dissociate into separate α and βchains when subjected to reducing conditions as reported by Hernandez *et al* (46). The final preparation of hpHGF contains 2 μg/ml hpHGF which is sufficient for the purposes of raising antibodies in rabbits and mice.

Figure 4.5 Western blotting of a concentrated preparation of human placental HGF and human recombinant HGF using polyclonal anti-HGF serum Lot No. 201

SDS-PAGE using 10% resolving gel and non reduced samples probed with rabbit polyclonal anti-HGF serum (Lot No. 201, 1/4000)

Lane 1 Molecular weight markers

2 Human placental HGF (20 ng)

3 Human recombinant HGF (100 ng)

Human placental HGF is visible as a band at 82 kDa, similar to that found in the lane containing human recombinant HGF.

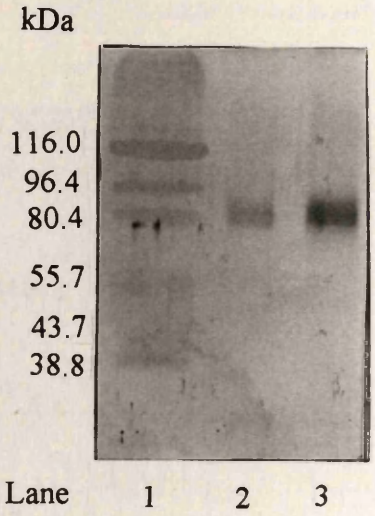

kDa
116.0
96.4
80.4
55.7
43.7
38.8
Lane
1
2
3

Figure 4.6 Western blotting using monoclonal antibody D9, of eluted fractions from hydroxylapatite column containing maximal human placental HGF content as determined by ELISA (c)

SDS-PAGE using a 7.5% resolving gel and non-reduced samples, probed with monoclonal antibody D9 culture supernatant (1/100)

Lane 1 { Fractions containing peak human placental HGF
2 { concentrations by ELISA

3 { Fractions containing no detectable human placental
4 { HGF
5 {

6 { Molecular weight markers

A band is seen adjacent to the molecular weight marker 80.4 kDa, corresponding to human placental HGF.

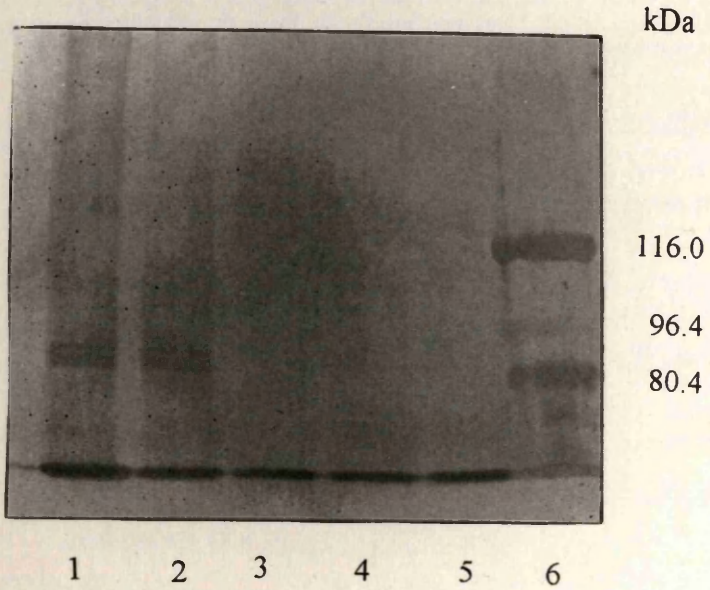

kDa
116.0
96.4
80.4
1
2
3
4
5
6

Figure 4.7 Western blotting of a concentrated preparation of human placental HGF and human recombinant HGF using monoclonal antibody B25

SDS-PAGE using a 12% resolving gel and non reduced samples, probed with monoclonal antibody to HGF (B25, 1/5000)

Lane 1 Human recombinant HGF (100 ng)

2 Molecular weight markers

3 Human placental HGF (20 ng)

An 82 kDa band is visible, corresponding to HGF, in lanes 1 and 3.

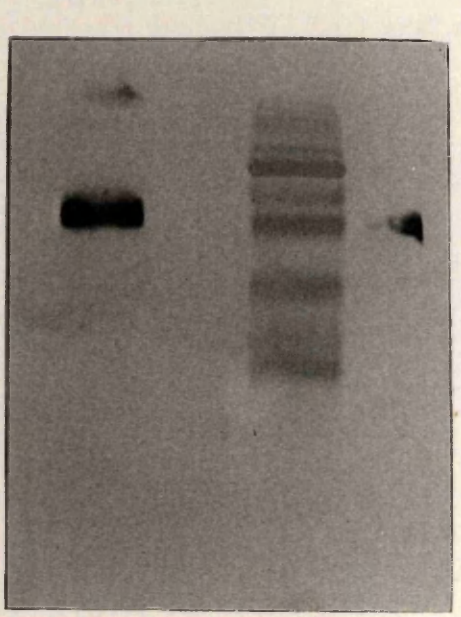

kDa
116.0
96.4
80.4
55.7
43.7
38.8
1
2
3
Lane

Figure 4.8 Western blotting of a concentrated preparation of human placental HGF and human recombinant HGF using monoclonal antibody B43

SDS-PAGE using a 12% resolving gel and non reduced samples, probed with monoclonal antibody to HGF (B43, 1/5000)

Lane 1 Molecular weight markers

2 Human recombinant HGF (100 ng)

3 Human placental HGF (20 ng)

An 82 kDa band is visible, corresponding to HGF in lanes 2 and 3.

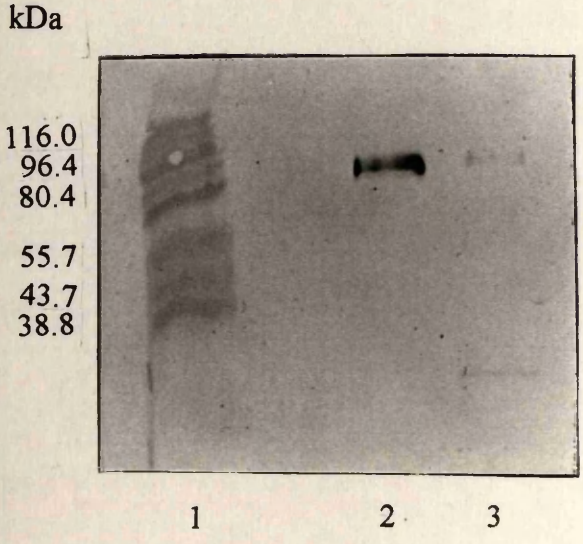

kDa
116.0
96.4
80.4
55.7
43.7
38.8
1
2
3

Figure 4.9 Western blotting of a concentrated preparation of human placental HGF and human recombinant HGF using monoclonal antibody A3.1.2-2

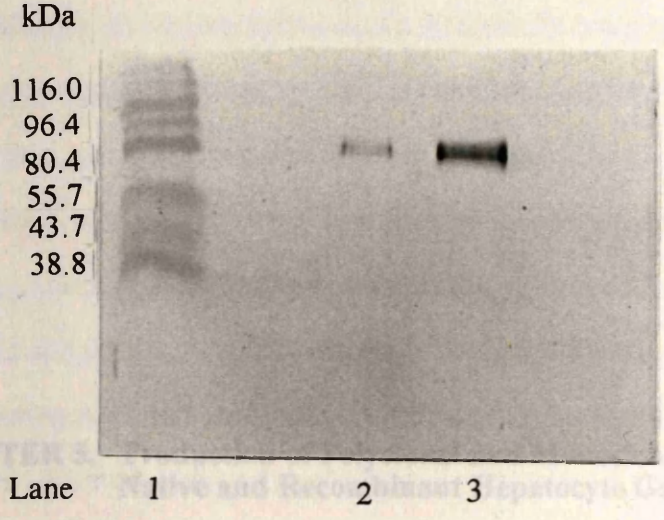
SDS-PAGE using a 12% resolving gel and non reduced samples, probed with monoclonal antibody to HGF (A3.1.2-2, 1/5000)

Lane 1 Molecular weight markers

2 Human placental HGF (20 ng)

3 Human recombinant HGF (100 ng)

An 82 kDa band is visible, corresponding to HGF, in lanes 2 and 3


kDa
116.0
96.4
80.4
55.7
43.7
38.8
Lane
1
2
3

CHAPTER 5. Production of Polyclonal and Monoclonal Antibodies to Native and Recombinant Hepatocyte Growth Factor Proteins

5. INTRODUCTION

5.1 Antibodies; monoclonal and polyclonal

In preparing antibodies to be used as investigative tools a choice is available between two very different types of reagent; conventional polyclonal antisera and monoclonal antibodies. A polyclonal antiserum contains many different antibody specificities to the various epitopes of a structurally complex immunogen. The ability to recognise different epitopes present on an antigen facilitates detection of that antigen, particularly in situations where the antigen is immobilised and only part of its structure is exposed. Examples include immunocytochemistry and ELISA. Another advantage includes the relative ease with which they may be prepared and their stability in laboratory storage (4°C or minus 20°C). Disadvantages relate mainly to the inherent heterogeneity of antibody present. Since an antiserum is the product of many responding classes of cells, this involves the classes and subclasses (isotypes) of antibody produced, as well as their specificity, titre and affinity. In practice this means that each batch of antiserum, even from the same animal requires to be assessed for its suitability in any particular assay. Unless the antibodies are affinity purified specific antibodies of interest account for a maximum of 20-30% of the immunoglobulin present.

Monoclonal antibodies have become increasingly popular since the method for their production was developed in 1975 (167). Monoclonal antibodies (MAbs) are derived from a single B cell clone which has been immortalised by fusion with a myeloma cell line. They are homogeneous in specificity, affinity and isotype. Each hybridoma clone produces an antibody which is specific to a single antigenic determinant of the immunogen (monospecific). Monoclonal antibodies are particularly useful in assays for soluble antigen in which they help overcome difficulties of sensitivity and specificity which may accompany the use of polyclonal antibody. Their production from cell culture may allow virtually unlimited amounts of antibody to be generated.

5.2 Preparation of Polyclonal Antibodies to Native Human Placental HGF and Recombinant Rat and Human HGF Peptides

Polyclonal antibodies are routinely produced in rabbits, sheep and goats. In this study rabbits have been used in view of the fact that other workers have successfully raised antibodies to HGF in this species (168). Moreover, rabbits are relatively easy to care for, robust, long lived and easy to handle, immunise and bleed. They give excellent IgG responses to a wide variety of immunogens.

5.3 Materials and Methods

Materials - see page 141

Methods

5.3.1 Immunisation of Rabbits

Prior to immunisation 5ml of preimmune blood was taken from the rear marginal ear vein of each animal, allowed to clot and serum separated and stored at -20°C.

Primary immunisation: Subcutaneous injection of protein was performed. hHGFα266 (100-500μg), a 56kDa recombinant bacterial fusion protein based on human HGFα-chain, produced as described in Chapter 2. rHGFβ121 (100-500μg), a 14kDa recombinant bacterial protein based on rat HGFβ chain, produced as described in Chapter 2. Native human placental HGF, preparation containing full length naturally occurring human placental HGF, as described in Chapter 4. Protein preparations were emulsified in PBS/Freunds complete adjuvant, to a final volume of 0.5-1ml. Two animals were immunised for each protein; Nos 340 and 341 - hHGFα266, Nos 38986 and 1700 - rHGFβ121, Nos 1699 and 1698 - human placental HGF.

Boost injections: Four subcutaneous injections of protein (100-500μg of hHGFα266, rHGFß121 or ~1-2μg of hpHGF emulsified in PBS/Freunds incomplete adjuvant to a final volume of 0.5ml) at intervals of 2 weeks.

Test bleeds of 5ml blood taken as above prior to each boost injection.

5.3.2 Western Blotting

Following SDS-PAGE (as described in Chapter 2) of non-reduced samples of hHGFα266 (12% gel), rHGFβ121 (15% gel) and human placental HGF (10% gel), proteins were blotted onto PVDF membranes using carbonate transfer buffer with BioRad Mini Protean II equipment as described in Chapter 3.

After transfer, membranes were incubated with PBS containing 0.1% (volume/volume) Tween 20 and 5% (weight/volume) powdered milk for 1 hour at room temperature. Membranes were then incubated with preimmune sera, antisera from immunised animals or positive control antibodies diluted in PBS containing 0.1% (volume/volume) Tween 20 (PBS-Tween) at room temperature overnight. Membranes were washed in PBS-Tween (3 x 10 minutes) and incubated with secondary antibody (alkaline phosphatase conjugated swine anti-rabbit immunoglobulins or, in the case of positive control murine monoclonal antibodies, in alkaline phosphatase conjugated rabbit anti-mouse immunoglobulins) diluted 1/1000 in PBS-Tween at room temperature for 1 hour. Membranes were washed 2 x 10 minutes in PBS-Tween, then 1 x 10 minutes in 100mM Tris buffer, pH 9.5 and exposed to nitro-blue tetetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP Appendix 3) and reaction stopped by washing with distilled water.

5.4 Results

Immunisation was tolerated well by each of the rabbits used. Adequate antibody titres were demonstrated in each animal after four subcutaneous boost injections at intervals of 2 weeks.

hHGFα266: sera from rabbits 340 and 341 were shown to detect the immunogen and full length recombinant human HGF in Western blotting studies. The antibody also cross-reacted with full length rat recombinant HGF (Fig 5.1).

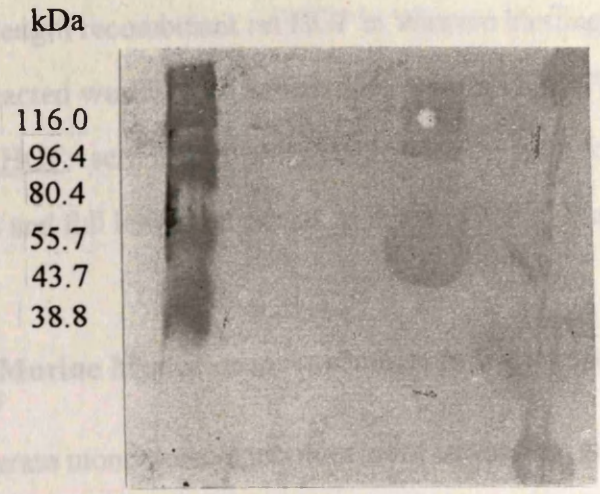
Figure 5.1 Western blotting of hHGFα266, recombinant human HGF and recombinant rat HGF using pre-immune and immune serum from rabbit 341

SDS-PAGE using a 10% resolving gel and non reduced samples probed with preimmune (a) and immune (b) serum (1/300) from rabbit 341 which had been immunised using the recombinant HGF protein hHGFα266.

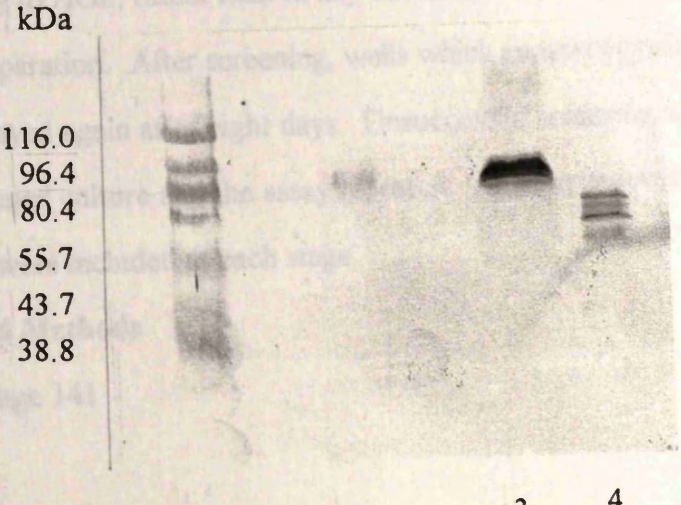
Lane	1	Molecular weight markers
	2	recombinant human HGF (100ng)
	3	recombinant rat HGF (100ng)
	4	partially purified hHGFα266 (100ng)

Background staining only is seen in the blot using preimmune serum (a). Using immune serum (b), bands are visible in lanes 2 and 3 of approximately 82 kDa corresponding to HGF. Several bands are visible in Lane 4 of approximate molecular weights 55.7-75 kDa. Immune serum thus recognises several proteins in the impure preparation of hHGFα266 and both recombinant human and recombinant rat HGF.

(a)


kDa
116.0
96.4
80.4
55.7
43.7
38.8
Lane 1 2 3 4

(b)


kDa
116.0
96.4
80.4
55.7
43.7
38.8
Lane 1 2 3 4

rHGFβ121: sera from rabbits 38986 and 1700 were shown to detect both the immunogen and full length recombinant rat HGF in Western blotting studies. The antibody also crossreacted weakly with human recombinant HGF (Fig 5.2).

Human placental HGF: sera from rabbits 1699 and 1698 were found to detect both the immunogen and full length recombinant human HGF in Western blotting studies (Fig 5.3).

5.5 Production of Murine Monoclonal Antibodies to Native Human Placental HGF

Attempts to generate monoclonal antibodies were carried out in collaboration with Dr Ailsa Campbell and Mr Pat Ferry of Glasgow University Department of Biochemistry. Mice were immunised according to a standard schedule, killed and spleen cells harvested for fusion with myeloma cells. At the same time serum was collected for assessment of polyclonal antibody response to the immunisation programme. Eight to ten days after plating of cells from fusion, hybridoma supernatants were assayed by ELISA for antibodies to human recombinant HGF. This was to allow selection of those clones formed following fusion of a cell producing antibody to HGF, rather than to any contaminant in the human placental HGF preparation. After screening, wells which appeared positive were subcloned and assayed again after eight days. Unsuccessful screening was followed by continued culture and the assay repeated. Appropriate positive and negative controls were included at each stage.

5.6 Materials and Methods

Materials - see page 141

Methods

5.6.1 Immunisation of Mice

Day 0; 100µl (~200ng) hpHGF in Freund's complete adjuvant injected sub-cutaneously. Subcutaneous booster injections of 100µl (~200ng) hpHGF in Freund's incomplete adjuvant given on days 10 and 20. On day 30 an intravenous

Figure 5.2(a) Western blotting of cell lysate of *E.coli* expressing the recombinant rat HGF protein rHGFβ121 using pre-immune and immune serum from rabbit 38986

SDS-PAGE using a 15% resolving gel and non reduced samples probed with pre-immune, lane 1, and immune, lane 2 serum (1/200) from rabbit 38986 which had been immunised using the recombinant HGF protein rHGFβ121.

Lane 1 Cell lysate of *E.coli* expressing rHGFβ121 probed with pre-immune serum

2 Cell lysate of *E.coli* expressing rHGFβ121 probed with immune serum

3 Molecular weight markers (pre-stained)

No staining is seen in lane 1. In lane 2 a band of 14 kDa is visible corresponding to rHGFβ121.

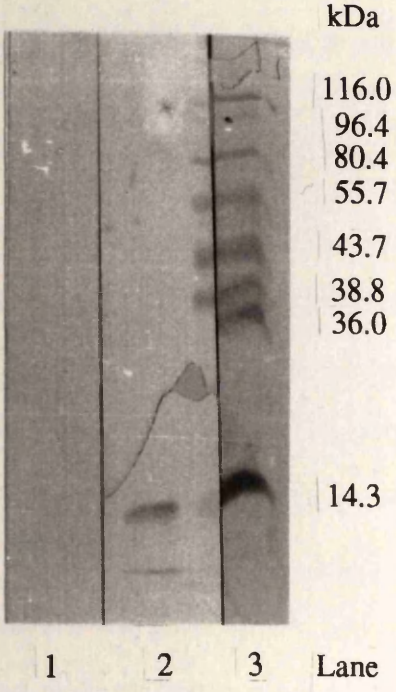

kDa
116.0
96.4
80.4
55.7
43.7
38.8
36.0
14.3
1
2
3
Lane

Figure 5.2(b) Western blotting of full length recombinant rat HGF using pre-immune and immune immunoglobulin from rabbit 38986

SDS-PAGE using a 10% resolving gel and non reduced samples probed with immunoglobulin purified from pre-immune, lane 2, and immune, lane 3, serum from rabbit 38986 which had been immunised against recombinant HGF protein rHGFβ121.

Lane 1 Molecular weight markers

2 Recombinant rat HGF (100 ng) probed with pre-immune immunoglobulin (0.232 mg/ml)

3 Recombinant rat HGF (100 ng) probed with immune immunoglobulin (0.232 mg/ml)

Background staining only is seen using pre-immune immunoglobulin (lane 2). Using immune immunoglobulin a band is visible in lane 3 of approximately 82 kDa corresponding to HGF.

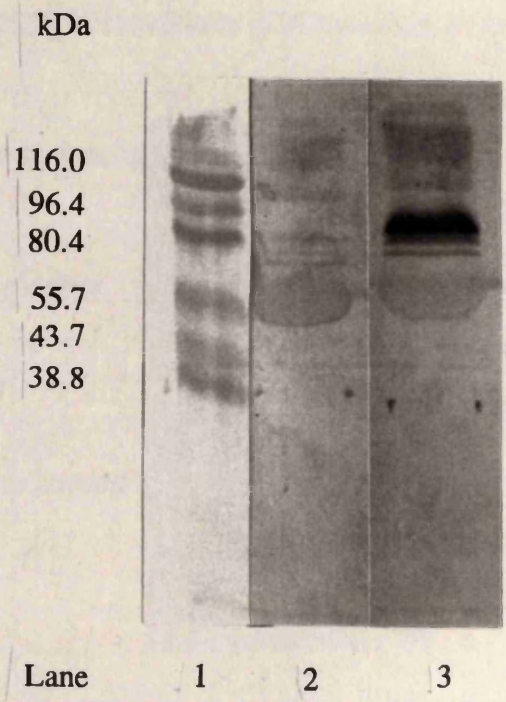

kDa
116.0
96.4
80.4
55.7
43.7
38.8
Lane
1
2
3

Figure 5.2(c) Western blotting of recombinant full length human HGF using pre-immune and immune serum from rabbit 38986

SDS-PAGE using a 12% resolving gel and non reduced samples probed with pre-immune, lane 1, and immune, lane 2, serum (1/100) from rabbit 38986 which had been immunised using recombinant HGF protein rHGFβ121.

Lane 1 Recombinant human HGF (100 ng) probed with pre-immune serum.

2 Recombinant human HGF (100 ng) probed with immune serum

3 Molecular weight markers

No staining is seen in lane 1. In lane 2 a band of approximately 82 kDa is visible corresponding to human recombinant HGF.

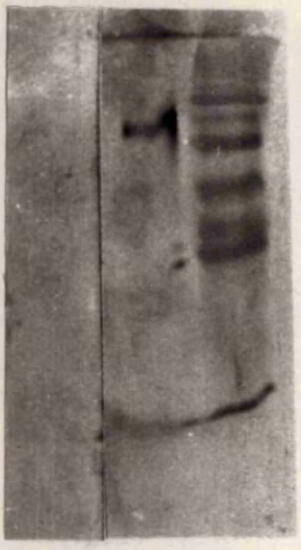

kDa
116.0
96.4
80.4
55.7
43.7
38.8
Lane
1
2
3

Figure 5.3(a) Western blotting of human recombinant HGF using pre-immune and immune serum from rabbit 1698

SDS-PAGE using a 10% resolving gel and non-reduced samples probed with pre-immune, lane 1, and immune lane, lane 2, serum (1/1000) from rabbit 1698 which had been immunised using purified human placental HGF.

Lane	1	Human recombinant HGF (200 ng)
	2	Human recombinant HGF (200 ng)
	3	Molecular weight markers

A band of approximately 82 kDa corresponding to human recombinant HGF is visible in lane 2 using immune serum. Serum from rabbit 1698 following immunisation is thus capable of detecting human recombinant HGF. No signal is seen in lane 1 using pre-immune serum.

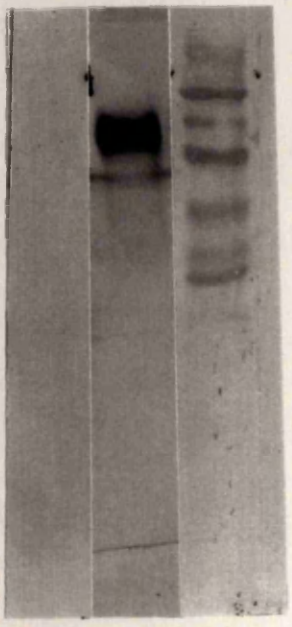

kDa
116.0
96.4
80.4
55.7
43.7
38.8
Lane 1 2 3

Figure 5.3(b) Western blotting of human placental HGF and human recombinant HGF using immune serum from rabbit 1698

SDS-PAGE using a 12% resolving gel and non-reduced samples probed with immune serum (1/200 and 1/1000) from rabbit 1698 which had been immunised using purified human placental HGF.

Lane 1 Human recombinant HGF (250ng) } serum 1698, 1/200

2 Human placental HGF (20 ng) }

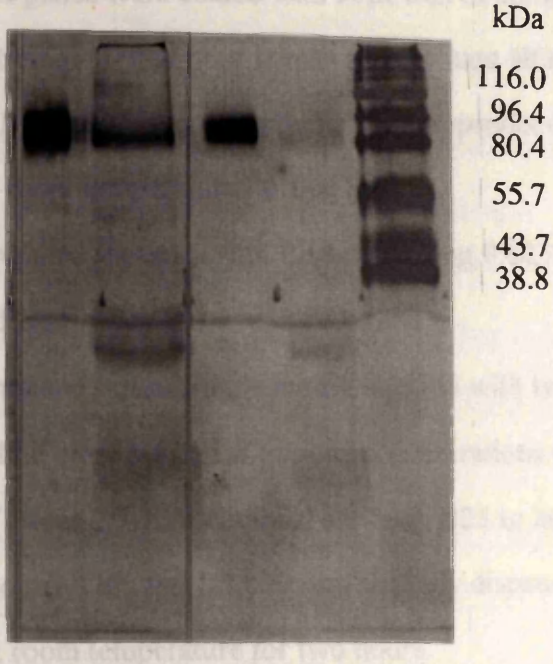
3 Human recombinant HGF (250 ng) } serum 1698, 1 /1000

4 Human placental HGF (20 ng) }

5 Molecular weight markers

In lanes 1 and 3 a band of approximately 82 kDa is visible, corresponding to human recombinant HGF. In lanes 2 and 4 a band is also visible at 82 kDa corresponding to human placental HGF. Further bands are evident in lanes 2 and 4 of approximately 10-20 kDa and 50-70 kDa corresponding to contaminating proteins in the human placental HGF protein.

Immune serum from rabbit 1698 thus recognises human placental HGF and human recombinant HGF


kDa
116.0
96.4
80.4
55.7
43.7
38.8
Lane 1 2 3 4 5

injection of 20µl (40ng) hpHGF in PBS was given. Four days later the animal was killed and spleen/serum harvested.

5.6.2 Assessment of Mouse Polyclonal Serum Response to Immunisation with Human Placental HGF

(1) Wells of microtitre ELISA plates were coated with 50µl/well of human placental HGF at a concentration of 100ng/ml or human recombinant HGF at a concentration of 2µg/ml in 0.1M $NaHCO_3$ coating buffer (see Appendix 4). Plates were then incubated at room temperature for one hour.

(2) The ELISA plates were washed six times with PBS containing 0.025% (volume/volume) Tween 20 (PBS/Tween 20).

(3) Pre-immune serum and immune serum from a mouse injected with two doses of purified human placental HGF were applied at various concentrations (1/100, 1/200, 1/400, 1/800) in PBS/Tween 20. Monoclonal antibody B25 to human recombinant HGF (positive control) diluted 1/5,000 was similarly dispensed. Plates were then incubated at room temperature for two hours.

(4) Plates were washed six times with PBS/Tween 20.

(5) Peroxidase conjugated goat anti-mouse immunoglobulin, diluted to 1/500 in PBS/Tween 20 was added and the plates incubated at room temperature for one hour.

(6) Plates were washed six times with PBS/Tween 20.

(7) Orthophenylene diamine substrate (see Appendix 3) was applied (100µl/well) and the plates developed in darkness for 10-15 minutes.

(8) Reaction was stopped by addition of 50µl/well 4N H_2SO_4 .

(9) Plates were read by automatic plate reader (Dynatech MR700, Dynatech Laboratories, Guernsey, Channel Islands, UK) at 490nm with a reference wavelength of 630nm.

5.6.3 Spleen Cell/Myeloma Fusion

Following removal the spleen was placed in 10ml RPMI medium in a sterile petri dish and cells released by teasing with needles. This cell suspension was passed twice through a 21 gauge needle and then through a 25 gauge needle into a sterile container.

Preparation of SP20 plasmacytoma cells

SP20 cells in logarithmic growth in RPMI containing 10% fetal calf serum were washed and re-suspended in 20ml RPMI containing penicillin and streptomycin to give approximately 2-5 x 10^7 cells in total.

Both spleen cells and SP20 cells were washed with 20ml RPMI 3 times. Control cells were taken from each and the cells were mixed and harvested at 300g as a compact pellet.

Controls from spleen cells and SP20 cells were diluted into HAT medium and 200µl of each were dispensed into 12 wells of a 96-well culture plate. Both spleen and SP20 cell controls were dispensed onto the same plate. Eight other plates received medium containing cells following fusion as described below -

Fusion protocol

The pellet of SP20 and spleen cells was gently mixed with 50% (weight/volume) polyethyleneglycol (1.0 ml)), prewarmed to 37°C, over 1-2 minutes. RPMI (5.0 ml) was then slowly added over 2 minutes to gently dilute out the polyethyleneglycol. The mixture of cells was centrifuged at 400 g for 5 minutes, the cell pellet was resuspended in 96 ml HAT medium and dispensed (200 µl/well) into the inner 60 wells of 8 microtitre plates. The plates were next incubated at 37°C in a humidified incubator with 5% CO_2.

5.6.4 Screening of Hybridomas for Antibodies to Human Recombinant HGF

Hybridomas were cultured for eight days prior to screening by ELISA of culture supernatants.

1) Wells of nine microtitre ELISA plates were coated with 50µl/well human recombinant HGF at a concentration of 2µg/ml in 0.1M NaHCO3 coating buffer (see Appendix 4) for 1 hour at room temperature.

2) The ELISA plates were washed 6 times with PBS containing 0.025% Tween 20.

3) Culture supernatant (100µl) from hybridomas, spleen cells and myeloma cells was dispensed into corresponding wells of ELISA plates. Normal and immune mouse serum diluted 1/1000 in PBS containing 0.025% Tween 20 and monoclonal antibody B25 to HGF (positive control) diluted 1/10,000 (i.e. final concentration 167ng/ml) in PBS containing 0.025% Tween 20 were similarly dispensed. Plates were then incubated at room temperature for 2 hours. Stages (4-9) were as described above in 'Assessment of mouse polyclonal serum response to immunisation with human placental HGF', section 5.6.2.

5.7 Results

5.7.1 Polyclonal Serum Response of Mice Immunised with Human Placental HGF

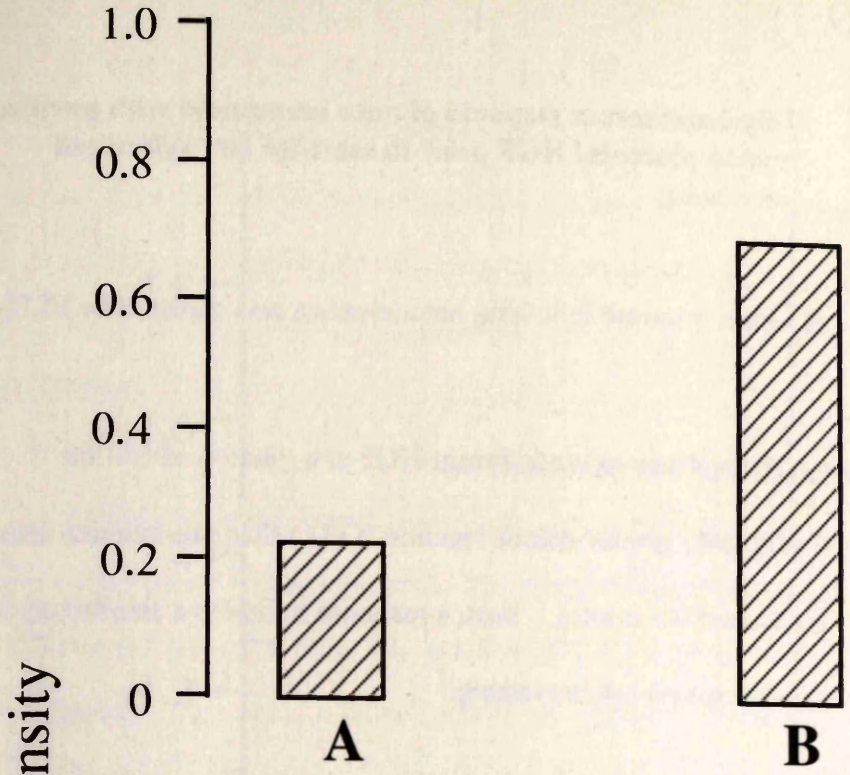
Sera from immunised mice detected both human recombinant HGF and human placental HGF. Figure 5.4 illustrates the difference between immune and pre-immune serum in the detection of human placental HGF and human recombinant HGF. Using wells coated with human placental HGF, pre-immune serum diluted 1/200 produced an ELISA Optical Density of 0.215. Immune serum gave an Optical Density of 0.691. When wells coated with human recombinant HGF were used, pre-immune serum diluted 1/200 produced an Optical Density of zero. Immune serum diluted 1/200 gave an Optical Density of 0.992.

Figure 5.5 illustrates the level of response produced in a mouse prior to sacrifice and spleen cell harvesting. Pre-immune serum diluted 1/1000 produced an ELISA Optical Density of 0.091. Immune serum diluted 1/1000 gave an Optical Density of 1.939 (maximum O.D. possible; 2.0).

Figure 5.4 Polyclonal serum response of mice immunised with purified human placental HGF

The polyclonal serum response following immunisation was assessed by ELISA as described.

