Proliferation and Maturation in Developing Human Liver

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List of Abbreviations.

α-1-AT	alpha-1-antitrypsin
Ab	Antibody
AFP	Alpha-fetoprotein
ANOVA	Analysis of variance
BMP	Bone Morphogenetic Protein
CPSI	Carbamoyl Phosphate Synthase I
CRP	C-reactive protein
DMEM	Dulbecco's Modification of Eagle's Medium.
DNA	Deoxyribonucleic Acid
DPPIV	Dipeptidyl-peptidase IV
EGF	Epidermal Growth Factor
ELISA	Enzyme-linked immunosorbent assay.
FACS	Fluorescence Activated Cell Sorting
FCS	Fetal Calf Serum
FGF	Fibroblast Growth Factor
FITC	Fluorescein Isothiocyanate
γGT	γ Glutamyl transferase.
GR	Glucocorticoid receptor
HBSS	Hank's Balanced Salt Solutiom
HGF	Hepatocyte Growth Factor
HRP	Horse Radish Peroxidase
IL-6	Interleukin 6
IL-6RE	IL-6 Response element
MAb	Monoclonal Antibody
mRNA	messenger Ribonucleic Acid
MTT	Thiazylol Blue Tetrazolium
NF κB	Nuclear Factor kappa B
OSM	Oncostatin M
PBS	Phosphate-buffered saline
PE	Phycoerythrin
PEPCK	Phosphoenolpyruvate carboxykinase
QC	Quality Control
Ra-H	Rabbit Anti-Human
RT-PCR	Reverse Transcriptase – Polymerase Chain Reaction
SEM	Standard error of the mean
Shh	Sonic hedgehog
SNBTS	Scottish National Blood Transfusion Service
T3	Tri-iodothyronine
TBPA	Thyroxine Binding Prealbumin
TGFα	Transforming Growth Factor α
TNFα	Tumour Necrosis Factor α
WME	William's Medium Eagle

Declaration

This thesis and the studies described herein are the unaided works of the author except where acknowledgement is made by reference. No part of this work has been accepted for any other degree, nor is any part being submitted concurrently in candidature for another degree.

Ian S. Currie
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Abstract

In the United Kingdom, the number of patients with liver failure awaiting transplantation now exceeds the capacity of the nation's donor pool, with the result that 20% of patients now die on the waiting list. The future for the treatment of liver failure lies in part with cell-based therapies, in which liver support may be provided on a short-term basis by biological liver-assist devices, or in which sufficient mass of liver tissue may be transplanted into patients to reverse liver failure as a graft. However, cell therapies are at a preliminary stage, due to a lack of basic understanding as to how human liver cells can be made to divide and to undergo functional maturation in vitro.

In order to understand how human liver cells can proliferate and differentiate in vitro, a culture system was developed to support second trimester fetal liver cells. Having characterised the culture system, and demonstrated viable hepatocytes after seven days in vitro, experiments were carried out to determine which circulating endocrine stimuli might initiate morphologic and functional maturation in the developing hepatocytes. Cells were then incubated with growth factors and cytokines and subject to two-colour flow cytometry to assess which cell fraction might proliferate in vitro. Finally, urea metabolism and protein secretion were assessed in the presence and absence of glucocorticoid and different growth factors, to assess the interactions of these various stimuli at a functional level.

The results showed that glucocorticoid alone brought about functional maturation in terms of increased protein secretion, with significant increases observed in α-fetoprotein, fibrinogen and α-1-antichymotrypsin secretion. This represented increased secretion per cell, as there was no effect of glucocorticoid on cell number. However, incubation with growth factors and cytokines showed that EGF stimulated cellular proliferation. This proliferation occurred within a primitive epithelial fraction, positive for cytokeratin 18, but negative for fibrinogen. Final experiments showed that EGF and HGF had modest stimulatory effects on urea synthesis. By contrast, KGF reduced urea synthesis by channelling ammonia into anabolic pathways. With regard to protein secretion, EGF inhibited fibrinogen and α-1-antichymotrypsin secretion, whereas, tumour necrosis factor inhibited fibrinogen alone. All of these observations were made only in the presence of dexamethasone.

These data show that a satisfactory method for fetal liver cell culture was developed. This model demonstrated that proliferation of liver epithelial cells was stimulated by EGF, whereas functional maturation of fetal liver cells could be brought about by exposure to glucocorticoid. Various growth factors and cytokines had modest effects on urea and protein secretion, but only in the presence of glucocorticoid. These experiments have provided new insights into the maturational and proliferative signals in developing human liver. These data

provide a frame of reference from which to develop cell-based therapies for the treatment of liver failure in clinical practice.

Chapter 1.

Review of the literature.

Section 1. Overview

1.1 Introduction

The presence of a functioning liver is central to homeostasis, and liver failure is ultimately incompatible with long term survival. Liver transplantation is presently the definitive treatment for acute and chronic liver failure in humans. However, the current supply of donor livers is insufficient to meet demand and the mortality rate whilst awaiting transplantation is presently 20% in the United Kingdom. In order to address this deficiency, it is necessary to develop new strategies to treat liver disease.

As a basis for therapy, the developing liver may exhibit properties which provide advantages and challenges compared to mature liver tissue (Mahli et al.,2002; Minguet et al., 2003). An understanding of the acquisition of liver mass and function during development is critically important to future cell-based therapies. To illustrate the functional capacities of the developing liver, a brief overview of fetal liver function is presented in this section.

1.2 The roles of the developing liver

Liver development provides the fetus with the necessary biochemical machinery to ensure homeostasis at the start of independent life. As the site for synthesis and storage of glycogen, the liver ensures that the fetus does not succumb to irretrievable hypoglycaemic injury during birth. Immediately after birth, hepatic gluconeogenesis provides the metabolic substrate to maintain viability until feeding is established. Bile secreted by the liver enables fat to be absorbed from milk taken into the gut, and the re-packaging and distribution of fats around the body is managed by the liver (Davenport, 1982).

Protein catabolism, particularly in the context of gluconeogenesis, results in the production of ammonia, a highly toxic by-product. The liver renders this potentially lethal agent harmless by incorporation into urea (Rodwell, 2000). Foreign substances, toxins and drugs, are rendered hydrophilic by liver oxidation and conjugation, and are harmlessly eliminated in bile and by the kidneys. Many such toxins, and numerous physiological molecules, are transported in blood bound to albumin, the major plasma protein, which is synthesised by the liver. A great many plasma proteins present at lesser concentrations subserve similarly important functions, as evidenced by uncommon but devastating deficiency diseases.

During development, and at parturition, the fetal liver ensures haemostasis by providing many of the coagulation factors in the blood, and many of the counter-

regulatory anti-coagulation factors. The synthesis of a large subset of these molecules requires vitamin K, a fat-soluble vitamin, the absorption of which is which is mediated by bile salts secreted by the liver (Davenport, 1982).

1.3 Rationale for studies of fetal liver.

Having reviewed the functionality of the fetal liver, it is necessary to justify whether studies of liver may be better addressed using fetal or adult liver cells. The developing liver may possess properties quite different to the adult organ. Proliferation of progenitor hepatocytes provides adequate functional liver mass at term, a process which may be reproducible in vitro, so providing the cellular basis for a liver support device, or the material for cell transplantation. Similarly, an understanding of the mechanisms which lead to hepatocyte proliferation and differentiation could lead to new treatments to prevent progression of liver disease, so avoiding or delaying liver transplantation. Furthermore, an understanding of biliary development may assist in therapies of transplant cholangiopathy, in which 'vanishing bile ducts' leads to intractable cholestasis and the need for re-transplantation.

Fetal tissues in general are recognised to show viability and proliferation in vitro, and function may be retained or improved with time in culture (Currie et al., 1994; Mahli et al., 2002), which contrasts with strictly short-term experiments in adult liver cells (Wigmore et al., 1997). However, there are no data which describe the behaviour of fetal liver cells in vitro. The introduction of

cell-based therapies in liver disease presently lacks any supportive data as to the functional attributes of developing liver cells. Knowledge of the mechanisms subserving proliferation and differentiation will be key to successful cell therapy.

1.4 Summary

The developing liver may possess many different competencies, the understanding of which renders this organ of central importance to cell-based therapies for liver disease. Whilst studies of adult human liver are not promising as material for cell therapy, insight into the developing capabilities of this organ may provide the required scientific background to develop life-saving novel treatments.

In order to understand how developing liver cells proliferate, and how they acquire their metabolic and synthetic competencies, this thesis set out to investigate novel properties of human fetal hepatocytes. The literature review describes salient evidence from published work, and the subsequent chapters describe the experiments which address the key issues of developing liver function in fetal human liver.

Section 2.

Introduction to liver development.

2.1 Embryogenesis.

Gastrulation, which results in the formation of the three embryonic layers of ectoderm, mesoderm and endoderm from the inner cell mass of the blastocyst, occurs between days 16 and 22 after fertilisation in the human. This establishes the overall body plan and irrevocably determines which cells in general will give rise to different tissues of the adult. Whilst cells of the blastocyst inner cell mass are totipotent (embryonic stem cells), those of the trilaminar embryo display lineage restriction, though a considerable degree of plasticity remains in many lineages until later in development.

2.2 Organogenesis of the liver.

The primordial liver can be defined anatomically during the third week of gestation, where it appears as an outgrowth of the ventral aspect of the distal foregut epithelium (Severn, 1971). The embryonic endodermal layer forms the hepatic diverticulum which penetrates the septum transversum as a series of proliferating fronds. The septum transversum is the mesenchymal layer lying between the vitelline duct (in free communication with the lining of the gut), and the cardiac mesoderm and is shown in stylised form in Figure 1.1.

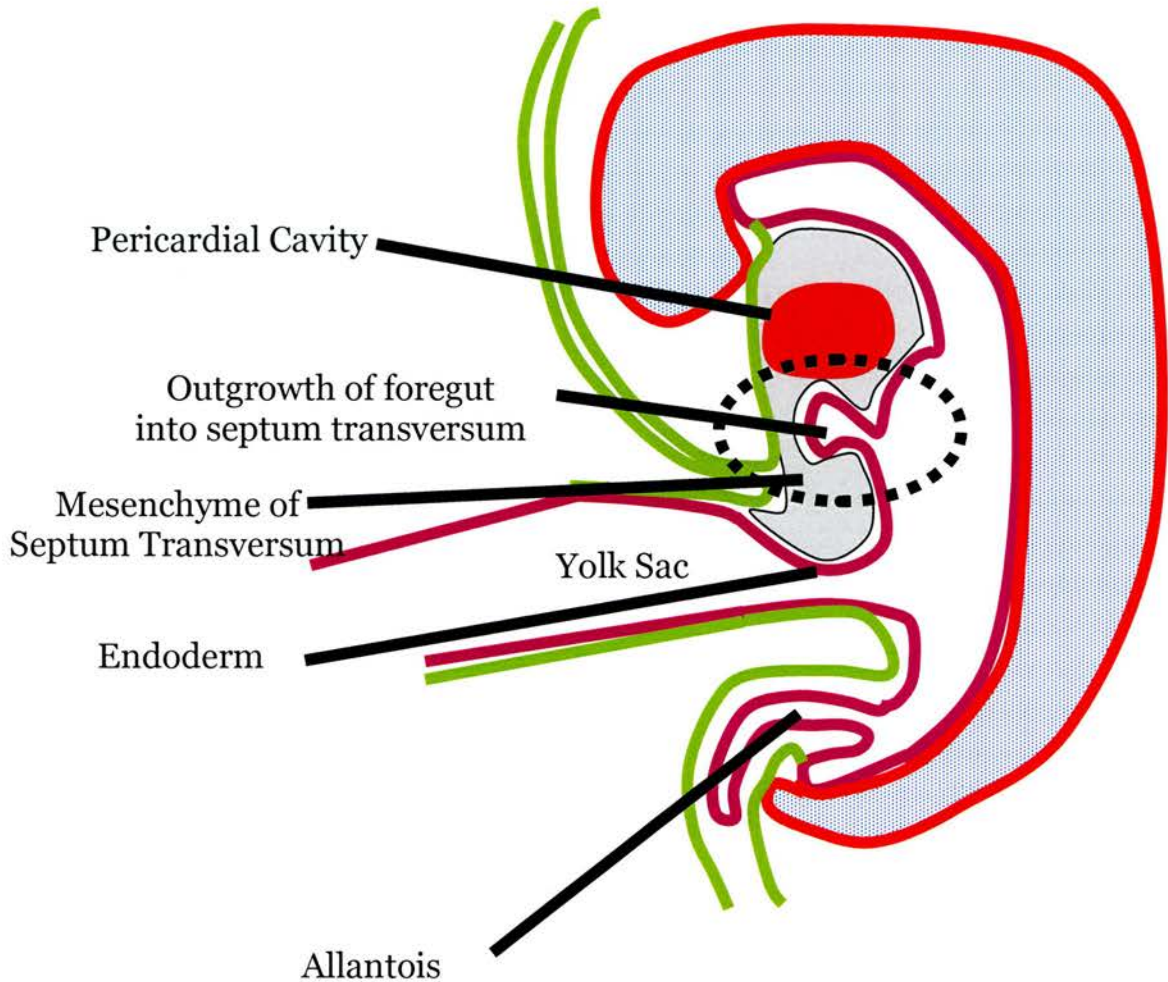


Figure 1.1. Outgrowth of foregut endoderm to form the presumptive liver. The human embryo is shown in sagittal section, corresponding to week 4 of development. The hepatic diverticulum is an out pouching of the endoderm growing into the septum transversum, directed by inductive signalling from this structure and from the immediately adjacent cardiac mesoderm. The hepatic diverticulum gives rise to the liver and to the extrahepatic biliary tree, including the common bile duct and gallbladder.

The interactions between the cardiac mesoderm, the septum transversum and the foregut endoderm provide an induction signal, triggering differentiation along the liver pathway, rather than pancreatic differentiation which is the endodermal default pathway.

Initially, proliferation of hepatoblasts, which are by definition the epithelial cells present prior to differentiation of the intrahepatic biliary tree, is accompanied by progressive enlargement of the presumptive liver, but with little anatomical sophistication until the biliary tree begins to develop at week 11. The steps which mediate liver cell proliferation and differentiation of the biliary tree are poorly understood, particularly so in the human.

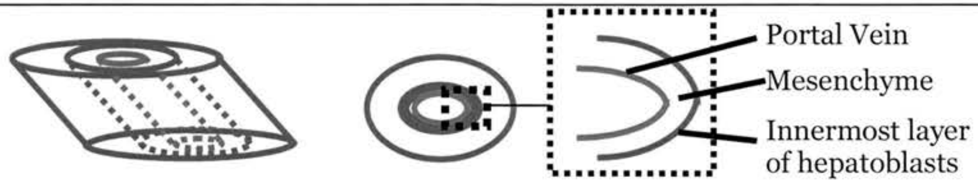
2.3 Development of the biliary tree

In human fetuses of 5mm crown-rump length, corresponding approximately to the end of the 4th week of development, liver tissue in the hepatic diverticulum is homogeneous, without evidence of either primitive intrahepatic bile ducts or hepatic arterial inflow. The caudal and cephalic regions of the hepatic diverticulum are embryologically distinct, and whilst the cephalic region interacts with the septum transversum mesenchyme to give rise to liver parenchyma, the caudal region gives rise to gall bladder, cystic duct and extrahepatic bile duct. Between the 4th week and 6th weeks of gestation, the extrahepatic bile duct develops from a primitive hollow tube a few cells thick into the definitive extrahepatic bile duct (Tan and Vijayan, 2001). By contrast,

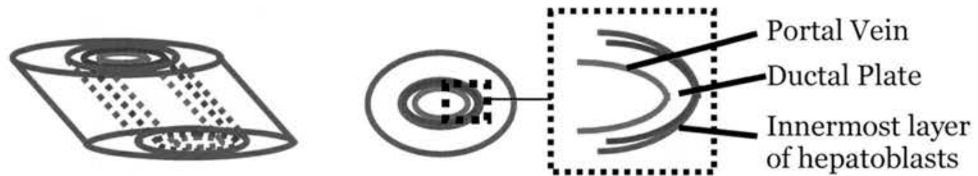
the ductal plate only begins to differentiate at the porta hepatis around 6-7 weeks developmental age (Figure 1.2) and extends throughout the liver subsequently (Ruebner et al., 1990). Remodelling of the ductal plate first occurs at the porta hepatis around week 11, such that the sleeve-like single cell layer which surrounds the portal tracts is reduplicated. This is followed by the formation of primitive tubules which will become the definitive intra-hepatic bile ducts (Crawford, 2002) . These develop progressively throughout the liver between weeks 11 – 16, and are connected to the extrahepatic biliary tree at the hilum from the very earliest developmental stage. Thus, the liver is competent to secrete bile from weeks 11-12 onwards. As development proceeds, the ductal plate degenerates, leaving successively fewer biliary tubules in each portal tract, until at birth one or perhaps two are left in each tract alongside the portal vein at birth.

2.4 Vasculature of the developing liver

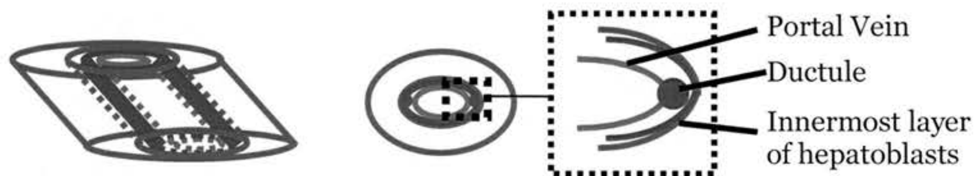
Vascular channels arise in the 3rd week of embryonic development, where precursor haemangioblasts give rise to endothelium and primitive blood cells (oligopotent haematopoietic stem cells). These lacunae of developing blood cells and endothelial cells are initially scattered and isolated, and have been described as 'blood islands'. They are found throughout the developing embryonic and extra-embryonic tissues. Extension of these islands, and fusion of their endothelial linings, gives rise to the primitive vasculature of the embryo. The



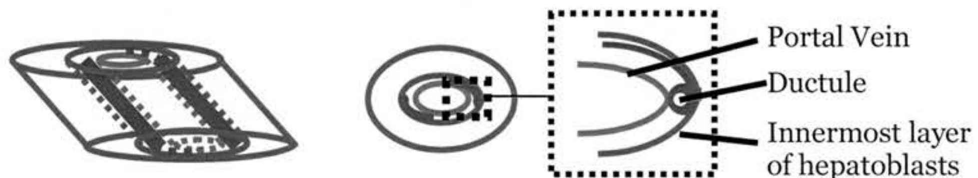
A. The primitive liver contains portal tract (vein alone) and hepatoblasts.



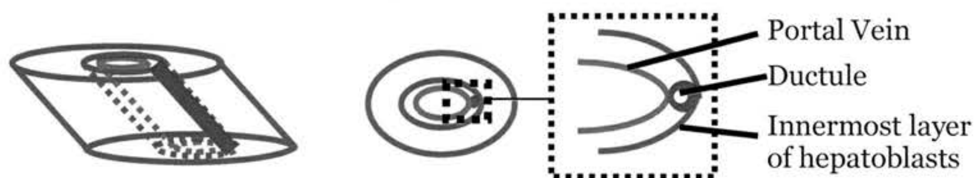
B. The ductal plate arises within the innermost layer of hepatoblasts.



C. Primitive tubules arise within the ductal plate and invade portal tract



D. The ductal plate degenerates, and the tubules develop lumina.



E. The ductal plate and excess tubules degenerate completely to leave one ductule per tract at birth.

Figure 1.2. The developmental anatomy of the biliary tract.
Prior to week 6 in development, the parenchymal cells are homogeneous hepatoblasts. Cytokeratin 19 expression becomes increasingly localised around the portal tract, indicating the morphogenesis of the ductal plate. The ductal plate reduplicatates, and from this double layered concentric structure arise multiple ductules. These are deleted during subsequent development to leave one ductule per tract at the time of birth.

subsequent ontogeny of the blood vessels related to the liver is complex, involving the vitelline veins, the umbilical veins and the hepatic veins.

2.4.1 The liver sinusoids

The vitelline veins drain blood from the yolk sac to the sinus venosus, the common venous channel of the primitive heart tube. Prior to entering the sinus, the vitelline veins pass through the septum transversum. The liver cords growing within the septum induce the formation of a dense plexus of veins, arising from the vitelline veins, which will ultimately form the hepatic sinusoids (Figure 1.3). This plexus is formed by vitelline vein-derived angioblasts proliferating within the septum transversum, probably in response to the immediate proximity of the presumptive liver cells. This observation invokes the presence of potential angioproliferative signals from hepatoblasts, and hepatoproliferative signals from angioblasts, which are as yet unidentified. Ultimately, the close approximation of these endothelial cells with hepatoblasts will result in the liver sinusoids, lined by endothelium, containing proliferating haematopoietic cells, and in immediate contact with hepatocytes. Although the accepted wisdom is that haematopoiesis proper begins in the paraaortic clusters in the splanchnopleuric mesoderm at week 6 which then seed the liver, it remains entirely possible that haematopoiesis is established during the formation of the vitelline capillaries and subsequent sinusoid proliferation. Similarly, it is possible that hepatic haematopoiesis occurring in adults as a

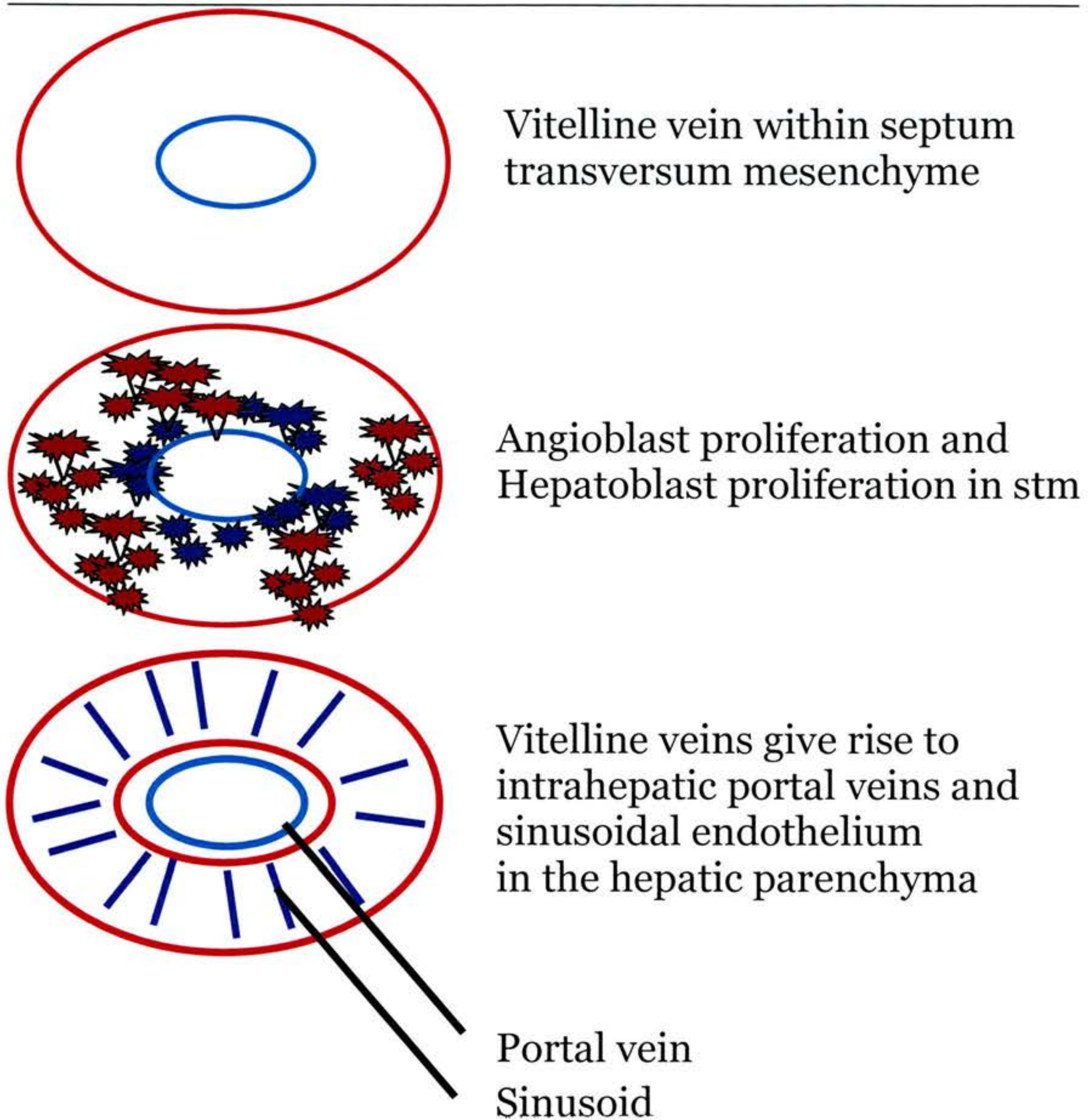


Figure 1.3. The development of sinusoidal endothelium from vitelline veins. Stm; septum transversum mesenchyme. Angioblasts are shown in blue, hepatoblasts in red. As the hepatoblasts proliferate within the septum transversum, angioblast proliferation occurs in parallel. Evidence suggests a bidirectional proliferative signalling mechanism. Cords of hepatoblasts intertwine with plates of endothelial cells, giving rise to the mature structure in which all hepatocytes have one face immediately adjacent to sinusoidal endothelium.

result of a marrow disorder may reflect a re-awakening of long-dormant haematopoietic stem cells resident in the liver since the early embryonic stage.

2.4.2 Portal venous system.

The developmental anatomy of the portal venous system is complex. In the early embryo, the right and left vitelline veins initially pass within the wall of the yolk sac, then within the mesenchyme of the embryo, through the septum transversum, and on to the sinus venosus. As the proximal components of these structures become involved in the development of the hepatic sinusoids, the main venous channels are forming a coarse anastomotic network around the duodenum. This undergoes further development, along with the rightward rotation of the duodenum, such that selected branches become dominant and others are lost. The result of these developmental events is the formation of the portal and superior mesenteric veins from the right vitelline vein and the splenic and inferior mesenteric veins from the left vitelline vein.

2.4.3 Umbilical veins

The right and left umbilical veins drain initially into the sinus venosus after passing through the septum transversum. In the early embryo, they are entirely separate from the developing liver sinusoids. With time, branches develop which join the hepatic sinusoids, contributing ever greater inflow to the developing liver. Subsequently, the original channels draining to the sinus

venosus become less and less significant until they are lost, and both the right and left umbilical veins drain only to the hepatic sinusoids.

The extrahepatic right umbilical vein degenerates completely, and the left establishes drainage through the liver, via the ductus venosus, which is initially formed by a confluence of vitelline vein capillaries. The ductus venosus enters the primitive left hepatic vein to drain to the inferior vena cava

In the adult, the left umbilical vein is observed as an obliterated remnant, the ligamentum teres, in the free edge of the falciform ligament which joins the left branch of the portal vein. The ductus venosus remnant forms the ligamentum venosum, passing between the left branch of portal vein and the inferior vena cava, lying in the groove between the caudate lobe and the left lobe of the liver.

2.4.4 Hepatic veins and inferior vena cava.

The drainage channels for the sinusoids in the early liver are the right and left hepato-cardiac channels. These are derived from the original drainage of the right and left vitelline veins into the sinus venosus. As the sinus venosus undergoes anatomical maturation, there is a generalised shift of venous drainage from a bilateral primitive pattern, to a right sided, mature pattern. In fact, the extrahepatic component of the left hepato-cardiac channel undergoes complete involution, and all the sinusoidal blood, plus that from the ductus venosus, drains through the right hepato-cardiac channel. This structure ultimately

forms the hepatic inferior vena cava, receiving blood from the hepatic veins, which are derived from the right and left vitelline systems.

2.4.5 Hepatic artery

The early fetal liver contains no branches of hepatic artery within the portal tracts. Within the portal tract mesenchyme, in parallel with the developing biliary system, capillary loops begin to develop from progenitor cells, reminiscent of the original development of the primitive embryonic circulatory system from haemangioblasts. These nascent capillary loops extend and fuse with their neighbours, around the time that the ductal plate arises between weeks 6-11 of development. These demonstrate the same pattern of anatomical maturation as the biliary tract, in that vessels are first seen at the hilum and then appear in a wave spreading from hilum to periphery as development progresses.

The development of the hepatic arterial inflow is coupled to the maturation of bile ducts, such that the mature hepatic artery complement in each portal tract is observed just around the time that the intrahepatic biliary radical has formed from the degenerating ductal plate. Abnormalities of ductal plate maturation, such as in the various congenital ductal plate malformations, are invariably associated with abnormal hepatic arterial complement in the portal tracts.

The developing hepatic arterial branches drain to the hepatic sinusoids, such that mixing of arterial and portal venous blood occurs here. In post-natal stages,

this results in blood of higher oxygen tension present in the region closest to the portal tract (zone 1), and blood of lowest oxygen tension closest to the central veins (zone 3). Of course, in the fetus, sinusoidal blood contains inflow from the umbilical vein, which has the highest possible oxygen tension in the fetus. Admixture with hepatic arterial blood paradoxically lowers oxygen tension, however, it is still the case that blood closest to the portal tract contains the highest oxygen tension.

Section 3.

Cell biology of liver organogenesis.

3.1 Introduction

The following section is focused on the development of hepatic parenchymal cells, namely hepatocytes and cholangiocytes, how they are induced to proliferate, and the signals inducing their maturation.

Prior to the morphological development of the primitive liver, the foregut endoderm lies in close contact with the mesenchyme of the septum transversum, and immediately caudal to the developing heart (Figure 1.1). Inductive signals pass between the septum transversum mesenchyme, the cardiac mesoderm, and the gut endoderm. These interactions, which involve soluble molecules diffusing along gradients to act on target tissues, result in a change of gene expression in the target. In this way, the endoderm begins to express albumin and AFP, the earliest indication that an inductive signal has been received, which confers a liver phenotype on this part of the foregut endoderm (liver specification).

Subsequently, the endoderm preferentially invades the septum transversum and proliferates within this structure. Again, this is a response to inductive signals passing between the various structures. It is clear that this simple

morphological event is exceptionally complex in terms of the signalling required to produce this sequence of proliferation, differentiation and morphogenesis. The default pathway for this part of the foregut is to develop along a pancreatic lineage, and so failure of this signalling interplay will prevent the development of the liver, which is not compatible with survival. These signalling networks are complex; however, an understanding of these pathways is important when contemplating the mechanisms which control proliferation and differentiation in the developing liver. This section describes in detail the various signalling pathways which are active in the earliest stages of liver development.

3.2 Specification of the liver and restriction to an hepatic fate

During the 3rd week of human development, or at the 6 somite stage in mice (~8.5 days gestation; term 20 days), the ventral endoderm undergoes a specification event which irrevocably commits a sub-region to an hepatic fate. Data in the human are absent from the literature, however, classical transplantation studies with chick and mouse embryos have shown that the initial stimulus to cause hepatic gene activation and proliferation of the hepatic diverticulum originates in the immediately adjacent cardiac mesoderm (Le Douarin, 1975, Gualdi etal., 1996).

3.2.1 Fibroblast Growth Factors and the cardiac mesoderm

Fibroblast Growth Factors (FGFs) are critical mediators signalling hepatic specification in gut endoderm. Jung et al. (1999) reported in mouse that cardiac

mesoderm is immunopositive for FGFs 1, 2 and 8 in early development. At the 7-8 somite stage, when liver specification occurs, all three are present. Notably, there was intense staining for FGF8 at the boundary of cardiac mesoderm and ventral endoderm, whereas FGF1 had just appeared at this stage. FGF receptors 1 and 4, which mediate the activities of FGF2 and FGF8b respectively, were expressed in the endoderm at the same time.

Incubation of endoderm isolated before hepatic specification with cardiac mesoderm or FGF1 at 50ng/ml, or FGF2 at 5ng/ml, but not FGF8b at either concentration, strongly induced expression of albumin in ventral endoderm. Confirmation of a physiological role for FGFs was obtained by adding soluble fusion proteins capable of binding FGFs to mesoderm-endoderm co-cultures; these experiments showed grossly attenuated albumin expression, in particular when FGF2 signalling was blocked.

Taken together, these data underscore a significant role for FGF2 in liver specification. Most likely, FGF2 released from cardiac mesoderm acts on FGF receptor 1 expressed by the ventral endoderm, a process which results in the stable expression of albumin by foregut endoderm and commits a specific area to an hepatic fate.

3.2.2 Fate determination and lineage restriction in the foregut endoderm

Elegant experiments have shown that the default pathway of differentiation in the endoderm destined to become liver is in fact pancreatic differentiation (Deutsch et al, 2001). Presumptive hepatic endoderm isolated from mouse embryos at the 4-6 somite stage and cultured in vitro for 2 days was demonstrated to express pdx1, a homeobox protein and one of the earliest known pancreas markers, but not albumin (Offield et al., 1996). By contrast, the same tissue isolated at the 7-8 somite stage stably expressed albumin in vitro, and not pdx1, confirming commitment to an hepatic lineage. The addition of fibroblast growth factor 2 or 8b, or cardiac mesoderm, to cultures of ventral foregut endoderm isolated at the 4-6 somite stage restricted the expression of pdx1, and promoted albumin expression, indicating the hepatic lineage specification induced by FGF release from cardiac mesoderm.