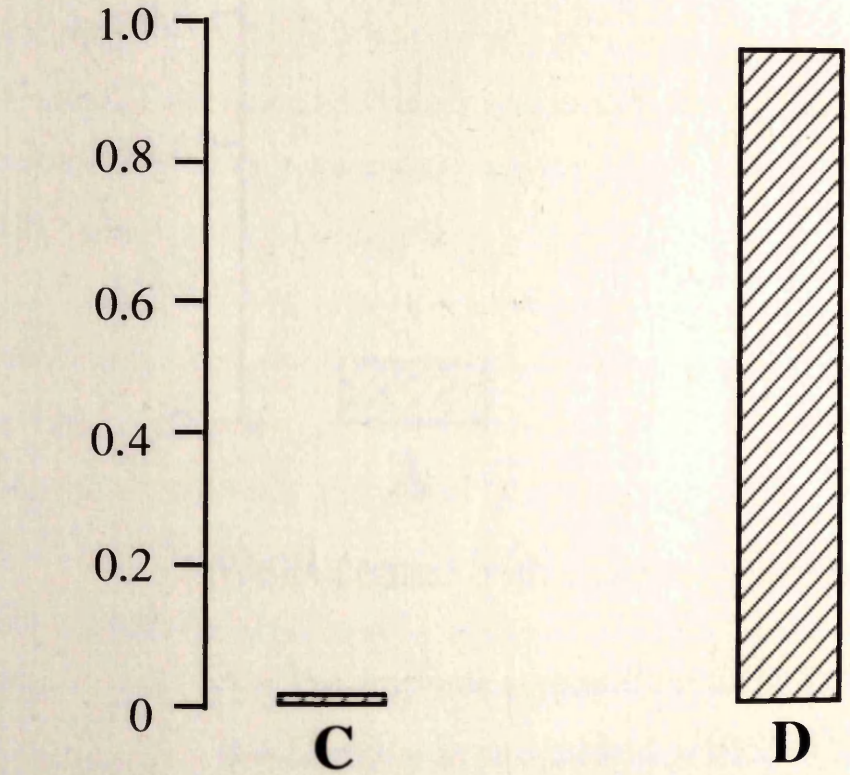
Immune serum detected both human placental and human recombinant HGF producing a significantly greater colour reaction (Optical Density) than pre-immune serum (from a non-immunised littermate).



Wells coated with human placental HGF

A = Pre-immune serum diluted 1/200 O.D. 0.215

B = Immune mouse serum diluted 1/200 O.D. 0.691



Wells coated with human recombinant HGF

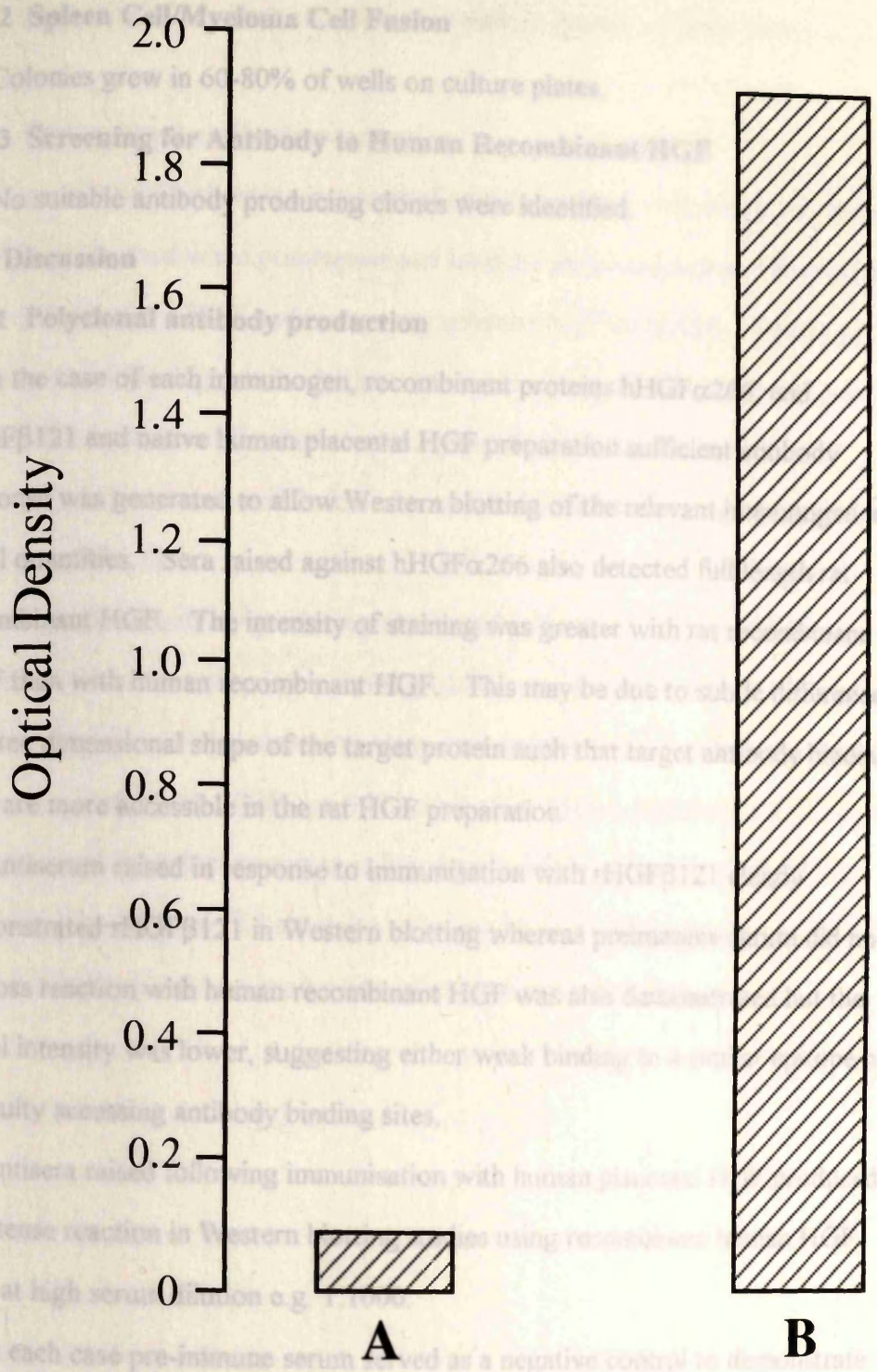
C = Pre-immune serum diluted 1/200 O.D. 0.000

D = Immune mouse serum diluted 1/200 O.D. 0.992

Figure 5.5 Polyclonal serum response of mice immunised with purified human placental HGF prior to sacrifice and spleen cell harvesting

The polyclonal serum response following immunisation was assessed by ELISA as described.

Immune serum detected human recombinant HGF at a dilution of 1/1000 producing a significantly greater colour reaction (O.D.) than pre-immune serum (from a non-immunised littermate). Such a response indicates a satisfactory level of immunity prior to spleen cell harvesting.



Wells coated with human recombinant HGF

A = Pre-immune serum diluted 1/100 O.D. 0.091

B = Immune serum diluted 1/1000 O.D. 1.939

5.7.2 Spleen Cell/Myeloma Cell Fusion

Colonies grew in 60-80% of wells on culture plates.

5.7.3 Screening for Antibody to Human Recombinant HGF

No suitable antibody producing clones were identified.

5.8 Discussion

5.8.1 Polyclonal antibody production

In the case of each immunogen, recombinant proteins hHGFα266, and rHGFβ121 and native human placental HGF preparation sufficient antibody response was generated to allow Western blotting of the relevant immunogen in small quantities. Sera raised against hHGFα266 also detected full length rat recombinant HGF. The intensity of staining was greater with rat recombinant HGF than with human recombinant HGF. This may be due to subtle differences in three dimensional shape of the target protein such that target antibody binding sites are more accessible in the rat HGF preparation.

Antiserum raised in response to immunisation with rHGFβ121 clearly demonstrated rHGFβ121 in Western blotting whereas preimmune serum did not. A cross reaction with human recombinant HGF was also demonstrated but the signal intensity was lower, suggesting either weak binding to a similar epitope or difficulty accessing antibody binding sites.

Antisera raised following immunisation with human placental HGF produced an intense reaction in Western blotting studies using recombinant human HGF even at high serum dilution e.g. 1:1000.

In each case pre-immune serum served as a negative control to demonstrate the effect of the immunisation process. These studies were useful in demonstrating that immunisation had produced a measurable antibody response and offered some suggestion as to the sensitivity of the antibody produced. Clearly, however, we could not exclude cross reaction with similar proteins i.e. the degree of antibody specificity was to be carried out if the antibody raised

proved capable of demonstrating target protein in immunocytochemistry or ELISA.

5.8.2 Monoclonal antibody production

A good polyclonal serum response of mice immunised with human placental HGF was obtained when preimmune and immune sera were assessed for reaction to human placental HGF and human recombinant HGF in ELISA. Before each mouse was killed, high antibody titre was confirmed by ELISA to allow optimal timing for production of suitable antibody producing hybridomas.

Spleen cell/myeloma cell fusion was successful with colonies growing in 60-80% of culture wells.

No suitable antibody producing clones were identified. Reasons for this remain unclear, the mice clearly developed an immune response to the immunogen and the sensitivity of the screening assay used is believed to be adequate (detects B25 at 1.67mg/ml x 1/10000 i.e. 167ng/ml). It is known that the yield of suitable hybridomas from initial screening is found to be of the order of 0-25% (169).

Following unsuccessful attempts with four mice insufficient time remained for further approaches to the production of monoclonal antibodies.

CHAPTER 6. Development of an ELISA for Detection of Hepatocyte Growth Factor in Human Serum

6. INTRODUCTION

6.1 Enzyme Linked Immunoabsorbent Assay (ELISA)

Since its development in the early 1970s (170,171), Enzyme Linked Immunosorbent Assay (ELISA) has become an established tool in a variety of fields of biological study. Ideally suited for the detection of minute quantities of material present in complex mixtures such as serum, these assays have been particularly useful in the detection of growth factors and hormones. One particular form of ELISA which is favoured for its combined simplicity, specificity and sensitivity is the 'sandwich' or two site antigen capture assay. In the basic form of this ELISA an antibody, frequently a monoclonal of high affinity is used to coat a solid phase. This is exposed to the test sample, then washed and a second, enzyme conjugated antibody is applied which binds to 'captured' antigen. After washing away unbound reagent the plate is exposed to substrate and developed. With the restriction that the antigen to be detected must have multiple or similar but spatially distant antibody binding epitopes such assays are potentially extremely sensitive. The use of monoclonal antibodies which confer features of specificity and reduced background signal as primary coating antibody together with a polyclonal second layer which amplifies the signal, helps to increase the sensitivity of these assays. Other means of improving sensitivity include the addition of further antibody layers, an avidin-biotin system, and the use of luminescence or enzyme cascades. However, clearly the performance characteristics of any ELISA are defined by the properties of the antibodies utilised.

The major difficulties encountered in the development of novel ELISAs are: (1) obtaining antibodies of high enough specificity and sensitivity for the antigen being studied, (2) obtaining antibodies which have long term stability (in particular this applies to monoclonal antibodies), (3) obtaining antibodies which do not bind in a non-specific manner, (4) obtaining antigen of known purity and concentration for assay standardisation.

In developing the ELISA for HGF, all of the above points had to be addressed. A number of antibodies were obtained for use in an HGF ELISA and their specificity and sensitivity assessed by Western blotting and ELISA. The stability of the antibodies was determined and cross reactivity to structurally/ immunologically similar molecules was examined by ELISA. Purified human recombinant full length HGF was obtained at a known concentration for purposes of calibration.

In these studies two main forms of ELISA sandwich were used comprising two or three antibody layers respectively. In the former, a monoclonal antibody was used to coat an ELISA plate, then samples (test or standard recombinant human HGF) were applied and incubated with the monoclonal antibody. A second antibody layer of purified, biotinylated rabbit IgG to HGF was applied and the antibody detected by addition of avidin-HRP (Horse-radish peroxidase) conjugates and the substrate orthophenylenediamine. The latter form of ELISA comprised a monoclonal coating antibody, then test or standard recombinant human HGF samples, detection with whole rabbit polyclonal antiserum to HGF, followed by biotinylated purified specific sheep antibody to rabbit immunoglobulins. (Cross reacting antibodies to murine immunoglobulins were removed prior to use). The detection system was by means of avidin-HRP conjugate and orthophenylenediamine (Figure 6.1).

The ELISA assays developed in this study may be summarised as illustrated in table 6.1.

6.2 Materials and Methods

Materials - see page 141.

6.2.1 Western Blotting

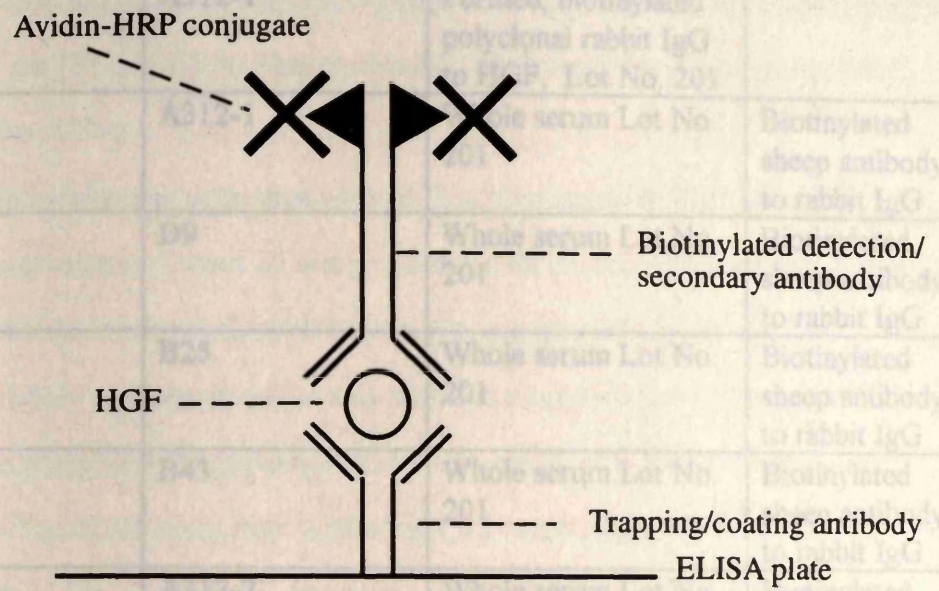
Blotting studies were performed as described in Chapter 3. Non-reduced samples of purified human recombinant HGF or purified native human placental HGF were subjected to SDS-PAGE and transferred to PVDF membrane and non-specific binding sites blocked by incubation with phosphate buffered saline (PBS)

Figure 6.1 Schematic representation of HGF ELISA methods

a) Two antibody layer ELISA sandwich: ELISA plate was coated with monoclonal anti-HGF trapping antibody, then samples (test sample or standard human recombinant HGF) applied. A second antibody layer of purified, biotinylated rabbit IgG to HGF was applied and the antibody detected by addition of avidin-HRP conjugate and the substrate orthophenylenediamine.

b) Three antibody layer ELISA sandwich: ELISA plate was coated with monoclonal anti-HGF trapping antibody, then samples (test sample or standard human recombinant HGF) applied. A second antibody layer of whole rabbit polyclonal antiserum to HGF was applied, followed by biotinylated purified specific sheep antibody to rabbit immunoglobulins. The detection system was by means of avidin-HRP conjugate and orthophenylenediamine.

a) Two antibody layer ELISA



b) Three antibody layer ELISA

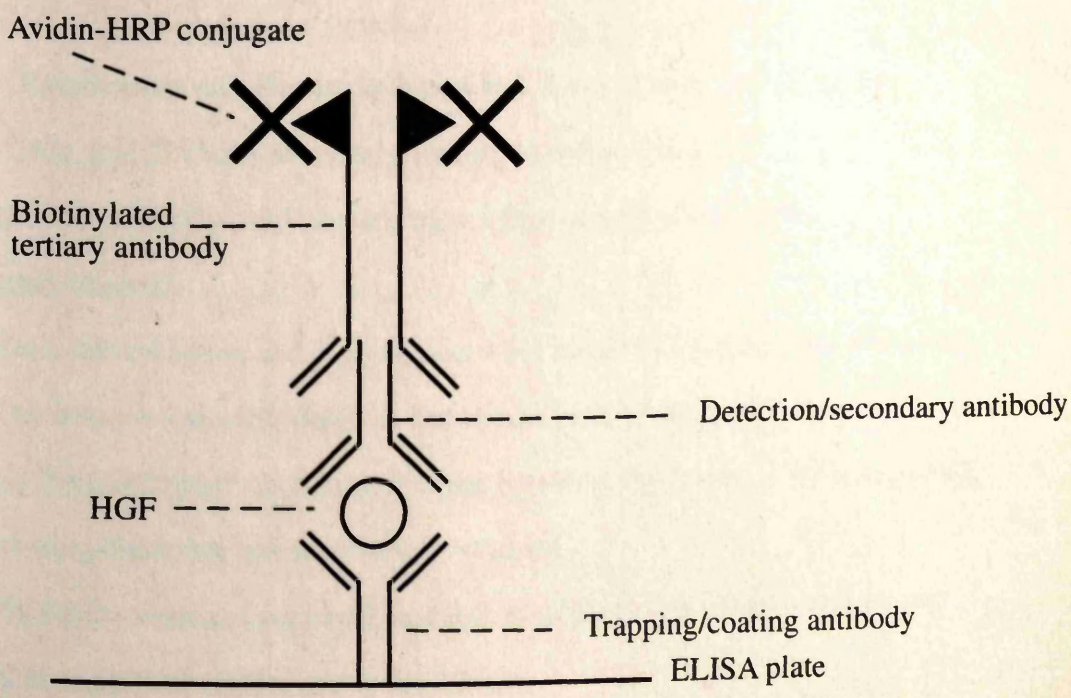


Table 6.1 Summary of ELISA Constituents and Structure

ELISA	Trapping/ Coating Antibody	Detection/ Secondary Antibody	Tertiary Antibody	Detection System
(a)	A312-1	Purified, biotinylated polyclonal rabbit IgG to HGF. Lot No. 201	-	Avidin-HRP OPD system
(b)	A312-1	Whole serum Lot No. 201	Biotinylated sheep antibody to rabbit IgG	Avidin-HRP OPD system
(c)	D9	Whole serum Lot No. 201	Biotinylated sheep antibody to rabbit IgG	Avidin-HRP OPD system
(d)	B25	Whole serum Lot No. 201	Biotinylated sheep antibody to rabbit IgG	Avidin-HRP OPD system
(e)	B43	Whole serum Lot No. 201	Biotinylated sheep antibody to rabbit IgG	Avidin-HRP OPD system
(f)	A312-2	Whole serum Lot No. 201	Biotinylated sheep antibody to rabbit IgG	Avidin-HRP OPD system

containing 3% (weight/volume) bovine serum albumin and 0.1% (volume/volume) Tween 20 at room temperature for 1 hour. The primary antibody under examination was diluted to 1/5000 in PBS containing 0.1% (volume/volume) Tween 20 (B25, B43, A312-2 and polyclonal rabbit antiserum Lot No 201) or 1/5 (D9, hybridoma culture supernatant) and incubated, shaking, at room temperature overnight (EURO BD60 Shaker, Stovall Life Science Inc. Greensboro, North Carolina, USA).

The membranes were then washed (3 x 10 minutes) in PBS containing 0.1% (volume/volume) Tween 20 and incubated with the secondary antibody (alkaline phosphatase conjugated rabbit anti-mouse immunoglobulins or alkaline phosphatase conjugated swine anti-rabbit immunoglobulins) diluted 1/1000 in PBS containing 0.1% (volume/volume) Tween 20 at room temperature for 1 hour. The membranes were washed in PBS containing 0.1% (volume/volume) Tween 20 (2 x 10 minutes) and finally in 100mM Tris buffer pH 9.5 (1 x 10 minutes).

The signal was developed by bathing the membranes in nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in 100mM Tris (pH 9.5) (Appendix 3). The reaction was stopped by washing the membranes in dH_2O.

6.2.2 Purification and Biotinylation of IgG from Whole Rabbit Serum

(1) Acetic acid (0.1M) was added to serum to reduce pH to 4.5.

(2) Undiluted caprylic acid was added to a final concentration of 5% (volume/volume).

(3) The acidified serum and caprylic acid were mixed by vortexing for 5 minutes.

(4) The mixture was centrifuged in 1ml aliquots at 13,000 rpm for 7 minutes using a "Microcentaur" microfuge (Fisons, Scientific Equipment), following which Pellets were discarded and supernatants retained.

(5) An equal volume of saturated ammonium sulphate was added step wise and mixed at room temperature gently for 2 hours.

(6) The mixture was centrifuged in 1ml aliquots in a "Microcentaur" microfuge at 13,000 rpm for 3 minutes.

(7) Pellets were retained, resuspended in PBS and the resulting solution dialysed against 2 changes of 2 litres of PBS for 1 hour.

(8) Protein (immunoglobulin) content of dialysed solution was estimated by absorbance of ultraviolet light at 280nm (OD_{280} = mg protein/ml).

(9) Protein concentration was adjusted to 5mg/ml using PBS.

(10) Biotin (2 mg) was weighed into glass bijoux bottle, then dissolved in 100µl dimethyl formamide.

(11) Antibody solution (1 ml i.e. 5 mg) was added to the biotin solution.

(12) Mixed gently by hand and allowed to stand at room temperature with occasional gentle mixing for 2 hours.

(13) The solution was dialysed against 2 litres PBS overnight.

6.2.3 Assessment of Untreated Biotinylated Sheep Polyclonal Anti-Rabbit Immunoglobulins for Cross Reactivity with Murine Monoclonal IgG Antibody

(1) An ELISA plate was coated with murine monoclonal antibody IgG2a CD72 at a concentration of 10µg/ml in 0.1M $NaHCO_3$, 50µl per well. The plate was sealed and incubated at 37°C overnight.

(2) The plate was washed 6 times with PBS containing 0.025% (volume/volume) Tween 20.

(3) Non-specific binding was "blocked" by addition of PBS containing 3% (weight/volume) BSA, at 100µl/well and incubation at 37°C for 2 hours.

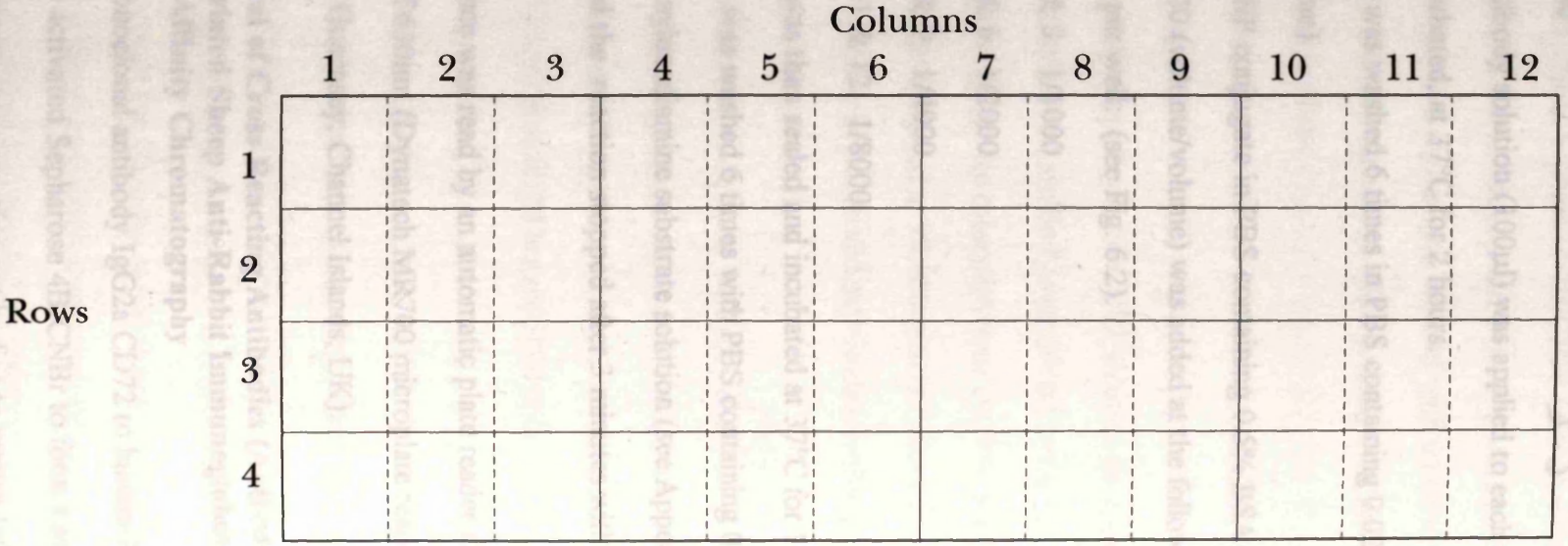
(4) The plate was washed 6 times with PBS containing 0.025% (volume/volume) Tween 20.

(5) Untreated biotinylated sheep polyclonal anti-rabbit immunoglobulins in PBS containing 0.5% BSA (weight/volume) and 0.5% Tween 20 (volume/volume) was added at the following concentrations (see Figure 6.2):

Row 1: 1/500 (neat solution = 1 mg/ml)

Row 2: 1/1000

Figure 6.2 **Plan of ELISA plate used in the assessment of untreated biotinylated sheep polyclonal anti-rabbit immunoglobulin for cross reactivity with murine monoclonal IgG antibody.**


Columns
1
2
3
4
5
6
7
8
9
10
11
12
Rows
1
2
3
4

Row 3: 1/2000

Row 4: 1/4000

Diluted antibody solution (100µl) was applied to each well, then the plate was sealed and incubated, at 37°C for 2 hours.

(6) The plate was washed 6 times in PBS containing 0.025% Tween 20 (volume/volume).

(7) Avidin-HRP conjugate in PBS containing 0.5% BSA (weight/volume) and 0.5% Tween 20 (volume/volume) was added at the following concentrations, 100 µl of solution per well: (see Fig. 6.2).

Columns 1,2 & 3: 1/1000

Columns 4,5 & 6: 1/2000

Columns 7,8 & 9: 1/4000

Columns 10,11 & 12: 1/8000

The plate was then sealed and incubated at 37°C for 1 hour.

(8) The plate was washed 6 times with PBS containing 0.025% Tween 20.

(9) Orthophenylenediamine substrate solution (see Appendix 4) was added at 100µl/well and the reaction stopped after 3 minutes with 50µl per well of 4N H_2SO_4.

(10) Absorbance was read by an automatic plate reader at 490nm with a reference wavelength of 630nm (Dynatech MR700 microplate reader, Dynatech Laboratories, Guernsey, Channel Islands, UK).

6.2.4 Removal of Cross Reacting Antibodies (Anti-Murine IgG) from Biotinylated Sheep Anti-Rabbit Immunoglobulins by Sepharose 4B-CD72 Affinity Chromatography

Murine monoclonal antibody IgG2a CD72 to human B-type lymphocytes was conjugated to activated Sepharose 4B-CNBr to form a sepharose-antigen column in order to remove cross reacting antibodies to mouse IgG by affinity chromatography from biotinylated sheep anti-rabbit immunoglobulins.

(a) Conjugation of murine monoclonal IgG2a antibody CD72 to sepharose 4B gel

(1) A volume of 1ml of sepharose 4B-CNBr (activated) beads was poured onto a 10ml centrifuge test tube (Falcon) and 5ml of 0.001M hydrochloric acid added. The rehydrated gel was shaken and washed with 0.001M hydrochloric acid, centrifuged and the acid discarded. The process was repeated, the supernatant discarded and gel retained.

(2) CD72 murine monoclonal IgG2a antibody was dialysed against 2 litres of 0.1M $NaHCO_3$ plus 0.15M NaCl at room temperature overnight.

(3) After removal of an aliquot of CD72 solution for estimation of protein concentration the dialysed antibody was added to the sepharose 4B-CNBr gel. The gel was mixed by gentle rotary inversion at 4°C overnight.

(4) The gel was centrifuged at 10,000 rpm for 10 minutes using a "Microcentaur" microfuge at room temperature and supernatant removed. Protein concentration in the supernatant was estimated.

(5) Gel was washed once in 0.1M $NaHCO_3$ plus 0.15M NaCl.

(6) Gel was mixed by gentle rotation at 4°C overnight in 0.1M Tris HCl, pH8.

(7) Gel was washed with 0.1M acetate pH 4.0 containing 1.0M NaCl.

(8) Gel was washed with 0.1M borate pH 8.0 containing 1.0M NaCl.

Steps 7 and 8 repeated.

(9) Gel was stored in PBS containing 0.01M EDTA at 5°C.

(b) Removal of swine antibodies to murine monoclonal IgG

(1) The Sepharose 4B-mouse CD72 IgG2a gel was washed in PBS containing 0.01M EDTA then centrifuged at 10,000 rpm using a "Microcentaur" microfuge at room temperature for 10 minutes and the supernatant discarded.

(2) Biotinylated sheep anti-rabbit immunoglobulins (1ml, 1 mg/ml) was added to the gel and gently mixed by repeated inversion at room temperature for 30 minutes, then centrifuged at 10,000 rpm using a "Microcentaur" microfuge and the supernatant retained for testing and use in ELISA.

6.2.5 ELISA Protocols

See table 6.1.

ELISA (a) (Monoclonal antibody to HGF A3.1.2-1, purified biotinylated polyclonal rabbit IgG (Lot No 201) to HGF, avidin-HRP conjugate/ orthophenylenediamine. (See Appendix 4 for buffers, solutions and reagents).

(1) The wells of a 96 well Dynatech microtitre ELISA plate were coated with 50µl monoclonal antibody to HGF (A312-1) diluted in coating buffer (0.1M $NaHCO_3$) to a concentration of 10µg/ml. The plate was then sealed and incubated overnight at 37°C.

(2) The plate was washed 6 times with phosphate buffered saline (PBS) containing 0.025% (volume/volume) Tween 20.

(3) Non-specific binding sites were "blocked" by addition of 100µl/well of PBS containing 3% bovine serum albumin (BSA) (weight/volume) and incubation for 2 hours at 37°C.

(4) The plate was washed 6 times with PBS containing 0.025% Tween 20.

(5) Serum samples and standard curve of reference serum diluted in PBS containing 0.5% BSA (weight/volume) and 0.5% Tween 20 (volume/volume) were applied, 100µl/well. Serum samples were diluted 1/500. Reference serum was diluted 1/40, 1/80, 1/160, 1/320, 1/640, 1/1280, 1/2560, 1/5120, 1/10240 and 1/20480. The plate was sealed and incubated at 37°C for 2 hours.

(6) The plate was washed 6 times with PBS containing 0.025% Tween 20.

(7) Biotinylated IgG purified from rabbit antiserum, (Lot No 201) to human recombinant HGF (5mg/ml), diluted 1/400 in PBS containing 0.5% BSA and 0.5% Tween 20 was applied, 100µl/well and the plate incubated at 37°C for 1½ hours.

(8) The plate was washed 6 times with PBS containing 0.025% Tween 20.

(9) Avidin-HRP conjugate diluted 1/3000 in PBS containing 0.5% BSA and 0.5% Tween 20 was applied, 100µl/well. The plate was sealed and incubated at 37°C for 1 hour.

(10) The plate was washed 6 times with PBS containing 0.025% Tween 20.

(11) Substrate solution (orthophenylenediamine) (100µl/well) was added and signal allowed to develop in darkness.

(12) The reaction was stopped by addition of 50µl per well of 4N H_2SO_4.

(13) Absorbence was read at 490nm by automatic plate reader with a reference wavelength of 630nm.

ELISAs (b), (c), (d), (e) and (f), were similar in format. In each case a different monoclonal trapping antibody was used to coat the plate. However, the same secondary antibody (whole anti-HGF rabbit serum Lot No 201), tertiary antibody (purified biotinylated sheep anti-rabbit immunoglobulin) and detection system (avidin-HRP/orthophenylenediamine) were used in each of these assays. The following describes ELISA (f), which was eventually chosen as the assay most suitable for the measurement of HGF in human serum.

ELISA (f)

(1) The inner 60 wells of a 96 well Dynatech microtitre ELISA plate were coated with 50µl per well of A3.1.2-2 monoclonal antibody to human recombinant HGF diluted in coating buffer (0.1M $NaHCO_3$) to a concentration of 5µg/ml sealed and incubated overnight at room temperature.

(2) The plate was washed 6 times with PBS containing 0.025% (volume/volume) Tween 20.

(3) Blocked for 1 hour at room temperature with 100µl/well of PBS containing 3% (weight/volume) bovine serum albumin (BSA).

(4) The plate was washed 6 times with PBS containing 0.025% Tween 20.

(5) Serum samples and standard recombinant human HGF diluted in PBS containing 0.5% BSA (weight/volume) and 0.5% Tween 20 (volume/volume) were dispensed into wells of the plate (100µl/well) and the plate incubated at room temperature for 2 hours.

(6) The plate was washed 6 times with PBS containing 0.025% Tween 20.

(7) Whole rabbit antiserum, Lot No 201, to HGF diluted to 1/5000 in PBS containing 0.5% BSA and 0.5% Tween 20 was dispensed into each well and the plate incubated, sealed, at room temperature for 1 hour.

(8) The plate was washed 6 times with PBS containing 0.025% Tween 20.

(9) Biotinylated sheep anti-rabbit immunoglobulins, diluted 1/500 (undiluted solution; 1 mg/ml) in PBS containing 0.5% BSA and 0.5% Tween 20 was dispensed into each well and plate incubated, sealed, at room temperature for 1 hour.

(10) The plate was washed 6 times with PBS containing 0.025% Tween 20.

(11) HRP-Avidin conjugate diluted 1/8000 in PBS containing 0.5% BSA and 0.5% Tween 20 was dispensed (100µl per well) and the plate incubated at room temperature for 1 hour.

(12) The plate was washed 6 times with PBS containing 0.025% Tween 20.

(13) Substrate solution (orthophenylenediamine), (100µl per well) was added to each well and signal allowed to develop in darkness.

(14) The reaction was stopped by addition of 50µl 4N H_2SO_4 to each well.

(15) Absorbence was read at 490nm by automatic plate reader with a reference wavelength of 630nm.