These observations corroborate other studies, showing that FGF signalling from cardiac mesoderm is necessary and sufficient to induce liver formation in the ventral endoderm (Gualdi et al, 1996; Jung et al., 1999). Interestingly, exposure of the presumptive pancreatic endoderm to FGFs caused stable liver specification. This suggests a degree of plasticity of the early endoderm in terms of lineage restriction and tissue specification. Immunostaining co-cultures of cardiac mesoderm and ventral endoderm for pdx1 or albumin consistently demonstrated that physical proximity to the cardiac mesoderm resulted in absent pdx1 expression and abundant albumin expression (Deutsch et al., 2001).

Tissues distal to cardiac mesoderm however, expressed pdx1 and not albumin, demonstrating the physiological pattern of endoderm development into a ventral pancreatic bud.

3.2.3 Sonic Hedgehog

Sonic Hedgehog (Shh) has a well described role as a signalling molecule in endoderm differentiation. Shh is expressed by dorsal gut endoderm, where it prevents expression of pancreatic lineage. Signalling by the overlying notochord locally inhibits Shh expression, relieving Shh inhibition, and permitting expression of the dorsal pancreatic bud (Kim and Melton, 1998). The role of Shh in the expression of the ventral pancreatic bud has recently been clarified, and it relates closely to the lineage restriction events of the developing liver (Deutsch et al., 2001). In foregut endoderm explants, isolated before liver specification, Shh peptide is absent, which permits development towards a pancreatic fate. By contrast, incubation with cardiac mesoderm, or FGF2, results in widespread immunostaining for Shh, suggesting that the mesodermal FGF signalling inhibits the pancreatic lineage by inducing Shh expression. Addition of recombinant Shh peptide to endoderm explants prevented the expression of pdx1. However, it did not induce albumin expression. This component of liver specification invariably required FGF.

Taken together, these data show that signalling from cardiac mesoderm to foregut endoderm is central to correct tissue patterning during development.

FGFs, originating in the cardiac mesoderm, signal the gut endoderm to express Shh, which inhibits the endodermal pancreatic lineage. FGFs also instruct the endoderm to activate the liver sequence, such that albumin and AFP immunopositivity are the first detectable signs of liver specification. Foregut endoderm, which is not immediately adjacent to the cardiac mesoderm, does not express Shh and is therefore permitted to develop towards a pancreatic lineage. Furthermore, this foregut endoderm receives insufficient FGF signalling. Thus, the liver pathway is not pursued, and the pancreatic lineage, which is the default pathway, is activated.

3.2.4 Bone Morphogenetic Proteins

Recent work has raised the question that septum transversum mesenchyme may also play a significant role in the earliest stages of liver development. Previous studies had not specifically excluded the possibility that septum transversum mesenchyme had accidentally been included with cardiac mesoderm or endoderm cultures. To investigate this potentially confounding issue, Rossi et al. (2001) used a co-culture technique to investigate the role of the septum transversum and Bone Morphogenetic Proteins (BMPs) in the earliest stages of liver development Immunostaining showed that the cardiac mesoderm and the septum transversum were positive for BMP4 at the at the 8 somite stage of liver specification in mice (Rossi et al., 2001). A lacZ reporter construct at the BMP4 locus confirmed that BMP4 was expressed in the cardiac mesoderm and the

septum transversum mesenchyme, but not in endodermal tissues destined to become liver.

In order to determine the physiological role for BMPs, BMP4 null homozygotes were followed through the somite stages during which the presumptive liver tissues invades the septum transversum mesenchyme to establish the liver bud. The BMP4 null homozygotes showed a marked delay in the normal endodermal thickening which precedes invasion of the septum transversum by the hepatic diverticulum, and gross impairment of the proliferation and invasion steps which occur subsequently. Despite this, differentiation of the presumptive liver cells occurred, as albumin mRNA expression was detected within the endoderm. However, the level of expression was much lower than normal, possibly due to the smaller number of presumptive hepatoblasts observed in this study.

To examine the relevance of BMPs in the context of FGF-mediated liver specification by the cardiac mesoderm, co-cultures (mesoderm, septum transversum and ventral endoderm) were incubated with FGF2 to induce liver development. Co-cultures incubated with Noggin protein, a non-selective BMP antagonist which binds BMPs, in addition to FGF2 did not demonstrate albumin expression in 7 out of 9 experiments, strongly implicating BMPs in the earliest stages of liver specification. To address this key issue, further experiments showed that BMP2, 4, 7 or 9 at 200ng/ml, or BMP 2 at 50ng/ml, could overcome the noggin-mediated inhibition of albumin expression.

The mechanism by which BMPs might influence specification of hepatocytes has not been clarified, however, the presence of Noggin protein vastly reduced the expression of GATA4, a transcription factor essential for albumin synthesis. This suggests that BMPs provide a critical permissive input to the expression of GATA4, so enhancing the differentiation of the hepatocellular phenotype. Interestingly, these cells expressed pdx1, the early pancreatic marker, suggesting that the presumptive liver tissue had not received the appropriate induction signal and was demonstrating the default lineage pathway. All of these observations are depicted in Figure 1.4, overleaf.

The data presented in this section demonstrate the levels of complexity in the specification and lineage restriction of the early liver. Whilst FGF2 from the cardiac mesoderm is critically important to induce liver specification in the endoderm, and rescue this tissue from a pancreatic fate, BMP2 is also required to permit the normal development of the liver. Noggin protein, the BMP-binding antagonist, was able to prevent the earliest liver marker expression, even in the presence of FGFs. Subsequently, BMP4 null homozygotes showed that BMPs were critically important to the endodermal thickening and migration into septum transversum mesenchyme which occurs as a normal part of liver development after specification.

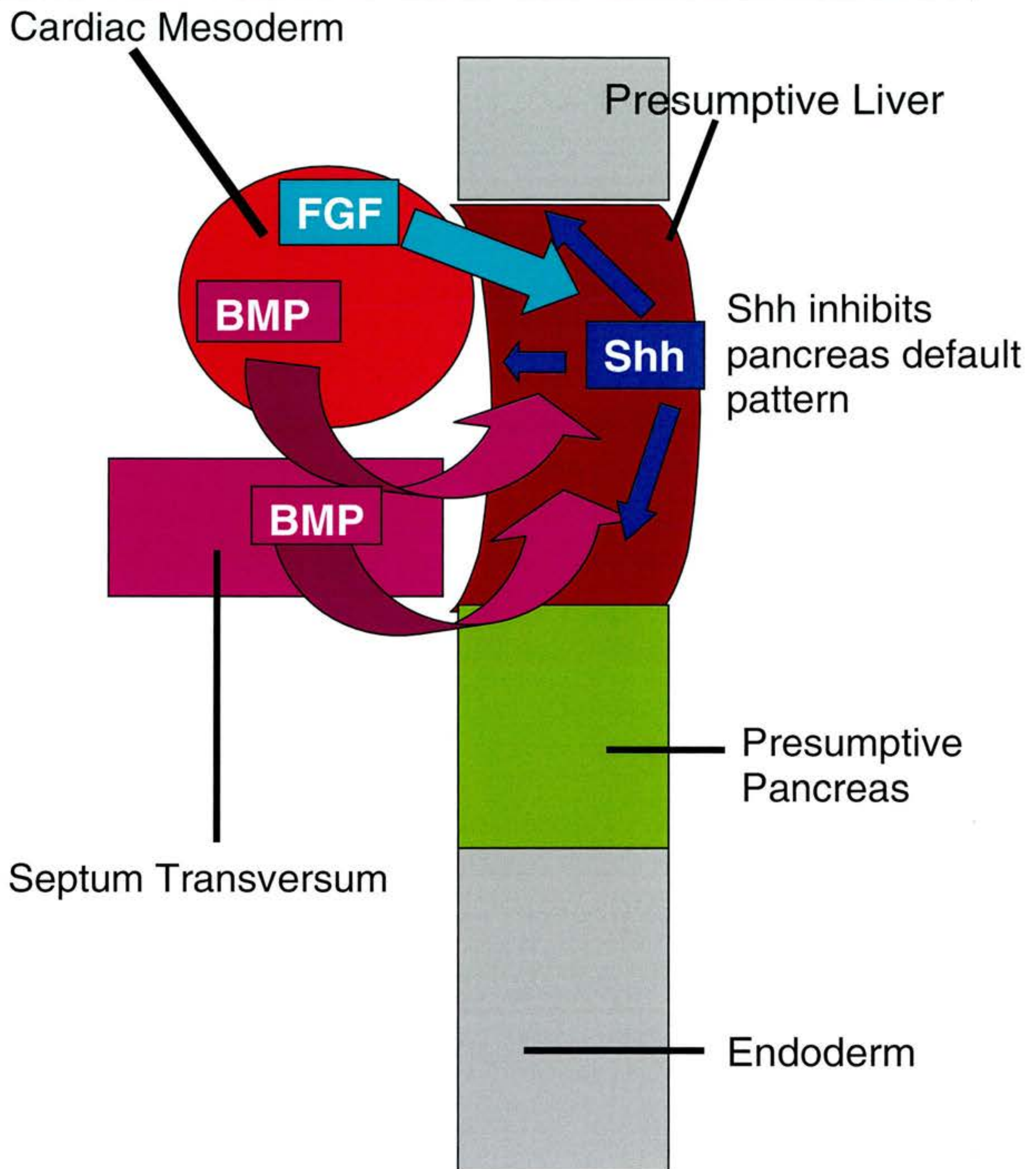


Figure 1.4. Liver specification in development. FGF induces Shh expression in the presumptive hepatic endoderm, which prevents expression of the pancreatic lineage. The presumptive pancreas is too distant to receive the FGF signal, and so develops along the default, pancreatic, lineage. In the developing liver, Shh by itself does not initiate hepatic gene expression. FGF from cardiac mesoderm, acting in concert with BMP4 as a permissive factor from septum transversum, activates the hepatic expression pattern.

Section 4.

Cell biology of liver development

4.1 Introduction

After liver specification has occurred, liver development is primarily directed towards cellular proliferation until morphogenesis of the biliary tree occurs at week 11. Prior to this point, the proliferating hepatoblasts, which are by definition the parenchymal cells present until differentiation into cholangiocytes and hepatocytes occurs, show evidence of bi-potentiality in terms of their cytokeratin complement. In this regard, there is considerable overlap between the phenotype of these developing liver cells, and liver progenitor cells detected in adult rat liver, so-called 'oval cells', which originate from the biliary tree and proliferate in response to injury, giving rise to ductules and hepatocytes (Alison et al., 1996; Petersen et al., 1998; Thiese et al., 1999).

As development progresses, biliary cytokeratins, which are initially detected in most hepatoblasts, become concentrated around the portal tract, and the morphology of the cells in this area changes from hepatocyte-like to cholangiocyte-like. Whilst this is only minimally discernable with simple stains such as Haematoxylin and Eosin, immunostaining provides clear evidence for this phenotypic change which heralds the formation of the ductal plate. At the

same time, the hepatic arterial inflow is established to the portal tract. This step relates closely to the development of the ductal plate and the biliary ductules. This section describes the nature of the early hepatoblasts, the potential signalling pathways active on these developing liver cells which allows proliferation and differentiation, and the early development of the biliary tree.

4.2 Hepatoblast Phenotype; Intracellular Markers

In order to study developing liver, it is critically important to be able to define the phenotype of the developing cells, so that differentiation can be followed in response to developmental cues and experimental treatments. The phenotype, which could be defined at the level of secreted proteins, cytoskeletal proteins, or tissue-specific transcription factors, might be expected to be fluid during development. This relates to the differing requirement for cells to undergo proliferation earlier in development, and to acquire mature biosynthetic capabilities later in development. The various phenotypic markers which might be used to detect liver cells during development, and to follow patterns of maturation in vivo and in vitro, are discussed below.

4.2.1 Intermediate Filaments

Intermediate filaments are primary structural proteins of cells, named for their size, which is intermediate between actin and myosin. In epithelia, heterodimers, but not homodimers, of cytokeratins form these intermediate filaments. Each pair is comprised of one member from type I (acidic) and one

from type II (neutral/basic) cytokeratins, each with a molecular weight of 40-70kD. Simple epithelia tend to express one pair alone, and mature hepatocytes express cytokeratins 8/18. By contrast, bile duct expresses 8/18 and 7/19, so containing two different intermediate filaments. In development, tissues can express different cytokeratin profiles, which may reflect potential for differentiation, or suggest a previous less differentiated state.

Mesenchymally-derived tissues, such as endothelium and fibroblasts, also contain intermediate filaments, however, in these tissues vimentin and related proteins comprise the building blocks. By contrast with cytokeratins, vimentin alone is sufficient to form filaments. Desmin is a related protein which is most often associated with muscle cells. Desmin and vimentin readily co-polymerise and so may be detected in the same cell type in the same filament. During development, vimentin may be expressed in epithelia along with cytokeratins. Should this occur, cytokeratins and vimentins do not form filaments together; rather, they each form their own intermediate filaments which can be detected separately in cells.

Cytokeratins

At the 0.5cm stage in the human fetus, which is approximately 4 weeks of age, nascent liver epithelial cells are immmunopositive for Cytokeratin 19 (Stosiek et al., 1990; Haruna et al.,1996). This observation indicates that cytokeratin 19 labelling is a marker for bi-potential hepatoblasts prior to ductal plate

differentiation, as cytokeratin 19 does not occur in maturing hepatocytes later in gestation. In addition, developing hepatoblasts, cholangiocytes and hepatocytes are all reported to be positive for cytokeratin 14 early in development, becoming negative by week 14 of gestation and remaining so (Haruna et al., 1996). Cytokeratin 18, which also labels adult hepatocytes, is present in developing hepatoblasts, hepatocytes and biliary cells at all gestational ages examined (Shimonishi et al., 2000; Aswathi et al., 2004).

Although the studies described above identify the fetal liver type II cytokeratins, data are scant with regard to the appropriate type I cytokeratin required to form the intermediate filament heterodimer. Human fetal liver cells are reported to be cytokeratin 8 positive, however, this was established only by western blot analysis of whole 2nd trimester liver, and did not address which cells might be positive (Mahli et al., 2002). A more detalailed study by Aswathi et al. (2004) demonstrated cytokeratin 8 immunoreactivity in hepatoblasts as early as week 10. Surprisingly, bile ducts are reported to become positive for cytokeratin 7 only after 20 weeks gestation, although these authors did not examine liver prior to 15 weeks gestational age (Sergi et al., 2000). These data were corroborated by Blakolmer et al. (1995), who noted cytokeratin 7 immunoreactivity developing from week 22 onwards. Cytokeratins 7 and 8 are detected in bile ducts in adult tissues, whereas hepatocytes are labeled by cytokeratin 8 antibody alone (Shimonishi et al., 2000).

The above observations allow a developmental cytokeratin phenotype to be surmised for developing human liver. In early liver, hepatoblasts are cytokeratin 8/14/18/19 positive. At this stage, no ductal plate is evident. As the ductal plate differentiates around week 10, ductal plate cells must also be cytokeratin 8/14/18/19 positive, however, they show an intense cytokeratin 19 reaction product (Haruna et al., 1996), whilst the remaining hepatocytes lose cytokeratin 19 immunopositivity (8/14/18/19+ve fading to 8/14/18+ve). When the biliary tract becomes morphologically distinct in mid-gestation, the neo-ductules are cytokeratin 8/18/19 positive, whilst hepatocytes are cytokeratin 8/18 positive. Latterly, bile ducts show the mature pattern of cytokeratin 7/8/18/19 staining, whereas hepatocytes still show cytokeratin 8/18 alone. These last two phenotypes are precisely the same as observed in mature adult liver tissue (Shimonishi et al., 2000).

Vimentin and desmin

Vimentin is considered to be a marker for mesenchymally-derived tissues such as fibroblasts and endothelium. However, in developing human liver, vimentin is detected within the portal tract mesenchyme generally, and in developing bile ducts specifically, between 9 and 36 weeks gestation, but not in hepatoblasts or hepatocytes (Haruna et al, 1996). These observations, in which a mesodermal marker appears in liver epithelium, have been corroborated in an adult rat model. Rats subject to partial hepatectomy and acetylaminofluorene treatment demonstrate an intense biliary proliferative response (Alison et al., 1996), which

provides a bipotential progenitor compartment to restore the hepatocyte and cholangiocyte complement. Proliferating ductules show a marked immmunopositive reaction for vimentin during the proliferative, migratory phase of this response, which subsequently disappears when tissue repair is complete (Alison et al., 1996). By contrast, desmin has been demonstrated in developing hepatic stellate cells, but not in hepatocytes, hepatoblasts or cholangiocytes, indicating a more restricted role for desmin during development (Nitou et al., 2000).