Serum samples were diluted 1:4 in PBS containing 0.5% BSA (weight/volume) and 0.5% Tween 20 (volume/volume). In those cases where a 1:4 dilution gave a signal greater than the highest point on the standard curve, the serum sample was retested at a higher dilution e.g. 1:10. Standard reference solutions of HGF were made up using recombinant human HGF (Genentech Inc, California, USA) in the following range:

50ng/ml, 25ng/ml, 12.5ng/ml, 6.25ng/ml, 3.125ng/ml, 1.56ng/ml

These solutions were stored as aliquots at -70°C until use.

In two of the ELISAs developed (ELISAs (a) and (c)) I was unable to use human recombinant HGF to draw standard curves or to calibrate the assay. In each of these cases I used two reference serum samples from a patient with liver

disease. Using this serum as a standard, sera from various patients were assayed and ranked in terms of activity in the assay. ELISA (a) method is described under ELISA Protocols; ELISA (a), page 92. The second assay, ELISA (c) was performed as follows:

Serum from a patient (E.O.) with acute hepatitis which reacted strongly (ELISA (c)) was used as a reference. A standard curve was constructed using this serum at the following dilutions: 1/15, 1/30, 1/60, 1/120, 1/240, 1/480, 1/960 and 1/1920. This reference serum was arbitrarily designated as containing 1000 units/ml serum. Other sera were defined in terms of these units by comparing their reaction in ELISA and that of the reference serum.

6.2.6 Levels of HGF in Serum Assayed by Otsuka Assay Laboratories ELISA and Comparison with Results Obtained using ELISA (c)

Dr. Fujiwara from the University of Tokyo kindly agreed to measure HGF levels in 22 samples using an assay undergoing development by Otsuka Assay Laboratories, Takushima, Japan (172).

Sera from 21 patients and one sample of placental homogenate (prior to purification of HGF) were assayed.

6.2.7 Assessment of Assays (d), (e) and (f) for Cross Reaction with Plasminogen

The wells of an ELISA plate were coated with monoclonal antibodies to HGF B25, B43 or A.3.1.2-2. After blocking the walls with BSA, samples of plasminogen, human serum or recombinant human HGF were dispensed into the wells and allowed to incubate. The assays were then completed as described previously. Using standard curves for HGF, cross reaction with plasminogen or reaction to HGF within that preparation was calculated.

Plasminogen levels in sera from 10 patients with various serum HGF levels (as determined by ELISA (f) were analysed (kindly performed by Dr J Conkie, Haematology Department, Royal Infirmary, Glasgow). The following methods

were used: (1) Amidolytic (streptokinase/plasminogen complex) chromogenic assay with plasmin specific substrate S-2251 (Kabi Diagnostica, Sweden) for determination of functional activity of plasminogen in sample. (2) Single radial immunodiffusion with M-Partigen(R) plasminogen immunodiffusion plates (Behringwerke AG, Marburg, Germany) for immunological determination of plasminogen including biologically inactive material.

The following sections relate to studies carried out using ELISA (f).

6.2.8 Stability of HGF in Serum: Collection of Serum

Blood taken from a patient with acute viral hepatitis was divided into six aliquots immediately and treated as follows:

(1) Separation of serum and storage until use at -70°C within 30 minutes.

(2) Storage of blood at room temperature for 24 hours, then separation and storage of serum at -70°C until use.

(3) Separation of serum within 30 minutes, storage of serum at room temperature for 24 hours, then storage at -70°C until use.

(4) Separation of serum within 30 minutes, storage at 4°C for 24 hours, then storage at -70°C until use.

(5) Separation of serum within 30 minutes, storage at 4°C for 1 week, then storage at -70°C until use.

(6) Storage of whole blood (clotted) at 4°C for 24 hours, then separation and storage of serum at -70°C until use.

6.2.9 Assay Precision

Intra-assay Precision

Serum from a patient known to have an elevated serum HGF level was used. The serum was divided into aliquots and stored at -70°C until use. To assess intra-assay variation 10 aliquots were thawed and HGF level determined using the assay and treating each aliquot as an individual sample.

Interassay Precision

In each of 10 assays standards of known human recombinant HGF concentration (3.125 ng/ml, 12.5 ng/ml and 25 ng/ml) were used to assess inter-assay precision at low, medium and high levels respectively.

6.2.10 Collection of Sera

See pages 147,148. Venous blood from patients and volunteers was allowed to clot at room temperature and serum obtained by centrifugation. Serum was stored in aliquots at -70°C until use. Serum samples were obtained from 33 healthy individuals (Median age 41 years, range 55) as a normal control group and from 11 patients (median age 58 years, range 36) following acute myocardial infarction as a disease group. Serum obtained from 221 patients (median age 55 years, range 78) attending outpatient clinics and from inpatients with liver disease was used as a liver disease group (Table 6.2).

Statistical Analysis

Statistical analysis of results was carried out by Dr Gordon Murray and Mrs Jan Love, Robertson Centre for Biostatistics, University of Glasgow.

6.3 Results

6.3.1 Western Blotting

In Western blotting studies using monoclonal antibodies B25, B43 and A3.1.2-2 to HGF, these antibodies detected an 82kDa band in lanes containing non-reduced recombinant human HGF. Similarly, the antibodies reacted specifically with an 82kDa band in a non-reduced preparation containing native human HGF purified from placenta (Figs 6.3, 6.4, 6.5). Antibody D9 did not detect human recombinant HGF on Western blotting studies but did detect an 82kDa band in a non-reduced preparation containing native human HGF purified from placenta (Fig 6.6).

Monoclonal antibody A312-1 was not analysed by Western blotting in our laboratory but was characterised by Professor T Nakamura and shown to react

Table 6.2 Patients and controls used in study of serum HGF levels

Diagnosis	Number of patients	Male	Female
Control: healthy volunteers	33	11	22
Control: acute myocardial infarction	11	7	4
Acute alcohol induced liver injury	19	14	5
Chronic alcohol induced liver injury	43	25	18
Acute hepatitis (non alcoholic)	43	26	17
Chronic active hepatitis	20	6	14
Chronic intrahepatic cholestasis	18	5	13
Extrahepatic biliary obstruction	6	2	4
Metastatic carcinoma involving liver with primary neoplastic disease elsewhere. Includes gastric adenocarcinoma (1), metastatic adenocarcinoma of other gastrointestinal tract origin (2), small cell bronchial carcinoma (3) and unknown primary disease (3)	9	5	4
Hepatocellular carcinoma	4	4	0
Paracetamol overdose	5	5	0
Liver transplant recipients	7	1	6
Chronic hepatitis C	10	8	2
Other miscellaneous conditions (including transient derangement of liver function tests, cause unknown (7), fatty liver (4), congenital hepatic fibrosis (3), haemochromatosis (3), cryptogenic cirrhosis (4), mild alcoholic liver disease (2), nodular regenerative hyperplasia (2), drug induced transient liver damage (3), squamous cell carcinoma of lung with nonspecific reactive liver changes (2), polyarteritis nodosa (1), non caseating epithelioid granulomatous liver disease (1), symptomatic gallstones without jaundice (4), disseminated lymphoma (1)	37	20	17
Total	265	139	126

Figure 6.3 Western blotting of human recombinant HGF and human placental HGF using monoclonal antibody B25

SDS-PAGE using a 12% resolving gel and non reduced samples, probed with monoclonal antibody to HGF (B25, 1/5000).

Lane	1	human recombinant HGF (100 ng)
	2	Molecular weight markers
	3	human placental HGF (20 ng)

An 82 kDa band is visible in lanes 2 and 3 corresponding to HGF

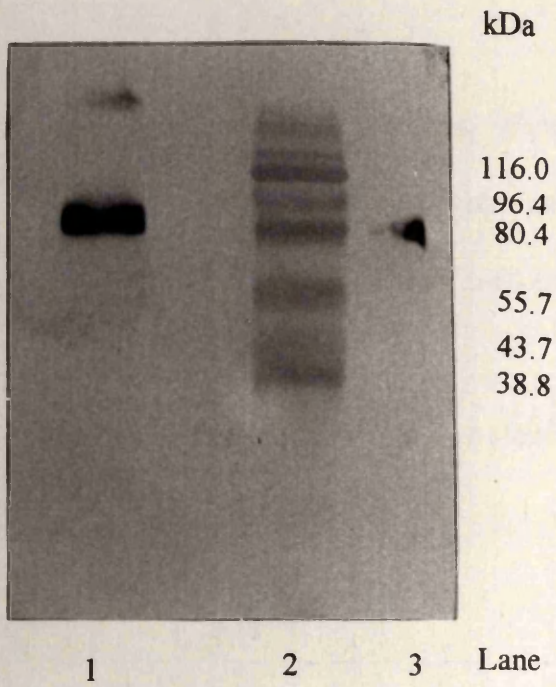

kDa
116.0
96.4
80.4
55.7
43.7
38.8
1
2
3
Lane

Figure 6.4 Western blotting of human recombinant HGF and human placental HGF using monoclonal antibody B43

SDS-PAGE using a 12% resolving gel and non reduced samples, probed with monoclonal antibody to HGF (B43, 1/5000).

Lane 1 Molecular weight markers

2 Human recombinant HGF (100 ng)

3 Human placental HGF (20 ng)

An 82 kDa band is visible in lanes 2 and 3 corresponding to HGF

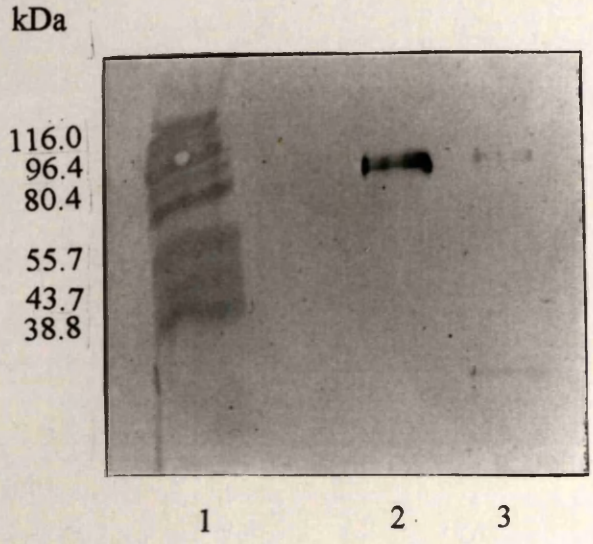

kDa
116.0
96.4
80.4
55.7
43.7
38.8
1
2
3

Figure 6.6 **Western blotting using monoclonal antibody D9, of eluted fractions from a hydroxylapatite column containing maximal human placental HGF concentration as determined by ELISA (c)**

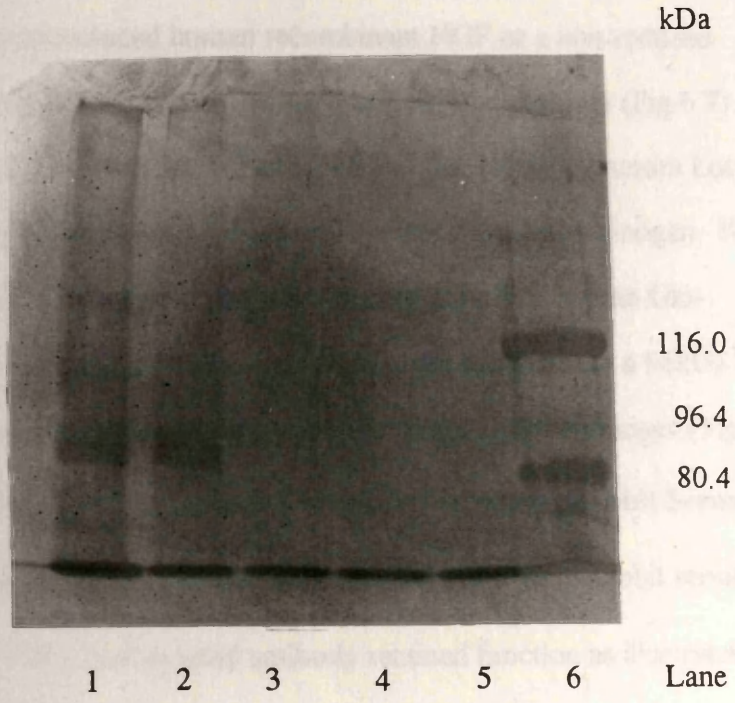
SDS-PAGE using a 7.5% resolving gel and non-reduced samples, probed with monoclonal antibody D9 culture supernatant (1/100).

Lane 1 { Fractions containing peak human placental HGF
2 { concentrations by ELISA

3 { Fractions containing no detectable human placental
4 { HGF
5 {

6 { Molecular weight markers

A band is seen in lanes 1 and 2 adjacent to the molecular weight marker 80.4 kDa, corresponding to human placental HGF.


kDa
116.0
96.4
80.4
1
2
3
4
5
6
Lane

with human recombinant HGF but not rat HGF or other growth factors (TGFα/ß, EGF, plasmin, insulin, tPA, acidic or basic FGF).

Polyclonal antiserum Lot No 201 to human HGF detected an 82kDa band in lanes containing non-reduced human recombinant HGF or a non-reduced preparation containing native human HGF purified from placenta (Fig 6.7).

Monoclonal antibodies A3.1.2-2 and B25 and polyclonal antiserum Lot No 201 to human HGF were tested for activity against human plasminogen. Neither of the monoclonal antibodies were shown to detect purified human Glu-plasminogen. Polyclonal antiserum Lot No 201 did demonstrate a 96kDa band, which approximates to the expected molecular weight of plasminogen (Fig 6.8).

6.3.2 Purification and Biotinylation of IgG from Whole Rabbit Serum

Immunoglobulin IgG was successfully purified from whole rabbit serum and biotinylated. Purified, biotinylated antibody retained function as illustrated by results of ELISA (a).

6.3.3 Assessment of Untreated Sheep Biotinylated Polyclonal Antibody (to Rabbit Immunoglobulins) for Cross Reactivity with Murine IgG antibody

Gross cross reaction was evident using the untreated biotinylated sheep anti-rabbit immunoglobulins. The signal was still greater than 1.0 when the sheep antibody was used at 1:4000 dilution with avidin-HRP conjugate dilution of 1/8000 (Table 6.3).

Cross reaction of sheep anti-rabbit immunoglobulins with murine immunoglobulin was dramatically reduced following removal of anti-murine IgG antibodies by Sepharose 4B-CD72 affinity chromatography. The signal was negligible when the sheep antibody was used at 1:4000, with avidin-HRP conjugate dilution of 1/8000 (compare with section preceding) (Table 6.4).

Figure 6.7 Western blotting of human recombinant HGF and human placental HGF using polyclonal anti-HGF serum Lot No. 201

SDS-PAGE using a 10% resolving gel and non-reduced samples, probed with rabbit polyclonal anti-HGF serum (Lot No. 201, 1/4000).

Lane 1 Molecular weight markers

2 Human placental HGF (20 ng)

3 Human recombinant HGF (100 ng)

A band of 82 kDa is visible in lanes 2 and 3 corresponding to HGF.

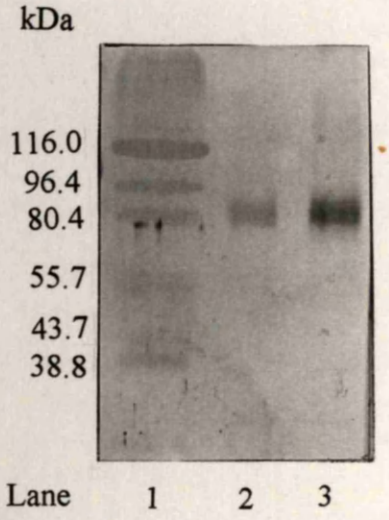

kDa
116.0
96.4
80.4
55.7
43.7
38.8
Lane
1
2
3

Figure 6.8 **Western blotting of human plasminogen using polyclonal anti-HGF serum Lot No. 201 and monoclonal antibodies B25 and A312-2**

SDS-PAGE using a 12% resolving gel and non reduced samples.

Lane 1 Molecular weight markers

2 Human plasminogen (200ng) probed with polyclonal anti-HGF serum, Lot No. 201 (1/4000)

3 Human plasminogen (200 ng) probed with monoclonal antibody B25 (1/5000)

4 Human plasminogen probed with monoclonal antibody A3.1.2-2-

A band of approximately 96 kDa is visible in lane 2 corresponding to plasminogen monoclonal antibodies D25 and A 3.1.2-2 do not demonstrate any protein bands in lanes 3 and 4.

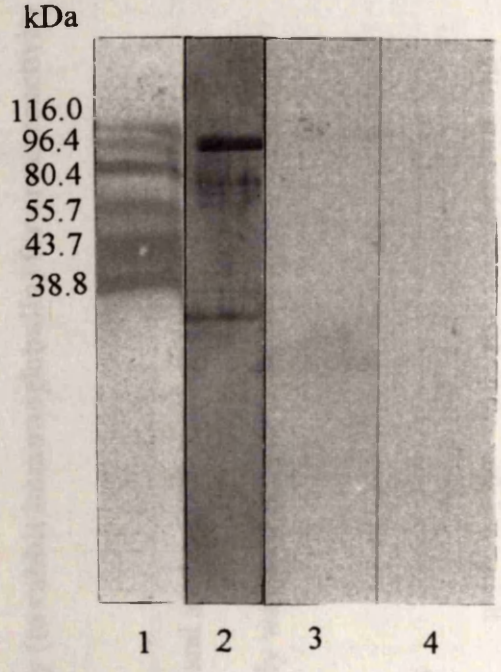

kDa
116.0
96.4
80.4
55.7
43.7
38.8
1
2
3
4

Table 6.3 Assessment of untreated sheep biotinylated polyclonal antibody (to rabbit immunoglobulin) for cross reactivity with murine IgG antibody

Gross cross reaction is evident using untreated sheep anti-rabbit immunoglobulins and avidin-HRP conjugate at low concentration. The signal was still greater than 1.0 when the sheep antibody was used at 1/4000 dilution with avidin-HRP conjugate dilution of 1/8000

		Columns													
		1	2	3	4	5	6	7	8	9	10	11	12		
Rows	1	Over	Over	Over	Over	Over	Over	Over	Over	Over	2.0148	Over	Over	{1/500	untreated sheep
	2	Over	Over	Over	Over	Over	Over	Over	Over	Over	Over	Over	Over	{1/1000	biotinylated
	3	Over	Over	Over	2.016	2.001	Over	1.848	1.943	1.860	1.595	1.646	1.641	{1/2000	polyclonal
	4	1.853	1.741	1.683	1.617	1.612	1.722	1.408	1.370	1.432	1.069	1.051	1.054	{1/4000	anti-rabbit immunoglobulin
		1/1000			1/2000			1/4000			1/8000				

Avidin-HRP conjugate

Table 6.4 Assessment of purified sheep biotinylated polyclonal antibody (to rabbit immunoglobulins) for cross reactivity with murine IgG antibody

A dramatic reduction in cross reactivity was achieved following removal of anti-murine IgG antibodies by

Sepharose 4B-CD72 affinity chromatography

		Columns												
		1	2	3	4	5	6	7	8	9	10	11	12	
	1	Over	Over	Over	Over	Over	Over	2.032	1.999	Over	0.296	0.241	0.239	{1/5000
Rows	2	Over	2.005	Over	2.024	1.990	1.985	1.804	1.772	1.814	0.108	0.101	0.083	{1/1000
	3	1.621	1.596	1.610	1.545	1.412	1.426	1.270	1.044	1.253	0.006	0.027	0.019	{1/2000
	4	0.734	0.880	0.914	0.889	1.001	0.602	0.557	0.589	0.682	0.003	0.021	0.033	1/4000
		1/1000			1/2000			1/4000			1/8000			

purified sheep biotinylated polyclonal anti-rabbit immunoglobulin

Avidin-HRP Conjugate

6.3.4 ELISA (a)

Using ELISA (a) a strong signal was detected in human serum and it was possible to construct a standard curve using various dilutions of a reference serum sample from a healthy volunteer.

The biotinylated antibody became degraded before the assay could be tested using purified recombinant human HGF. By comparing signal detected using other sera, samples could be ranked in terms of "HGF" activity.

If the highest point (1/40 dilution) on the graph is designated as 100 units/ml, then whole neat reference serum = 4000 units/ml. Since test samples are applied at 1/500 reading these points off the graph gives their value for HGF in relation to the reference serum sample (Figure 6.9).

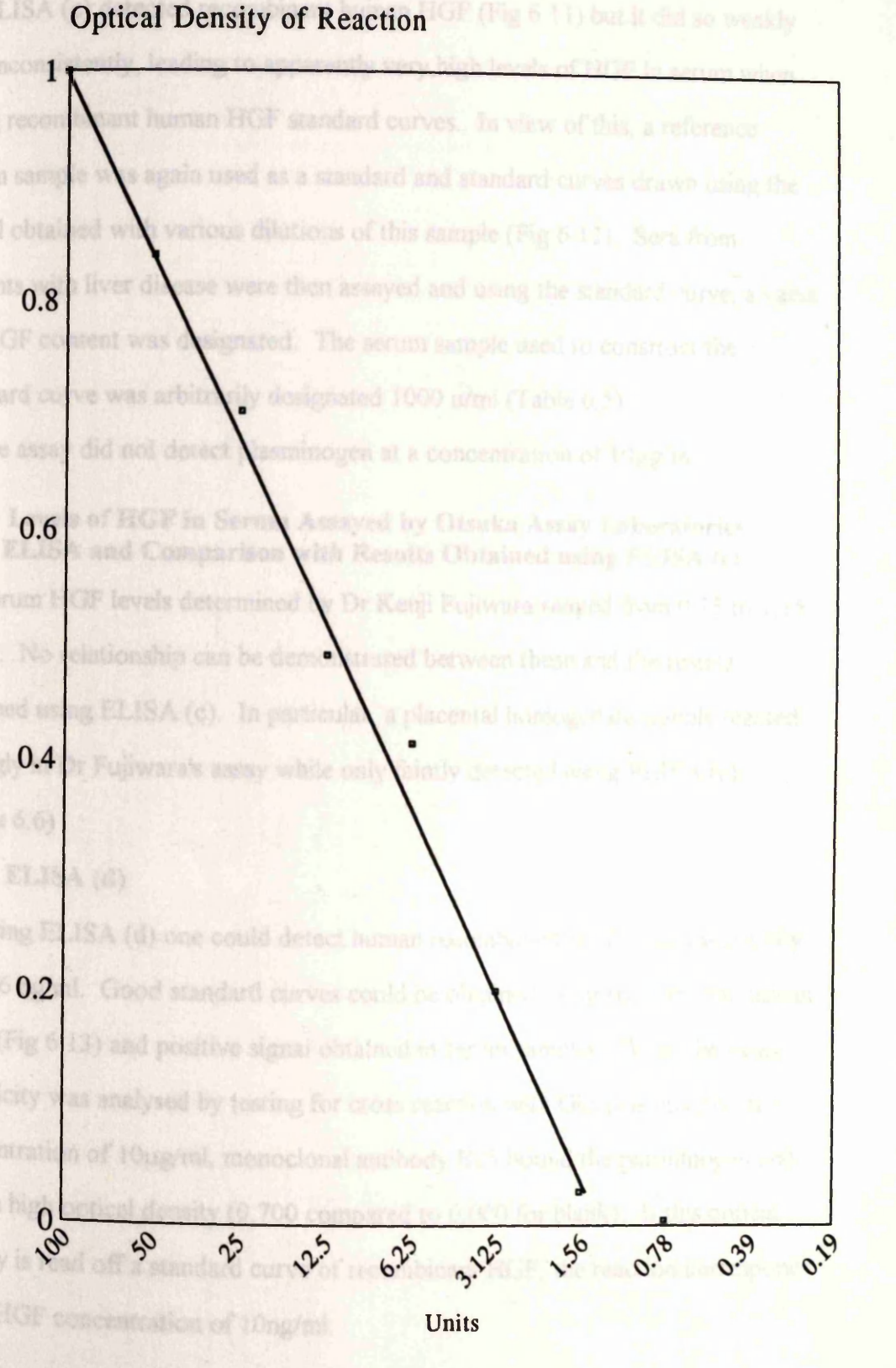
		O.D.	Units on Graph	Serum Level (units/ml)
Patient	1	0.547	13.8	6,900
	2	0.950	76.0	38,000
	3	0.586	16.3	8,150
	4	0.496	11.2	5,600
	5	0.538	13.3	6,650
	6	0.631	19.8	9,400
	7	0.533	13.1	6,550
	8	0.552	14.1	7,050
	9	0.573	15.5	7,750
	10	0.472	10.0	5,000
	11	0.460	9.6	4,800
	12	1.066	Greater than 125	Greater than 62,000

6.3.5 ELISA (b)

ELISA (b) detected recombinant human HGF and a standard curve of signal intensity for various concentrations of recombinant human HGF could be drawn. Background was negligible - unfortunately insufficient supplies of monoclonal

Figure 6.9 ELISA (a). Standard curve using various dilutions of a reference serum sample

The x-axis also shows assigned units, where dilution of reference serum 1/40 = 100 units. The curve is linear in the range 100 units to 1.56 units.


Optical Density of Reaction
1
0.8
0.6
0.4
0.2
0
100
50
25
12.5
6.25
3.125
1.56
0.78
0.39
0.19
Units
1/40
1/80
1/160
1/320
1/640
1/1280
1/2560
1/5120
1/10240
1/20480
Dilution of Reference Serum

antibody A3.2.1-1 remained for the purposes of measuring serum levels to HGF (Fig 6.10).

6.3.6 ELISA (c)

ELISA (c) detected recombinant human HGF (Fig 6.11) but it did so weakly and inconsistently, leading to apparently very high levels of HGF in serum when using recombinant human HGF standard curves. In view of this, a reference serum sample was again used as a standard and standard curves drawn using the signal obtained with various dilutions of this sample (Fig 6.12). Sera from patients with liver disease were then assayed and using the standard curve, a value for HGF content was designated. The serum sample used to construct the standard curve was arbitrarily designated 1000 u/ml (Table 6.5).

The assay did not detect plasminogen at a concentration of 10μg/ml.

6.3.7 Levels of HGF in Serum Assayed by Otsuka Assay Laboratories ELISA and Comparison with Results Obtained using ELISA (c)

Serum HGF levels determined by Dr Kenji Fujiwara ranged from 0.15 to 1.15 ng/ml. No relationship can be demonstrated between these and the results obtained using ELISA (c). In particular, a placental homogenate sample reacted strongly in Dr Fujiwara's assay while only faintly detected using ELISA (c). (Table 6.6)

6.3.8 ELISA (d)

Using ELISA (d) one could detect human recombinant HGF with a sensitivity of 1.56 ng/ml. Good standard curves could be obtained using recombinant human HGF (Fig 6.13) and positive signal obtained in serum samples. When the assay specificity was analysed by testing for cross reaction with Glu-plasminogen at a concentration of 10μg/ml, monoclonal antibody B25 bound the plasminogen and gave a high optical density (0.700 compared to 0.000 for blank). If this optical density is read off a standard curve of recombinant HGF, the reaction corresponds to an HGF concentration of 10ng/ml.

Figure 6.10 ELISA (b). Standard curve using various concentrations of recombinant human HGF (ng/ml)

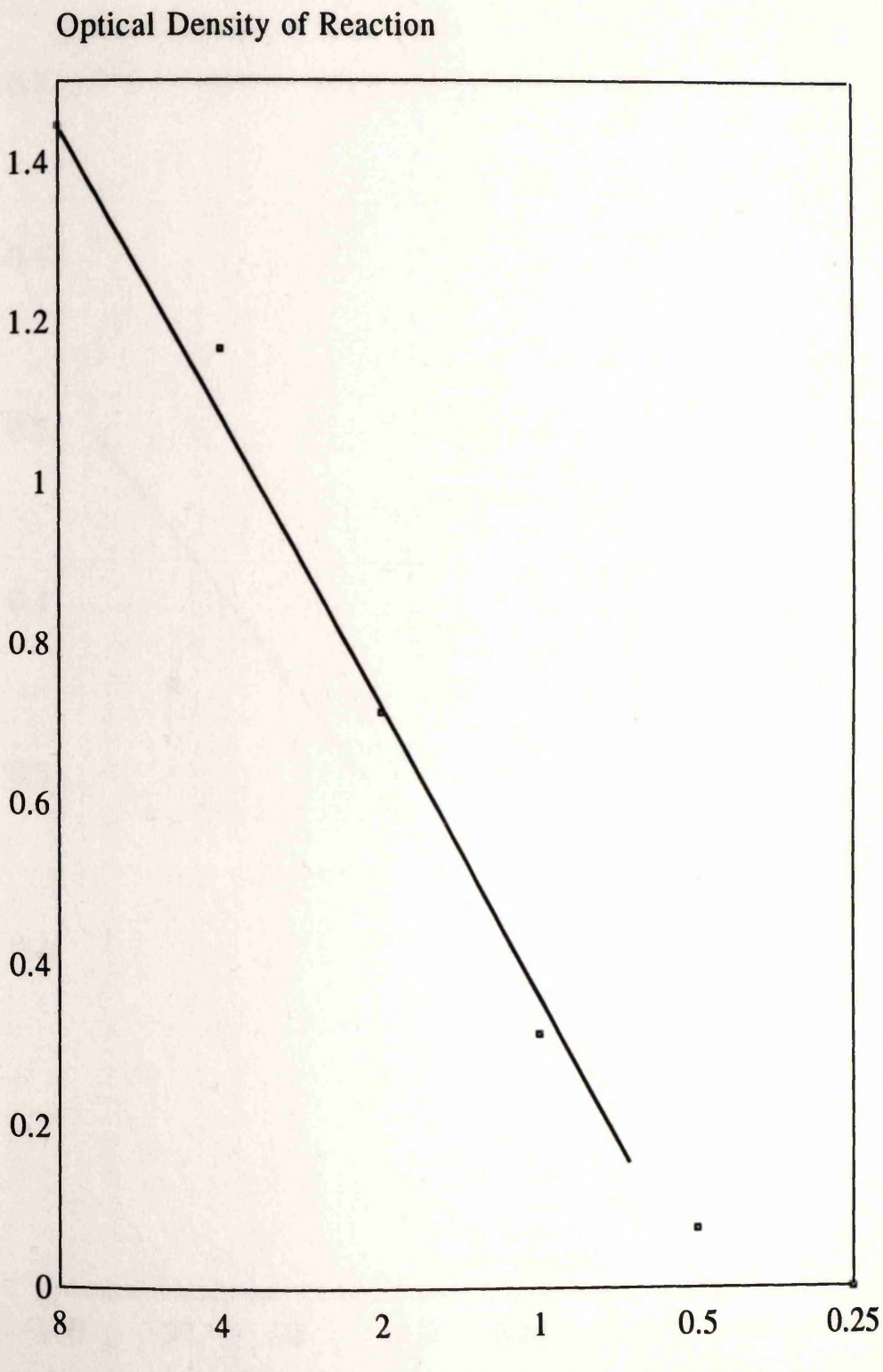

Optical Density of Reaction
1.4
1.2
1
0.8
0.6
0.4
0.2
0
8
4
2
1
0.5
0.25
Concentration of Recombinant Human HGF (ng/ml)

Figure 6.11 **ELISA (c). Standard curve using various concentrations of recombinant human HGF) (ng/ml).**

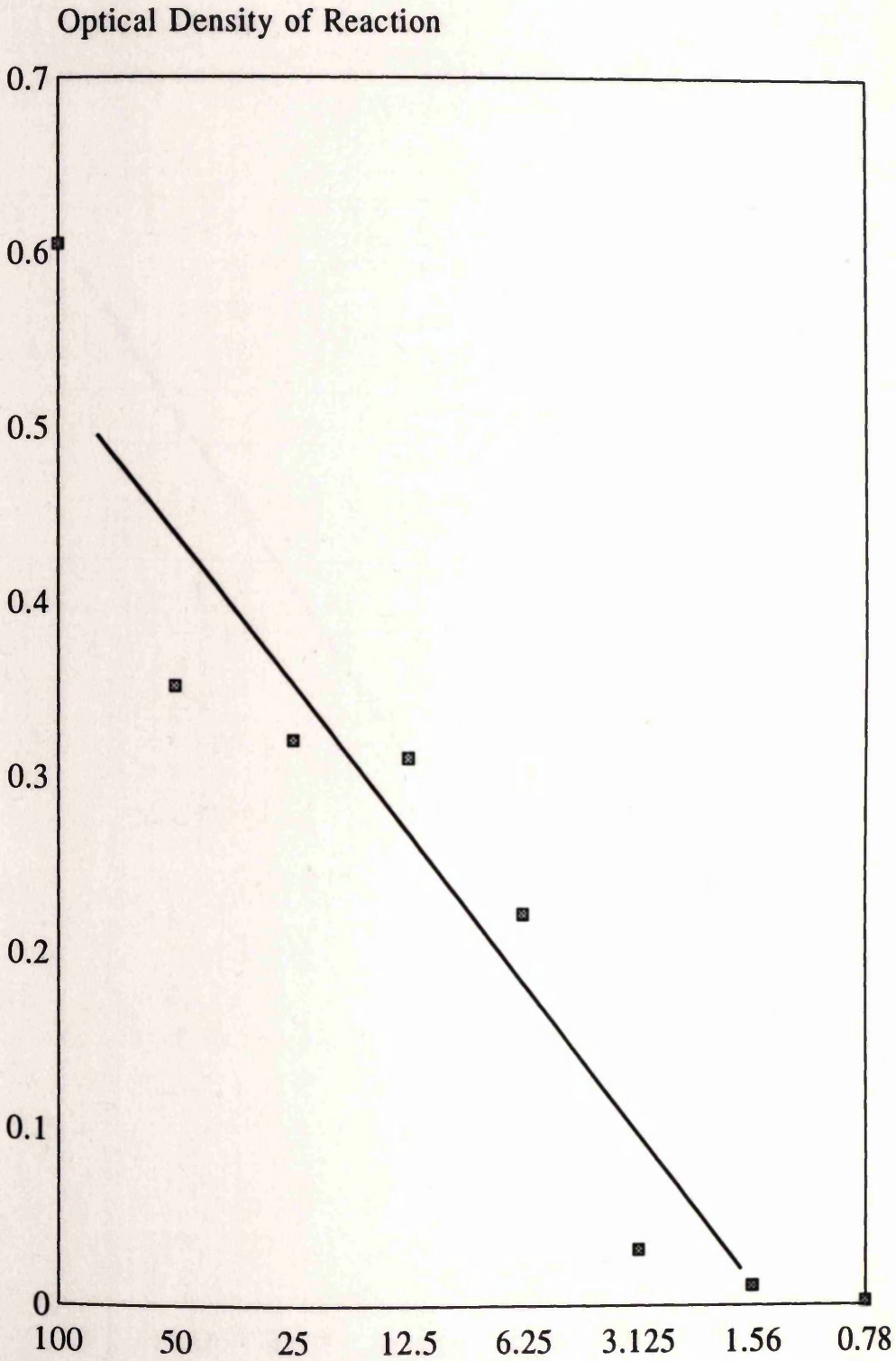

Optical Density of Reaction
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
100
50
25
12.5
6.25
3.125
1.56
0.78
Concentration of Recombinant Human HGF (ng/ml)

Figure 6.12 **ELISA (c). Standard curve using various diluations of a reference serum sample**

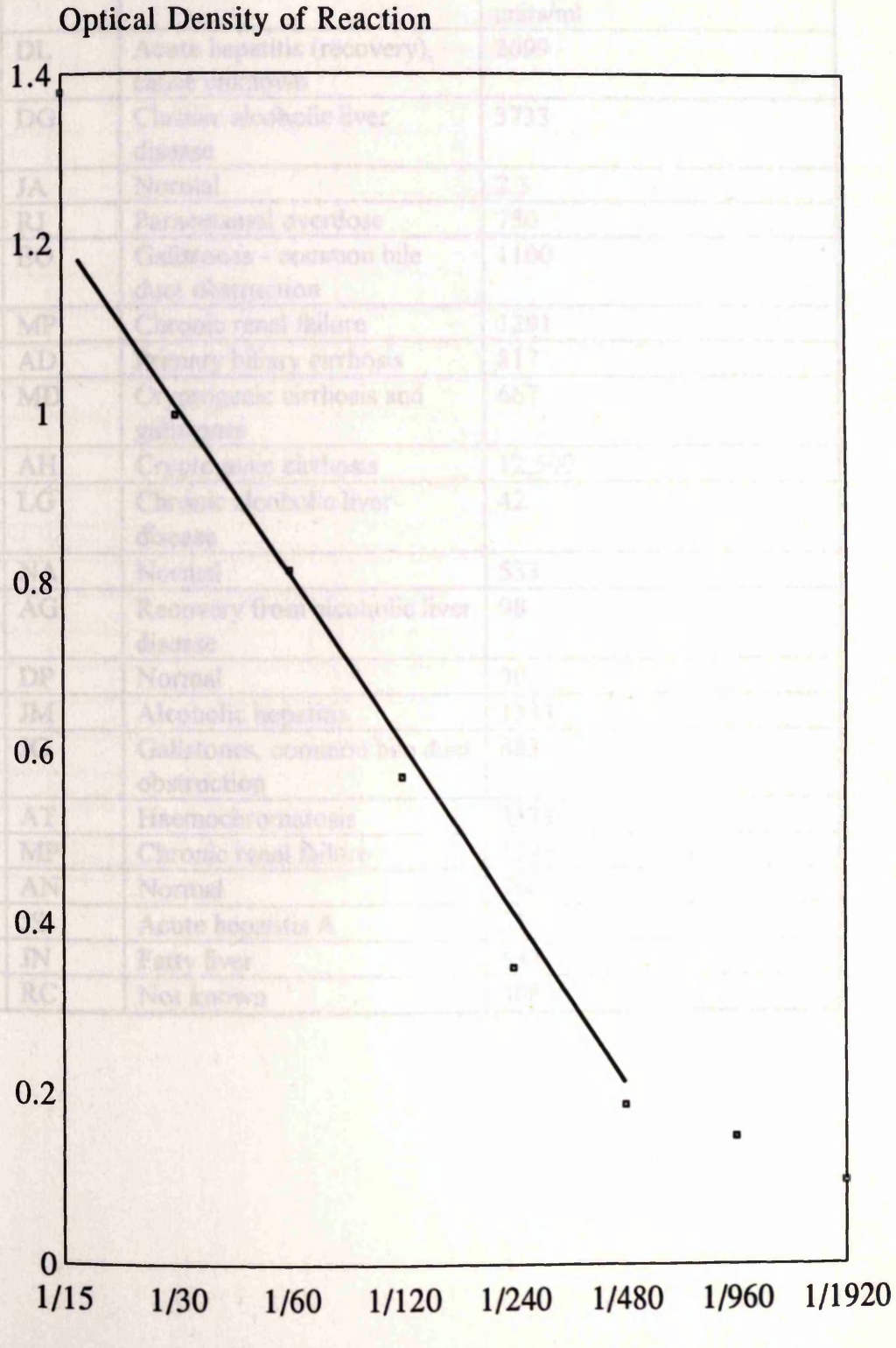

Optical Density of Reaction
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1/15
1/30
1/60
1/120
1/240
1/480
1/960
1/1920
Dilution of Reference Serum

Table 6.5 Serum HGF level determined using ELISA (c) and a reference serum sample

Sera from healthy controls and from patients with various liver diseases were compared to the reference serum

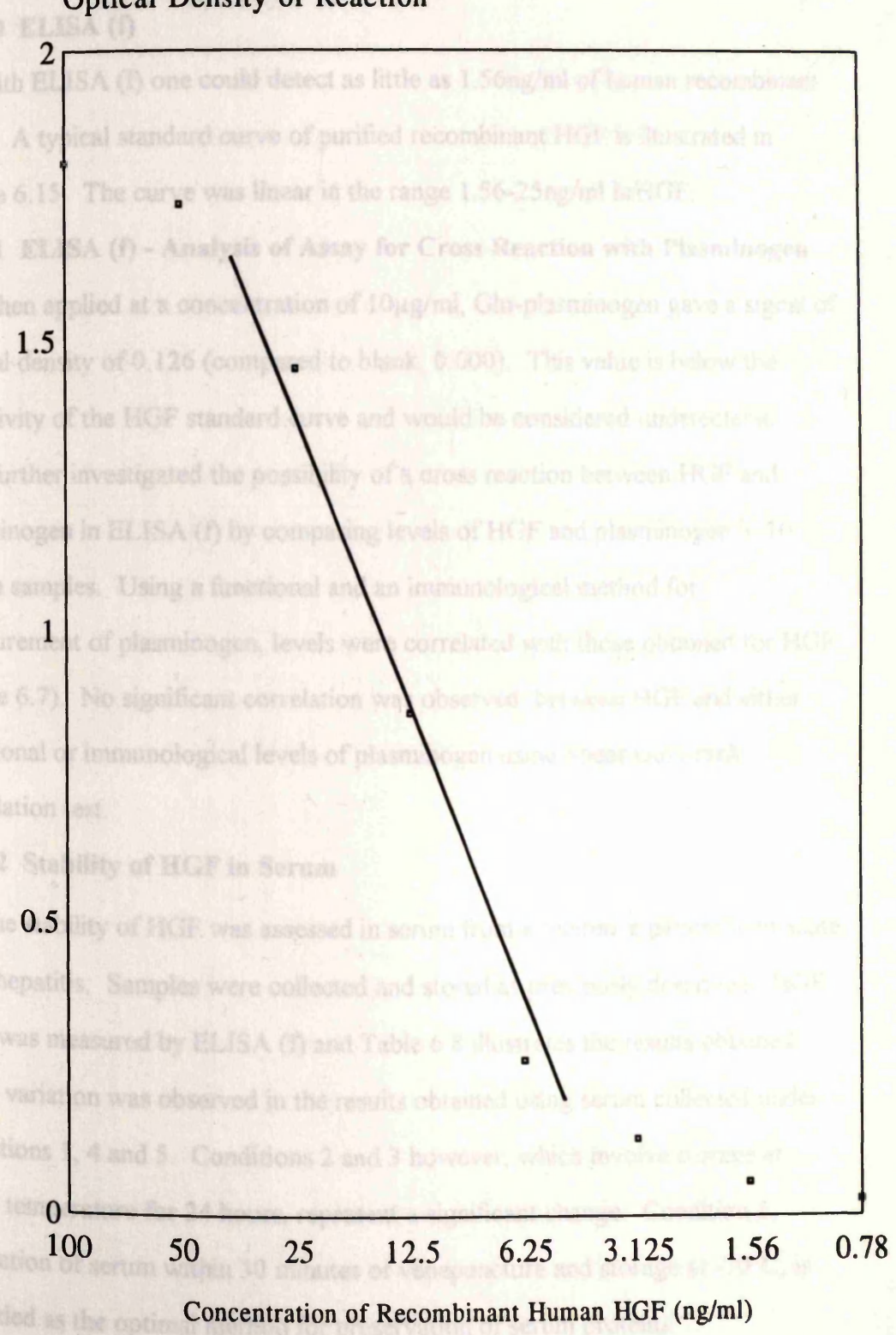
Patient	Initials	Diagnosis	Serum HGF (units/ml) reference serum = 1000 units/ml
1	DL	Acute hepatitis (recovery), cause unknown	2699
2	DG	Chronic alcoholic liver disease	3733
3	JA	Normal	2.3
4	RJ	Paracetamol overdose	750
5	EO	Gallstones - common bile duct obstruction	1100
6	MP	Chronic renal failure	1291
7	AD	Primary biliary cirrhosis	817
8	MD	Cryptogenic cirrhosis and gallstones	667
9	AH	Cryptogenic cirrhosis	12,500
10	LG	Chronic alcoholic liver disease	42
11	NA	Normal	533
12	AG	Recovery from alcoholic liver disease	98
13	DP	Normal	30
14	JM	Alcoholic hepatitis	1333
15	JG	Gallstones, common bile duct obstruction	883
16	AT	Haemochromatosis	3333
17	MP	Chronic renal failure	1375
18	AN	Normal	766
19	JS	Acute hepatitis A	57
20	JN	Fatty liver	633
21	RC	Not known	308

Table 6.6 **Comparison of HGF content of sera/placental homogenate determined by ELISA (c) and by Dr. Kenji Fujiwara using Otsuka Assay Laboratories ELISA**

No relationship can be shown between the results obtained using ELISA (c) and the Otsuka Assay Laboratories ELISA

Patient	Initials	HGF content ng/ml K. Fujiwara Assay	HGF content, standard units, in assay (c)
1	DL	0.49	2699
2	DG	0.18	3733
3	JA	0.38	23
4	RJ	0.51	750
5	EO	0.14	1100
6	MP	0.82	1291
7	AD	0.32	817
8	MD	0.47	667
9	AH	0.58	12,500
10	LG	0.47	42
11	MA	0.23	533
12	AG	0.24	98
13	PP	0.27	30
14	JM	0.29	1333
15	JG	0.32	883
16	AT	1.15	3333
17	MP	1.05	1375
18	AN	0.17	766
19	JS	0.33	57
20	JN	0.48	633
21	RC	0.50	308
22	Placental homogenate	>10	53

Figure 6.13 ELISA (d). Standard curve using various concentrations of recombinant human HGF (ng/ml)


Optical Density of Reaction
2
1.5
1
0.5
0
100
50
25
12.5
6.25
3.125
1.56
0.78
Concentration of Recombinant Human HGF (ng/ml)

6.3.9 ELISA (e)

Using ELISA (e) human recombinant HGF could be detected with a sensitivity of 6.25 ng/ml (Fig 6.14). The assay did not cross react with plasminogen in that no signal was detected using 10μg/ml plasminogen. The assay did not however detect HGF in human serum samples.

6.3.10 ELISA (f)

With ELISA (f) one could detect as little as 1.56ng/ml of human recombinant HGF. A typical standard curve of purified recombinant HGF is illustrated in Figure 6.15. The curve was linear in the range 1.56-25ng/ml hrHGF.

6.3.11 ELISA (f) - Analysis of Assay for Cross Reaction with Plasminogen

When applied at a concentration of 10μg/ml, Glu-plasminogen gave a signal of optical density of 0.126 (compared to blank, 0.000). This value is below the sensitivity of the HGF standard curve and would be considered undetectable.

I further investigated the possibility of a cross reaction between HGF and plasminogen in ELISA (f) by comparing levels of HGF and plasminogen in 10 serum samples. Using a functional and an immunological method for measurement of plasminogen, levels were correlated with those obtained for HGF (Table 6.7). No significant correlation was observed between HGF and either functional or immunological levels of plasminogen using Spearman's rank correlation test.

6.3.12 Stability of HGF in Serum

The stability of HGF was assessed in serum from a reference patient with acute viral hepatitis. Samples were collected and stored as previously described. HGF level was measured by ELISA (f) and Table 6.8 illustrates the results obtained. Little variation was observed in the results obtained using serum collected under conditions 1, 4 and 5. Conditions 2 and 3 however, which involve storage at room temperature for 24 hours, represent a significant change. Condition 1, separation of serum within 30 minutes of venepuncture and storage at -70°C, is regarded as the optimal method for preservation of serum proteins.

Figure 6.14 ELISA (e). Standard curve using various concentrations of recombinant human HGF (ng/ml)

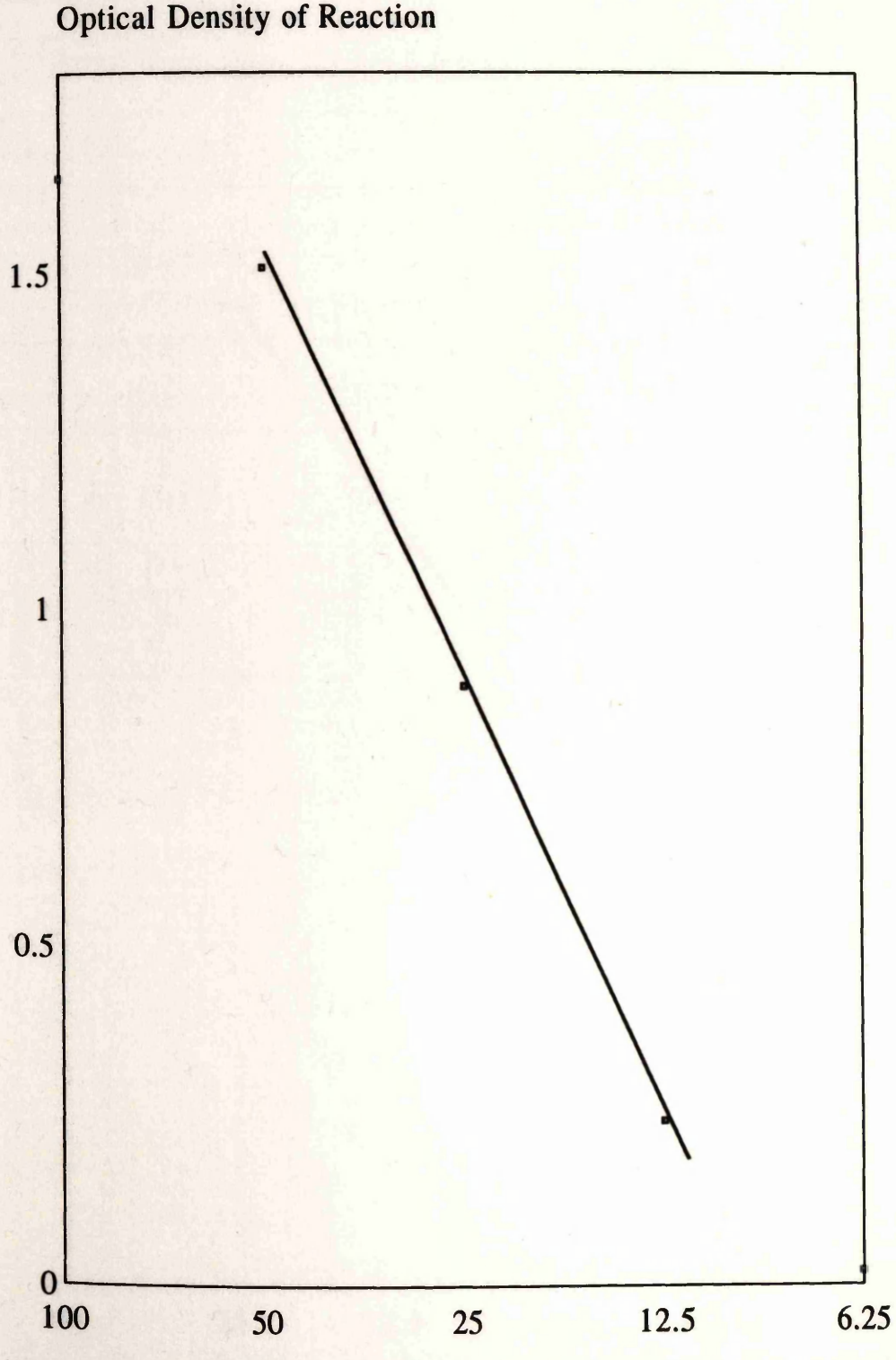

Optical Density of Reaction
1.5
1
0.5
0
100
50
25
12.5
6.25
Concentration of Recombinant Human HGF (ng/ml)

Figure 6.15 ELISA (f). Standard curve using various concentrations of recombinant human HGF (ng/ml)

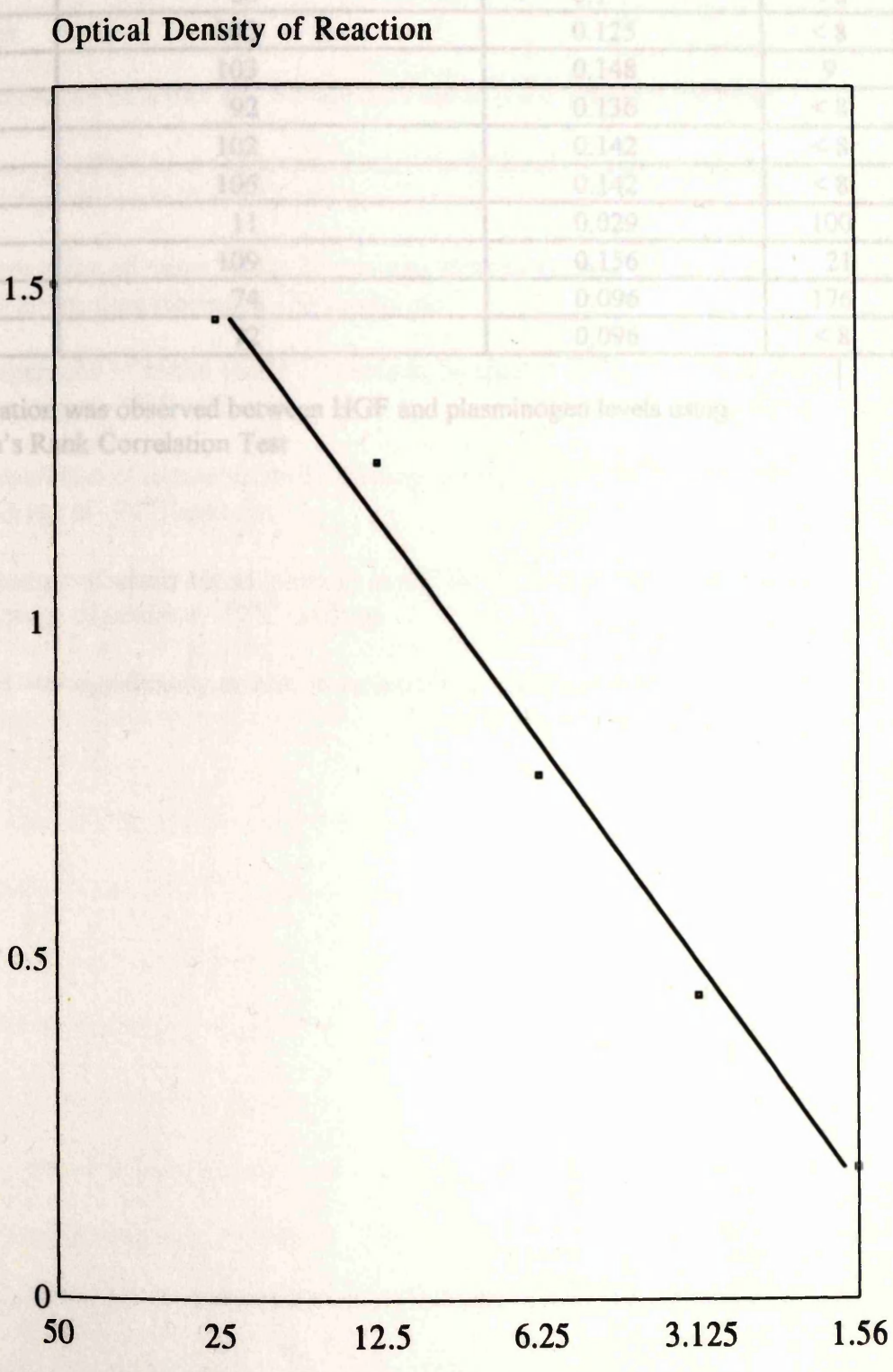

Optical Density of Reaction
1.5
1
0.5
0
50
25
12.5
6.25
3.125
1.56
Concentration of Recombinant Human HGF (ng/ml)

Table 6.7 Comparison of serum levels of plasminogen and HGF (determined by ELISA (f))

Sample No.	Functional Plasminogen Assay (% of normal activity)	Immunological Plasminogen Assay	HGF (ng/ml)
1	80	0.1	< 8
2	100	0.125	< 8
3	103	0.148	9
4	92	0.136	< 8
5	102	0.142	< 8
6	105	0.142	< 8
7	11	0.029	100
8	109	0.156	21
9	74	0.096	176
10	72	0.096	< 8

No correlation was observed between HGF and plasminogen levels using Spearman's Rank Correlation Test

Table 6.8 Stability of HGF in Serum

Condition	(1)	(2)	(3)	(4)	(5)	(6)
Serum HGF (ng/ml)	9.6	14.8	13.2	9.8	11.2	10.4

Serum was stored under the following conditions and HGF level determined using ELISA (f)

Conditions

(1) Separation of serum and storage until use at -70°C within 30 minutes

(2) Storage of blood at room temperature for 24 hours, then separation and storage of serum at -70°C until use

(3) Separation of serum within 30 minutes, storage at room temperature for 24 hours, then storage at -70°C until use

(4) Separation of serum within 30 minutes, storage at 4°C for 24 hours, then Storage at -70°C until use

(5) Separation of serum within 30 minutes, storage at 4°C for 1 week, then storage at -70°C until use

(6) Storage of whole blood (clotted) at 4°C for 24 hours, then separation and storage of serum at -70°C until use

HGF level was significantly greater in serum stored under conditions (2) and (3)

6.3.13 Assay Precision

Intra-assay precision using ELISA (f)

The results obtained using ELISA (f) and 10 aliquots of a known serum sample assayed during one run of the ELISA are shown in table 6.9.

The intra-assay coefficient of variation was 4.85% (mean value 108.85ng/ml, range 12ng/ml).

Interassay Precision using ELISA (f)

The results obtained using standards of known human recombinant HGF concentration at low (3.125ng/ml), medium (12.5ng/ml) and high (25ng/ml) levels in 10 assays were as shown in table 6.10.

The interassay coefficient of variation at low (3.125ng/ml), medium (12.5ng/ml) and high (25ng/ml) HGF levels were 8.2% (mean value 3.2ng/ml, range 0.7ng/ml), 9.7% (mean value 13.3ng/ml, range 4.5ng/ml) and 10.0% (mean value 24.7ng/ml, range 9.0ng/ml) respectively.

The sensitivity of the assay was 2ng/ml and the maximum serum concentration was 1/4, thus the minimum detectable concentration of HGF in serum was 8ng/ml.

6.3.14 Measurement of Serum HGF Levels in Health and Disease using ELISA (f)

Thirty-three healthy controls were assayed for HGF by ELISA (f), only two had detectable levels of HGF. A group of patients with a diagnosis of acute myo-ardial infarction (n = 11) were used as a disease control group and none had detectable levels of HGF. Figure 6.16. As only two normals had detectable levels normal levels could not be constructed against which patients could be compared. However, when the healthy control group was compared with groups of patients with various liver diseases, significant differences in HGF serum level were found in a number of cases. Serum HGF concentration was statistically significantly

Table 6.9 Intra-assay precision of ELISA (f)

Aliquot Number	1	2	3	4	5	6	7	8	9	10
HGF level ng/ml	105	105	104	105	105	112.5	115	116	116	105

Results obtained using ELISA (f) and 10 aliquots of a known serum sample during one run of the assay

Table 6.10 Interassay precision using ELISA (f)

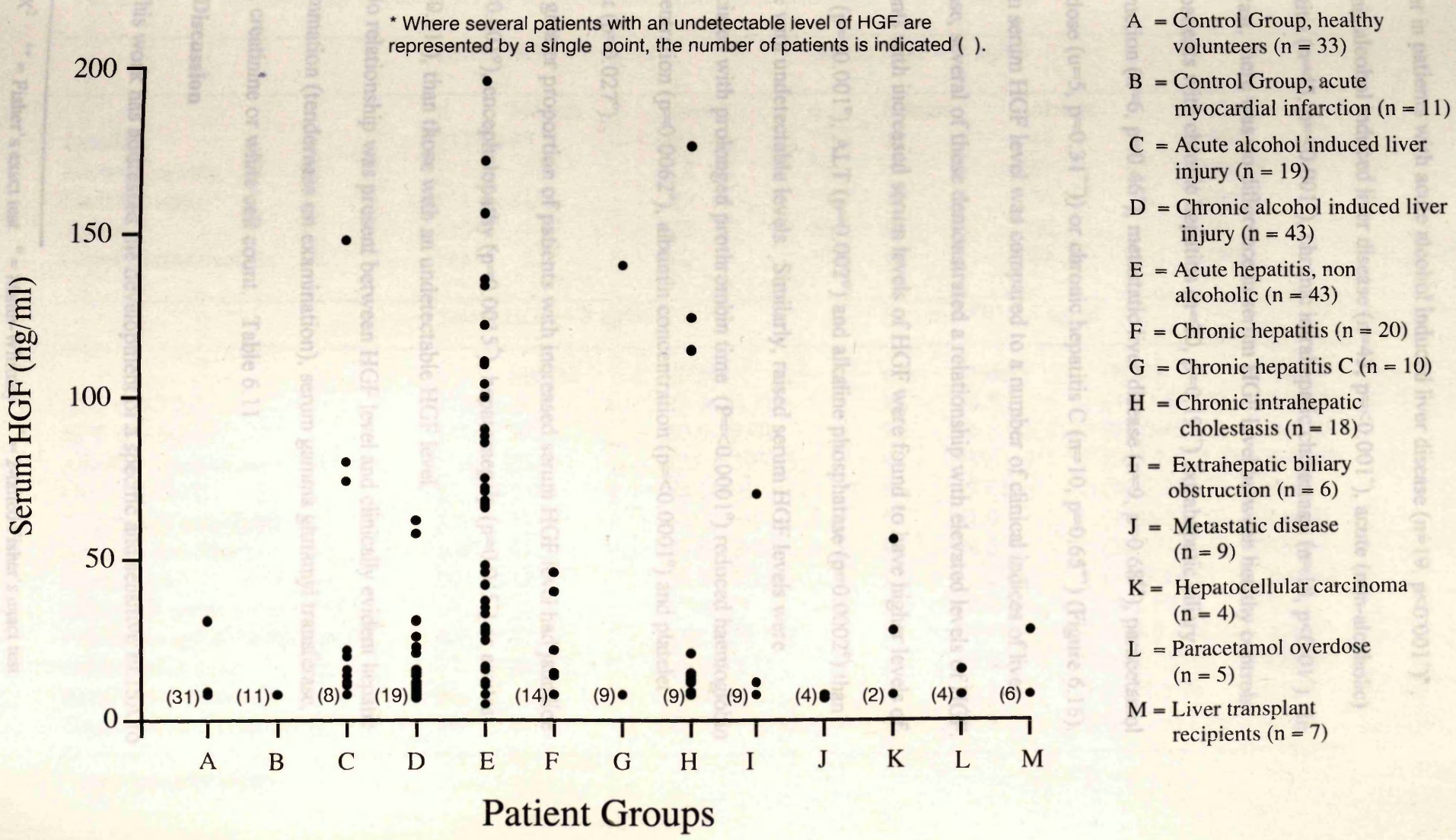
		ng/ml	ng/ml	ng/ml
Standard		3.125	12.5	25
Assay Number	1	3.125	13.5	25
	2	3.7	12.5	24
	3	3.0	12.5	26
	4	3.125	16.5	27
	5	3.125	12.5	25
	6	3.1	12.5	26
	7	3.0	13.5	18
	8	3.125	13.5	25
	9	3.7	12.0	25.5
	10	3.1	13.8	25

Results obtained using standard of known human recombinant HGF at low, medium and high levels in 10 runs of ELISA (f)

Figure 6.16 Serum Level of HGF in Liver Disease and Controls

This figure illustrates the range of HGF levels in controls and patients with various liver diseases

Serum Level of HGF in Liver Disease and Controls



A = Control Group, healthy volunteers (n = 33)

B = Control Group, acute myocardial infarction (n = 11)

C = Acute alcohol induced liver injury (n = 19)

D = Chronic alcohol induced liver injury (n = 43)

E = Acute hepatitis, non alcoholic (n = 43)

F = Chronic hepatitis (n = 20)

G = Chronic hepatitis C (n = 10)

H = Chronic intrahepatic cholestasis (n = 18)

I = Extrahepatic biliary obstruction (n = 6)

J = Metastatic disease (n = 9)

K = Hepatocellular carcinoma (n = 4)

L = Paracetamol overdose (n = 5)

M = Liver transplant recipients (n = 7)

higher in patients with acute alcohol induced liver disease (n=19, p<0.001^{+})$^{\bullet}$, chronic alcohol induced liver disease (n=43, p=<0.001^{+}), acute (non-alcoholic) hepatitis (n=43, p=<0.001^{+}), chronic intrahepatic cholestasis (n=18, p<0.01^{+}). In contrast, there was no difference in serum HGF level between healthy controls and patients with chronic hepatitis (n=20, p=0.19^{++}) extrahepatic biliary obstruction (n=6, p=0.46^{+}), metastatic liver disease (n=9, p=0.68^{++}), paracetamol overdose (u=5, p=0.31^{++})) or chronic hepatitis C (n=10, p=0.65^{++}) (Figure 6.16). When serum HGF level was compared to a number of clinical indices of liver disease, several of these demonstrated a relationship with elevated levels of HGF. Patients with increased serum levels of HGF were found to have higher levels of AST (p=<0.001°), ALT (p=0.002°) and alkaline phosphatase (p=0.0002°) than those with undetectable levels. Similarly, raised serum HGF levels were associated with prolonged prothrombin time (P=<0.0001°) reduced haemoglobin concentration (p=0.0062°), albumin concentration (p=<0.0001°) and platelet count (p=0.027°).

A greater proportion of patients with increased serum HGF level had jaundice (p=<0.001°), encephalopathy (p=0.0015*), hepatomegaly (p=0.045^{+}) or ascites (p=<0.01^{0}), than those with an undetectable HGF level.

No relationship was present between HGF level and clinically evident hepatic inflammation (tenderness on examination), serum gamma glutamyl transferase, urea, creatinine or white cell count. Table 6.11

6.4 Discussion

This work has addressed the development of a specific and sensitive ELISA to

$^{\bullet +} = X^2$ $^{++}$ = Fisher's exact test $^{\circ}$ = Mann-Whitney * = 2-tailed Fisher's exact test

Table 6.11 **Relationship of raised serum levels of HGF and clinical features of liver disease**

	Serum HGF < 8 ng/ml			Serum HGF > 8 ng/ml			
	n	Present	Absent	n	Present	Absent	P value
Jaundice	112	15	97	77	31	46	**<0.0001**
Encephalopathy	112	0	112	76	7	69	**0.0015**
Hepatomegaly	111	42	69	76	40	36	**0.0452**
Ascites	110	7	103	75	17	58	**<0.01**
Hepatic inflammation	111	9	102	73	12	61	0.0821

	Serum HGF < 8 ng/ml			Serum HGF > 8 ng/ml			
	n	Median	*(IQR)	n	Median	(IQR)	P value
AST (10-35u/l)	112	46.0	(29.5-76.7)	94	96.5	(46.0-437.8)	**<0.0001**
ALT (10-50u/l)	111	59.0	(30.0-92.0)	95	86	(33.0-456.0)	**0.002**
Alkaline phosphatase (70-260u/l)	112	222.5	(125.2-395.5)	92	359	(233.7-621.5)	**0.0002**
GGT (5-50u/l)	81	191.0	(63.5-401.0)	56	177.5	(89.2-356.6)	0.72
Bilirubin (3-18µmol/l)	111	11.0	(8.0-21.0)	95	41.0	(15.0-122.0)	**<0.0001**
Albumin (36-50g/l)	108	41.5	(37.0-45.0)	92	36.0	(30.25-41.0)	**<0.0001**
Haemoglobin (11.5-18g/dl)	101	13.6	(11.8-15.2)	72	12.6	(10.9-14.2)	**0.0062**
White cell count 4-11x10^9/l)	101	6.9	(5.5-9.3)	72	6.9	(5.0-10.0)	0.94
Platelet count (150-400x10^9/l)	100	238	(172.2-287.3)	72	202.5	(138.2-276.5)	**0.027**
Prothrombin time (11-15s)	73	14.0	(13.0-15.5)	50	17.0	(14.0-20.0)	**<0.0001**
Urea (2.5-7.5mmol/l)	95	4.8	(3.6-6.3)	77	4.3	(3.05-5.9)	0.22
Creatinine (60-110µmol/l)	94	81.0	(70.75-101.25)	72	83.5	(64.25-97.0)	0.52

*Inter Quartile Range

measure serum levels of HGF. ELISA has been established as a powerful tool in the investigation of biological systems, allowing specific detection of proteins and their measurement within complex mixtures such as serum. However, major problems are often encountered in the development of such assays in ensuring assay specificity and sensitivity.

HGF is a potent growth factor, and to date work has mainly been concentrated on its hepatocyte mitogenic activity, however, the descriptions of raised levels of HGF in a number of liver disease types including fulminant hepatic failure, acute hepatitis, liver cirrhosis and hepatocellular carcinoma (119,122) have raised interest in its use in the assessment of liver disease. To this end a sensitive and specific ELISA was developed for HGF. Both monoclonal and polyclonal antibodies were evaluated for use in the ELISA.