4.2.2 Secreted proteins and functional markers

A key feature of liver function is the secretion of a broad spectrum of plasma proteins. Albumin and AFP are well recognised as proteins indicative of liver differentiation and function. At the 0.5cm stage in the human fetus, which is approximately 4 weeks of age, liver cells are positive for albumin and AFP (Rossi et al. 2001; Haruna et al. 1996; Jones et al., 2001). In fact, these studies showed that albumin and AFP are detected in the very earliest hepatoblasts. A functional correlation of these data has previously been obtained by Gitlin and co-workers. AFP was detected in supernatant from cultured fetal liver cells collected in week 6 of gestation (Gitlin and Boesman, 1967), fibrinogen from livers cultured at 5.5 weeks, and albumin from liver cultures at 32 days (Gitlin and Biasucci, 1969).

The identification of a secreted protein, along with an intermediate filament, allows the identification of epithelial subtype, namely hepatocellular, cholangiocyte or bi-potential progenitor. Such data permit the phenotyping of cells in vitro, so that differentiation may be pursued during development.

4.3 Hepatoblast Phenotype; Cell Surface Markers

4.3.1 Thy-1

Thy-1 (CD 90) is a transmembrane protein of molecular weight 95kD, the various motifs of which suggest a binding site on the extracellular moiety for which the ligand is unknown. Thy-1 surface antigen has been well described as a marker for adult liver progenitor cells ('oval cells') in mature rat liver (Petersen et al., 1998), and is also a marker used to purify primitive haematopoietic stem cells in clinical bone marrow transplantation (Shimazaki et al., 2004).

No data regarding purification are yet available in the fetal human liver, however, fetal rat studies report the use of Thy-1 to purify putative liver stem cells (Fiegel et al., 2003). Cells purified using magnetic beads coated with anti-Thy-1 monoclonal antibody were positive for cytokeratin 18, albumin, AFP and Thy-1 mRNA by RT-PCR. Immunostaining these cells showed positive reaction product using anti-albumin, anti-AFP and anti-cytokeratin 18 antibodies, which strongly supports the hypothesis that Thy-1 positive cells are closely related to epithelial hepatocytes or hepatoblasts. Sadly, no data were presented to illustrate which compartment in the developing liver the Thy-1 positive cells

originated from. Interestingly, Thy-1 positive cells from fetal mouse liver did not express markers of epithelial differentiation, rather, mesenchymal markers such as vimentin and α-smooth muscle actin were noted (Hoppo et al., 2004). These authors were satisfied that liver progenitor cells in fetal mouse did not express Thy-1, and that the Thy-1+ve fraction in fetal mouse liver was in fact a mesenchymal stem cell which supported the hepatic stem cell compartment.

In adult rat liver, oval cells are capable of proliferation and differentiation so as to regenerate hepatocytes and biliary structures (Alison et al., 1996). These cells are thought to derive from the smallest biliary radicles, the Canals of Hering (Thiese et al., 1999). Responding to signals which are not yet defined, the stem cells proliferate as an expanding oval cell fraction, in particular when liver injury is massive or when mature hepatocytes have been prevented from proliferation, for example using 2 acetylaminofluorene. Most data are available from the adult rat, where Petersen has shown that these oval cells express the surface marker Thy-1 (Petersen et al., 1998). Thus, Thy-1+ve oval cells can be selected by flow cytometry or magnetic separation techniques, and subsequent immunostaining reveals cytokeratin 18, AFP and albumin, confirming a primitive liver epithelial phenotype in the injured adult rat liver. These cells undergo initial morphological differentiation which renders them hepatocyte-like, before expressing markers of hepatocyte maturity, such as P450 enzymes (Alison et al., 1996).

Thy-1 was not identified as a liver stem cell marker by Petersen's group in the adult mouse (Petersen et al., 2003). Rather, a complex phenotype, Sca 1/CD34/CD45/A6 +ve, was likely to be the stem cell fraction, as most were AFP +ve. These observations were corroborated in fetal mouse liver when Thy-1 antibody was used to purify fetal mouse Thy-1+ cells. Whilst CD49f+ cells (alpha-6 integrin) were positive by RT-PCR for AFP, albumin and cytokeratin 19 mRNA species, Thy-1 positive cells were found to express mesenchymal markers, such as vimentin, desmin and alpha-smooth muscle actin (Hoppo et al., 2004). The presence of the Thy-1+ mesenchymal cells greatly enhanced the hepatocellular differentiation of the CD49f+ liver epithelial progenitor fraction. In parallel studies of 1st trimester human liver, bone marrow and blood, a mesenchymal progenitor was identified which was immmunopositive for similar markers to the above mouse Thy-1+ cell; this cell type was an effective support for haematopoietic stem cell proliferation and was thought to be the fetal equivalent of the adult human mesenchymal stem cell isolated from bone marrow (Campagnoli et al., 2001). These authors did not, however, determine whether their stem cells were Thy-1 positive. From the present literature, it is not clear whether a proportion of fetal Thy-1+ cells in the human liver are of mesenchymal origin.

With regards to the role of Thy-1+ cell in the epithelial lineage in humans, Thy-1 positive cells have been detected in long term culture of fetal human liver, where mixed cell cultures were characterised by flow cytometry (Lazaro et al., 2003).

This must be regarded with some suspicion as no further characterisation was carried out in the Thy-1 +ve fraction.

As a parallel to the data available, Fiegel et al. (2004) have studied hepatoblastoma tumour cell types from human paediatric liver resection specimens. Interestingly, they found cells expressing Thy-1 and either hepatocyte or biliary lineage markers within abnormal ducts of the primitive tumours. These data support a relationship between Thy-1 and primitive liver progenitors, and further suggest a link between liver stem cells and cancer.

It would seem that the biology of Thy-1 + cells in the mouse, rat and human is not identical, and that whilst generalisations can be made about the roles of Thy-1+ cells across the species, the precise biology may be quite different in each. At present, it seems likely that Thy-1 may represent a surface marker for hepatoblasts or liver stem cells in the rat. In the mouse, data indicate that Thy-1 is not a hepatoblast marker, nor an epithelial stem cell marker. In the human, data are too scarce to provide a useful guide. However, Thy-1 co-localisation with cytokeratins in hepatoblastoma provides some evidence that Thy-1 is at least relevant to liver progenitor biology in humans.

4.3.2 Dlk

Delta-like leucine zipper kinase (Dlk; preadipocyte factor 1, the full length form of fetal antigen 1 (FA1)), is a transmembrane protein which contains 6 EGF-like

sequences in the extracellular domain, a transmembrane region and a small intracellular component (Laborda, 2000). It is a member of the EGF homeotic protein family, which includes the Notch receptor, and the Notch ligands such as Delta, Serrate, and their mammalian counterparts Jagged 1 and Jagged 2. It has been implicated in haematopoiesis and the development of myelodysplasia (Miyazato et al., 2001), which extends observations that Notch is expressed by human CD34+ haematopoietic progenitors (Milner et al., 1994). Dlk is expressed in the human central nervous system, in particular in the monoaminergic nuclei (Jensen et al., 2001). It is associated with inhibition of maturation of bone marrow osteoblasts and adipocytes (Abdallah et al., 2004), and is found in high levels in amniotic fluid, where it represents a significant fraction of non-maternal proteins (Drohse et al., 1998).

In developmental studies, FA1, which is the truncated from of Dlk corresponding to the extracellular component alone, was detected by immunostaining in human fetal liver. At week 5 of gestation, immunostaining was confined to hepatoblasts, whereas haematopoietic cells were negative (Floridon et al., 2000). As gestation progressed, staining intensity fell until by week 15, little immunoreaction was observed. FA1 staining was observed in the fetal pancreas in this study, with all glandular cells strongly labelled. In both human and rat, immunoreactive FA1/dlk was found latterly in gestation to be associated with insulin-secreting pancreatic beta cells alone (Tornehave et al., 1996: Carlsson et

al., 1997), prompting suggestions that this pathway was important in beta cell differentiation.

In rat liver, Dlk is expressed on the surface of hepatoblasts and antibodies directed against the extracellular moiety have been used to purify hepatoblasts from day 21 of gestation fetal rats (Jensen et al., 2004) and from E14.5 fetal mouse liver (Tanimizu et al., 2003; 2004). No such attempts have been described using human tissues. In rat and mouse, hepatoblasts purified using anti-Dlk antibodies and magnetic separation express alpha-fetoprotein and albumin mRNA and/or protein. Interestingly, very few cells were immmunopositive for both albumin and cytokeratin 19, the biliary marker, in the mouse cells, and intra-splenic transplantation of these cells in a mouse model resulted in donor hepatocytes alone (Tanimizu et al., 2003). These data suggest that the dlk+ cells may be at a more differentiated stage than those cells isolated using more traditional stem cell markers (see Suzuki et al., 2000, 2002). However, further studies by the same group refuted this observation. Using magnetic separation to purify dlk+ cells, these authors isolated pure cultures from E14.5 day mouse livers. Cells were cultured on laminin and incubated with oncostatin M and Matrigel. A second group of cells were grown in a collagen gel (Type I) and treated with HGF. A third group of cells were cultured on plastic in the absence of dexamethasone, but in the presence of HGF and retinoic acid. These different culture conditions gave rise to hepatocyte, cholangiocyte and pancreatic β-cell differentiation, underscoring the developmental relationships

between all three cell types, which are of course all derived from gut endoderm. With regard to the phenotype of the cultured liver progenitors, all expressed albumin and cytokeratin 19 when cultured at confluence in non-differentiating conditions, however, the expression of dlk rapidly fell to undetectable levels within days (Tanimizu et al.; 2004). It is worthy of note that the original purified dlk+ cells were stated to be negative for cytokeratin 19. This in some way contrasts with the human data, where very early liver cells are noted to be albumin+ and cytokeratin 19+ (Stosiek et al.; 1990: Rossi et al.; 2001).

Taking all of the data together, Dlk is a surface marker of hepatoblasts in rats and mice, and the developmental data although scant in human fetal liver, supports the hypothesis that Dlk is a hepatoblast marker in the fetal human liver.

4.3.3 E-Cadherin

E-Cadherin is a cell surface calcium-dependent binding molecule which mediates intercellular adhesion in epithelia, and is expressed in human hepatocytes (Ogou et al., 1983). In the developing mouse liver, it is present on the hepatocellular membrane (Nitou et al., 2000). This property has been used to purify presumptive hepatoblasts from the fetal mouse liver at E12.5 by magnetic bead separation. E-cadherin positive cells were found to express cytokeratins, albumin, AFP and E-Cadherin, but were negative for Desmin, by immunofluorescence (Nitou et al., 2002). Isolated culture of hepatoblasts

rapidly lead to loss of albumin, AFP and E-cadherin staining, an effect which was reversed by co-culture with the E-cadherin negative fraction (non-parenchymal cells), strongly suggesting a mutually supportive role for parenchymal and non-parenchymal cells. This was previously alluded to by Hoppo et al. (2004).

Studies in the human fetal liver have been carried out to examine the developmental profile of E-cadherin and the intracellular species which mediate the linkage between E-cadherin and actin filaments, α and β-catenins (Terada et al., 1998). E-cadherin was immunostained in livers as early as 8 weeks, but the authors only describe findings from 10 weeks onward. E-cadherin was expressed in the ductal plate cells, and not in hepatocytes or hepatoblasts, and the staining suggested a cytoplasmic localisation, rather than the membranous location as demonstrated for adult human hepatocytes in the same study. However, the livers studied were at a more advanced stage in development compared to the mouse studies of Nitou et al (2002) where E-cadherin was a hepatoblast surface marker. There is, as yet, no good evidence in the human that early hepatoblasts express E-cadherin. Nonetheless, E-cadherin is a component of intercellular junctions, particularly tight junctions mediated by cellular desmosomes, and would be expected to be expressed in liver epithelium. Further studies are required to establish definitively whether E-cadherin plays a role in human liver hepatoblast development. Nonetheless, the expression of E-cadherin by maturing biliary tract, which finally becomes membranous at the

immature bile duct stage (Terada et al; 1998), suggests a method to identify purified populations of this cell type.

4.3.4 Integrins

Integrins are widely implicated in the structural integrity of epithelia. The integrin complex β1α6 (CD29CD49f) is expressed by proliferating mouse liver progenitors in vitro (Tanimizu et al., 2004). This complex binds laminin, a substrate demonstrated to enhance survival of mouse liver progenitors (Tanimizu et al., 2004). Mouse chimaeras created with β1-integrin null ES cells resulted in progeny which demonstrated β1-integrin null cells present in mesodermal structures, but a striking absence of null cells in any endodermally-derived epithelia, for example liver (Berger et al., 2003). β-1 integrin is critically involved in maintenance of human hepatocyte viability, and activation of β-1 integrins using the activating antibody TS2/16 reduces apoptosis caused by loss of the integrin in adult human hepatocytes (Newsome et al., 2004), possibly via a MAP kinase dependent pathway (Zhang et al., 2002). No data exist describing the presence or role of β-1 integrins in the early human liver, however, it is likely that the absence of beta-1 integrin cells in endodermally derived epithelia relates to apoptotic deletion, underscoring the importance of intercellular integrity in liver development (Couvelard et al., 1998).

4.4 The proliferation of liver progenitors

Early hepatoblasts undergo proliferation without appreciable differentiation until the appearance of the ductal plate as detected initially by immunostaining around week 6 of gestation. Even at this point, most hepatoblasts remain undifferentiated until much later, when the primitive cytokeratin profile changes to that of hepatocytes. This proliferative phase is likely dependent, in part, on the absence of mediators of differentiation and the presence of specific growth factors which support proliferation without differentiation.

In order to understand the biology of developing liver cells, and to harness their potential for clinical treatment, it is necessary to determine which factors might be responsible for initiating proliferation without differentiation in the progenitor pool. This section seeks to examine several factors previously identified as promoting liver cell proliferation which may be active in fetal life.

4.4.1 Epidermal Growth Factor (EGF)

Of various growth factors reputed to cause hepatocyte proliferation, significant data have accumulated to support a role for EGF. Epidermal growth factor is a 53 amino acid cytokine of 6.2kDa, which binds with highest affinity to EGF-R1, also known as erb1 (c.f. erb 2-4). In human hepatocytes, data suggest that EGF is a potent mitogen (Engl et al., 2004), and is important for histogenesis of liver tissue (Michalopoulos et al., 2003). In normal human liver, hepatocytes strongly express EGF receptor mRNA, in contrast to the biliary system, which is

negative (Milani et al., 1991, Komuves et al., 2000). TGF-α, which is a potent physiological agonist at the EGF receptor, is present in fetal human hepatocytes (Yasui et al., 1992) and may act in an autocrine manner (Harada et al., 1996). In the proliferating liver cells found in cirrhotic nodules, hepatocytes are strongly positive for EGF pre-pro-peptide, the EGF precursor, whereas accompanying bile ducts are only very faintly labelled, despite the high levels of EGF mRNA in the biliary system (Komuves at al., 2000). In further support of a role for EGF in physiological liver cell proliferation, data show that EGF expression is upregulated within 30 minutes of partial hepatectomy (Mullhaupt et al., 1994). EGF also induces proliferation in hepatoma cells, acting via a genistein-sensitive pathway (Wu et al., 2003).

In developing human liver, the EGF receptor gene is substantially hypomethylated compared to adult tissue, implying increased gene expression (Kaneko et al., 1985). To complement this observation, Terada et al. (1994) documented the ontogeny of Transforming Growth Factor-α (TGF-α) and its receptor (the EGF receptor) in human fetal liver. During fetal life, bile ducts contained the strongest staining for both TGF-α and receptor, whereas hepatocytes were moderately immunopositive for TGF-α and only mildly positive for receptor. By contrast, adult liver showed strong staining in hepatocytes and bile ducts for receptor, with only bile ducts staining for TGF-α. Disappointingly, these authors did not study this pathway prior to the

emergence of bile ducts in development. It therefore remains unclear as to whether the EGF pathway is present earlier in development, and if so, what its role might be with regard to developing hepatoblasts.

In studies of cultured human fetal liver cells, EGF has been shown to induce proliferation of cells in vitro (Mahli et al, 2002), which is associated with increased γ Glutamyl transferase (γ–GT) and tyrosine aminotransferase activities (Rehman et al., 2004). However, these studies did not identify the specific cell types involved, and so the conclusion that hepatocyte proliferation *per se* was induced by EGF cannot be supported by this work.

With regard the tissue responses to EGF, and the developmental time frame for EGF activity, EGF receptor immunoreactivity was first detected on day 17 of fetal development in the rat, with intrinsic kinase activity appearing by day 18 (De et al., 1991). These data were complemented by the high levels of TGF-α, an EGF receptor ligand, found in fetal rat liver at day 20 of gestation (Brown et al., 1990). Such data argue against a role for EGF in early liver development, as this time period occurs considerably after the time of liver specification and initial hepatoblast proliferation. Rather, such EGF activity may be involved in hepatocyte proliferation occurring as a liver growth phenomenon immediately prior to birth. On the other hand, these observations reveal the possibility of a further level of control in the EGF pathway during development.