All of the antibodies assessed for possible use in ELISA were analysed by Western blotting studies in order to confirm activity against HGF and to investigate activity against the related protein plasminogen. (Monoclonal antibody A312-1 was characterised by Professor T. Nakamura and shown to react with recombinant HGF but not with rat HGF or other growth factors or plasmin). All of the tested antibodies which had been raised against recombinant human HGF (A312-2, B25, B43 and polyclonal antiserum Lot No 201), detected an 82 kDa band in non reduced samples of human recombinant HGF or purified native human placental HGF. Monoclonal antibody D9 which had been raised against a synthetic 11-amino acid peptide based on rat α-chain did not detect recombinant human HGF in Western blotting, but did detect an 82 kDa band in non reduced native human placental HGF. These results suggest that antibodies

A312-2, B25, B43 and polyclonal serum Lot No 201 are capable of detecting both native and recombinant forms of full length, non reduced HGF. Monoclonal antibody D9 however seems capable of detecting native placental HGF, but not recombinant human HGF, a finding which has implications for the use of recombinant HGF in an assay based on this antibody. This point is significant, since it would not be possible to calibrate an ELISA based on this antibody using recombinant HGF.

When antibodies A312-2, B25 and polyclonal antiserum Lot No 201 to human recombinant HGF were assessed for activity against plasminogen by Western blotting, only the polyclonal antiserum demonstrated a band of approximately the expected weight of plasminogen. These results suggest specificity of A312-2 and B25 for human HGF, native or recombinant and cross reactivity of polyclonal antiserum Lot No 201 for human plasminogen. Although ideally one would hope for no antibody cross-reaction with proteins other than target protein, the ELISA developed utilises a sandwich technique in which the first, monoclonal, antibody layer confers specificity to the assay, allowing the use of secondary antibody which may have some cross reacting features. The use of secondary polyclonal antibody helps to amplify the assay signal. In assays (b), (c), (d), (e) and (f) this principle is taken a step further with the addition of a third, biotinylated, polyclonal antibody which is used to detect the secondary antibody. This antibody is detected by means of avidin peroxidase conjugates with the application of orthophenylene diamine substrate which produces an orange/brown signal in the presence of peroxidase. The adverse effect of the use of additional antibody layers is the problem of cross reaction with protein in the assay other than the

target protein. In these studies biotinylated polyclonal sheep anti-rabbit immunoglobulin was used and found to strongly cross react with murine IgG monoclonal antibody. The cross reacting anti-mouse immunoglobulin activity was successfully removed by affinity chromatography using murine monoclonal IgG antibody bound to CNBr-activated Sepharose 4B; a dramatic reduction in background signal was achieved.

Several assays, using different antibody combinations, were used to develop the most sensitive and specific ELISA for the measurement of serum HGF.

ELISA (a): Although ELISA (a) was capable of developing a strong signal using human serum degradation of the purified biotinylated rabbit anti-HGF immunoglobulin prevented calibration of the assay and measurement of serum HGF levels. The antibody instability may be related to the purification and biotinylation of the polyclonal anti-HGF antibody. It is of interest that the few sera with the greatest activity as detected by ELISA (a) were also found to react strongly in ELISA (f) (greater than 62,000 units and 38,000 units in ELISA (a); 124 ng/ml and 11 ng/ml respectively, in ELISA (f)).

ELISA (b): The standard curve of recombinant human HGF illustrates that the assay could detect HGF. Unfortunately insufficient monoclonal antibody was available to measure serum levels of HGF. The process of establishing the assay and reagent conditions involved consumption of considerable antibody stock.

ELISA (c): this assay involved monoclonal antibody D9 which on Western blotting studies demonstrated an 82 kDa band in a non reduced preparation of native human placental HGF, but did not detect human recombinant HGF. Although the assay could detect recombinant human HGF it did so weakly and

inconsistently. The use of recombinant human HGF standards with the ELISA led to apparently very high levels of HGF in serum. It was believed that the problem involved differences in recognition of native and recombinant human HGF by monoclonal antibody D9, a view supported by the Western blotting studies. In an attempt to overcome this difficulty serum from a patient with acute hepatitis which reacted strongly in the assay (ELISA (c)) was used as a reference and arbitrarily designated as containing 1000 units per millilitre of serum. Other sera were defined in terms of these units by comparing their reaction in ELISA (c) and that of the reference serum. A range of levels was obtained (Table 6.5). The highest level was seen in a patient with cryptogenic cirrhosis with end stage liver disease who was later to be considered for liver transplant surgery. Liver biopsy from this patient demonstrated established macronodular cirrhosis. Serum obtained from another patient, who had recently sustained minor hepatic injury following paracetamol overdose was found to have a modest level of HGF. Such findings required confirmation, in particular the assay required to be calibrated against purified HGF. I sought to achieve this by sending a number of serum samples to Dr. Kenji Fujiwara, of the University of Tokyo (Dr. Kenji Fujiwara *et al* reported measurement of serum HGF levels using an ELISA kit undergoing development by Otsuka Assay Laboratories, Tokushima, Japan (172)).

It was hoped that if the HGF content of the reference serum and a number of patient's sera could be determined, one might derive HGF levels for the other tested samples. No relationship could be demonstrated between results obtained using ELISA (c) and those produced by Dr. Fujiwara. While this finding was considered significant, a more important difference was found in the result

obtained using human placental homogenate, which is used as a starting material for the purification of HGF as described in Chapter 4. The material was strongly positive in Dr. Fujiwara's assay, while only weakly detected in ELISA (c). In view of this fact, together with the poor sensitivity of ELISA (c) for human recombinant HGF and the inability of the monoclonal antibody D9 to detect recombinant human HGF on Western blotting analysis, ELISA (c) was deemed unsuitable for precise quantitative assessment of HGF levels in serum.

ELISA (d): The assay developed using monoclonal antibody B25 illustrates the difficulty in achieving specificity in an ELISA even when the assay is based on a monoclonal antibody. This assay demonstrates cross reaction with plasminogen, the plasma protein bearing closest resemblance to HGF (amino acid sequence homology of 37%). It is also important to note that the cross reactivity was not evident on Western blotting studies using B25 and purified native human Glu-plasminogen. The degree of cross reaction was such that the assay could not be used to measure serum HGF levels.

ELISA (e): This assay was also unsuitable for measurement of HGF levels. Although no cross reaction was evident with plasminogen and suitable standard curves could be drawn using human recombinant HGF, the assay failed to produce a signal when serum samples or native human placental HGF were tested. An explanation for this finding may be that subtle differences in structure between native and recombinant human HGF prevent recognition of the former by monoclonal antibody B43.

ELISA (f): This assay which is based on monoclonal antibody A312-2 has been shown to detect both native and recombinant human HGF. Clearly one major aim

in the development of an ELISA for HGF was the achievement of a sufficient degree of sensitivity. Previously reported levels of HGF are very low with quoted values comparable to those observed for the interleukins (119,122), hence a high level of sensitivity in the ELISA was required. The use of a sandwich assay with an additional layer of polyclonal antibody and an avidin/biotin peroxidase detection system greatly enhanced the assay sensitivity. The detection limit was 0.2 ng of HGF per well, taking into account the serum dilution used for the assay this is equivalent to 8 ng/ml.

Another important factor when establishing a new ELISA is confirmation of assay specificity. As has been described, cross reaction with structurally similar proteins may occur in an ELISA. No significant cross reaction was seen in assay (f) with purified native human Glu-plasminogen at 10 μg/ml. The inability of the assay to detect plasminogen was confirmed by a lack of correlation between HGF levels as measured in the assay and plasminogen levels as measured by conventional biochemical techniques (Table 6.7).

We sought to determine whether the conditions under which serum is collected and stored would affect the value for HGF concentration in serum using the ELISA. Table 6.8 illustrates that some storage dependent variation does occur. Condition (1) in which serum was separated and frozen within 30 minutes was the optimal method for preservation of serum proteins. There was little difference between the figure for HGF concentration under this condition and that produced when serum was separated and stored at 4°C before freezing or where whole blood was allowed to clot and stored at 4° prior to separation and freezing. The results in Table 6.8 suggest that under storage at room temperature serum

undergoes changes that result in detection of increased levels of HGF. Possible reasons for this include alteration in molecular structure, such that more epitopes are readily detected by antibodies in the ELISA. In view of these findings, only sera which had been collected under the following conditions were used when comparing HGF serum levels in patients with liver disease and controls: (1) Separation of serum and storage until use at -70°C, within 30 minutes of venepuncture. (2) Separation of serum within 30 minutes, storage at 4°C for 24 hours then storage at -70°C until use. (3) Storage of blood at 4°C for 24 hours then separation and storage of serum at -70°C until use.

Under these conditions, the assay had an intra-assay coefficient of variation of 4.85% and inter-assay coefficients of variation at low (3.125 ng/ml), medium (12.5 ng/ml) and high (25 ng/ml) levels of human recombinant HGF of 8.2%, 9.7% and 10.0% respectively. These figures are consistent with a high degree of intra- and interassay precision and fall within acceptable limits for assay performance (173). In view of the results of testing for assay specificity, sensitivity and precision, I believe there was sufficient evidence to conclude that ELISA (f) is capable of reliably detecting HGF in human serum. I did not, therefore, seek to compare serum HGF levels obtained by Dr Kenji Fujiwara with figures obtained using ELISA (f).

Three groups of serum samples were studied to establish whether serum levels of HGF as measured by ELISA have potential clinical value. Thirty three normal individuals (median age 41 years, range 55) were used as a negative control group. Only two normal individuals had detectable HGF levels, and these observed levels were low. A patient group in whom no liver damage was

anticipated; patients recovering from uncomplicated myocardial infarction (n=11, median age 58 years, range 36) had no detectable HGF levels. However, comparison of serum HGF levels in normals and in patients with a variety of liver diseases (n=221, median age 55 years, range 78) demonstrates an increased HGF serum level in patients with liver damage and in particular disease states where considerable hepatic regeneration would be predicted.

A number of groups of patients with liver disease were observed to have significantly higher levels of serum HGF than controls viz. acute ($p=<0.001$) and chronic ($p=<0.001$) alcohol induced liver disease, acute (non-alcoholic) hepatitis ($p=<0.001$) and chronic intrahepatic cholestasis ($p=<0.01$). Other groups of patients were identified which did not have significantly increased levels of HGF. These include patients with chronic hepatitis, extrahepatic biliary obstruction, metastatic liver disease, paracetamol overdose and chronic hepatitis C. Our findings are similar to those previously reported by other groups, although there are a number of differences (119,122,172,174). All of these reports describe low serum HGF levels in controls, with raised levels in various groups of patients with liver disease. Tsubouchi *et al* (119) noted HGF levels in serum from patients with fulminant hepatitis, acute hepatitis, chronic hepatitis and cirrhosis to be higher than controls. However, only the increase in serum HGF of patients with fulminant hepatitis and acute hepatitis was statistically significant. Tomiya *et al* (172) found raised levels of HGF in nineteen percent of 112 patients with chronic liver disease. Hioki *et al* (122) recorded high levels of serum HGF in patients with acute hepatitis, fulminant hepatitis, liver cirrhosis and hepatocellular carcinoma. Our findings differ in that HGF levels were not increased in chronic

hepatitis or hepatocellular carcinoma. We did not find increased HGF levels in patients following paracetamol overdose. Possible reasons for these findings may include differences in population groups and perhaps differences in disease aetiology e.g. chronic alcohol excess and viral hepatitis. In addition several of the groups described involve small patient numbers. All of the patients in the small group in whom we measured HGF level following paracetamol overdose made a rapid recovery. None experienced major hepatic injury. Shiota *et al* (174) have recently reported similar results following measurement of serum HGF in patients with liver disease using an immunoradio-metric assay (IRMA). However, their results differ from those described here in that they found increased serum levels of HGF in patients with chronic hepatitis and hepatocellular carcinoma. These groups describe lower figures for HGF in serum than those reported here. Reasons for such differences may be related to the mechanism of HGF detection with antibodies detecting different epitopes upon the HGF molecule. Thus, it is difficult to compare exact values obtained with different assays.

Serum HGF may be elevated as a result of increased production (80) or reduced clearance. The liver is the major organ for clearance of exogenously administered HGF (175,176,177) and clearance of HGF decreases in rats following carbon tetrachloride induced liver injury (176) or partial hepatectomy (178). Tsubouchi *et al* have suggested that serum HGF may be useful in predicting outcome in patients with fulminant hepatic failure (120).

It has also been suggested that HGF serum level may be an index of the severity of liver disease (122,172,174). These reports have also proposed that HGF levels may be related to conventional indices of liver disease. We have

found that patients with increased serum levels of HGF were likely to have higher levels of AST, ALT and alkaline phosphatase. Raised serum HGF levels were also associated with prolonged prothrombin time, reduced haemoglobin concentration, albumin concentration and platelet count. A greater proportion of patients with increased HGF levels had jaundice, encephalopathy, hepatomegaly or ascites. These findings would be consistent with the suggestion that increased HGF levels are to be found in patients with more advanced liver disease. As discussed above, mechanisms for this increase in HGF level may include increased production in an attempt to repair the hepatic damage or reduced clearance by the injured liver.

This study has highlighted some of the difficulties encountered in the development of an ELISA for measurement of minute quantities of target material in complex biological fluids such as serum. The work has also extended the variety of liver diseases in which serum HGF has been measured and reinforces the concept that HGF is related to severity of liver disease and may be useful as an index of degree of hepatic dysfunction.

CHAPTER 7. Expression of Hepatocyte Growth Factor and Hepatocyte Growth Factor mRNA in Human Liver Biopsies

7. INTRODUCTION

7.1 Immunocytochemistry and *in situ* Hybridisation for HGF and HGF mRNA

HGF has been localised in non-parenchymal liver cells immunocytochemically in man and rats (93) and its mRNA detected by *in situ* hybridisation in normal rats (88) and following hepatic injury (81). In order to examine the role of HGF in human liver disease I sought to investigate whether changes in expression of HGF mRNA and its protein product in the liver could be related to disease type or severity. All available antibodies to HGF, except A3.1.2-1, were assessed for suitability in immunocytochemistry for HGF using a variety of techniques including indirect alkaline phosphatase, indirect immunoperoxidase and alkaline phosphatase-anti-alkaline phosphatase (APAAP), in fresh frozen and formalin fixed paraffin embedded tissue.

To examine the expression of HGF mRNA non-isotopic *in situ* hybridisation techniques have been employed. Initially a digoxigenin labelled riboprobe based on rat HGF was assessed in view of sequence homology of greater than 90% between rat and human HGF. In addition a human specific digoxigenin labelled riboprobe was synthesised and tested.

7.2 Immunocytochemistry - Localisation of HGF in Human Liver Tissue

Materials - see page 141

Methods

7.2.1 Frozen Tissue

Sections of 5μm thickness were cut from blocks, placed on poly-L-lysine coated slides and allowed to dry for 1 hour at room temperature. Sections were then fixed in acetone for 10 minutes and allowed to air dry.

A solution of Tris buffered saline, containing 20% (volume/volume) normal swine serum, was applied for 10 minutes to reduce non-specific binding of antibody. Primary antibody was then applied.

7.2.2 Paraffin Section

Liver biopsies were fixed in 10% buffered formalin and processed for paraffin embedding. Sections of 3µm thickness were cut onto poly-L-lysine coated slides and dried for 1 hour at 60°C. Sections were dewaxed in xylene for 10 minutes, washed with three changes in absolute alcohol, followed by two washes in methanol. After being rinsed in methanol the sections were hydrated in distilled water. In some experiments sections were trypsinised (Tris buffered saline containing 0.1% weight/volume and 0.1% calcium chloride) for 10 minutes. Slides were then washed in phosphate buffered saline and 'blocked' by addition of phosphate buffered saline containing 20% (volume/volume) normal swine serum for 10 minutes.

7.2.3 Indirect Alkaline Phosphatase Detection Method

This method was used with each of the available antibodies. Various dilutions of antibody were used, starting from a minimum of 1 in 5. Each antibody was incubated for both 1 hour at room temperature and overnight at 4°C. Monoclonal antibodies were diluted in phosphate buffered saline containing 2% (weight/volume) bovine serum albumin (2% BSA/PBS) and polyclonal antibodies in phosphate buffered saline containing 4% (volume/volume) normal swine serum (4% NSS/PBS). Sections were washed 3 x 10 minutes in phosphate buffered saline and secondary antibody applied (alkaline phosphatase conjugated rabbit anti-mouse immunoglobulins diluted 1:25 in 2% BSA/PBS or alkaline phosphatase conjugated swine antibody to rabbit immunoglobulins diluted 1:75 in 4% NSS/PBS). After incubation for 1 hour at room temperature sections were washed 3 x 10 minutes in phosphate buffered saline and exposed to substrate (NBT/BCIP or fast red, Appendix 3) for 5-10 minutes. Sections were mounted under glycerol gelatin and examined under the light microscope.

7.2.4 Alkaline Phosphatase Anti-Alkaline Phosphatase Detection Method (APAAP)

This method was used with monoclonal antibodies.

Secondary antibodies: rabbit anti-mouse immunoglobulins diluted 1:30 in 2% BSA/PBS was applied to sections and incubated for 1 hour at room temperature. Sections washed 3 x 10 minutes with phosphate buffered saline and murine monoclonal alkaline phosphatase-anti-alkaline phosphatase complexes, diluted 1:50 in 2% BSA/PBS, added. After incubation for 1 hour at room temperature, slides were washed 3 x 10 minutes with phosphate buffered saline and exposed to substrate, mounted and examined as described above in the Indirect Alkaline Phosphatase Detection Method.

7.2.5 Indirect Immunoperoxidase Detection Method

This technique was used with all polyclonal antibodies. Each primary antibody, diluted in phosphate buffered saline containing 20% (volume/volume) normal swine serum, was incubated for both 1 hour at room temperature and overnight at 4°C. Various dilutions of antibody were used, starting from a minimum of 1:5.

Sections were washed 3 x 10 minutes in phosphate buffered saline and secondary antibody applied; peroxidase conjugated swine anti-rabbit immunoglobulin, diluted 1:50 in 4% NSS/PBS. After incubation for 1 hour at room temperature slides were washed 3 x 10 minutes in phosphate buffered saline.

Signal was developed by incubation of sections in 3' diamino-benzidine tetrachloride (DAB) solution. DAB is converted by peroxidase to an insoluble brown precipitate. The slides were washed in water and counterstained with

haematoxylin, dehydrated through graded alcohols, cleared in xylene and mounted with Hystomount.

7.2.6 Preparation of Polymorphonuclear Leucocyte (Polymorph) Neutrophil Proteins

Venous blood (20ml) was taken from a healthy volunteer into a heparinised tube. A volume of 5ml of this blood was layered on top of 3.5ml "Polymorph prep" (Nycomed A.S. (UK) Ltd, Birmingham, UK) and centrifuged at 500g for 15 minutes. This step leads to separation into layers of plasma, monocytes, polymorphs and erythrocytes. The layer containing neutrophil polymorphs was removed and mixed with an equal volume of 0.45% NaCl and 0.9% NaCl added to bring the volume up to 15ml. This preparation was then centrifuged at 400g for 10 minutes. The pelleted cells were resuspended and made up to 15ml volume with PBS and centrifuged for a further 10 minutes at 400g. The pellet was re-suspended and mixed with 0.5ml dH_2O until red cells lysed. Double concentrate PBS (0.5ml) was added and volume made up to 15ml with PBS. The sample was centrifuged again at 400g for 10 minutes. The cellular pellet was resuspended in a total volume 1-2ml of PBS and cells sonicated for 10 seconds to disrupt the cells and solubilise the cellular proteins (Branson Sonifier 250, Dane Ultrasonics, Hayes, Middlesex, UK).

7.2.7 Western Blotting of Polymorph Proteins

Non-reduced samples of polymorph neutrophil cell lysate, (8.8 μg/lane) recombinant human HGF (each 200ng/lane) and purified native human placental HGF (20ng/lane) were subjected to SDS-PAGE on a 10% gel and blotted onto PVDF membrane as described in chapters 2 and 3. Following transfer, membranes were "blocked" by incubation with phosphate buffered saline containing 3% (weight/volume) bovine serum albumin and probed with:

a) Pre-immune serum from rabbit 1699, diluted 1/1000

b) Immune serum from rabbit 1699, diluted 1/1000

c) Rabbit anti-HGF serum, Lot No. 201 diluted 1/4000

d) Mouse monoclonal antibody to human recombinant HGF, B25, diluted 1/5000

These primary antibodies were detected using alkaline phosphatase conjugated swine anti-rabbit immunoglobulins or alkaline phosphatase conjugated rabbit anti-mouse immunoglobulins and NBT/BCIP (Western blotting as described in Chapter 4).

7.2.8 Absorbance of Antibody with Antigen

Rabbit antiserum 1699 was diluted to 1/400 in Tris buffered saline and 400µl incubated with either 5.75µg of polymorph proteins or 20µg of recombinant human HGF (Genentech) at room temperature overnight.

Immunocytochemistry using the antiserum was then performed as described above using the indirect immunoperoxidase detection method.

7.3 Results

7.3.1 HGF Immunocytochemistry

The following antibodies failed to demonstrate HGF in frozen human liver biopsies.

Monoclonal: A321-2
B25
B43
D9

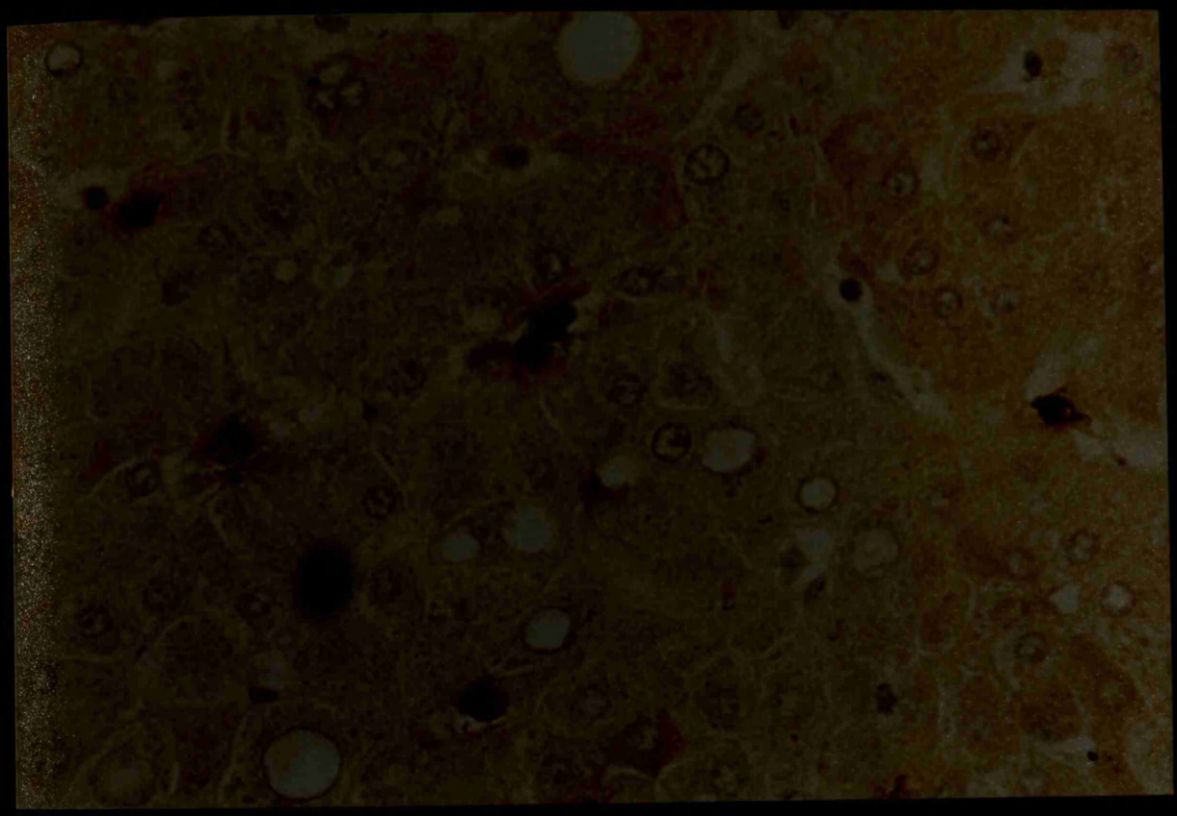
Polyclonal: Lot No 201
640/641 (rabbit antisera to HGF4, as described in section 4.

Polyclonal rabbit antiserum 38986 failed to demonstrate HGF in frozen rat liver following carbon tetrachloride administration.

Polyclonal rabbit antiserum 1699 localised polymorph neutrophil leucocytes and some non-parenchymal cells in fresh frozen and formalin fixed, paraffin embedded tissues with no staining of parenchymal cells (Figure 7.1). No staining was evident using preimmune serum.

Figure 7.1 Human liver from a patient with clinical evidence of chronic alcohol induced liver disease stained with polyclonal rabbit antiserum 1699

Primary antibody used at a dilution of 1/400. Secondary antibody; Peroxidase conjugated affinity purified swine anti-rabbit immunoglobulins. Substrate; diaminobenzidine tetrachloride (DAB) solution. Haematoxylin counterstain Polymorph neutrophil leucocytes are positively stained as shown.



When antiserum which had been preabsorbed with polymorph proteins was used, virtually all cellular staining was abolished (Figure 7.2). Incubation of antiserum with bovine serum albumin (as a control) did not affect staining (Figure 7.3). Preabsorption of antiserum with human recombinant HGF similarly did not affect staining (Figure 7.4).

7.3.2 Western Blotting of Polymorph Proteins

Immune serum, but not pre-immune serum, from rabbit 1699 detected several protein bands in the polymorph neutrophil lysate and detected human recombinant and native human placental HGF. Polyclonal antiserum Lot No. 201 and monoclonal antibody B25 both detected human recombinant HGF, but failed to detect any protein band in polymorph neutrophil lysate (Figure 7.5).

7.4 Discussion

Immunolocalisation of HGF in tissue sections has proved difficult to achieve. Although some work has been published on this subject, differing results have been obtained by independent groups. Wolf *et al* (93) reported localisation of HGF in a wide variety of human and rat tissues. In human liver bile duct epithelium and endothelial cells of central veins and portal vessels, moderate staining for HGF was obtained. Sakaguchi *et al* (178) found HGF staining in polymorphonuclear leucocytes and biliary epithelial cells but not in the endothelial cells of hepatic or portal veins. In this study, none of the antibodies that detected human recombinant HGF in Western blotting or ELISA demonstrated HGF immunohistochemically.

Fresh frozen tissue was used in an attempt to avoid some of the known difficulties associated with immunocytochemistry in fixed tissues. Fixation alters tissue antigenicity by, for example, crosslinking proteins. Although important for morphological preservation, crosslinking may reduce the availability of antigen within tissue; epitopes may be denatured, antigen conformation altered or accessibility to immunoreagents reduced (180).

Figure 7.2 Human liver from a patient with clinical evidence of chronic alcohol induced liver disease stained with polyclonal rabbit antiserum 1699, which had been pre-absorbed with polymorph proteins

Formalin fixed, paraffin embedded liver tissue. Primary antibody used at a dilution of 1/400. Secondary antibody; peroxidase conjugated affinity purified swine anti-rabbit immunoglobulins. Substrate; diaminobenzidine tetrachloride (DAB) solution. Haematoxylin counterstain.

Virtually all cellular staining has been abolished following incubation of antiserum 1699 with polymorph proteins.

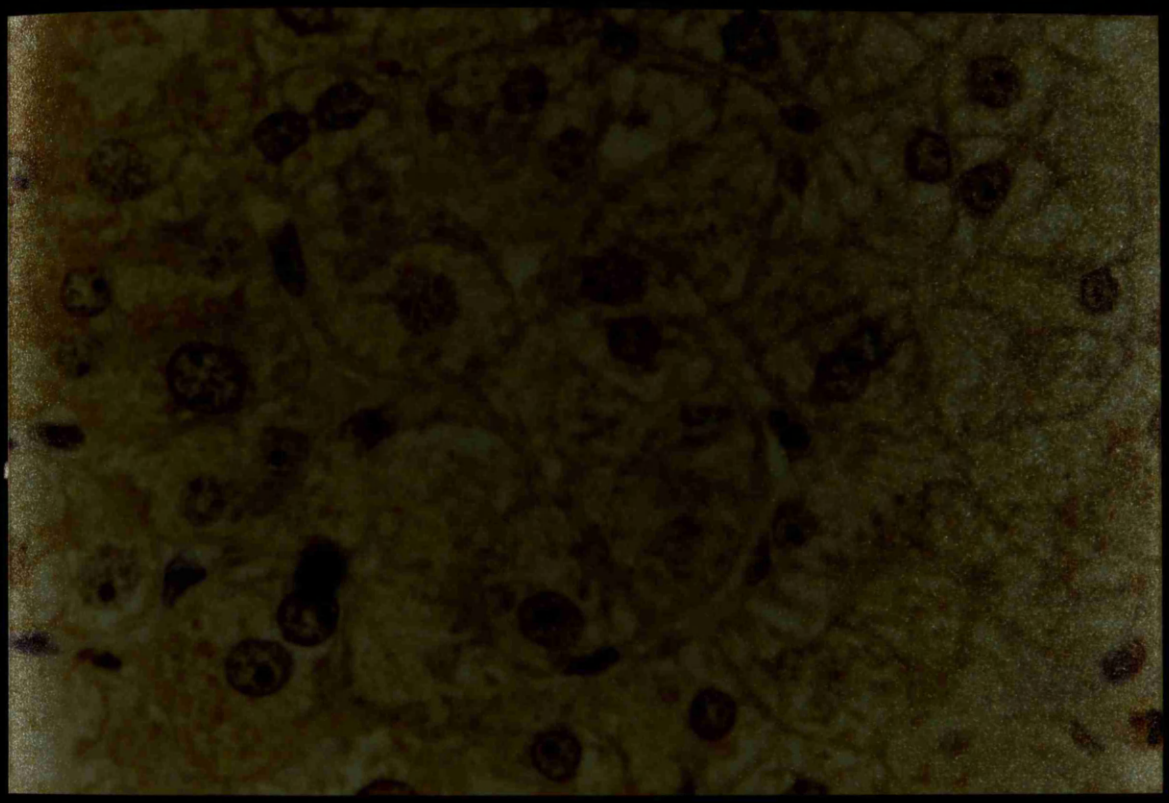


Figure 7.3 Human liver from a patient with clinical evidence of chronic alcohol induced liver disease stained with polyclonal rabbit antiserum 1699, which had been incubated with bovine serum albumin

Formalin fixed, paraffin embedded liver tissue. Primary antibody used at a dilution of 1/400. Secondary antibody; peroxidase conjugated affinity purified swine anti-rabbit immunoglobulins. Substrate; diaminobenzidine tetrachloride (DAB) solution. Haematoxylin counterstain.

Incubation of antiserum with bovine serum albumin does not affect staining.

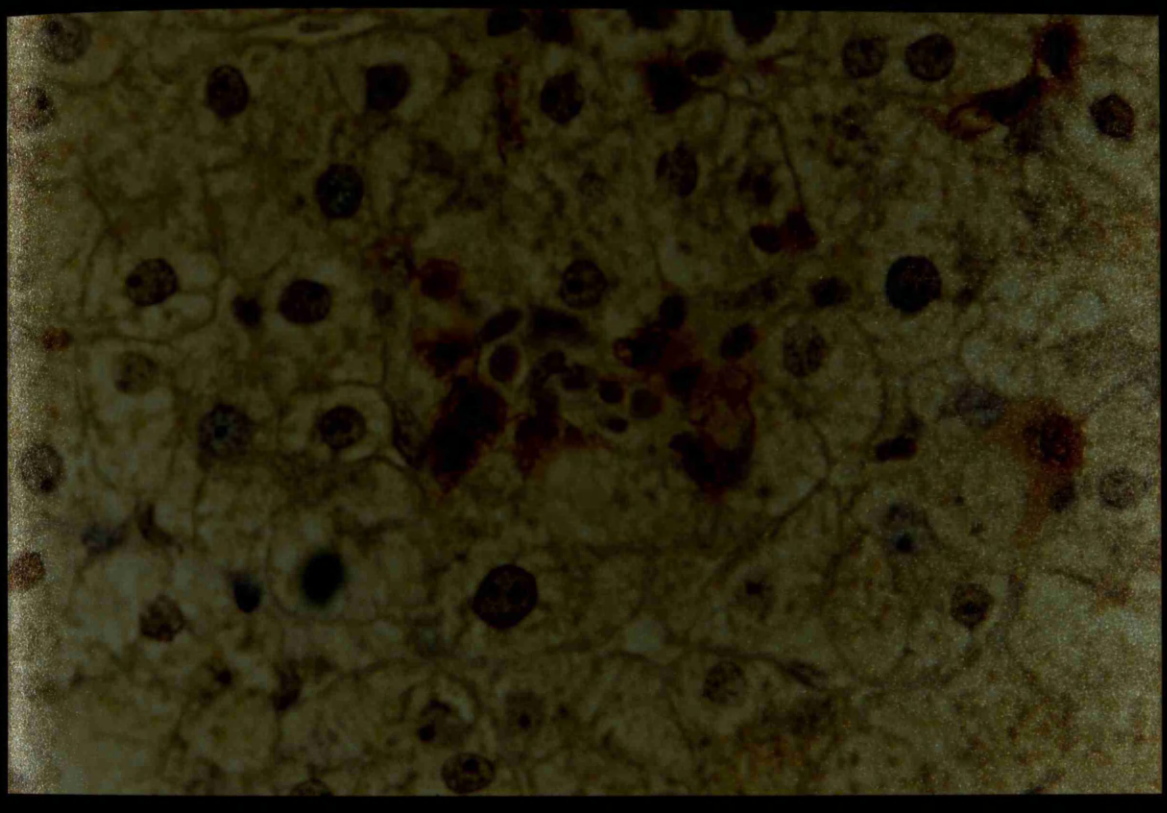


Figure 7.4 Human liver from a patient with chronic alcohol induced liver disease stained with polyclonal rabbit antiserum 1699, which had been incubated with human recombinant HGF

Formalin fixed, paraffin embedded liver tissue. Primary antibody used at a dilution of 1/400. Secondary antibody; peroxidase conjugated affinity purified swine anti-rabbit immunoglobulins. Substrate; diaminobenzidine tetrachloride (DAB) solution. Haematoxylin counterstain.

Incubation of antiserum with human recombinant HGF does not affect staining.

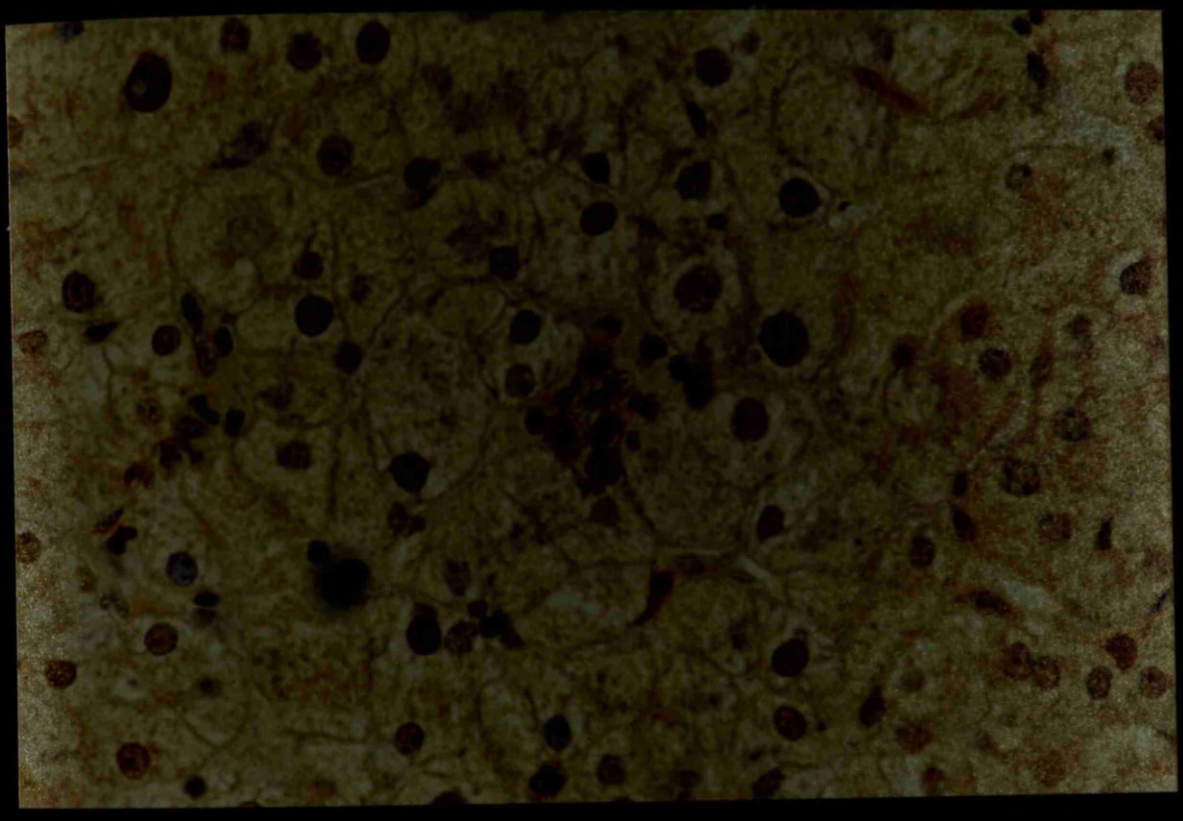


Figure 7.5 Western blotting of human recombinant HGF and polymorph proteins using pre-immune and immune serum from rabbit 1699, rabbit anti-HGF serum Lot No. 201 and monoclonal antibody B25

Lane 1 Human placental HGF (20 ng/Lane)

2 Human recombinant HGF (200 ng/Lane)

3 Polymorph neutrophil cell lysate (8.8 μg/Lane)

4 Human recombinant HGF

5 Polymorph neutrophil cell lysate

6 Human recombinant HGF

7 Polymorph neutrophil cell lysate

8 Human recombinant HGF

9 Polymorph neutrophil cell lysate

10 Molecular weight markers

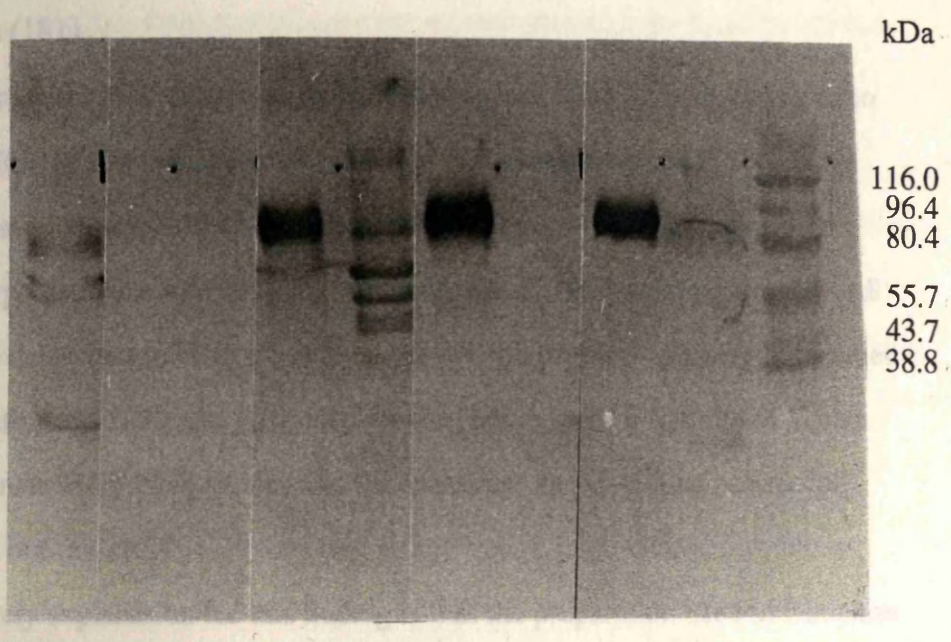
Lanes 1,4 & 5 probed with immune serum from rabbit 1699 diluted 1/1000

Lanes 2 & 3 probed with pre-immune serum from rabbit 1699, diluted 1/1000

Lanes 6 & 7 probed with rabbit anti-HGF serum Lot No. 201, diluted 1/4000

Lanes 8 & 9 probed with mouse monoclonal antibody to human recombinant HGF B25 diluted 1/5000

Immune serum only from rabbit 1699 detected several protein bands in the polymorph neutrophil cell lysate and detected human recombinant and native human placental HGF. Rabbit anti-HGF serum Lot No. 201 and mouse monoclonal antibody B25 detected human recombinant HGF but did not demonstrate any protein bands in polymorph neutrophil lysate.


kDa
116.0
96.4
80.4
55.7
43.7
38.8
Lane 1 2 3 4 5 6 7 8 9 10

Similarly, the alkaline phosphatase-anti-alkaline phosphatase (APAAP) method was adopted in view of the known improvements in sensitivity which may be achieved with the technique. Mason has reported APAAP to be four or five times more sensitive than conventional two and three stage indirect immunoperoxidase methods (181).

Antiserum 1699, raised against human placental HGF, initially appeared to detect HGF in non-parenchymal cells of liver and in neutrophils. To test the hypothesis that polymorphs contained HGF, Western blotting studies of polymorph proteins with a number of antibodies to HGF were performed. All failed to demonstrate HGF among the polymorph proteins. Absorption studies using antiserum 1699 and polymorph lysate, bovine serum albumin or human recombinant HGF have shown that the antiserum was detecting polymorph proteins but not HGF.

A likely explanation for this finding is that the preparation used to immunise rabbit 1699 was not pure (see Chapter 3). This work underlines the importance of clearly validating the specificity of staining produced in immunohistochemical studies. Notwithstanding the work by Sakaguchi *et al* (179) which describes immunolocalisation of HGF in polymorphonuclear leucocytes within liver, our results indicate that immunocytochemical staining in our study is due to reaction with polymorph proteins, not HGF.

7.5 HGF mRNA *in situ* Hybridisation Studies

In situ hybridisation methods allow the examination of specific nucleic acid sequences in biological preparations in which the structure of the material present is preserved intact. The preparation may consist of tissue sections, cells or chromosomes. When combined with immunocytochemistry, *in situ* hybridisation offers the possibility of investigating gene activity, in terms of DNA, mRNA and protein expression and relating this to histological findings of the tissue in question.

The technique of *in situ* hybridisation was first developed in 1969 (182,183) and initially could only be performed by radioisotopic labelling of complementary nucleic acid sequences and detection with autoradiography. More recently considerable advances have been made in the field of probe detection by non-isotopic methods. Such methods are for several reasons considered to represent significant improvements in the area of *in situ* hybridisation technology.
(1) Detection of hybrids can be achieved in a shorter time than is required for autoradiography, (2) Cellular resolution is improved compared to that achieved using radiolabelled systems, (3) The biohazards and expense associated with radioisotope work are avoided, (4) Non-radioactive probes have a long shelf life.

Two disadvantages have been identified with the use of non-radioactive probes:
(1) sensitivity of the probe may be less than that of a radiolabelled probe. However, this problem has in many cases been overcome following refinement of non-radioactive labelling and detection methods, (2) standardisation of the procedure of non-isotopic *in situ* hybridisation and of the intensity of the reaction product is difficult to obtain, preventing quantitative analysis.

In general one may consider two types of non-radioactive hybridisation method - direct and indirect. In the direct approach the reporter (detectable) molecule is attached directly to the nucleic acid probe and detection of probe is possible immediately after hybridisation. Examples of this approach include terminal fluorochrome RNA probe labelling (184) and direct enzyme labelling of nucleic acids (185). In the indirect techniques probes contain a reporter molecule which is subsequently detected by affinity cytochemistry. Examples of this method include biotin labelling (186) and, more recently, the digoxigenin system (187).

When embarking on the study of a particular nucleic acid species by *in situ* hybridisation one may choose from three main types of probe; cDNA probes,

cRNA probes and synthetic oligonucleotide probes. The first type produced were cDNA probes and for many years represented the only kind available. However, cRNA probes provide a number of advantages over cDNA probes.

(1) cRNA probes are single stranded and therefore are not affected by problems of self annealing in solution.

(2) Hybrid stability is greater; allowing post-hybridisation washing to be more stringent.

(3) Unhybridised probe may be destroyed by addition of RNase which does not affect cRNA hybrids, leading to reduced background signal.

In addition to these methods one may now choose the option of using synthetic oligonucleotide probes which have been successfully used to localise mRNA by *in situ* hybridisation (188). However, the synthetic oligonucleotides cannot readily be labelled to the same degree of specific activity possible with cRNA probes. In addition, hybrids involving cRNA probes are more stable and allow more thorough post hybridisation removal of unhybridised probe. For these reasons cRNA probes are preferred over synthetic oligonucleotide probes in the study of low abundance mRNAs.

In this study two riboprobes were prepared - pRBC-1, based on rat HGF cDNA sequence, and hHGFα266, based on human HGF nucleotide sequence. *In situ* hybridisation to HGF mRNA was attempted using these two probes in rat and human tissue.

7.6 Materials and Methods

Materials - see page 141.

Methods

7.6.1 Probe Preparation

Probes were prepared by Mr R. Ferrier and Dr K. Hillan, Department of Pathology, Western Infirmary, Glasgow.

pRBC-1

To transcribe anti-sense probes plasmid DNA was linearised with XhoI then incubated with a transcription buffer containing T3 RNA polymerase and digoxigenin labelled UTP: sense probes were produced by linearising plasmid DNA with PstI and transcribing with T7 polymerase. The resultant probe was purified then identified by blotting onto Hybond C-membrane and probing with alkaline phosphatase conjugated sheep antibodies to digoxigenin with NBT/BCIP substrate.

hHGFα266

In a similar fashion, plasmid DNA was linearised using Hind III cDNA fragment hHGFα266 was cloned into pGEM II (Promega) and specific antisense single stranded RNA probes transcribed using T7 polymerase in the presence of digoxigenin-II-UTP (Boehringer-Mannheim).

7.6.2 Partial Alkaline Hydrolysis of cRNA Riboprobe to Produce a Cocktail of 100-150 Base Probes

After testing of full length probes, both were subjected to partial alkaline hydrolysis treatment (Mr R. Ferrier, Department of Pathology, Western Infirmary, Glasgow).

Regulation of RNA Probe Length by Alkaline Hydrolysis

Carbonate buffer: 60mM Na_2CO_3/40mM $NaHCO_3$, pH 10.2.

Neutralisation buffer: 200mM acetate: 1% (volume/volume) acetic acid, pH 6.

(1) RNA (1μg) was hydrolysed by adding an equal volume of DEPC (Diethylpyrocarbonate) treated water and 2 volumes of carbonate buffer. The solution was incubated at 60°C for 10 minutes.

(2) An equal volume of neutralisation buffer was added to stop hydrolysis.

(3) RNA ethanol was precipitated with sodium acetate.

(4) The precipitate was centrifuged and the pellet washed in 70% spirit.

(5) The probe was reconstituted in DEPC-treated water.

7.6.3 HGF mRNA Detection - *In situ* Hybridisation

(1) Sections of 3μm thickness were cut onto 3-aminopropyl tri-ethyoxysilane (APES) coated slides. The sections were dewaxed in xylene and hydrated through alcohols to diethylpyrocarbonate treated phosphate buffered saline (PBS/DEPC).

(2) Sections were incubated in 0.2M HCl for 20 minutes at room temperature and washed in PBS/DEPC.

(3) Sections were incubated in 0.3% (volume/volume) Triton X-100 for 15 minutes at room temperature and washed in PBS/DEPC.

(4) Proteinase K digestion was carried out for 15 minutes at 37°C (100μg/ml in PBS/DEPC).

(5) Sections were fixed in 4% (weight/volume) paraformaldehyde for 5 minutes and washed in PBS/DEPC.

(6) Sections were bathed in prehybridisation buffer for 2 hours at 37°C (2 x SSC/50% formamide).

(7) The probe, diluted in hybridisation buffer, was denatured by heating at 70°C for 2 minutes, then quenched on ice and applied to section, coverslip mounted and hybridised overnight at 42°C.

(8) Coverslips were removed in 2 x SSC and sections washed twice in 2 x SSC for 30 minutes at room temperature.

(9) Sections were washed in 0.1% SSC for 30 minutes at room temperature, then for 30 minutes at 45°C and finally in digoxigenin buffer 1 for 5 minutes at room temperature.

(10) Sections were incubated with alkaline phosphatase conjugated anti-digoxigenin antiserum for 2 hours at room temperature (1:2000 in digoxigenin buffer 1 plus 20% normal swine serum).

(11) Sections were washed twice in digoxigenin buffer 1 for 15 minutes at room temperature.

(12) Washed in digoxigenin buffer 3 for 5 minutes at room temperature.

Finally, the sections were:

(13) Incubated in NBT/BCIP (Appendix 3) at room temperature overnight.

(14) Washed in water.

(15) Counterstained with haematoxylin and mounted with glycerol gelatin.

7.7 Results

The alkaline hydrolysed pRBC-1 probe based on rat HGF molecule sequence failed to localise HGF mRNA in human liver tissue. Using rat liver obtained following partial hepatectomy and common bile duct ligation the probe demonstrated HGF mRNA in cells which, on morphological grounds, resembled Ito cells (81) (Figures 7.6, 7.7).

The alkaline hydrolysed hHGFα266 probe based on human HGF nucleotide sequence failed to localise HGF mRNA in normal or diseased human liver tissue.

7.8 Discussion

The pRBC-1 probe based on rat HGF nucleotide sequence was used in an attempt to localise human HGF mRNA since there is known to be 90% homology between human and rat HGF sequence (49). It is possible that the small differences which exist in sequence between the two species are responsible for the failure of the probe to detect human HGF mRNA.

The HGFα266 probe was produced in an attempt to overcome this problem by generating a probe based on the human HGF sequence. Possible reasons for the failure of this probe and pRBC-1 may include the large size of the probes. pRBC-1 is 1200 bases long and hHGFα266 is 796 bases long. However, following alkaline hydrolysis in which the probes were reduced to 100-150 bases the probes still failed to detect human HGF mRNA. The use of the digoxigenin system may also be a factor since this is a large molecule and may affect nucleic acid hydridisation by steric hindrance.

Figure 7.6 Expression of HGFmRNA demonstrated by *in situ* hybridisation using pRBC-1 probe in rat liver, day 1 following common bile duct ligation.

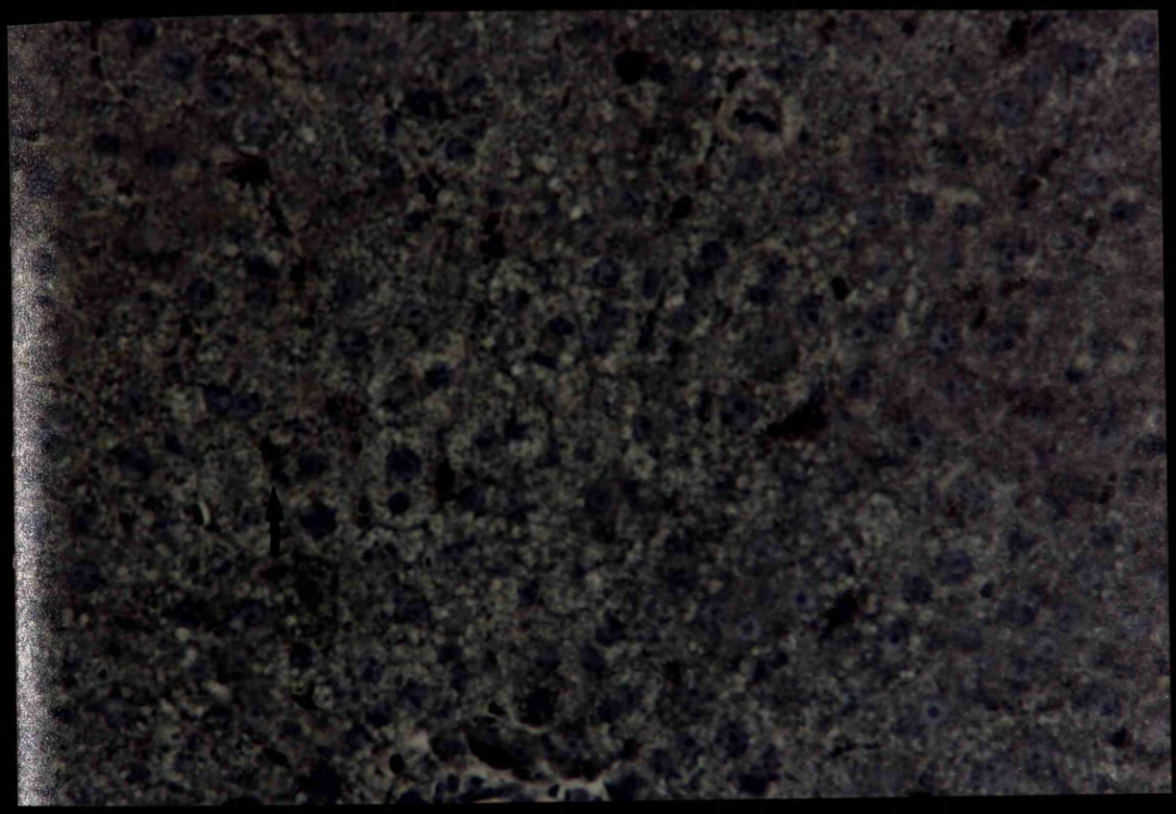
Formalin fixed, paraffin embedded rat liver tissue. Probe pRBC-1 detected using alkaline phosphatase conjugated antidigoxigenin antiserum and NBT/BCIP substrate.

Positive staining is present in cells corresponding to Ito cells.

Figure 7.7 Expression of HGF mRNA demonstrated by *in situ* hybridisation using pRBC-1 probe in rat liver, day 1 following partial hepatectomy.

Glutaraldehyde fixed, paraffin embedded rat liver tissue. Probe pRBC-1 detected using alkaline phosphatase conjugated antidigoxigenin antiserum and NBT/BCIP substrate.

Positive staining is present in cells corresponding to Ito cells.



CHAPTER 8. Examination of the Expression of Hepatocyte Growth Factor Receptor (c-*met*) in Diseased Human Liver by Immunocytochemistry

8. INTRODUCTION

8.1 HGF Receptor (c-*met*) Immunocytochemistry

The cell surface receptor for HGF was identified in 1991 as the product of the proto-oncogene c-*met* (65,66). The proto-oncogene codes for a hetero-dimeric protein consisting of two dilsulphide bonded chains, an α-chain of 50kDa and a ß-chain of 145kDa. The α and ß chains are both exposed at the cell surface and the ß-chain spans the cell membrane. The protein is widely distributed in various tissues (71) and cell types (189) and is a member of the family of protein tyrosine kinases. The tyrosine kinase part of the receptor is present on the intracellular part of the ß-chain (70).

One way in which to determine the role of HGF in liver disease is to examine expression of its receptor. In 1991 Prat *et al* described a murine monoclonal antibody to the extracellular domain of c-*met* (71). The antibody was obtained following immunisation with living cells from the human gastric carcinoma cell line GTL-16 in which the gene is amplified and over-expressed (68). The antibody, DO-24, had been used to examine expression of the *met* protein in frozen sections of various tissues including liver and gastrointestinal tract. Using this antibody on frozen sections of normal liver and on biopsies from patients with various liver diseases the expression of HGF receptor was examined by immuno-histochemistry.

8.2 Materials and Methods

Materials - for all materials including fresh frozen normal and diseased human liver tissue see page 141.

Methods

8.2.1 Immunocytochemistry

Sections of 5μm thickness were cut onto poly-L-lysine coated slides, allowed to air dry at room temperature for 1 hour and fixed in cold acetone for 10 minutes.

After washing sections in phosphate buffered saline (2 x 1 minute) non-specific binding sites were blocked by incubation with phosphate buffered saline containing 2% bovine serum albumin (weight/volume) (2% BSA/PBS). Blocking solution was poured off and DO-24 monoclonal antibody (culture supernatant) or control antibody (culture supernatant) applied neat to sections and incubated at room temperature for 1 hour.

Sections washed 3 x 10 minutes in phosphate buffered saline.

Rabbit polyclonal antibodies to murine immunoglobulins diluted 1:30 in 2% BSA/PBS applied to sections and incubated at room temperature for 1 hour.

Slides were washed, 3 x 10 minutes, in phosphate buffered saline, incubated with murine monoclonal alkaline phosphatase - anti-alkaline phosphatase complexes diluted 1:50 in 2% BSA/PBS for 1 hour, washed 3 x 10 minutes in phosphate buffered saline and incubated with substrate (Fast red, see appendix 3). Sections were lightly stained with haematoxylin and mounted under glycerol gelatin.

8.3 Results

Staining was present in the sections with DO-24 antibody but of low intensity, and associated with a considerable degree of non-specific background staining. No staining was evident using control antibody. Histologically normal liver tissue

demonstrated the presence of fine hepatocyte membranous staining consistent with the presence of membrane bound HGF receptor on hepatocytes. Biopsies in which histological, particularly architectural, abnormalities were present demonstrated absence or reduced intensity of staining (Figures 8.1,8.2,8.3)

8.4 Discussion

The effects of HGF are believed to be mediated by its cell surface receptor, the c-*met* proto-oncogene product (65,66,69).

Immunohistochemical localisation of c-*met* in frozen liver biopsy material has shown that in diseased liver of various aetiologies, the level of expression of HGF receptor appears to be reduced. An explanation for this finding may be receptor internalisation following binding to ligand (61), i.e. down regulation of the receptor in response to increased availability of ligand. There is some evidence for down regulation of c-*met* following binding of HGF by hepatocytes *in vitro* (190). These changes may be related to the role of HGF in liver disease. Evidence supporting this proposal includes the finding that HGF receptor numbers are reduced in liver following partial hepatectomy (61,63) and carbon tetrachloride induced hepatitis (61). Interestingly, the numbers of HGF receptors remain unchanged in uninjured organs following partial hepatectomy. Similarly, if unilateral nephrectomy is performed, rapid down regulation of HGF receptors is noted in the remaining kidney without evidence of change in other organs (63). This has led to the suggestion of two types of HGF receptor, functional and non functional, both of which may bind HGF, but only one of which may be activated by such binding (63).

Figure 8.1 Human liver (normal) stained with monoclonal antibody DO-24

Acetone fixed, frozen liver tissue. Primary antibody DO-24 culture supernatant. Detection by alkaline phosphatase-anti-alkaline phosphatase method. Haematoxylin counterstain.

Hepatocyte membranes are stained, consistent with the presence of membrane bound HGF receptor.

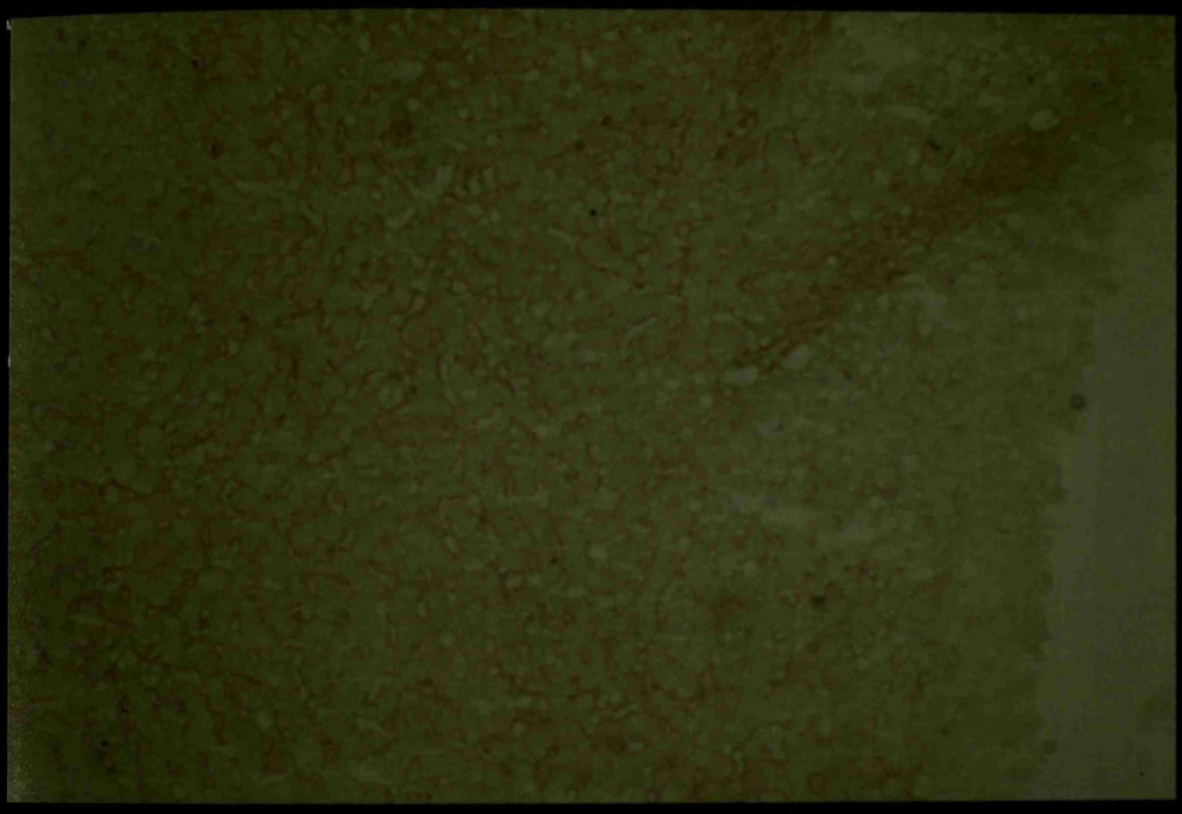


Figure 8.2 Human liver from a patient with cirrhosis stained with monoclonal antibody DO-24

Acetone fixed, frozen liver tissue. Primary antibody; DO-24 culture supernatant. Detection by alkaline phosphatase-anti-alkaline phosphatase method. Haematoxylin counterstain.

Hepatocyte membrane staining is present, although less intense than that seen in histologically normal tissue.

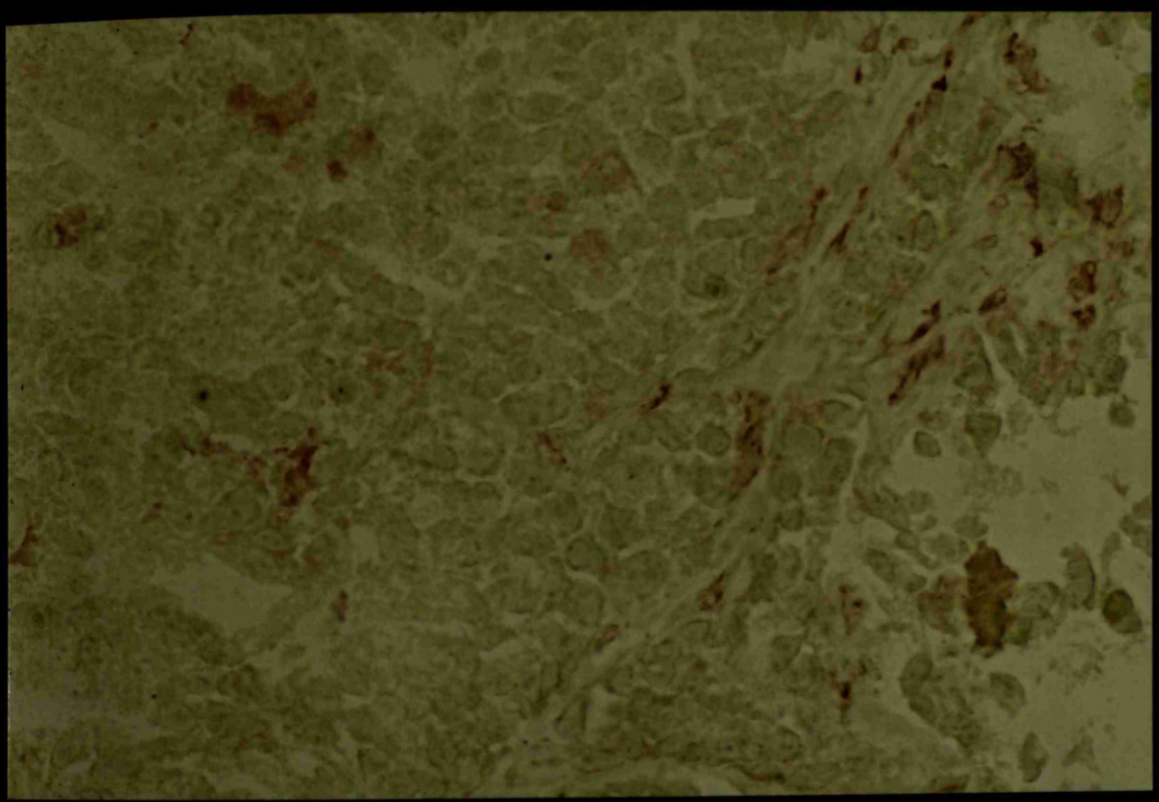


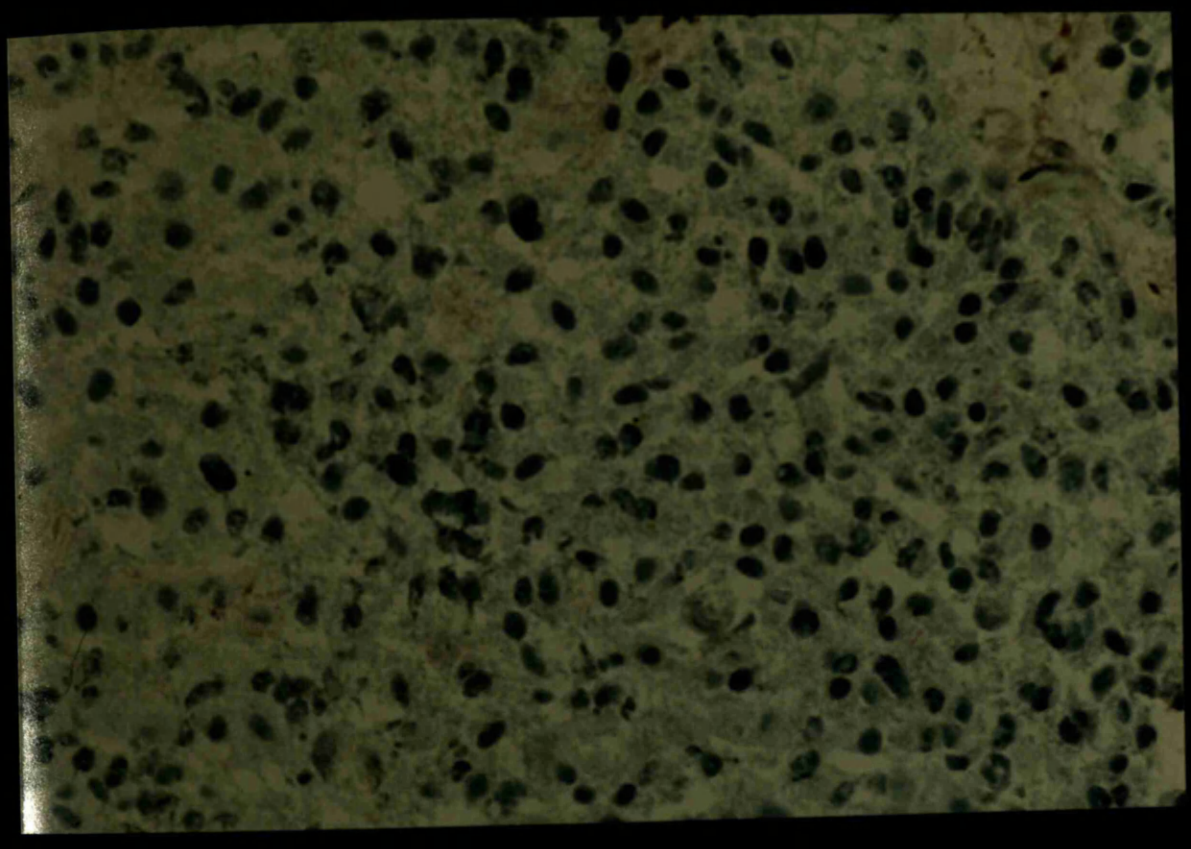
Figure 8.3 Human liver from a patient with hepatocellular carcinoma stained with monoclonal antibody DO-24

Acetone fixed, frozen liver tissue. Primary antibody; DO-24 culture supernatant.