In summary, the evidence implicating EGF in the developing human liver is circumstantial. In the rat, data support a role late in development. The time frames for human and rat liver development are very different in the two species, and it is probably unwise to over-extend observations from one to the other. A putative role for EGF in developing human liver awaits dynamic studies in vitro to determine which cell types might respond to EGF.

4.4.2 Hepatocyte Growth Factor

Hepatocyte Growth Factor (HGF) is a glycosylated protein heterodimer comprised of alpha and beta chains bound by a disulphide bond. This growth factor is secreted as an inactive precursor (pro-HGF) then cleaved by a urokinase-like activity to yield an alpha-beta HGF structure capable of binding and activating the HGF receptor tyrosine kinase (Vigna et al., 1994). HGF is well recognised as the 'scatter factor', and is now thought to mediate significant aspects of the switch from non-invasive to invasive phenotypes of certain cancers (De Luca et al., 1999: Medico et al., 2001). The role of HGF to induce a morphological and phenotypic change in liver cells from a stable epithelial pattern to a mobile mesenchymal immature cell type during development has been well established (Pagan et al., 1999).

In early human liver development, HGF is expressed by the septum transversum mesenchyme, and c-Met, the HGF receptor, is located on hepatoblasts (Schmidt et al., 1995; Bladt et al., 1995). HGF is also found in the haematopoietic cells of the liver (Defrances et al., 1992). Inactivation of either of these molecules causes liver hypoplasia as a result of significantly increased apoptosis The mechanism which subserves this cell death has not been described, but it is tempting to invoke failure of HGF-dependent synthesis of Bcl-2 or Bcl-xl as the cause of apoptotic cell death. Interestingly, HGF knockout is lethal in utero, and fetuses show grossly hypoplastic livers with parenchymal cell depletion and, in addition, severely malformed placentas leading to intrauterine death (Schmidt et al., 1995). To complement these data, c-met positive cells, but not CD45, the leukocyte common antigen, positive cells, from fetal liver were able to repopulate the injured liver with hepatocytes in a retrorsine/two-thirds hepatectomy rat model (Suzuki et al., 2004). These observations establish that, the HGF/c-met pathway is central to normal liver development. In support of a role in the acquisition of liver mass, adult mouse studies show that c-met/HGF is critical to liver regeneration after liver resection or liver injury (Huh et al., 2004).

In the developing human, c-met and HGF have been detected in fetal liver between 6 and 13 weeks of gestation, suggesting a role continuing beyond the earliest specification and invasion phases of liver development (Kolatsi-Joannou et al., 1997). Later, c-met is expressed in the ductal plate and in the developing

bile ducts, as well as in hepatocytes, but only at low level; subsequently c-met protein is not detectable after 30 weeks gestation nor in normal adult liver (Terada et al., 1998). These findings go along with the general hypothesis that the HGF/c-met pathway may be required as an anti-apoptosis mechanism in development. This may act in concert with the NF-κB pathway which also prevents apoptosis in development (see below).

A simple role for HGF/c-met in development, as previously discussed, may be complemented by a role in hepatocellular maturation (Spijkers etal., 2001). In the rat, c-met was shown to be weakly positive throughout the liver early in development, however, at day 13 of gestation, expression rose sharply, whilst the localisation moved rapidly to the hepatic periphery. At the same time, the urea cycle enzyme carbamoylphosphate synthase appeared at the porta hepatis. The authors concluded that the change in localisation of the c-met protein heralded the end of the embryonic phase and the beginning of the fetal, maturational phase. There may be some relationship in this between the expression of c-met protein and the appearance of the Notch/Jagged pathway which is thought to mediate ductal plate differentiation (see section 4.5.2, below).

Taken together, the data presented show that the c-met/HGF pathway is critically important in the normal development of the liver. Parenchymal cell complement is very much dependent on HGF/c-met, as demonstrated in the

knockout studies. Conversely, later development appears not to require HGF in quite the same way. However, there are species differences, and the data in humans in particular are scant. An understanding of the role of HGF in the developing human liver awaits further studies.

4.4.3 Tumour Necrosis Factor

In adult liver, proliferation of mature hepatocytes readily occurs in response to liver injury, as clearly documented in historical (Higgins and Anderson, 1931; Lawrence et al., 1959) and contemporary studies (Boulton et al., 1998). Mature hepatocytes enter S phase within 16-17 hours of two-thirds hepatectomy, with a proliferative response approximately 15-fold that of control levels (Gerlach et al., 1997).

In mature animals, Tumour Necrosis Factor α (TNFα) priming leads to a 2 -4 fold greater hepatocyte proliferation in response to growth factors (Webber et al., 1998). Antibodies to TNF inhibit the IL-6 rise and inhibit hepatocyte proliferation after partial hepatectomy (Akerman et al,. 1992). TNFR1 gene knockout leads to severely impoverished hepatocyte proliferation as a response to partial hepatectomy (Yamada et al., 1997). These data confirm that in mature animals, in addition to a major role in the sepsis response, TNF is central to the reparative proliferation seen in liver regeneration after injury.

The developing mouse liver at day 10-13.5 of gestation expresses TNFα in a majority of cells, although the immaturity of the liver makes morphological identification of the specific cell types problematic (Doi et al., 1999). Knockout mice lacking the gene for TNFα show no ill effects in liver development and are born with normal livers, demonstrating that TNFα is not required. Conversely, knockout mice lacking the relA subunit of Nuclear Factor kappa B (NF κB), or the I kappa B kinase gamma subunit, demonstrate embryonic lethality accompanied by massive liver apoptosis (Doi et al.; 1999: Rudolph et al., 2000). Mice with relA deficiency can be rescued from embryonic lethality by a further knockout of the TNFα gene (Doi et al., 1999). These data demonstrate that TNFα is responsible for cell death in the knockout fetal liver, and that NF κB normally protects the developing liver from TNFα -induced apoptosis. Although double knockout mice are born with no evidence of liver abnormality, they later succumb to an episode of what appears to be sepsis around day 40 of life, having been made profoundly susceptible to infection by the relA knockout.

In adult animals, the evidence supports a role for TNFα in triggering the wave of cell proliferation that occurs in response to liver injury or liver resection. However, the evidence cited from developmental studies suggests that TNFα is not a major factor, and may be responsible for parenchymal cell apoptosis in particular situations.

4.4.4 Gp130 receptor ligand family

This cytokine family includes oncostatin M (OSM), Interleukin 6 (IL-6), IL-11, Leukaemia inhibitory factor, ciliary neurotrophic factor and cardiotrophin-1. All family members interact with a receptor, comprising a ligand-specific component and gp130, the common signalling component. This leads to common effects observed in the presence of different ligands. Acting via Janus Kinase activation, STAT3 is phosphorylated, and the resulting dimer then passes to the nucleus to bring about various transcriptional effects. In parallel, SH2 is bound and activated by the gp130 subunit, which then recruits the Raf/MAPK pathway as part of the pleiotropic effects of these ligands (Taga and Kishimoto, 1997).

The greatest evidence base which supports a role for gp130 ligands during liver development concerns Oncostatin M (OSM) and the effects on liver maturation, rather than proliferation. In very early embryogenesis, OSM is responsible for the induction of haematopoietic stem cells from the primitive haemangioblasts of the aorta-gonad-mesonephros area in E11 mouse embryos (Mukouyama et al., 1998), cells which then invade the liver to form the definitive haematopoietic tissues of the fetus. OSM is then produced by the haematopoietic cells of the fetal mouse liver, whereas the OSM receptors are predominantly expressed by hepatocytes, strongly supporting a paracrine role for OSM during liver development (Kamiya et al., 1999). Gp130 null mice do not show the large accumulation of glycogen in late fetal stages, again supporting a role for OSM in

hepatic maturation (Kamiya et al., 1999). Despite the weighty assertions made by Kamiya et al. (1999), careful examination of their data reveal that the experiments were not specifically designed to answer the questions they posed. For example, low glycogen levels in gp130 null liver are attested to by low magnification and poor quality micrographs. The alternative hypothesis, that hepatocyte apoptosis explains the lack of glycogen storage, is not rejected by the data presented. Similarly, their data suggesting that OSM is expressed by haematopoietic cells and OSM receptor by hepatocytes is based on mRNA data alone, and on a method of cell purification for which no evidence of actual cell purification is provided. This paper in particular must be regarded as provisional until stronger data are reported.

By contrast to the OSM data, IL-6 added to cell cultures had little effect on indices of liver maturation. However, addition of soluble IL-6 receptor to the cultures allowed IL-6 to induce effects similar to OSM (Kamiya et al 1999). These data suggest that IL-6, another gp-130 family member, could have significant effects in developing liver, provided that soluble IL-6 receptor is present.

Despite certain weaknesses in the literature, there seems little doubt that gp130 ligands are significant in hepatocyte maturation. OSM at a level of 10 ng/ml was required along with glucocorticoid (Dex; 10-7M) to induce the polygonal

epithelial phenotype associated with developing hepatocytes (Kamiya et al.; 1999); neither alone was sufficient in this regard. OSM is implicated in the development of E-cadherin-mediated adherens junctions between hepatocytes, a mechanism which is critically dependent on the K-Ras, but not H-Ras nor N-Ras, pathway (Matsui et al., 2002a). OSM down regulates Cyclin D in liver development, supporting the idea that OSM favours maturation rather than proliferation (Matsui et al., 2002b).

Despite the significant literature in fetal rat and mouse liver, little data are available in humans. Attempts to develop artificial liver devices using commercially available human fetal liver tissue have shown that OSM can induce albumin production and P450 enzyme activity, whilst suppressing alpha fetoprotein. However, the cell cultures expressed 10 fold less functionality than Hep G2 tumour cells, strongly suggesting a profound difficulty with these commercial preparations (Hanada et al., 2003).

4.5 The induction of the ductal plate and bile duct maturation.

4.5.1 Introduction

The ductal plate first appears at the hilum of the liver in the 1.8 cm human embryo (Ruebner et al., 1990), which corresponds approximately to 6 weeks gestational age, the corresponding figure in the rat being day 15 of gestation (Shiojiri et al., 1991). The ductal plate arises from the hepatoblasts which are in contact with the mesenchyme surrounding the portal tract, which at this stage

contains only portal vein. Initially, the ductal plate forms a sleeve-like interface between portal tract and hepatocytes (See Figure 1.2). The hepatic artery branches are absent when the ductal plate forms, but are fully developed before the primitive tubules differentiate. Clearly, there are many potential mesenchymal-epithelial interactions which may co-ordinate the induction and differentiation of the biliary tree.

4.5.2 Induction of the ductal plate.

The induction event responsible for the appearance of biliary tract precursors has only recently been investigated in animal models, and has not so far been studied in detail in the human. In humans, available data strongly implicate the Jagged/Notch pathway in the commitment of hepatoblasts to a biliary fate and the morphogenesis of the biliary tree. Alagille syndrome, which is known to result from an allelic null mutation of the Jagged1 gene (JAG1), the ligand for the receptor protein Notch, is observed to cause developmental abnormalities in the liver, a primary feature of which is impaired differentiation of the intrahepatic bile ducts from ductal plate. Sequencing analysis of the genotype in patients with Alagille syndrome indicates that various genetic lesions, all of which are within the Jagged 1 gene, may result in the phenotypic expression of the disease (Yuan et al., 1998). Mouse studies indicate, as one might expect, that abnormalities in Notch may also result in the disease phenotype. Mice heterozygous for the Jagged1 null allele and the Notch2 hypomorphic allele demonstrate the characteristic developmental abnormalities of Alagille

syndrome, including those of the intrahepatic biliary tree (McCright et al., 2002).

These data suggest a role in human liver development for the Jagged/Notch pathway. This has been pursued at a preliminary level using histological studies of developing human liver (Flynn et al., 2004). Notch 1-4 mRNA was present in fetal liver during development. Notch 3 receptor protein was expressed in the portal tract mesenchyme, whereas Jagged 1 was localised to the ductal plate. No other developmental data were offered in this study, and so the dynamic developmental interactions for this pathway in the human remains obscure.

In the fetal mouse at 15.5 days gestational age, Notch 2 mRNA was most abundant, whereas mRNA species for Notch 1, 3 and 4 were, by comparison, barely detectable (Tanimizu and Miyajima; 2004). Only Notch 2 protein was visualised by immunocytochemistry. In early development, Notch 2 was detected in a diffuse pattern which as development progressed, rapidly concentrated in the ductal plate and in parenchymal cells close by. By contrast, Jagged 1 was detected in abundance in the mesenchyme surrounding portal vein. Immediately adjacent to this Jagged 1 positive layer lay the ductal plate. Given that both Jagged and Notch are cell surface molecules it may be that direct contact mediates this induction, so leading so a single layer of ductal plate cells abutting the portal tract mesenchyme. Further experiments by the same group showed that Notch inactivation, either by siRNA directed against Notch 2 or

pharmacological inhibition with the γ-secretase inhibitor L685,458, caused up-regulation of liver specific genes such as tyrosine aminotransferase and carbamoyl phosphate synthase.

By contrast, activation of this pathway by constitutive expression of the intracellular signalling domain of the Notch molecule, lead to expression of cholangiolar markers, such as CK19, CK7, HNF-1β and integrin B4. These data are supported by observations that overexpression of Jagged 1 in mouse liver drives expression of CK19 and gamma-glutamyl-transpeptidase via transactivation of the Hes 1 promoter (Kodama et al., 2004).

Taken together, these findings strongly suggest in the mouse that the Jagged 1 ligand, found in cells of the portal vein mesenchyme, acts on immediately neighbouring parenchymal cells to induce ductal plate phenotype. By contrast, the situation in the human fetal liver seems less clear, as the Jagged 1 lay in the ductal plate and Notch 3 in the portal tract mesenchyme. The inversion of the ligand/receptor pair in the human suggests a more complex mechanism. It remains possible that mesenchymal Notch activation causes a second signal to pass to the nascent ductal plate from the portal tract mesenchyme to induce ductal plate differentiation. Such mechanisms require dynamic studies in vitro to determine the interactions of various pathways.

4.5.3 Remodelling the ductal plate

The signalling mechanism which mediates formation of primitive biliary tubules from the ductal plate is unknown. The addition of HGF to cultures of human biliary cells induced invasion of a collagen gel in vitro to yield an anastomosing network of biliary cords (Ishida et al., 2001), much like the migration of ductal plate cells into the portal mesenchyme during remodelling. No data were advanced by these authors beyond these observations, and no other groups have studied this phenomenon. Other groups have made important observations regarding the signalling required to initiate ductal plate differentiation. Fetal rat liver cells collected at day 14 of development transplanted into syngeneic adult rat spleen demonstrated orderly differentiation of ductal plate and biliary ductules (Notenboom et al., 2003). The authors made the observation that this may be an intrinsic property of hepatoblasts exposed to mesenchyme. This is a very important observation, as it suggests that clues from placenta or gut venous blood are unimportant, compared to the presence of mesenchymal tissue. Clearly, further study is required in this area, in particular as these mechanisms may underlie disorders of the biliary tree observed in infantile biliary dysgenesis and in post-transplant cholangiopathy.

4.6 Development of the vascular structures of the liver

4.6.1 Portal vein and sinusoids

At the very earliest stages of liver development, the endodermal bud grows into the septum transversum, at which time there is no vascular supply. Within the septum transversum, primitive angioblasts proliferate and coalesce to form a capillary endothelial plexus which intermingles with the developing hepatic epithelium to form the hepatic sinusoids. The importance of these endothelial cells has been thrown into sharp relief by data from Matsumoto et al. (2001). They prepared flk-1-/- mutant mice with absent endothelial cells and demonstrated that the nascent endothelial cells are responsible for the demarcation of the tissues into which the liver bud will grow, and that deletion of endothelium causes failure of outgrowth of the liver bud. These data suggest developmental signalling between the two compartments well before other studies had previously implied.

4.6.2 Hepatic artery

Until week 10 of development in the human, no hepatic arterial representation exists within portal tracts. Subsequently, portal tract capillaries form in situ by a process of vasculogenesis from CD34+ve mesenchymal stem cells which are detected in the portal tract around this time (Terada and Nakanuma, 1993; Nakanuma et al., 1993: Gouysse et al., 2002). There is a topographic sequence of maturation, exactly as for the ductal plate, with vessels first appearing in the

hilar region around week 10, with successively maturing vessels developing towards the periphery of the liver up to week 15.