Detection by alkaline phosphatase-anti-alkaline phosphatase method.

Haematoxylin counterstain.

No hepatocyte membrane staining is demonstrated.



However, there is also evidence of increased hepatic expression of c-*met* gene during liver regeneration in rats following carbon tetrachloride treatment (190). Similarly, studies using epithelial cell lines *in vitro* have demonstrated low levels of expression of c-*met* in confluent cell cultures, with high levels of expression in cells cultured at low density. Moreover, it has recently been shown that HGF itself may induce expression of c-*met* (192).

Reasons for this apparent disparity between HGF receptor gene expression and expression of HGF receptor may relate to the rate at which HGF receptor is internalised i.e. rapid generation of further mRNA in order to replace internalised receptor protein at the plasma membrane of the cell.

Unfortunately, the staining produced with this antibody was of low intensity with considerable background signal and limited availability of the antibody precluded large scale study of c-*met* expression. The original report of c-*met* localisation using DO-24 described the use of a fluorescein labelled secondary antibody. This may allow clearer definition of membranous staining than the alkaline phosphatase-anti-alkaline phosphatase method, since the exquisite sensitivity of the immunofluorescence of the technique allows identification of very small quantities of antigen within a tissue section. Unfortunately, in immuno-fluorescence, the signal is viewed against a dark background in which little histological detail is discernible. The alkaline phosphatase method was chosen since in this work I was attempting to relate the staining pattern of c-*met* to the histological findings in each biopsy.

CHAPTER 9. Hepatocyte Growth Factor *In vivo* Inhibition Studies

9. INTRODUCTION

9.1 Cytokine and Growth Factor Inhibition

Considerable evidence has accumulated in favour of the hypothesis that HGF is a mediator of hepatic regeneration following injury (Chapter 1 section 1.7.6). However, the hypothesis has not been clearly tested. A number of agents are implicated in the hepatic regenerative response. Examples in which the activity of each agent is neutralised include tumour necrosis factor alpha (inhibition by neutralising antibody) (193), catecholamines (α_1 adrenergic receptor blockade by prazosin) (138) and oestrogen (inhibition by tamoxifen) (142).

In a similar fashion, the biological effects of other cytokines have been investigated by experiments in which inhibition is induced by administration of neutralising antibody (194,195). To help define the significance of the contribution made by HGF to the hepatic regenerative response I sought to inhibit the effect of HGF *in vivo* by administration of neutralising antibodies to HGF.

In this study two test antibodies were used, a monoclonal (D9), raised against a synthetic 11 aminoacid peptide based on rat HGF (see page 141) and a rabbit polyclonal antibody raised against a synthetic 14 kDa protein based on rat HGF (38986, as described in Chapter 4) with appropriate control antibodies. Prior to undertaking HGF inhibition studies *in vivo* I attempted to assess antibody suitability using *in vitro* HGF inhibition with primary isolated rat hepatocytes in culture. Isolated hepatocytes were cultured in the presence or absence of HGF which had been preincubated with test antibody or control. DNA synthesis was assessed by incorporation into DNA of tritiated thymidine. Unfortunately, as a result of technical difficulties the assay described here involved only one antibody and could not be successfully repeated in the time remaining for the project.

In the *in vivo* study the antibodies were administered before and after partial hepatectomy and the animals killed at 24 and 72 hours following surgery. One hour prior to death bromodeoxy-uridine (BrdU) was administered. Liver

regeneration was assessed by histology and DNA synthesis determined by BrdU uptake by liver cells.

9.2 Materials and Methods

Materials - see page 141.

Rabbit polyclonal antiserum to HGF peptide rHGFβ121 and normal preimmune rabbit serum 951 see Chapter 5, section 5.4. In each case, antibody was purified from culture supernatant or serum by caprylic acid/saturated ammonium sulphate, dialysed against PBS and filter sterilised.

9.2.1 Methods - *In vitro* HGF inhibition study

9.2.1.1 Hepatocyte isolation

Hepatocytes were isolated from an adult male Wistar rat (350g) using the collagenase perfusion method of Seglen (196). Animals were maintained in 12:12 light/dark cycle and given laboratory and water *ad libitum*. All solutions, glassware and tubing were sterilised prior to use in the perfusion. The rat was anesthetised (4% halothane, 800ml/min nitrous oxide, 800ml/min oxygen) and laparotomy performed. The portal vein was cannulated and the superior and inferior vena cava divided. Calcium free buffer was used to perfuse the liver (via portal vein cannula) for at least 12 minutes.

The pump was switched to collagenase buffer and this was recycled allowing reperfusion of the liver for 12-15 minutes until the liver softened. The liver was removed from the animal and transferred to warm washing buffer wherein the liver capsule was removed and cells released with scissors. The cell suspension was filtered through sterile gauze and then cells washed by pelleting by microfuge (Microcentaur: Fisons Scientific Equipment) at 500rpm for two minutes and resuspended in 30ml PBS. This step was repeated and the final cell pellet resuspended in Williams medium E supplemented with 10^{-9} M insulin, 10^{-9} M dexamethasone, 2mM glutamine, 50 units/ml penicillin, 50μg/ml streptomycin and 5% fetal calf serum. Cell numbers were determined in a haemocytometer and

viability was calculated by trypan blue (0.05%) exclusion. Cell viability was greater than 80%.

9.2.1.2 Hepatocyte culture

The isolated hepatocytes were diluted with medium to give appropriate cell numbers for culture. The cells were plated at 6 x 10^4 cells per well on Costar 24 well culture plates. The cells were incubated at 37°C in a humidified atmosphere of 95% air/5% carbon dioxide for a period of 20 hours. At this stage the medium was changed to Williams medium E containing additives as above but without fetal calf serum. To this medium had been added combinations of HGF and antibody as described below. Three wells were used for each combination.

	HGF	Antibody	Antibody Dilution
(1) Medium	-	-	-
(2) Medium	10ng/ml	-	-
(3) Medium	10ng/ml	D9	1/50
(4) Medium	10ng/ml	D9	1/100
(5) Medium	10ng/ml	D9	1/500
(6) Medium	10ng/ml	Control	1/50
(7) Medium	10ng/ml	Control	1/100
(8) Medium	10ng/ml	Control	1/500

The hepatocytes were cultured for a further 24 hours, then DNA synthesis was determined by pulse labelling cells with [^{3}H] thymidine (2.5µCi/well) for a further 6 hours in the presence or absence of 10mM hydroxyurea. The cells were washed once with cold PBS and solubilised with 500µl PBS containing 2% (volume/volume) triton X-100. After scraping the cells from the wells, they were transferred to 1.5ml Eppendorf tubes on ice. 500µl ice cold trichloroacetic acid (TCA) was added to each tube and the cells centrifuged at 13,000rpm ('Microcentaur' centrifuge) for 2 minutes at 4°C. The samples were washed twice with 200µl of 5% trichloroacetic acid and the final pellets resuspended in 200µl 1.5M tris base at 4°C. These samples were placed in glass scintillation vials

containing 5mls of scintillant (Ecocint) and 2mls of water and counted in a liquid scintillation counter (Packard Tri-Carb (B) 300C, Canberra Packard, Pangbourne, Berkshire, UK). The difference between the values for [^{3}H] thymidine present in acid precipitable material from cells incubated with and without hydroxyurea was taken to represent DNA synthesis. All results are expressed as the mean value of triplicate wells.

9.2.2 Methods - *In vivo* HGF Inhibition Study

9.2.2.1 Purification of monoclonal antibodies from culture supernatant

0.1M acetic acid was added to 20ml of culture supernatant to lower pH to 4.5. Neat caprylic acid was then added to a final concentration of 5% (volume/volume) and the solution mixed by vortexing for 5 minutes. Suspension centrifuged at 13,000rpm ('Microcentaur' centrifuge) for 7 minutes, pellets discarded and supernatant retained. An equal volume of saturated ammonium sulphate was added in aliquots and the solution mixed at room temperature for 2 hours. Solution centrifuged ('Microcentaur') at 13,000rpm for 3 minutes. A floating fatty layer formed on the surface of the solution, this was retained and dissolved in PBS and dialysed against several changes of PBS at 4°C overnight. After dialysis the solution was removed, filter sterilised and the protein content estimated by absorption of ultraviolet light at 280nm. Aliquots stored at -70°C until use.

9.2.2.2 Purification of polyclonal IgG from serum

Procedure as for monoclonal antibody until saturated ammonium sulphate step. After mixing with saturated ammonium sulphate for 2 hours at room temperature the solution was centrifuged at 13,000rpm ('Microcentaur' centrifuge) for 3 minutes. The pellet formed was reconstituted with PBS and the supernatant discarded. Solution containing pelleted material dialysed against PBS sterilised, protein content estimated then sterilised and stored as for monoclonal IgG.

9.2.2.3 *In vivo* inhibition

36 adult male Lewis rats (n=36, mean weight 250g) were housed with a 12:12 hour light/dark cycle and permitted *ad libitum* consumption of water and a standard rat pellet diet. After a one week equilibration period, the animals were separated into 6 groups of 6 animals identified as "A" to "F". Twelve hours prior to partial hepatectomy, groups A & E and groups B & F were injected intra-peritoneally with purified D9 and 3A1 antibodies respectively (dose 1.52mg/kg body weight). Similarly, rats in group "C" were injected intra-peritoneally with polyclonal antibody 38986 (dose 5.85mg/kg body weight) and those in group "D" received antibody 951 (dose 5.85mg/kg body weight). The animals underwent a 70% partial hepatectomy (4) under halothane anaesthesia in mid-morning. Each group received further intraperitoneal injections of antibody (D9 & 3A1; dose 1.52mg/kg body weight, 38986 & 951; dose 5.85mg/kg body weight) at 12, 36 and 60 hours post hepatectomy as appropriate. At 24 hours post hepatectomy groups C, D, E and F were killed by cervical dislocation. Groups A and B were killed at 72 hours post hepatectomy (see Table 9.1). One hour prior to death, bromodeoxyuridine (dose 50mg/kg body weight) was administered by intraperitoneal injection. Aliquots of liver tissue were placed in tissue fixative (mercuric chloride, buffered formalin, glutaraldehyde) and remaining liver tissues snap frozen in liquid nitrogen.

9.2.2.4 Bromodeoxyuridine (BrdU) immunocytochemistry

Liver tissue fixed in buffered formalin was routinely processed, paraffin embedded and 5 μm sections cut onto glass slides coated with 3-aminopropyltriethoxysilane. Sections were dewaxed to water and incubated in methanol and hydrogen peroxide (0.5%) at room temperature for 15 minutes to inactivate endogenous peroxidase. The sections were exposed to 0.1% porcine trypsin pH 7.8, followed by 2M HCl for 30 minutes at 37°C to denature DNA.

After incubation with 20% (volume/volume) normal rabbit serum in Tris buffered saline pH 7.6 at room temperature for 10 minutes primary antibody (B44

Table 9.1 HGF *in vivo* inhibition; animal groups antibodies and time intervals

Group	Antibody given	Specificity of antibody for HGF	Time interval between partial hepatectomy and killing (hours)	No of rats surviving surgery
1 A(1-6)	D9 monoclonal	YES	72	6
2 B(7-12)	3A1 monoclonal	NO	72	5
3 C(13-18)	38986 polyclonal	YES	24	6
4 D(19-24)	951 polyclonal	NO	24	6
5 E(25-30)	D9 monoclonal	YES	24	6
6 F(31-36)	3A1 monoclonal	NO	24	5

mouse anti-BrdU, diluted 1/40 in Tris Buffered saline containing 4% (volume/volume) normal rabbit serum) was added and sections incubated overnight at 4°C.

A secondary peroxidase-conjugated rabbit anti mouse immunoglobulin diluted 1/20 in Tris buffered saline containing 4% normal rabbit serum, was applied and incubated for 30 minutes at room temperature.

Labelled cells were detected using 3,3'-diaminobenzidine (DAB) substrate (0.1%) in presence of hydrogen peroxide (0.02%).

Sections were counterstained with Mayer's haematoxylin and mounted in DPX. One thousand nuclei were counted for each animal, 250 nuclei per field at x200 magnification using an E11A 19mm eyepiece graticule (Graticules Ltd, Towbridge, Kent, UK). Four fields were examined per slide, two centred on a portal tract and two centred on a hepatic vein.

9.2.2.5 HGF mRNA detection - *In situ* hybridisation

See Chapter 7, section 7.6.3 for methods.

9.3 Results

9.3.1 *In vitro* HGF Inhibition

Counts for tritiated thymidine were low but the stimulatory effect of human recombinant HGF was evident (ten-fold increase in DNA synthesis). Incubation with monoclonal antibody D9 inhibited this effect in a dose dependent manner (see Table 9.2). After 24 hours in culture, the characteristic hepatocyte morphology was clearly visible. Following administration of test antibodies a toxic effect was observed on the cells incubated with control antibody α66. These cells assumed granular, degenerate appearance and demonstrated very low levels of DNA synthesis at all antibody concentrations.

9.3.2 *In vivo* HGF Inhibition

Thirty four of the thirty six rats tolerated antibody administration, anaesthesia and partial hepatectomy without ill effect. One animal, no. 11, died under

Table 9.2 DNA synthesis by isolated rat hepatocytes in culture - effect of human recombinant HGF, monoclonal antibody D9 and control antibody

Culture of hepatocytes with 10ng/ml human recombinant HGF led to a ten-fold increase in tritiated thymidine incorporation (DNA synthesis). Antibody D9 inhibited this effect in a dose dependent manner. Wells containing control antibody demonstrated very low levels of DNA synthesis at all concentrations.

	[^{3}H] thymidine incorporation (cpm)
No HGF, No antibody	48
HGF (10ng/ml), No antibody	479
HGF (10ng/ml), D9 at 1/50	206
HGF (10ng/ml), D9 at 1/100	277
HGF (10ng/ml), D9 at 1/500	478
HGF (10ng/ml), control at 1/50	41
HGF (10ng/ml), control at 1/100	25
HGF (10ng/ml), control at 1/500	12

anaesthesia (anaesthetic death) and one animal, no. 35, died in the immediate post operative period.

No gross difference was evident between each group on examination of harvested liver. Histology of liver tissue confirmed marked proliferative activity in all groups. No direct toxic effects were seen in any group. Liver tissue from all animals showed considerable DNA synthesis.

The proportion of BrdU positive hepatocyte nuclei in partially hepatectomised rats (see Tables 9.3,9.4.9.5)

The overall proportion of BrdU positive cells (perivenular and periportal) showed highly significant variation between groups ($p<0.001$), all of the significance being attributable to the use of two different time intervals. The overall proportion of BrdU positive cells showed no significant differences among groups 3 to 6 ($p=0.345$ by one-way analysis of variance), or between groups 1 and 2 ($p=0.546$ by t-test).

The periportal to perivenular ratio of BrdU positive cells differed significantly from unity ($p=1.37\times10^{-5}$, single-sample t-test), but did not differ significantly among groups (Figures 9.1, 9.2, 9.3, 9.4, 9.5, 9.6).

Evidence of HGF gene expression in the liver was identified by HGF mRNA in situ hybridisation using pRBC-1 digoxigenin labelled riboprobe (Fig 9.7).

9.4 Discussion

9.4.1 *In vitro* HGF Inhibition Study

Low counts for tritiated thymidine were noted, this could be due to using a larger volume of medium per well, leading to dilution of tritiated thymidine prior to uptake. In addition, the hepatocytes were cultured on plastic without collagen coating of wells. Although cell adhesion had been satisfactory, it is known that coating growth surfaces with collagen leads to enhanced cell attachment and growth (197).

Subsequent attempts to culture rat hepatocytes included the use of wells coated with rat tail collagen type I.

Table 9.3 **Proportion of BrdU positive hepatocyte nuclei in partially hepatectomised rats (Labelling index; results expressed as a percentage of total nuclei)**

Rat	Group	Specific	Hours	PV1	PA1	PV2	PA2	PA+PV	PA/PV
1	1	1	72	4.8	5.2	2.8	4.0	4.2	1.21
2	1	1	72	4.0	11.2	5.6	11.2	8.0	2.33
3	1	1	72	7.2	10.0	6.0	12.8	9.0	1.73
4	1	1	72	8.4	10.8	12.0	8.0	9.8	0.92
5	1	1	72	11.2	12.0	11.2	9.6	11.0	0.96
6	1	1	72	12.4	13.2	7.6	8.8	10.5	1.10
7	2	0	72	4.0	11.2	5.6	11.2	8.0	2.33
8	2	0	72	3.6	3.6	3.2	2.8	3.3	0.94
9	2	0	72	18.0	11.6	9.6	16.8	14.0	1.03
10	2	0	72	11.6	7.6	6.8	8.4	8.6	0.87
11	2	0	72	*	*	*	*	*	*
12	2	0	72	2.4	6.4	2.4	1.6	3.2	1.67
13	3	1	24	25.6	44.0	30.8	35.2	33.9	1.40
14	3	1	24	42.0	51.2	51.2	45.2	47.4	1.03
15	3	1	24	46.4	60.0	42.8	67.2	54.1	1.43
16	3	1	24	29.6	48.4	22.0	34.4	33.6	1.60
17	3	1	24	50.0	40.8	46.8	47.6	46.3	0.91
18	3	1	24	14.8	56.4	32.4	44.0	36.9	2.13

Abbreviations; Rat: Individual identities. Group - see Table 9.1. Specific: 0 = antibody not specific for HGF. 1 = specific for HGF. Hours: Time interval between partial hepatectomy and killing. PV1: Labelling index of first perivenular field. PA1: Labelling index in first periportal field. PV2: Labelling index in second perivenular field. PA2: Labelling index in second periportal field. PA+PV: Overall labelling index for each animal. PA/PV: Ratio of periportal to perivenular labelling indices.

Table 9.3 (continued) **Proportion of BrdU positive hepatocyte nuclei in partially hepatectomised rats (Labelling index; results expressed as a percentage of total nuclei)**

Rat	Group	Specific	Hours	PV1	PA1	PV2	PA2	PA+PV	PA/PV
19	4	0	24	44.0	54.05	44.8	52.0	48.7	1.19
20	4	0	24	37.2	41.2	28.4	31.6	34.6	1.11
21	4	0	24	36.8	61.2	34.8	40.0	43.2	1.41
22	4	0	24	40.0	56.8	40.4	58.0	48.8	1.43
23	4	0	24	33.2	60.0	35.6	53.6	45.6	1.65
24	4	0	24	36.0	44.4	40.4	38.4	39.8	1.08
25	5	1	24	36.4	38.0	40.0	38.4	36.0	0.88
26	5	1	24	29.6	34.4	24.4	31.2	29.9	1.21
27	5	1	24	44.8	38.8	34.4	60.8	44.7	1.26
28	5	1	24	28.0	33.6	35.6	29.6	31.7	0.99
29	5	1	24	32.0	28.8	25.6	54.0	35.1	1.44
30	5	1	24	46.4	62.8	31.2	42.8	45.8	1.36
31	6	0	24	30.0	34.4	32.4	46.8	35.9	1.30
32	6	0	24	31.2	52.8	39.6	54.8	44.6	1.52
33	6	0	24	43.6	48.8	33.6	45.64	42.9	1.22
34	6	0	24	24.8	43.6	16.4	56.8	35.4	2.44
35	6	0	24	*	*	*	*	*	*
36	6	0	24	20.0	40.0	32.8	38.4	32.8	1.48

Abbreviations; Rat: Individual identities. Group - see Table 9.1. Specific: 0 = antibody not specific for HGF. 1 = specific for HGF. Hours: Time interval between partial hepatectomy and killing. PV1: Labelling index of first perivenular field. PA1: Labelling index in first periportal field. PV2: Labelling index in second perivenular field. PA2: Labelling index in second periportal field. PA+PV: Overall labelling index for each animal. PA/PV: Ratio of periportal to perivenular labelling indices.

Table 9.4 **BrdU hepatocyte labelling index in partially hepatectomised rats; descriptive statistics for all 34 animals**

	n	Mean labelling index	Median	S.D.	Minimum	Maximum
PV	34	25.87	28.50	14.58	2.40	48.40
PA	34	33.97	40.10	18.96	3.20	63.60
PA+PV	34	29.92	34.85	16.34	3.20	54.10
PA/PV	34	1.37	1.28	0.42	0.87	2.44

Abbreviations:
PV: Perivenular region labelling index
PA: Periportal region labelling index
PA+PV: Overall labelling index
PA/PV: Ratio of periportal to perivenular labelling indices
n: Number of animals
S.D.: Standard deviation

Table 9.5 BrdU positive hepatocyte nuclei in partially hepatectomised rats; descriptive statistics for the 6 groups of animals

	Group	n	Mean	Median	S.D.	Minimum	Maximum
PV	1	6	7.77	8.30	3.12	3.80	11.20
	2	5	6.72	4.80	4.73	2.40	13.80
	3	6	36.20	36.40	11.48	23.60	48.40
	4	6	37.63	37.00	4.24	32.80	44.40
	5	6	34.03	35.00	5.53	27.00	39.60
	6	5	30.44	31.20	7.16	20.60	38.60
PA	1	6	9.73	10.90	2.61	4.60	11.40
	2	5	8.12	8.00	4.68	3.20	14.20
	3	6	47.87	46.20	8.68	39.60	63.60
	4	6	49.27	51.80	8.56	36.40	57.40
	5	6	40.37	37.60	9.19	31.60	52.80
	6	5	46.20	47.20	6.23	39.20	53.80

Abbreviations:

PA+PV: Overall labelling index
PA/PV: Ratio of periportal to perivenular labelling indices
n = number of animals in each group
S.D.: Standard Deviation

Table 9.5(continued) BrdU positive hepatocyte nuclei in partially hepatectomised rats; descriptive statistics for the 6 groups of animals

	Group	n	Mean	Median	S.D.	Minimum	Maximum
PA+PV	1	6	8.75	9.40	2.47	4.20	11.00
	2	5	7.42	8.00	4.47	3.20	14.00
	3	6	42.03	41.60	8.44	33.60	54.10
	4	6	43.45	44.40	5.52	34.60	48.80
	5	6	37.20	35.55	6.63	29.90	45.80
	6	5	38.32	35.90	5.13	32.80	44.60
PA/PV	1	6	1.37	1.15	0.55	0.92	2.33
	2	5	1.37	1.03	0.62	0.87	2.33
	3	6	1.42	1.41	0.44	0.91	2.13
	4	6	1.31	1.30	0.22	1.08	1.65
	5	6	1.19	1.23	0.22	0.88	1.44
	6	5	1.59	1.48	0.49	1.22	2.43

Abbreviations:

PA+PV: Overall labelling index
PA/PV: Ratio of periportal to perivenular labelling indices
n = number of animals in each group
S.D.: Standard Deviation

Figure 9.1 DNA synthesis, demonstrated by BrdU uptake in liver tissue from rat No 2, killed 72 hours after partial hepatectomy (monoclonal antibody D9 administered intraperitoneally)

Formalin fixed, paraffin embedded liver tissue. Section stained with murine monoclonal anti-BrdU antibody. Secondary antibody; peroxidase-conjugated rabbit anti-mouse immunoglobulin. Substrate; diaminobenzidine tetrachloride (DAB) solution. Haematoxylin counter stain.

Scattered BrdU positive nuclei are present.

Top (a) x500

Bottom (b) x1200

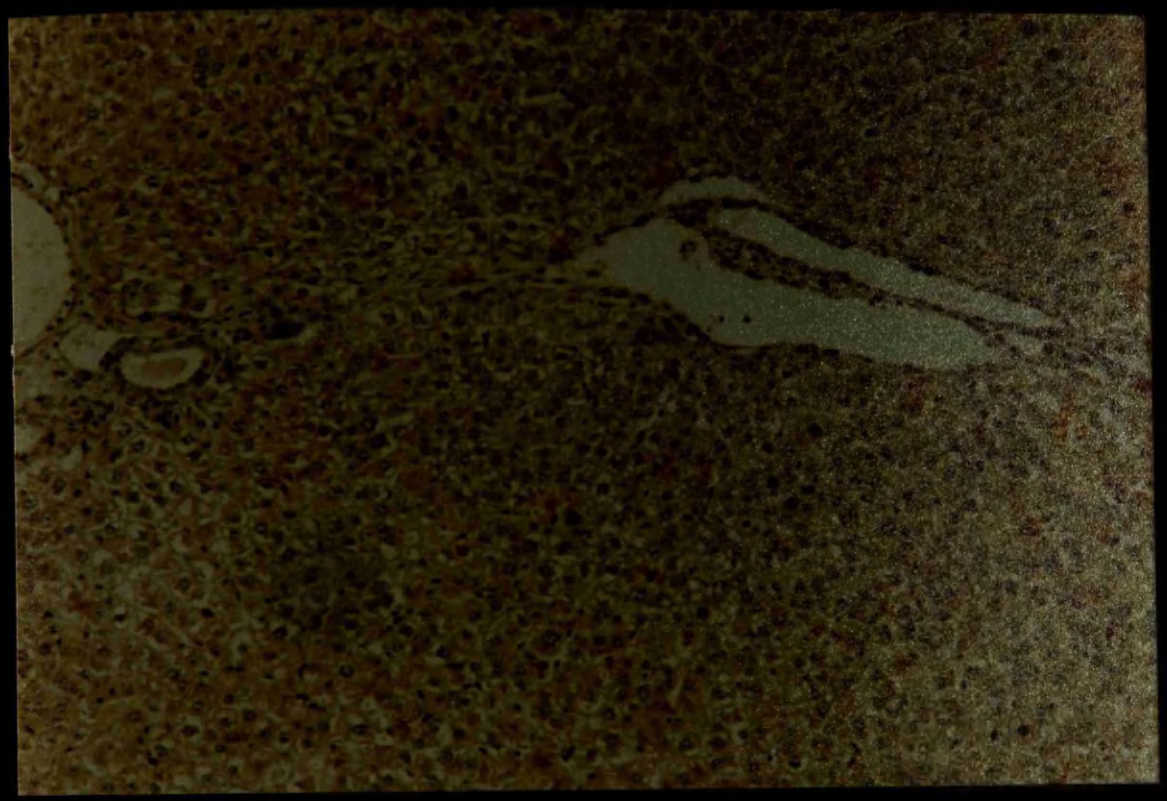


Figure 9.2 **DNA synthesis, demonstrated by BrdU uptake, in liver tissue from rat No 10, killed 72 hours after partial hepatectomy (monoclonal control antibody 3A1 adminstered intra-peritoneally)**

Formalin fixed, paraffin embedded liver tissue. Section stained with murine monoclonal anti-BrdU antibody. Secondary antibody; peroxidase-conjugated rabbit anti-mouse immunoglobulin. Substrate; diaminobenzidine tetrachloride (DAB) solution. Haematoxylin counter stain.

Scattered BrdU positive nuclei are present.

Figure 9.3 DNA synthesis, demonstrated by BrdU uptake, in liver tissue from rat No 15, killed 24 hours after partial hepatectomy (polyclonal antibody 38986 administered intraperitoneally)

Formalin fixed, paraffin embedded liver tissue. Section stained with murine monoclonal anti-BrdU antibody. Secondary antibody; peroxidase-conjugated rabbit anti-mouse immunoglobulin. Substrate; diaminobenzidine tetrachloride (DAB) solution. Haematoxylin counter stain.

A greater proportion of nuclei are stained positively for BrdU in this section compared to Figure 9.2.

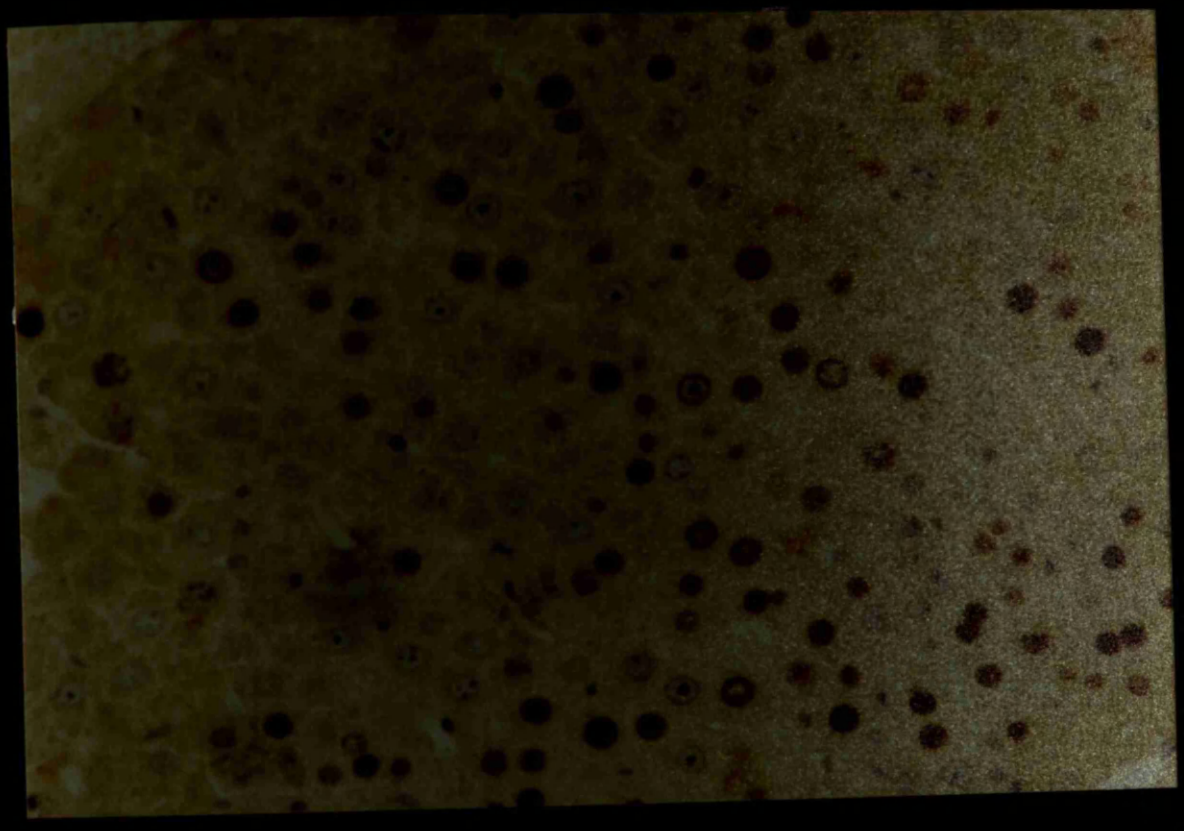


Figure 9.4 DNA synthesis, demonstrated by BrdU uptake, in liver tissue from rat No 22, killed 24 hours after partial hepatectomy (polyclonal control antibody 951 administered intra-peritoneally)

Formalin fixed, paraffin embedded liver tissue. Section stained with murine monoclonal anti-BrdU antibody. Secondary antibody; peroxidase-conjugated rabbit anti-mouse immunoglobulin. Substrate; diaminobenzidine tetrachloride (DAB) solution. Haematoxylin counter stain.

A large proportion of nuclei are stained positively for BrdU. A greater proportion of periportal nuclei demonstrate BrdU uptake than perivenular.

Top (a) x1200

Bottom (b) x500

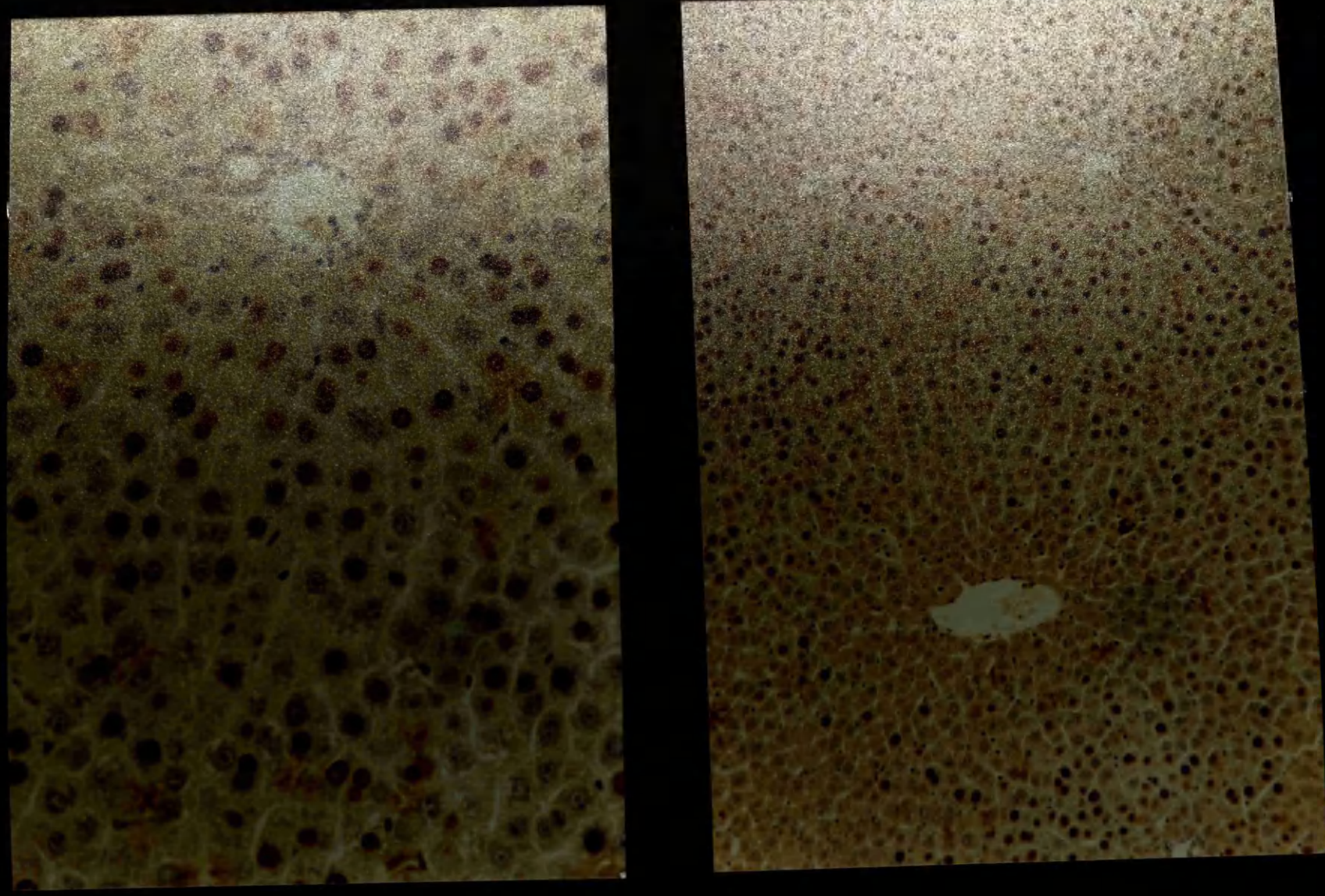


Figure 9.5 DNA synthesis, demonstrated by BrdU uptake in liver tissue from rat No 27, killed 24 hours after partial hepatectomy (monoclonal antibody D9 administered intraperitoneally)

Formalin fixed, paraffin embedded liver tissue. Section stained with murine monoclonal anti-BrdU antibody. Secondary antibody; peroxidase-conjugated rabbit anti-mouse immunoglobulin. Substrate; diaminobenzidine tetrachloride (DAB) solution. Haematoxylin counter stain.

BrdU positive nuclei are present diffusely the hepatic acinus, but are more abundant in the periportal region.

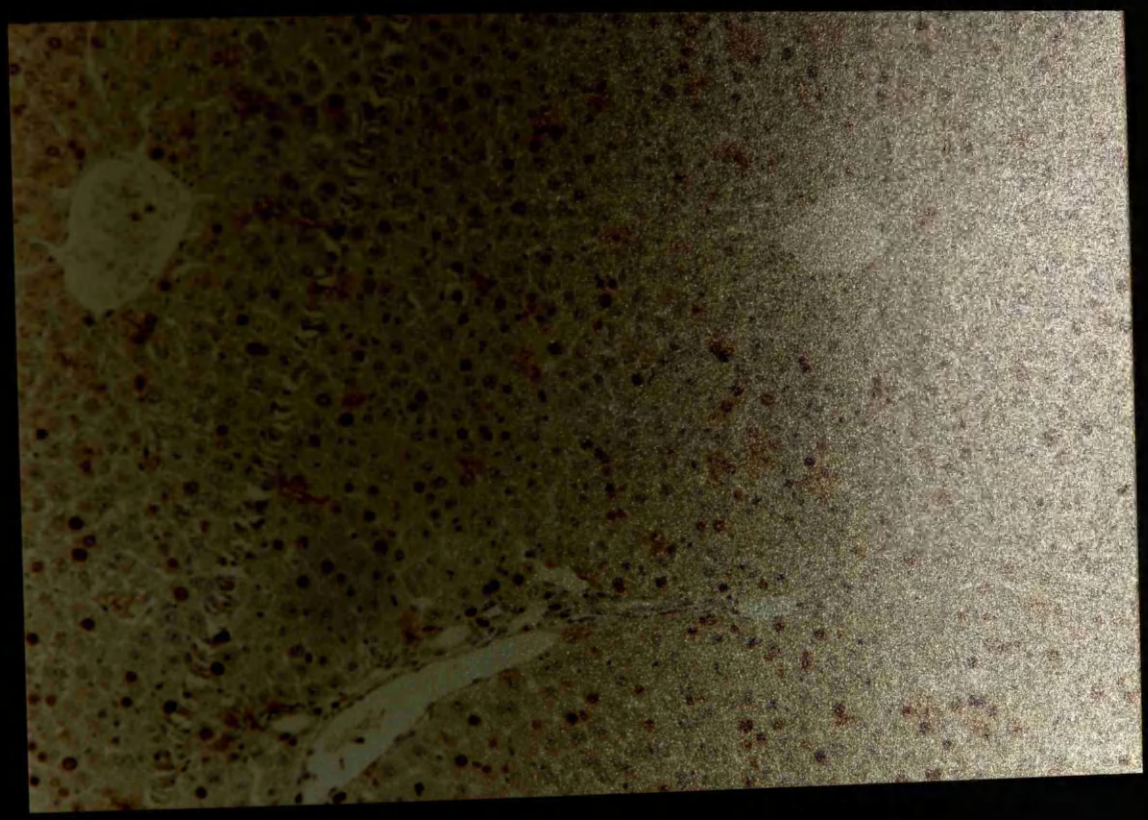


Figure 9.6 DNA synthesis, demonstrated by BrdU uptake, in liver tissue from rat No 34, killed 24 hours after partial hepatectomy (monoclonal control antibody 3A1 administered intra-peritoneally)

Formalin fixed, paraffin embedded liver tissue. Section stained with murine monoclonal anti-BrdU antibody. Secondary antibody; peroxidase-conjugated rabbit anti-mouse immunoglobulin. Substrate; diaminobenzidine tetrachloride (DAB) solution. Haematoxylin counter stain.

A large proportion of nuclei demonstrate BrdU uptake. A greater proportion of periportal than perivenular nuclei are stained positively for BrdU.

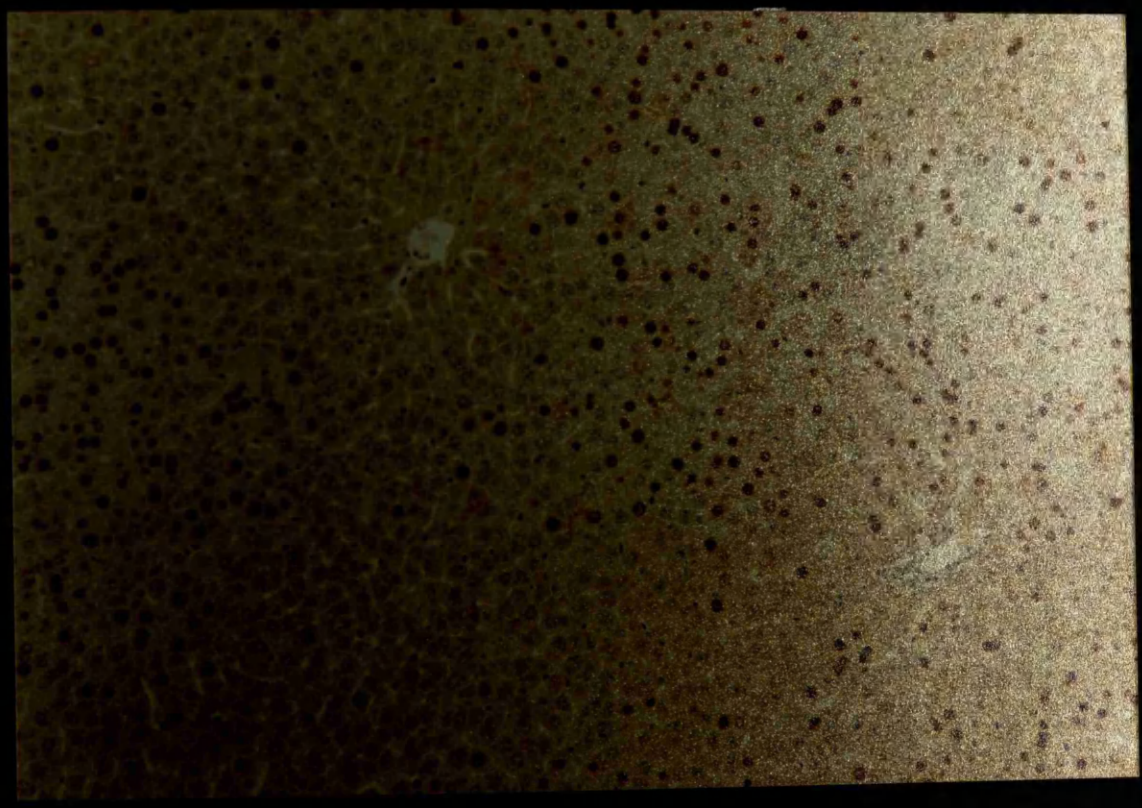
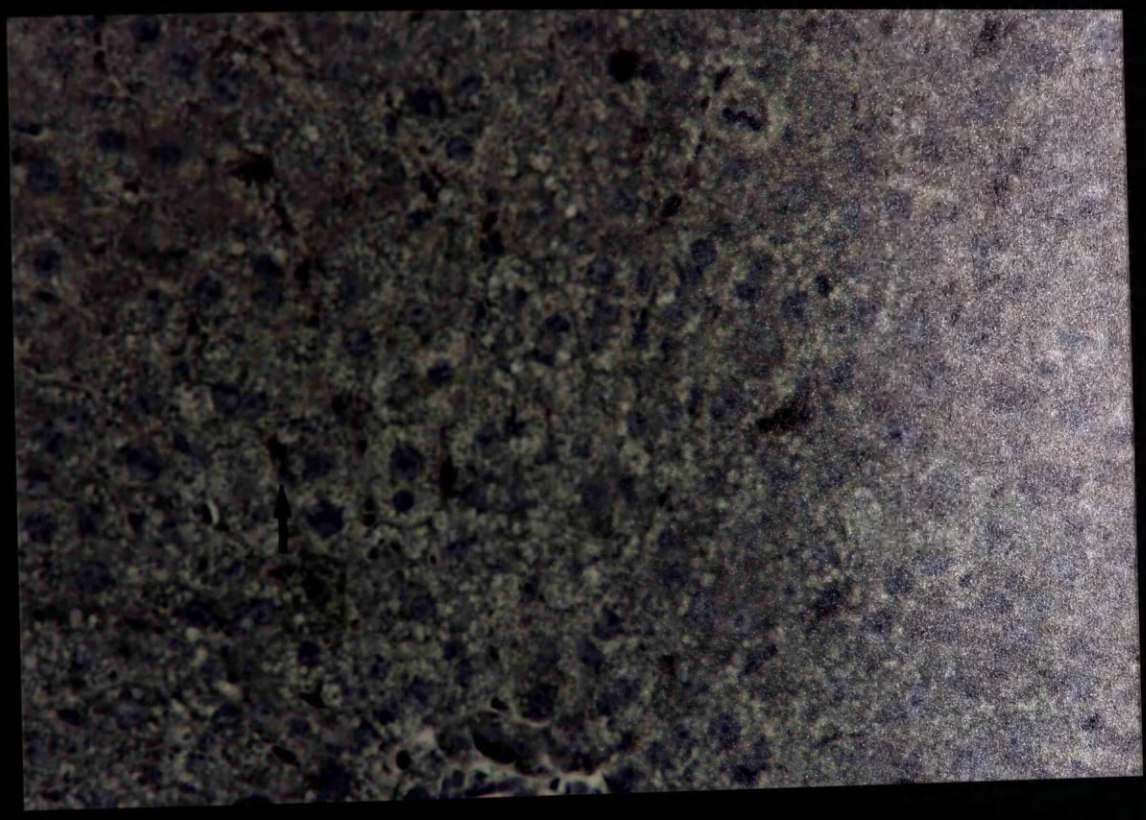


Figure 9.7 Expression of HGF mRNA in liver tissue from rat No 13, killed 24 hours after partial hepatectomy

Glutaraldehyde fixed, paraffin embedded liver tissue. *In situ* hybridisation using pRBC-1 probe, alkaline phosphatase conjugated anti-digoxigenin antiserum and NBT/BCIP substrate. Positive staining is present in cells corresponding to Ito cells.



The control antibody α66 is likely to have been an inappropriate choice for this study. Although the antibody is a murine monoclonal of the same class as D9 and is present in the form of culture supernatant, this antibody may cross react with rat hepatocytes. This may result in a direct toxic effect on the affected cells. Human recombinant HGF was used in this study since rat recombinant HGF was initially unavailable.

Several attempts were made to repeat these experiments, but as a result of technical difficulties these studies could not be completed. In one case collagen in a dilute acetic acid solution was used to coat the wells of the culture plates. Inadequate washing of the plates is the likely cause of a direct toxic effect on the hepatocytes. Two other attempts were marred by bacterial contamination of the cell cultures.

9.4.2 *In vivo* Inhibition

The aim of this study was to establish clearly the significance of HGF in the hepatic regenerative response following injury. The procedures of antibody administration, anaesthesia and partial hepatectomy were tolerated well by the animals with the exception of the two deaths described.

Liver tissue from all groups showed evidence of considerable DNA synthesis, demonstrated by BrdU uptake. A highly significant difference was seen between animals killed 24 hours after partial hepatectomy and those killed after 72 hours. Much greater levels of DNA synthesis were evident in animals killed at 24 hours, consistent with the phenomenon described in which the peak of DNA synthesis occurs at 24 hours (see Chapter 1, section 1.4.1). The demonstration that a greater proportion of periportal then perivenular nuclei were BrdU positive is also consistent with the known distribution of initial waves of DNA synthesis within the hepatic acinus (22).

No difference was seen between groups of animals given anti-HGF antibody (monoclonal or polyclonal) compared to animals given control antibody.

Possible reasons for a failure to demonstrate a difference between the groups may include -

(1) Use of antibodies which may not be capable of neutralising HGF *in vivo*.

(2) Antibodies may have been given in insufficient quantity, or the route of administration (intraperitoneal) may have hindered antibody access.

(3) Antibodies may neutralise circulating HGF without effect on HGF provided locally within the liver.

(4) Antibodies are neutralising HGF but HGF is not required for the initial waves of DNA synthesis within the liver.

On the basis of the methods used and results obtained, no further conclusion may be reached with regard to the role of HGF in DNA synthesis following partial hepatectomy.

Other approaches were considered including -

(1) administration of the naturally occurring HGF antagonist HGF/NK2 (56,57).

(2) administration of antisense oligonucleotides to inhibit translation of HGF from mRNA. However, neither of the two groups who have produced HGF/NK2 are currently able to synthesise sufficient quantities of protein for such experiments. Antisense oligonucleotides could not be used since targetting to the appropriate cells is currently not possible.

MATERIALS

Animals

Young adult male New Zealand white rabbits (Hyline Rabbits Ltd, Statham, Cheshire, UK).

BALB/C mice were supplied by Glasgow University Department of Biochemistry.

Adult male Wistar rats (350g body weight) were supplied by Glasgow University Department of Pharmacology.

Antibodies - monoclonal

D9, culture supernatant, to an 11 amino acid synthetic peptide based on rat HGF α chain. Supplied by Professor A D Burt, Royal Victoria Infirmary, Newcastle-upon-Tyne, UK.

A312-1, purified antibody to human recombinant HGF, IgG subclass 2a. The antibody reacts with human recombinant HGF but not with rat HGF or other growth factors (TGFα/β, EGF, plasmin, insulin, tPA, acidic or basic FGF). Supplied by Professor Toshikazu Nakamura, Osaka University School of Medicine, Osaka, Japan.

B25, B43 and A312-2, purified IgG subclass 2a, to human recombinant HGF, supplied by Dr P Godowski and Dr Filip Roos, Genentech Inc, California, USA.

Murine monoclonal antibody IgG subclass 2a CD72 to human B type lymphocytes (The Binding Site Ltd, Birmingham, UK).

DO-24 culture supernatant, to extracellular domain of c-*met*, characterised and supplied by Dr Maria Pratt, University of Turin, Turin, Italy.

Murine monoclonal IgG antibody 341, culture supernatant, to sheep urinary antigen, supplied by Miss Andrea Brown, Glasgow University Department of Biochemistry.

Monoclonal α66, culture supernatant, antibody to human hepatocyte antigen. Supplied by Dr K Hillan, Glasgow University Department of Pathology, Western Infirmary, Glasgow.

Murine monoclonal clone B44 anti-BrdU antibody (Becton Dickinson, Cowley, Oxford, UK).

Antibodies - polyclonal

Rabbit polyclonal antiserum, Lot No 201, to full length human recombinant HGF. The antiserum reacts with human recombinant HGF (α and β chains) but does not react with rat HGF. Characterised and supplied by Professor Toshikazu Nakamura, Osaka University School of Medicine, Osaka, Japan.

Alkaline phosphatase conjugated affinity purified swine anti-rabbit immunoglobulins, alkaline phosphatase conjugated affinity purified rabbit anti-mouse immunoglobulins, rabbit polyclonal affinity purified antibodies to murine immunoglobulins, murine monoclonal alkaline phosphatase-anti-alkaline phosphatase soluble complexes, peroxidase conjugated affinity purified swine anti-rabbit immunoglobulins and peroxidase conjugated rabbit anti-mouse immunoglobulins (Dakopatts, Denmark).

Peroxidase conjugated gamma chain specific goat antibodies to mouse immunoglobulins (Sigma Chemical Co Ltd, Poole, Dorset, UK).

Biotinylated sheep antibodies to rabbit immunoglobulins (The Binding Site Ltd, Birmingham, UK).

Sheep alkaline phosphatase conjugated antibodies to digoxigenin (Boehringer Mannheim Biochemica, Lewes, East Sussex, UK).

Cell Culture Reagents

DH5 and BL21 (DE3), *Escherichia coli* were supplied by the MRC Virology Institute, Western Infirmary, Glasgow.

SP20 plasmacytoma cells supplied by Glasgow University Department of Biochemistry.

2-YT broth, ampicillin, RPMI 1640 with L-glutamine, penicillin, streptomycin, William's medium E, glutamine and fetal calf serum (Gibco BRL, Paisley, UK).

Collagenase (Boehringer Mannheim Biochemica, Lewes, East Sussex, UK).

Isopropyl-β-D-thiogalactopyranoside (IPTG), hypoxanthine, thymidine, aminopterin, 2-mercaptoethanol, fetal calf serum, insulin, dexamethasone and hydoxyurea (Sigma Chemical Co Ltd, Poole, Dorset, UK).

Polyethylene glycol (PEG) 1500 (BDH Ltd, Warwick, UK).

Chemicals

Sodium dodecyl sulphate, acrylamide, N,N'-methylenebisacrylamide, ammonium persulphate, N,N,N',N'-tetramethyl-ethylenediamine (TEMED), glycine, isobutanol, glycerol, 2-mercaptoethanol, coomassie brilliant blue, glutathione, sodium thioglycolate, amido black, nitro blue tetrazolium (NBT), 5-bromo-4-chloro-3-indolylphosphate (BCIP), Freunds complete adjuvant, Freunds incomplete adjuvant, orthophenylenediamine (1,2-benzenediamine), trichloroacetic acid and bromodeoxyuridine (Sigma Chemical Co Ltd, Poole, Dorset, UK).

Bromophenol blue and triton X-100 (BDH Chemicals Ltd, Poole, Dorset, UK).

Acetic acid, caprylic acid and ammonium sulphate (BDH Laboratory Supplies, Merck Ltd, Leicestershire, UK).

Digoxigenin-II-UTP and all enzymes used for *in situ* hybridisation (Boehringer Mannheim Biochemica, Lewes, East Sussex, UK).

Glu-plasminogen (Enzyme Laboratories (Europe), Sketty, Swansea, UK).

Horseradish peroxidase-avidin D (HRP) conjugate (Vector Laboratories, Peterborough, UK).

Tritiated thymidine (Amersham, Aylesbury, UK).

'Marvel' powdered milk (Premier Beverages, Adbaston, Stafford, UK).

Chromatography Reagents

Glutathione sepharose 4B, heparin agarose (Pharmacia Biotech Ltd, Herts, UK).

Hydroxylapatite (Bio-Rad Laboratories Ltd, Herts, UK).

CNBr-activated sepharose 4B (Pharmacia LKB, Biotechnology AB, Uppsala, Sweden).

Molecular weight markers (SDS-PAGE)

'Electron' MW range 14,300-71,500 Da (BDH Chemicals Ltd, Poole, Dorset, UK).

Prestained molecular weight markers MW range 38,800-116,000 and unstained MW range 29,000-205,000 Da (Sigma Chemical Co Ltd, Poole, Dorset, UK).

'Rainbow' coloured prestained molecular weight markers MW range 14,300-200,000 Da (Amersham, Buckinghamshire, UK).

Plasmids

pGEX-2T 266 plasmid containing an 800 base pair cDNA fragment coding for a 26.7kDa peptide based on human HGFα-chain formed as a fusion with glutathione-s-transferase; pET-121 plasmid containing a 365 base pair cDNA fragment coding for a 14kDa peptide based on rat HGFβ-chain: These plasmids were prepared by Dr K Hillan and Dr C Preston, MRC Virology Institute, Western Infirmary, Glasgow. pGEX-2T (Pharmacia Biotech Ltd, Herts, UK); pET-8C (supplied by Dr C Preston).

pRBC-1 containing a 1.4kbase EcoRI rat HGF cDNA fragment coding for the 3' portion of the α-subunit, the β-subunit and a portion of the 3' untranslated region (supplied by Professor T Nakamura, Osaka University School of Medicine, Osaka, Japan).

hHGFα266. The PCR amplified cDNA fragment of 796 base pairs coding for part of the human α-subunit, generated as described in Chapter 2, was cloned into the BamHI site of the pGEM II2f plasmid (Promega, Southampton, UK).

'Polymorph prep' (Nycomed A.S. (UK) Ltd, Birmingham, UK) contains sodium metrizoate 13.8% (weight/volume) and dextran 500 8.0% (weight/volume).

Recombinant HGF

Human recombinant HGF - full length recombinant human HGF supplied by Dr P Godowski, Genentech Inc, California, USA. Full length recombinant rat HGF supplied by Professor T Nakamura, Osaka University School of Medicine, Osaka, Japan.

Scintillation fluid

Ecocint A scintillation fluid (National Diagnostics, Mannville, New Jersey 08835, USA).

Tissue fixatives

Glutaraldehyde, buffered formalin and mercuric chloride were provided by Glasgow University Department of Pathology, Western Infirmary, Glasgow.

Equipment, plasticware and disposables

Chromatography columns, length 42cm, diameter 3.3cm and length 40cm, diameter 2.2cm (Pharmacia Biotech Ltd, Herts, UK).

BioRad Mini-Protean II SDS-PAGE and blotting equipment (BioRad Laboratories, Hertfordshire, UK).

Large scale (20cm x 2cm) SDS-PAGE equipment (Scotlab, Coatbridge, UK).

'Atomix' MSE blender, Fisons Instruments, Crawley, Sussex, UK.

Conductivity meter CDM3 (Radiometer Copenhagen, Denmark).

Beckman DU-64 spectrophotometer, Beckman RIIC Ltd, Glenrothes, UK.

Jenway 3061 pH meter (Scotlab, Coatbridge, UK).

Petri dish, 5cm culture plates (Sterilin Ltd, Hounslow, Middlesex, UK).

Tissue culture 96-well plates 'Costar' (Northumbria Biologicals Ltd, Cramlington, UK).

Tissue culture 24-well plates (Costar UK Ltd, High Wycombe, Buckinghamshire, UK)

Dynatech microtitre/ELISA plates (Dynatech Laboratories, Chantilly, Virginia, USA).

Dialysis tubing, Visking (Medicell International Ltd, London, UK).

"Problott" transfer membrane (Applied Biosystems)

PVDF (Polyvinylidene difluoride) membrane (Millipore Corporation, Bedford, MA 01730, USA).

Hybond C extra-nitrocellular membrane (Amersham International plc, Buckinghamshire, UK).

Human tissue and serum

Placenta Fresh normal term placentas were obtained with the assistance of Dr John Kingdom, Glasow Royal Maternity Hospital.

Serum and liver biopsies

Serum from healthy volunteers and patients with various liver diseases was collected prospectively, with fresh frozen liver biopsies from August 1991 to June 1993. Full ethical approval was obtained from the appropriate local Ethical Committee prior to the collection of serum and liver biopsies (see Appendix 5).

Healthy Controls

Volunteers were recruited from staff in the University Department of Pathology, Western Infirmary, Glasgow. A number of volunteers were recruited from among patients of the University Department of Surgery, Western Infirmary following admission to surgical wards for minor elective surgical procedures e.g. groin hernia repair, varicose vein surgery.

Serum was obtained from 33 normal healthy controls, 11 male, 22 female, Mean age 43.0 years, Median 41.0, Range 55.0.

Disease Controls

A second control group was included, comprising patients admitted to the Coronary Care Unit of the Western Infirmary, Glasgow following an acute myocardial infarction confirmed by serial electrocardiographs and cardiac enzyme measurements.

Serum was obtained from 11 patients with acute myocardial infarction, 7 male, 4 female. Mean age 52.0 years, median 58.0, range 36.0.

Patients with Liver Disease

Serum was obtained from patients with liver disease attending outpatient Gastroenterology and Hepatology Clinics or following admission to medical or surgical wards at the following Glasgow hospitals: Southern General Hospital, Gartnavel General Hospital, Western Infirmary and Stobhill Hospital.

Additional, retrospective sera were obtained from patients with acute viral hepatitis from the Regional Virus Laboratory, Ruchill Hospital, Glasgow.

Venous blood was obtained by venepuncture from patients and volunteers, allowed to clot at room temperature and serum obtained by centrifugation at 1000rpm for 3 minutes and stored at -70°C until use.

Wherever possible, blood was obtained at the time of routine venepuncture. In those cases where separate venepuncture was required informed consent was obtained from each patient and the General Practitioner informed by letter of the patient's involvement in the study.

Serum was obtained from 221 patients with liver disease, 121 male, 100 female. Mean age 52.0 years, median 55.0, range 83.0. Table 6.2 illustrates the various conditions included.

Liver Biopsies

Liver biopsies were obtained at the time of diagnostic liver biopsy performed on patients admitted to the above hospitals. At the time of biopsy, a 3-5mm length of tissue was taken, immersed in 'Tissue Tek' OCT compound (Miles Inc Diagnostics Division, Elkhart, USA), then immediately snap frozen in liquid nitrogen. Biopsies were stored in liquid nitrogen. In addition, formalin fixed, paraffin embedded tissue from each case was available from the pathology department of each hospital.

Normal human liver tissue; fresh frozen normal human cadaver (organ donor) liver was supplied by Dr K Hillan, Department of Pathology, Western Infirmary, Glasgow.

Medical Records

In each case of liver disease medical records were obtained and examined. Information thus obtained was used to compile a database using the Paradox 3.5 program at the Western Infirmary PC cluster. In addition to standard biographical data, each record included clinical history and examination findings together with the results of investigations.

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Appendix 1

SDS-PAGE gels

Stacking gel; 4%, 0.125M Tris, pH 6.8

Reagent	Volume
dH_2O	6.1 ml
0.5M Tris Hcl, pH 6.8	2.5 ml
10% SDS (weight/volume)	100 µl
30% Acrylamide/0.8% N′ N′-bis-methylene acrylamide	1.3 ml
10% ammonium persulphate	50 µl
TEMED (N′ N′ N′ N-tetramethylenediamine)	10 µl

Stacking gel (protein sequencing) 5%, 0.125M Tris, pH 8.8

Reagent	
dH_2O	5.7 ml
0.5M Tris Hcl, pH 8.8	2.5 ml
10% SDS	100 µl
30% Acrylamide/0.8% bis-acrylamide	1.65 ml
10% ammonium persulphate	50 µl
TEMED	10 µl

Resolving gels

Reagent	15%	12.5%	12%	10%	7.5%
dH_2O	2.3 ml	3.15 ml	3.35 ml	4 ml	4.85 ml
1.5M Tris Hcl, pH 8.8	2.5 ml	2.5 ml	2.5 ml	2.5 ml	2.5 ml
10% SDS	100 µl	100 µl	100 µl	100 µl	100 µl
30% Acrylamide/0.8% bis-acrylamide	5 ml	4.2 ml	4 ml	3.3 ml	2.5 ml
10% ammonium persulphate	100 µl	50 µl	50 µl	50 µl	50 µl
TEMED	5 µl	5 µl	5 µl	5 µl	5 µl

Appendix 2

SDS-PAGE and Western blotting buffers

Electrophoresis buffer

25mM Tris
250mM glycine
0.1% SDS

Upper electrophoresis buffer (protein sequencing, 4x)

12 µg Tris base and 0.8 g SDS in 150 ml dH_2O, titrated to pH 6.8 with 6N HCl, dH_2O added to a final volume of 200 ml.

Lower electrophoresis buffer (protein sequencing, 4x)

36.34 g Tris base and 0.8 g SDS in 150 ml dH_2O, titrated to pH 8.8 with 6N HCl, dH_2O added to a final volume of 200 ml.

Pre-run electrophoresis: Glutathione solution, 10mM (350 µl) added to 70 ml upper electrophoresis buffer.

Sample loading buffer: (non reducing)

dH_2O	4 ml
0.5M Tris-HCl, pH 6.8	1 ml
Glycerol	800 µl
10% SDS	1.6 ml
0.05% bromophenol blue	200 µl

For reducing conditions, 50 µl β-mercaptoethanol added to 1 ml loading buffer. Samples diluted with an equal volume of loading buffer and heated at 95°C for 4 mins prior to use.

Western Blot Transfer buffer: 2.52g $NaHCO_3$
0.954g Na_2CO_3
600ml Ethanol made up to 3L with dH_2O

CAPS Transfer buffer (blot transfer buffer for protein sequencing):
10mM 3-[cyclohexylamino]-1-propansulphonic acid, 10% (volume/volume) methanol; pH 11.0

Appendix 3

Staining solutions/chromogens

Coomassie Blue stain solution

500 ml 100% methanol
100 ml glacial acetic acid
400 ml dH_2O
2.5 g Coomassie brilliant blue

Coomassie Blue destain solution

500 ml 100% methanol
100 ml glacial acetic acid
400 ml dH_2O

Amido Black stain solution

Amido Black 0.1% solution in 45% methanol and 10% acetic acid

Amido Black destain solution

2% acetic acid in 90% methanol

NBT/BCIP: Nitro-Blue-Tetrazolium/S-Bromo-4-chloro-3-Indolyl Phosphate

Stock solutions:

NBT: 70% Dimethylformamide containing 7.5% nitroblue tetrazolium

BCIP: 100% Dimethylformamide containing 5% 5-bromo-4-chloro-3-indolyl phosphate

NBT/BCIP: 100 mM Tris, pH 9.5, containing 0.44% Stock solution NBT and 0.33% stock solution of BCIP

Fast Red: 20 mM naphthol AB-TR, 10 mM Fast Red TR, 15mM Levamisole in veronal acetate buffer pH 9.2

Appendix 4

ELISA solutions and buffers

Coating buffer 0.1M $NaHCO_3$

8.4 g $NaHCO_3$ dissolved in 1 Litre dH_2O

Phosphate Buffered Saline (PBS) x 20 stock solution

320 g NaCl, 48.4 g K_2HPO_4 and 13.6 g kH_2PO_4 dissolved in 1.5 Litre dH_2O (boiling),

made up to 2 Litres with dH_2O

Blocking solution: PBS containing 3% (weight/volume) Bovine Serum Albumin

Washing solution: PBS containing 0.025% (volume/volume) Tween 20

Sample dilent: PBS containing 0.5% (volume/volume) Tween 20 and 0.5%

(weight/volume) Bovine Serum Albumin.

Orthophenylenediamine substrate solution

Twenty-five millilitres 0.2M citrate adjusted to pH 5.0 with Potassium hydroxide made up to 50ml with dH_2O orthophenylenediamine (100 mg) dissolved in solution and 25µl of 31% H_2O_2 added immediately prior to use.

Appendix 5

Approval for these studies was obtained from the appropriate local Ethical Committee.

Western Infirmary, Glasgow, G11 6NT Tel 041-339 8822

Please Quote Ref: 90/75
JKF/NS

HOSPITAL ADMINISTRATION

25 April 1990

Professor R MacSween
Department of Pathology
Western Infirmary

Dear Professor MacSween

THE ROLE OF HAPATOCYTE GROWTH FACTOR IN LIVER REGENERATION AND DISEASE

I refer to the above and write to confirm that at the Ethical Committee meeting held on 17 April 1990 full Ethical approval to the proposed study was given. The Committee asked me to point out, however, that Ethical approval is given to this particular study only and that any intention to use any of the tissue for alternative studies would require a separate approach for Ethical approval.

Yours sincerely

JOHN K FLOOD
Secretary
Ethical Committee

Western Infirmary, Glasgow, G11 6NT Tel. 041 339 8822

HOSPITAL ADMINISTRATION

Please Quote Ref: 91/144
JKF/CD

2 October 1991

Dr K Edward McLaughlin
Pathology Department
WESTERN INFIRMARY

Dear Dr McLaughlin

HEPATOCYTE GROWTH FACTOR - SERUM LEVELS IN HEALTH DISEASE

I refer to the above and I am pleased to advise that ethical approval to the proposed study was granted at the meeting of the West Ethical Committee held on 17 September, 1991, but this was subject to your agreement to modify the patient consent to include the standard General Practitioner notification clause. I would appreciate, for our files, a copy of the amended consent taking account of this point.

Yours sincerely

JOHN K FLOOD
Secretary
Ethical Committee

Appendix 6

In-situ hybridisation solutions/buffers

Pre hybridisation buffer

2 x SSC/50% formamide

hybridization buffer	volume
2M Tris pH 7	5 µl
100x Denhardts	1.25 ml
20 x SSC	1 ml
Formamide	5 ml
20% SDS	0.25 ml
50% Dextransulphate	0.5 g
Salmon sperm DNA (10 mg/ml) denatured and sonicated	0.25 ml
DEPC treated H_2O	2.25 ml
	10 ml

Digoxigenin buffer 1
12.7 g Tris Hcl
2.36 g Tris base
8.77 g NaCl
makes 1 Litre, pH to 7.5 at 20°C

Digoxigenin buffer 3
12.6 g Tris base
5.84 g NaCl
10.16 g $MgCl_26H_2O$
Tris base diluted in950 ml H_2O and pH adjusted to 9.5 after addition of salts, pH checked (9.5) and volume made up to 1 Litre

Appendix 7

Rat Hepatocyte Isolation Buffers

Calcium free buffer (x 10)

41.5 g NaCl
2.5 g KCl
12.0 g HEPES
dissolved in 500 ml dH_2O pH adjusted to 7.4

Collagenase buffer (x 10)

9.75 g NaCl
1.25 g Kcl
6.0 g HEPES
dissolved in 250 ml dH_2O pH adjusted to 7.4
Collagenase (60 mg) added to 120 ml collagenase buffer prior to use

Washing buffer (x 10)

16.3 g NaCl
1.0g Kcl
4.8 g HEPES
dissolved in 200 ml dH_2O pH adjusted to 7.4, then 2.45 ml
1M $CaCl_2$ solution added