The relationship between hepatic artery and ductal plate is a complex one. Observations indicate that the hepatic arterial inflow to a portal tract is always completed before the biliary radical proper arises from the ductal plate (Libbrecht et al., 2002). In developmental disorders of the intrahepatic biliary tree, anomalies of the hepatic arterial inflow within portal tracts are strongly associated with abnormal differentiation of the bile ducts (Awasthi et al., 2004). In fact, whilst no specific differences were observed in portal veins, hepatic arteries were observed to be hypertrophied and hyperplastic in cases of intrahepatic bile duct dysgenesis. Given that Jagged 1 is synthesised in portal vein, hepatic artery and ductal plate (Tanimizu and Miyajima, 2004: Flynn et al., 2004), and that Notch 3 receptor protein lies within the portal tract mesenchyme (Flynn et al., 2004), there may be a reciprocal signalling mechanism between mesenchyme, ductal plate and vessels. As yet, there are no data in the literature to indicate any mechanistic interaction between bile duct cells and hepatic artery branches. Nonetheless, an interdependence is strongly suggested by available data.

4.7 Functional studies of liver progenitors

Hepatoblasts potentially could form the basis for cell therapy of liver disease. Clearly, it is critical to identify which cell fraction in the developing liver might

possess the requisite proliferative capabilities and biochemical maturity. The developmental potential of hepatoblasts has been investigated mostly through transplantation experiments, principally in the rat. Dabeva et al. (2000) utilised a very rough preparation of day 14 fetal rat liver, with the dipeptidyl-peptidase IV (DPPIV) positive F344 rat as a donor. At this developmental stage, liver epithelial cells are hepatoblasts by definition, as the primitive biliary tract is yet to develop (Van Eyken et al., 1988). In the study by Dabeva et al (2000), the liver was subject to collagenase digestion and selective removal of red cells alone, resulting in a purity of AFP positive cells estimated at 15%. This mixed preparation was then infused into portal vein of DPPIV negative F344 recipients either immediately after two thirds partial hepatectomy or after no procedure. The data clearly show that the liver cells differentiated into both hepatocytes and biliary tract cells. Further work suggested that as much as 60-80% repopulation may occur (Sandhu et al., 2001). Animals not subject to hepatectomy demonstrated minimal engraftment of transplanted cells. In fact, a proliferative signal to the native liver was shown to be critical to engraftment, proliferation and differentiation, and that whilst hepatectomy provided the most potent stimulus, tri-iodothyronine treatment could also stimulate these processes. Interestingly, animals left for several months after cell transplantation demonstrated continuing proliferation of the transplanted cells in contrast to adult hepatocytes, confirming the long-term proliferative capacity of fetal liver epithelial cells (Sandhu et al., 2001).

Human fetal liver cells have recently been investigated in a similar manner. Mahieu-Caputo et al. (2004) took liver from fetuses 11-13 weeks gestational age, isolated the epithelial cells and, after cryopreservation, transplanted them into athymic mice. Up to 10% engraftment was reported, and human liver serum proteins were detected by immunostaining in the mouse liver. This is potentially a very important study, however, the authors failed to take the opportunity to determine the functional competence of the transplanted cells. For example, 70% hepatectomy after engraftment would potentially have lead to an expansion of the human liver cell fraction, to the degree that the animal was arguably dependent on the presence of the human liver cells, demonstrable by plasma protein concentrations. This would have strengthened the importance of this paper considerably. Therefore, reports of credible functional data concerning human fetal liver cell transplantation remain absent from the literature.

In mouse studies, liver removed from day 11 post coitus embryos was subject to Fluorescence Activated Cell Sorting (FACS) to provide a fraction characterised as c-kit^{low} (CD45/TER119)$^{-}$, which was demonstrated to repopulate all epithelial lineages (cholangiocytes and hepatocytes) of depleted liver tissue in vitro (Minguet et al., 2003). Although these authors made no attempt to specify exactly which cells were responsible for repopulation, they made the point that the specific cell type was contained in their FACS-isolated fraction. Of course, this experiment did not assess functionality in vivo, nonetheless, these data strongly suggest that this phenotype may be important in cell transplantation.

These data were extended by further studies of a similar fraction from mouse liver, c-kit^{low} (CD45/TER119) - α6-integrin+/β1-integrin+. These authors transplanted the cells into adult mouse spleen, whereupon cells migrated to liver and differentiated into liver parenchymal cells (Suzuki et al, 2000).

In order to purify mouse hepatoblasts, E-cadherin antibodies coupled to magnetic beads were used with liver cells from day 12.5 of gestation. (Nitou et al., 2002). These cells were isolated and cultured, and found to express albumin, AFP and carbamoylphosphate synthetase. Expression was supported only when non-parenchymal cells were allowed to interact with the purified hepatoblasts, an effect which was mimicked by conditioned medium from the non-parenchymal cells.

Taken together, these data show that hepatoblasts are competent to provide functional hepatocytes and biliary epithelial cells in vivo, at least in the rat. Surprisingly, a method as coarse as portal venous infusion appears sufficient to deliver cells which can occupy any liver epithelial compartment. Whilst published data concerning hepatoblasts seems relatively unrefined at present, a separate population of cells, hepatic stem cells, is presently being pursued as a potential source of liver cells. Of note, highly purified CD34+ve human haematopoietic stem cells isolated from umbilical cord blood or from adult human bone marrow have been transplanted into mice. Liver histochemistry

one month post transplant demonstrated the incorporation of hepatocytes immunopositive for human albumin, and the presence of detectable quantities, albeit very small, of circulating human albumin in these animals (Wang et al., 2003). Such data imply that engraftment of human stem cells in the liver has occurred, and that differentiation to form hepatocytes has also been confirmed.

These studies are dissimilar to the work described above, in that these authors sought to identify precursors to hepatoblasts, rather then hepatoblasts *per se*. Essentially, two approaches are slowly arising in the attempt to understand developing liver biology; the first is utilising hepatoblasts and the second hepatoblast stem cells. Clearly, these fields are highly complementary, and knowledge of both disciplines will be required to develop cell therapies into the clinical domain.

Whilst the observation that highly purified liver stem cells can be transplanted into mice and engraft is very exciting, some very basic data are clearly lacking from the literature. No critical studies of the functionality of developing human liver cells in vitro or in vivo have been attempted, in particular, no studies have described protein secretion or urea synthesis, or whether certain agents can bring about maturation or proliferation of hepatoblasts, or even whether functional status is altered by, for example, proliferation signals. Without knowledge of these factors, the observation of engraftment becomes strictly academic. In fact, in studies reported so far, no evidence has been provided that

fetal human liver cells retain any competence after transplantation; for example, no metabolic diseases have been corrected by human fetal liver cell transplant. Without key understanding of the relevant maturation and proliferation signals for human fetal liver cells, the tremendous promise of these cells as a therapeutic tool remains to be realised.

Section 5

Functional maturation of the liver

5.1 Introduction

During the embryonic period, which is up to the end of the second month in humans, the development of the liver is primarily directed towards tissue morphogenesis and lineage development, so as to deliver the cell complement appropriate to the functional maturation of the liver. Development during the embryonic stage is strictly limited to an increasing complement of genes expressed and cytoplasmic proteins relevant to this proliferative stage.

The anatomical maturation of the liver, which predominantly relates to development of bile ducts and hepatic arterial inflow, clearly has implications for developing function. All of these developmental steps have at least delivered an organ capable of secreting bile into the duodenum by week 11-12. Further development in utero features this anatomical maturation moving out progressively from hilum to periphery, a phase which lasts some way into the third trimester.

At birth, the liver must be capable of gluconeogenesis, ammonia detoxification, plasma and coagulation protein synthesis and metabolite conjugation. Whilst the neonatal liver is not fully mature, it must have a level of function consistent

with survival whilst further maturation occurs. This section reviews the rather scant but important literature describing functional development of the liver.

5.2 Plasma Proteins

Albumin mRNA and protein are detected within the cytoplasm of presumptive hepatoblasts during the 4th week. Albumin synthesis and secretion is a unique biological function of this organ in the adult. At the same stage of development, alpha-fetoprotein expression is recognized in hepatoblasts (Jones et al., 2001). Whilst albumin is regarded as the major component contributing to plasma oncotic pressure in adults, as well as a transport medium for various physiological and xenobiotic species, AFP is thought to be the corresponding plasma protein during fetal development. AFP is secreted by liver cell cultures as early as 4-5 weeks of gestation, as well as by cultures of yolk sac and gastrointestinal tract cell preparations (Gitlin et al., 1972).

Parallel studies have described albumin, AFP, alpha-1-antitrypsin, transferrin, and alpha-2 macroglobulin production by the yolk sac in first trimester fetuses (Gulbis et al., 1998). These authors considered that the yolk sac was the primary site of protein synthesis until the liver had sufficiently matured. Of course, evidence from Gitlin et al. (1972) demonstrates that the liver is already competent to secrete AFP at least, although other viscera may contribute to the developing plasma protein complement.

In support of the hypothesis that the early liver is competent to secrete many proteins, a pioneering study by Gitlin and Biasucci (1969) combined tissue culture with radioimmunoelectrophoresis to demonstrate the breadth of plasma proteins which were synthesised in the human embryo as early as day 29 of gestation. These authors documented secretion of transferrin, albumin, prealbumin, α1-antitrypsin, C'1 esterase inhibitor, hemopexin, β-lipoprotein and α2-macroglobulin from embryo cultures at day 29, and caeruloplasmin and orosomucoid by day 32. Later, fibrinogen secretion was documented, but not until 5.5 weeks. All of these proteins were synthesised by the liver. IgG and IgM synthesis was also detected at 10.5 and 12 weeks respectively. However, these proteins were synthesised by the developing spleen. Although protein synthesis in general increased remarkably during gestation, most remained well short of maternal levels by parturition. These observations suggest that synthetic capacity of the liver matures considerably during gestation; however, the liver in the newborn is still immature.

In second trimester fetuses, albumin levels increase from approximately 4 g/l to over 10 g/l by week 23 (Christiansen et al., 2000). By the third trimester, levels of albumin are virtually identical to adult levels, approximately 35-45 g/l (Nayak and Mital, 1977). By contrast to the upward trends in fetal albumin, AFP at term has fallen to approximately 10% of the level observed 7 weeks previously (Bader

et al., 2004). AFP continues to fall post partum, consistent with further maturation of the fetal liver.

Albumin and AFP synthesis and secretion are clearly differently regulated. Immunocytochemistry reveals that these proteins may be found separately in different hepatocytes, but may also be detected simultaneously in the same cell. The plasma concentrations of each appear to be related to the total number of cells synthesising the particular protein. In each case, cells distributed around central veins appear to be the preferred location for synthesis (Bader et al., 2004).

5.3 Coagulation Factors and Fibrinogen

The coagulation pathway in neonates has long been recognized as immature, and bleeding diatheses in newborns have prompted the introduction of routine vitamin K injections within the first few days of life for all British children. Although there are no published data for the first trimester, vitamin K-dependent coagulation factor activities in the second trimester are just 9-28% of adult levels (Forestier et al., 1985). More detailed study of healthy fetuses in which cord blood samples were collected under ultrasound guidance from the second trimester until the neonatal stage showed progressive gestational changes. The vitamin K-dependent factors, II, VII, IX and X, generally trend upwards during fetal life (Reverdiau-Moalic et al., 1996). Interestingly, the prothrombin time and the activated partial thromboplastin time fell from 32 to

17 seconds and 169 to 44 seconds respectively over the period from 19 weeks to newborn; at the same time, fibrinogen levels rose from 0.85 to 1.7 g/l. These data are consistent with developmental maturation which provides a functional coagulation system at birth. By way of physiological compensation, coagulation inhibitor concentrations were also very low during development, providing a balance for the low levels of coagulation factors.

During development, adequate fetal haemostasis is essential to prevent fetal loss or neonatal death (Sun et al., 1998). The majority of coagulation factors are synthesized in the liver, and so liver maturation has a further role to protect the fetus against spontaneous, catastrophic, haemorrhage. Fibrinogen null/null mice demonstrate normal survival during gestation, but only half survive 2 weeks post-natally, with the remainder dying as a result of intraperitoneal bleeding (Camerer et al., 2004). This is contrasted with pro-thrombin deficiency which leads to mid-gestation loss or death by haemorrhage in the immediate neonatal period (Sun et al., 1998). In humans, the recessive genetic condition afibrinogenaemia results in an absence of detectable fibrinogen and affected children may demonstrate spontaneous haemorrhage (intracranial bleeding, gastrointestinal bleeding, haemarthrosis, splenic rupture) at any time in gestation or post-natal life (Neerman-Arbez et al., 2003).
These data indicate how important a functioning coagulation cascade is for fetal and neonatal survival. During fetal life, there is a progressive increase in fibrinogen levels, although there is a marked discrepancy between functional

and total fibrinogen and the levels at birth are still low compared to adult (Reverdiau-Moalic et al., 1996). The maturational signal which mediates this important gestational change is unknown.

5.4 Maturation of bile synthesis in fetal life.

The anatomical development of the biliary tree is clearly a pre-requisite to bile secretion in fetal life. At week 11 in the human fetus, the hollow extrahepatic bile duct connects with the sleeve-like ductal plate which surrounds the portal tracts at the hilum. The ductal plate then begins to remodel in a centripetal manner, such that intrahepatic bile ducts form first at the porta hepatis. Having established a communication between parenchymal hepatocytes and the lumen of the gut, the way is paved for bile secretion into the gut to commence at week 12.

Cell cultures prepared from second trimester human livers excrete fluorescein, permitting the authors to hypothesise that the liver at this stage was competent to secrete bile (Bauer et al., 1991). However, no evidence was provided to demonstrate that bile secretion actually occurred in this model. Dumaswala et al. (1989) had already shown that human fetal gallbladder bile contained a broad spectrum of bile acids from weeks 16 to 19 of gestation. Later work demonstrated bile acid secretion as early as week 12 (Itoh and Onishi, 2000).

The question as to whether such bile acids might possibly have been of maternal origin was refuted comprehensively in two studies which showed a very different spectrum of bile acids in fetal bile as compared to the adult. These studies identified novel pathways of bile acid synthesis in human fetal development; C-1 and C-6 hydroxylation was observed by Colombo et al. (1987) and C-4 hydroxylation by Dumaswala et al. (1989), indicating a relative immaturity of the adult C-12 pathway. Interestingly, the C-4 hydroxylation pathway had not previously been described in mammalian bile acid synthesis (Dumaswala et al., 1989; Nakagawa and Setchell; 1990).

The predominant bile acid in the mid trimester liver is chenodeoxycholic acid, with cholic acid second in abundance. However, concentrations are extremely low prior to week 17 (less than 0.05mM; Colombo et al., 1987). Thereafter, concentrations began to rise sharply, possibly reflecting the anatomical maturation of the intrahepatic bile ducts at that stage (Crawford, 2002). Whilst chenodeoxycholic acid predominated over cholic acid in early development, full term fetuses demonstrated an inversion of this finding, undoubtedly reflecting the maturation of the C-12 pathway later in development (Nakagawa and Setchell; 1990). At birth, bile secretion is critical to survival; fat absorption from milk, and the avoidance of steatorrhoea, is critically dependent on a well-functioning enterohepatic bile salt circulation.

5.5 Glucose homeostasis.

Not until parturition is the fetus dependent on endogenous glucose. At this time, the relative hypoxia of labour places significant pressure on anaerobic metabolism and, therefore, on hepatic glucose production. This becomes critical to survival during parturition.

5.4.1 Glycogen metabolism

The key enzymes involved in glycogen metabolism are glycogen synthase and phosphorylase, responsible respectively for synthesis and breakdown of glycogen. Glucose-6-phosphatase, the enzyme releasing glucose to blood, catalyses the final step in glucose release from hepatic stores.

Glycogen is detectable in the fetal human liver at week 8 of gestation and builds slowly thereafter, with levels of glycogen synthase increasing in parallel (Schwartz et al., 1975). Glycogen synthase activity continues to rise as late as 36 weeks (Devi et al., 1992). Indirect measurements of human fetal liver glycogen, using the ultrasound attenuation coefficient of fetal liver, have shown that glycogen levels increase towards a maximal pre-parturient level, then fall sharply during labour (Carson et al., 1990; 1991). These data suggest that increasing glycogen stores relate to increasing activity in the synthetic pathway. Animal studies confirm that glucocorticoids and insulin produce a synergistic effect in stimulating glycogen synthesis in fetal hepatocytes, with no effect of

insulin alone in rats (Plas and Nunez, 1976), although human fetal liver responds by increasing glycogen content (Schwartz et al., 1975).

Early experiments with fetal liver slices made the observation that glucagon, acting via cAMP, caused glycogenolysis (Schwartz et al., 1975). In a parallel paper, these data were refined to show that the cAMP pathway could be augmented by the glucocorticoid agonist triamcinolone (Schwartz and Rall, 1975). Notably, agonists working through cAMP (glucagon, β-agonists) or IP3 (ATP, vasopressin, angiotensin) are able to induce glycogenolysis in human liver, with a slightly greater potency ascribed to the former group (Keppens et al., 1993). Physiological agonists acting via these pathways are present in elevated concentrations in fetal plasma during labour (Pohjavouri et al., 1985), supporting the hypothesis that fetal stress may provide the trigger for glycogenolysis at term.

Glucose-6-phosphatase is the specific liver enzyme which permits glucose to be released into blood. This enzyme has been documented as early as 7.5 weeks gestation in human liver (Simpson et al., 1987). Interestingly, no change was observed in activity between weeks 7.5 and 24, with levels approximately 30% of postnatal controls throughout. These data imply that the change from fetal to neonatal levels may occur late in development (Girard, 1986). The most detailed studies have been carried out by Burchell and Hume (1995), whose data

reinforce historical studies, and demonstrate a marked, developmentally regulated, change in tissue distribution and levels.

5.4.2 Gluconeogenesis.

Gluconeogenesis is dependent on a supply of precursors, usually lactate and glucogenic amino acids, of which alanine is most important. These are funnelled after transamination, for example by alanine aminotransferase, either through pyruvate or via the citric acid cycle so as ultimately to provide oxaloacetate. This is the common pathway feeding in to phosphoenolpyruvate carboxykinase (PEPCK), which is the rate-limiting step in gluconeogenesis. Latterly, glucose-6-phosphatase releases free glucose from hepatocytes into blood.

Neonatal hypoglycaemia is a significant issue in paediatric practice; published studies reveal that 37-43% of neonates show hypoglycaemia within 2 hours of birth (Cole and Peevy, 1994). The absolute requirement for hepatocellular cytoplasmic PEPCK to protect against hypoglycaemia in neonatal life is well shown in studies of human neonates lacking this enzyme; such newborns rapidly succumb to hypoglycaemia within hours of birth (Vidnes and Sovik, 1976). Of the four key enzymes of gluconeogenesis, only cytoplasmic PEPCK is absent from fetal liver. This enzyme is repressed by the physiological levels of insulin in the fetus, and it is the fall in insulin levels in the immediate postnatal period which induces PEPCK expression rather than hypoglycaemia (Yeung and Oliver, 1968).

Importantly, fetal cortisol appears to protect against hypoglycaemia (Economides et al., 1988), and cortisol levels are higher and the incidence of hypoglycaemia lower in those babies born by vaginal delivery (Economides et al., 1988; Cole and Peevy, 1994). PEPCK activity rises dramatically in the 24 hours after delivery which may relate to increased cortisol levels post-partum, however the role of glucagon (and of insulin) cannot be ignored (Girard, 1986). Molecular studies of PEPCK show a cAMP response element, a Glucocorticoid response element and a thyroid hormone response element in the promoter region of PEPCK. In addition, C/EBPα has a critical role, as null homozygous mice with a C/EBPα deletion die of hypoglycaemia within hours of birth (Hanson and Reshef, 1997). It is clear from these data that despite the critical importance of PEPCK in gluconeogenesis, the role for PEPCK in preventing hypoglycaemia is strictly in the post-natal period. Similarly, it is clear that hepatic gluconeogenesis cannot form the primary mechanism to support glucose levels prior to birth. Therefore, fetal glycogen synthesis and glycogenolysis must be the most important mechanism to maintain glucose levels before gluconeogenesis is fully functional.

Taking these data together, the fetal liver is able to deliver glucose during birth as a result of a progressive maturation of pathways subserving glycogen synthesis and breakdown. Postnatally, gluconeogenesis is activated in the immediate post-partum stage to maintain glucose levels at physiological levels.

Clearly, maturation of these pathways during fetal life is central to survival in the perinatal stage.

5.6 The urea cycle and organification of ammonia.

The incorporation of ammonia into urea converts a highly toxic species into a non toxic, highly soluble by-product which may be easily excreted. This pathway protects the fetus from irretrievable neurological injury which would supervene should ammonia levels rise much above the physiological range. Maturation of this pathway in development is necessary for fetal survival beyond the first few days (Schofield et al., 1999).

During fetal development, urea cycle enzyme activity increases to approximately 90% of adult levels by week 36 (Mukarram Ali Baig et al., 1992). Some authors have documented the full complement of urea cycle enzymes as early as week 9 (Karsai and Elodi, 1982). Carbamoyl phosphate synthase I is the rate limiting enzyme for the urea cycle, and is present at the highest level compared to other enzymes in 1st trimester liver (Karsai and Elodi, 1982). Immunostaining for CPSI demonstrates reaction product in human liver by day 31, only 5 days after liver specification (Van Beers et al., 1998). Detectable urea synthesis occurs in the second half of gestation, doubtless related to increasing enzyme activity (Mukarram Ali Baig et al., 1992; Karsai and Elodi, 1982).

Urea cycle enzymes have been studied in greatest detail in animal models. Fetal rat liver has very low synthetic capacity, which rapidly rises to ¼ of adult levels within 24 hours of birth (Kadowaki et al., 1983). Corticostreroids and glucagon have central roles in this induction of urea cycle activity (Husson and Vaillant, 1982), which has a striking similarity to the induction of glycogenolysis in term rat fetuses as described in previous sections. CPSI mRNA and protein are absent at day 16 of development, and only appear in significant amount by day 20, 1 day before birth (Van Beers et al., 1998). The late appearance of this rate-limiting enzyme in urea synthesis, coupled with the low fetal urea cycle activity and a rapid neonatal increase in activity contrasts sharply with the situation in the human. As alluded to above, urea cycle activity is at 90% of adult levels by week 36 of gestation. No detailed data are available which illuminate the mechanisms whereby this gestational rise occurs. However, it may be that the human neonate has a lesser tolerance for ammonia toxicity than the rat, hence the differing perinatal urea cycle capacities.

This section sought to review the functional maturation of fetal liver in vivo, so as to provide insight to the potential liver function at different gestational ages. It is clear that the functionality of early liver, at the time when scientific studies of liver tissue have been undertaken, is modest. Certainly, those studies which have examined trends in liver function during gestation show very large changes this time frame, however, the neonatal liver just barely has the requisite function to ensure survival. Whilst there is credible data showing that differentiated fetal

liver cells survive in culture, at least in the rat, there is no credible data to describe specific mediators of maturation and proliferation, and no data to show enhanced functional status as a response to intervention. Transplantation of fetal liver cells, as presently understood, could not be a therapeutic intervention, until more data are available to clarify how these cells can be made suitable for such a purpose.

5.7 Maturation signals in the fetal liver.

5.7.1 Introduction.

During fetal life, organ growth and physiological maturation occur, in order to equip the fetus with such physiological reserve as appropriate to the rigours of parturition and the initial stages of independent life. Maturation of the fetal liver is central to survival during this phase.

5.7.2 Glucocorticoids

In animal studies, glucocorticoids in the fetal circulation increase markedly during late gestation (Norman et al., 1985), in parallel with glucocorticoid receptor expression (Speirs et al., 2004) in fetal liver. Although glucocorticoid receptors have been detected in human fetal liver (Ballard and Ballard, 1974), the action of glucocorticoids has not been documented in developing humans. Cell culture work using animal tissue reveals that glucocorticoids promote urea synthesis from ammonia by enhancing activity of all five urea cycle enzymes (Ulbright and Snodgrass, 1993) in fetal rat. Maturation of the urea cycle is an

absolute requirement for perinatal survival (Schofield et al., 1999) and for perinatal gluconeogenesis, which is also under direct stimulatory input from glucocorticoids (Hanson and Reshef , 1997), as approximately 2 moles of ammonia are generated for every mole of glucose synthesised from amino acids. Glucagon is reported as being of increasing importance in the immediate perinatal phase of gluconeogenesis (Hanson and Reshef, 1997), with some evidence for glucagon resistance in fetal life (Devaskar et al., 1984; Vinicor et al., 1976). Together, glucocorticoids and glucagon may have a potent role to bring about the functional development of the liver.

5.7.3 Thyroid hormones

In parallel with glucocorticoids, thyroid hormone in fetal liver may exert developmental effects. T3 causes a re-prioritisation of plasma protein synthesis in fetal rat liver; albumin mRNA and protein synthesis is increased in a dose-dependent manner by T3, whilst AFP mRNA and protein is co-ordinately decreased (Anteby et al., 1993). In human HepG2 cells, AFP and alpha-1-antitrypsin secretion is similarly decreased, whilst alpha-1-acid glycoprotein was increased by T3 (Kobayashi and Horiuchi, 1995). T3 is also noted in animal studies for a potent proliferative effect in hepatocytes (Cubero et al., (2005), and a role to support glycogen synthesis (Betley et al., 1993), both of which are features of the developing liver. These data suggest that T3 may be important in human liver development, but do not clearly indicate whether proliferation or function will be supported by this hormone.

5.7.4 Sex steroids

Oestrogens and progesterone are present in umbilical vein blood where they are conducted directly to the fetal liver. As gestation progresses, a complex interplay between fetal liver and placenta develops which is responsible for the synthesis of oestriol, the predominant oestrogen towards term in the human. Whether sex steroids have a role in liver development is not known, as the data from the literature regarding sex steroids and liver development are extremely scant. Simpson and Carr (1984) demonstrated increased synthesis of cholesterol from human fetal liver cells in response to high levels of oestradiol, which occurred in a dose-responsive manner. In adult humans, exogenous sex steroids are associated with the formation of liver adenomas, which are thought to have malignant potential. The data suggest a proliferative response, or a change in differentiation status, as a result of oestrogens or androgens (Belghiti and Farges, 2001). Given the extremely high levels of sex stroids in the liver inflow, it remains entirely possible that these hormones may have significant effects on liver function, however, there are no published data.

Section 6.

Summary and Objectives

The fetal liver provides significant potential for the treatment of liver failure. During fetal life, the developing liver is required to attain sufficient mass and biochemical competence to support neonatal survival at term. These various competencies, which include plasma protein secretion, ureagenesis, gluconeogenesis, bile secretion and conjugation, are precisely those required in adults with liver failure. In contrast to the failing liver, the fetal liver demonstrates increasing functional competence over time, presumably in response to various signals provided by the fetal environment. It is this capacity for proliferation and functional maturation which makes the developing liver so important in the search for new modalities of treatment in liver disease.

It is clear from the review of the literature that there is considerable depth of knowledge regarding the cell biology controlling the earliest stages of liver development. Similarly, there is considerable knowledge which relates to the developmental anatomy. Despite this, the functional status of the liver cells, which cannot be determined by PCR or immunocytochemistry, has been neglected, despite the critical observation that it is the functionality of the liver rather than its fascinating organogenesis which sustains life. In particular, the

environmental cues which trigger functional liver development in humans are unknown.

In order to address these issues, an hypothesis was formed to focus specifically on the acquisition of liver function in development. This hypothesis states that 'Soluble factors which mediate tissue development act on fetal liver to induce proliferation and maturation of liver tissue'.

To address this hypothesis, the following aims were identified;

1. To develop a culture system which could support developing human liver tissue in vitro, and to confirm that liver tissue retained measurable functionality in vitro (Chapter 3).
2. To determine candidate maturation signals using functional assays of liver cells in the presence and absence of putative signalling ligands (Chapter 4).
3. To investigate prolifcration signals in developing liver, and to assess the cellular phenotype responding to the individual proliferation signals (Chapter 5).
4. To assess the effects of maturation and proliferation signals on functional aspects of developing liver (Chapter 6).

The purpose of this thesis is to develop a suitable model of developing human liver, and to examine progenitor hepatocyte proliferation and maturation. Further, this thesis sought to determine how biological competence varied after exposure to these various agents which are capable of inducing proliferation and maturation.

A series of experimental chapters follows which describes these avenues of investigation. A final chapter presents a unified view of the experimental findings, and suggest areas for further study.

Chapter 2

General materials and methods

2.1 Introduction

This chapter describes techniques common to a number of studies in this thesis. Methods which relate to a specific chapter are elaborated upon in the relevant chapters. The specific methods for fetal liver tissue culture are discussed in chapter 3, where the various steps required to develop the technique are also presented.

2.2 Urea assay.

The kit commercially available to measure urea from ammonia (Sigma) is based on the Berthelot reaction. This reaction results in the formation of indophenol, a highly coloured blue product, from ammonia, hypochlorite and phenol in the presence of nitroprusside, which acts as a catalyst. This reaction is shown in full below (Figure 2.1). In order to measure urea with this reaction, urease is added initially, converting all urea to ammonia which then participates in the Berthelot reaction. A problem arises in cell cultures to which free ammonia has been added to provide a precursor for urea synthesis. In order to measure urea alone, the samples are assayed twice, once with urease and once without. The

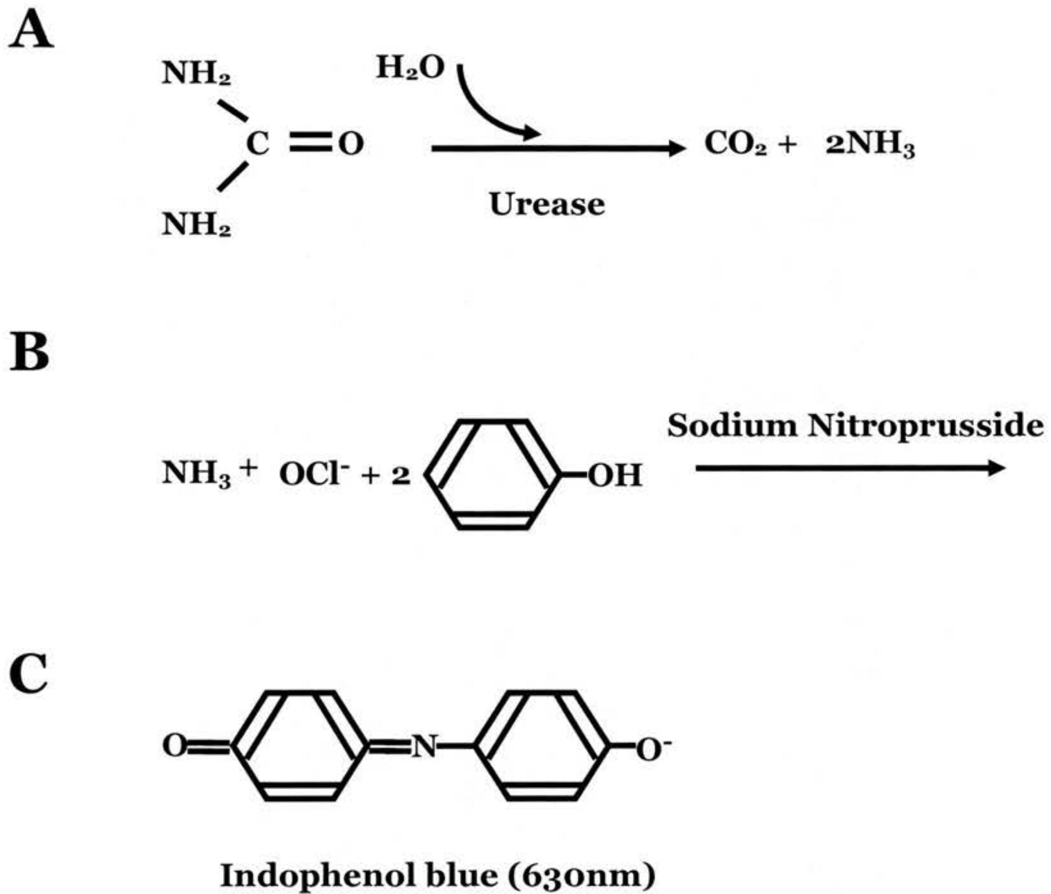


Figure 2.1. The Berthelot reaction to detect urea by colorimetry
A; Urea is converted to ammonia by enzymatic hydrolysis (urease). **B**; Ammonia, hypochlorite and phenol are mixed. **C**; In the presence of nitroprusside, indophenol blue is formed and detected at 630nm.

difference between the two estimations reveals the amount of ammonia derived from urea, whereas the estimation in the absence of urease shows the total amount of free ammonia remaining in the sample.

The kit available from Sigma was designed to utilise a sample size of 10 µl, with a final reaction volume of 7.5ml in a 10ml test tube. As every single experiment would generate a minimum of 48 samples, which would be prohibitively time consuming and inaccurate to manage in individual test tubes, it was decided to re-develop the assay on a semi-micro basis which would allow handling of large numbers of samples relatively easily.

The Sigma urea assay kit No. P 640 was used throughout as the basis for this technique. Costar 96-well ELISA low protein binding plates (Corning Inc.) were used throughout. Samples were kept frozen at -20°C prior to assay. On the day of assay, samples were thawed, kept on wet ice, and divided into two aliquots which were assayed separately in triplicate, with aliquot 1 assayed in the presence of urease and aliquot 2 in its absence. So as to reduce the assay from tube to microplate level, the assay volumes were divided by 40 and the assay replicated directly, giving a final reaction volume of 190µl.

2.2.1 Standards

Standards were made from analytical grade urea (Sigma) dissolved in ureagenesis buffer (Table 2.1). Other constituents are shown in Table 2.2.

2.2.2 Method

To each well was added 12.5μl of urease in buffer or buffer alone and 2.5μl of sample, standard or blank in triplicate. The plate was briefly agitated on a plate shaker, and then incubated for 30 minutes at 37°C in a humidified incubator. The phenol/nitroprusside, alkaline hypochlorite and water were then added; the plate was again agitated and incubated at room temperature for 30 minutes. The absorbance of each well was then measured at 630nm on a Dynex MRX II platereader (DYNEX Technologies, USA)., using the mean of triplicate readings with blank subtraction.

It was not clear as to what the range of the assay would be in microplate format, and so an experiment was designed to establish the concentrations of urea which might be detected by the assay. A range of standards was made up using serial dilutions. The absorbance readings relating to the various standards are shown in Figure 2.2 below.

Table 2.1 Ureagenesis Buffer.

10 ml of 10x Earle's Balanced Salt Solution (Invitrogen) with calcium and magnesium

0.5 ml of 20% human serum albumin

1 ml of Penicillin/Streptomycin

1 ml of sodium pyruvate stock (100mM)

1 ml of 1M HEPES

1 ml of 2 M glucose stock

3ml of 7.5% $NaHCO_3$ stock

Ammonium chloride 1M stock (sterile filtered) 1:1000 dilution as required.

Make up volume to 100ml with sterile culture-grade water.

Table 2.2. Substituents of urea assay buffer.

Reagent	Volume
Urease	12.5μl
Standard/Sample/Blank	2.5μl
Phenol Nitroprusside	25μl
Alkaline Hypochlorite	25μl
Deionised Water	125μl
Total assay volume;	190μl

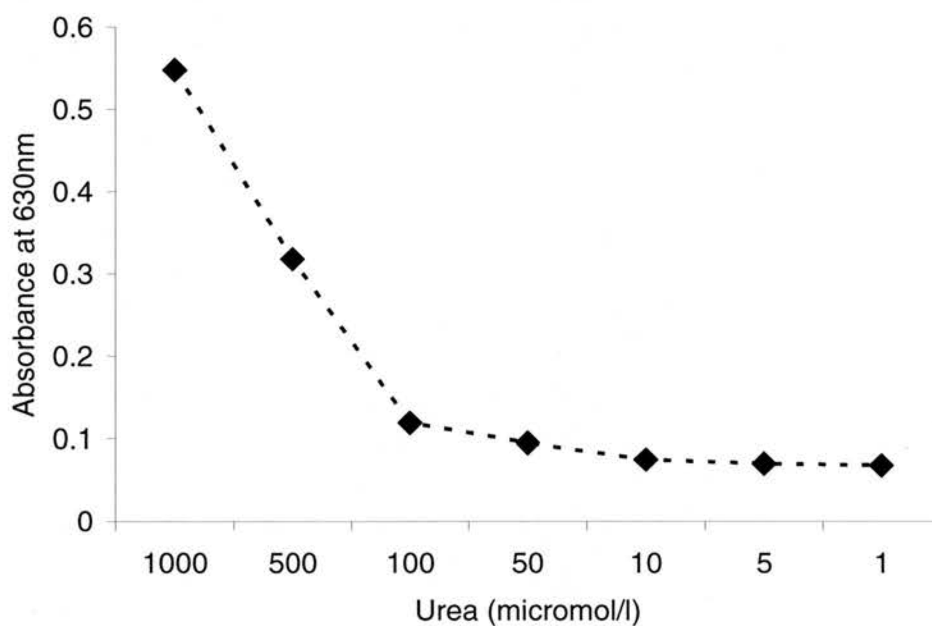


Figure 2.2. Titration of the range for the urea microplate assay.
In triplicate wells, urea standards were prepared in assay buffer from 1000 down to 1 micromol/l. The assay protocol was carried out and the colour reaction product indophenol blue was measured by absorption at 630nm. Absorption minus reagent blank was plotted against urea standard concentration. From the curve plotted above, the useful range of the assay appeared to be from 100 to 1000micromol/l, and further modification was required to deliver a more sensitive assay technique.

The limit of detection appeared to be between 50 and 100 μmol/l, which was felt to be insensitive. As it was likely that the amounts of urea synthesis by the cultures would be small, the sample volume was increased 10 times to 25μl and the urease volume was increased to 50μl. Other volumes remained the same. The duration of urease incubation at 37°C was set at 30 minutes or 60 minutes, and the assay was then repeated. The results are shown below in Figure 2.3. As can be readily seen, the assay appears more sensitive than previously. Incubation for 60 minutes gives a curve which lay on the horizontal axis after blank subtraction, and was felt to give better assay performance. These data were re-plotted to demonstrate the linearity of the relationship, which was entirely acceptable, as shown in Figure 2.4, below.

Ultimately, the decision was taken to utilise standards between 0 and 500μmol/l in 100μmol/l increments. As can be seen in the figures, this appears to be the most linear part of the curve.

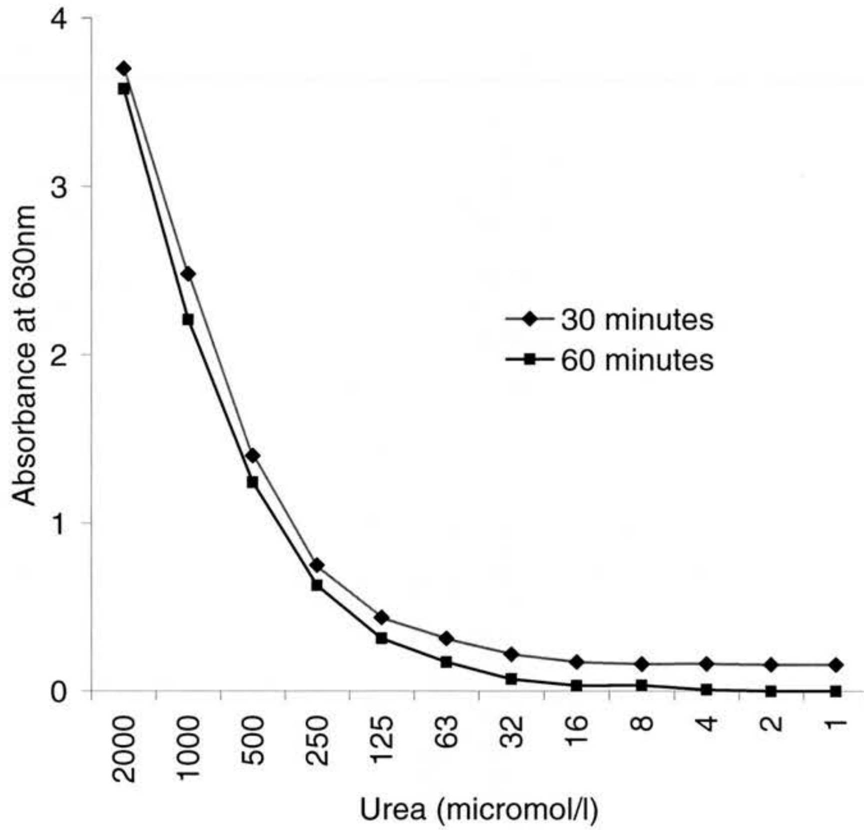


Figure 2.3. Determination of the incubation period at 37°C.
In triplicate, urea standards were prepared in assay buffer from 2000 to 1 micromol/l by serial dilution. The volume of standard in the assay was increased to 25 microlitres, and the volume of urease solution to 50 microlitres. The assay was carried out as previously except that the incubation at 37°C was extended to 30 minutes or 60 minutes. The reaction product indophenol blue was measured by absorption at 630nm and the absorption minus reagent blank was plotted against urea standard concentration. From the curves above, the assay appeared to be more sensitive than previously. A 60 minute incubation was preferred as it allowed an asymptotic approach to the x-axis.

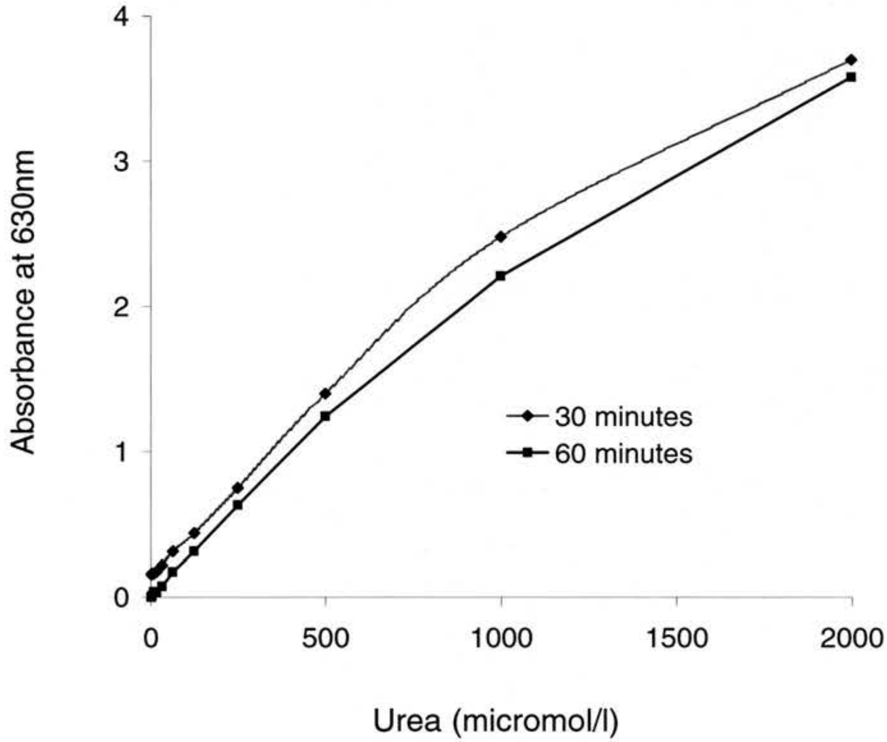


Figure 2.4. Linearity of the urea microplate assay.
In triplicate, urea standards were prepared in assay buffer from 2000 to 1 micromol/l by serial dilution. With 25 microlitres of standard, and 50 microlitres of urease, the assay was carried out with the incubation at 37°C of 30 or 60 minutes. The reaction product indophenol blue was measured by absorption at 630nm and the absorption minus reagent blank was plotted against a linear scale of urea standard concentration. The 60 minute incubation was chosen as the assay reagent blanks approached the origin as shown above.

2.3 General methods for tissue culture

Whilst different cell types and culture methods are used in this thesis, certain techniques are common throughout and are described here.

2.3.1 Materials

All culture media and balanced salt solutions were obtained from Invitrogen, as were fetal calf serum, Penicillin/Streptomycin mix (10,000 Units/ml + 10mg/ml respectively) and Insulin/Transferrin/Selenium supplement (ITS-X). L-glutamine, 1M HEPES, and human serum albumin were obtained from Sigma. Type II collagenase was supplied by Worthington (Lorne Laboratories, Reading) and trypsin EDTA solution by Invitrogen. Type I Rat tail collagen solution was obtained from Roche Diagnostics (East Sussex). All other biochemicals were culture grade or better, and were obtained from Sigma. All plasticware was sterile, disposable, and was supplied by Invitrogen.

2.3.2 Cell culture

Cells were cultured in Costar polystyrene flasks, or in 6, 12, 24 and 96 well plates. The culture medium depended on the cell type used, however, culture of HepG2 cells (hepatoma cell line) required 10%FCS in DMEM with antibiotics (1:100 dilution of stock) and L-glutamine (2mmol/l final concentration).

2.3.3 Trypsinisation

Cells were trypsinised by pouring off culture medium, washing twice in an appropriate volume of warm Hank's Balanced Salt Solution without calcium or magnesium, then adding an aliquot of 5ml trypsin/EDTA solution warmed to 37°C, and pouring off the excess. After this step was repeated, cultures were incubated at 37°C until the cells were released from the substrate, as demonstrated by loosening after a sharp tap. Ice cold culture medium containing 10% fetal calf serum was added to the suspension to neutralise the trypsin, and the cells were poured off into a 20 ml universal container and spun down at 1500 rpm (320g) for 5 minutes (MSE Centaur I, Thermo Life Sciences). The supernatant was poured off, and cells were then resuspended as appropriate in a specific volume of medium or buffer. An aliquot was then removed for cell counting using a trypan blue technique as described below.

2.3.4 Cell counting.

An aliquot of cell suspension with a known volume was removed and added to a known volume of 4% trypan blue (Sigma) and mixed on a vortex mixer, providing a dilution between 1:2 to 1:20 as desired. This suspension was added to the counting chamber of a Neubauer haemocytometer and viewed at high power under an Olympus CHA microscope (Olympus UK). Five small squares from the field of 25 were counted, with those cells touching the upper or right sided dividing lines being counted, and those crossing the lower or left sided lines excluded. The total count and the number of blue (dead) cells were noted,

and the various calculations were carried out as described below to provide an indication of viability and cell density.

Viability = total cells – dead cells/total cells x 100%

Mean live count per square = total live count/5 = A

Dilution factor = B

Number of cells/ml = $A*B*10^4$

Total number of cells = $A*B*10^4$*volume of suspension

Cells were then replated as appropriate in warmed culture medium at a specific density/cm^2, or otherwise processed for experiments.

2.4. Characterisation of liver cells by flow cytometry

Flow cytometry utilises specific antibodies linked to fluorescent labels to identify cell populations expressing the antigen of interest. In brief, a cell suspension containing cells which have bound antibody is treated with a fluorescently labelled second antibody. This cell suspension is fed through the cytometer, and laser excitation at specific wavelengths induces fluorescence at different wavelengths. This fluorescence is detected as the cell passes a photomultiplier, and the cell is 'counted' as immmunopositive/negative for the antigen of interest.

Most commonly, cell surface antigens are first choice when developing a method for flow cytometry. However, the cell surface antigens for human fetal hepatocytes were not recorded in the literature, and so methods were developed to detect the known intracellular antigens of developing hepatocytes and biliary tract cells.

These techniques were not available in the laboratory, and so were developed as part of the preliminary characterisation of the fetal cell cultures. All antibodies were obtained from DakoCytomation. In the first instance, Hep G2 cells were used to assess which fixation and permeabilisation protocol would be suitable. Subsequent studies utilised either HepG2 or fetal liver cells to validate the technique. Flow cytometry to assess cell surface antigens, as described in chapter 3, was carried out in exactly the same manner, except that the permeabilisation step was omitted.

2.4.1 Fixation and Permeabilisation for Flow Cytometry.

40g Paraformaldehyde (Sigma) was weighed out accurately and added to 850ml deionised water. This was heated to 70°C for 30 minutes with stirring. A few drops of 1M NaOH were added to solubilise the paraformaldehyde, after which 100ml 10x phosphate buffered saline was added (Table 2.3). The solution was then allowed to cool, made up to 1000ml with deionised water, the pH adjusted to 7.4 as required and stored for up to 3 weeks at 4°C. This stock solution of 4% formaldehyde was used to prepare more dilute fixatives as required.

Table 2.3 10x Phosphate buffered saline

NaCl	80g
KH_2PO_4	0.2g
Na_2HPO_4	14.4g
KCl	2g

To make 10X PBS, the salts were made up to 1 litre with deionised water, and the pH was adjusted with concentrated HCl or NaOH to pH7.4 (pH meter, model 410, Thermo Orion, UK). This was diluted 10 fold with deionised water for use.

In order to fix cells for flow cytometry, cell cultures were trypsinised and washed as described above. Cell suspension in cold culture medium was placed in 3ml polypropylene tubes (100,000 cells per tube; Sarstedt, Cologne) and cells were spun down at 200g for 5 minutes. Cells were resuspended in 0.5ml fixative and incubated at 4°C for 15 minutes. To compare fixatives, this technique was carried out in two concentrations of formaldehyde in PBS, 0.05% and 0.5%. Fixatives for this experiment were prepared by diluting stock fixative (4% paraformaldehyde in 10mM PBS, pH 7.4), with 10mM PBS.

2 ml 1%BSA/0.1% sodium azide in PBS pH 7.4 (wash buffer) was subsequently added to each tube to neutralise fixative and block non-specific binding sites. Cells were then spun down at 320g for 5 minutes as before and the inactivated fixative was poured off. Having fixed and washed the cells, cell membranes were permeabilised with either 0.1% triton X (Sigma) in blocking solution (10% normal swine serum (DakoCytomation), in wash buffer) or blocking solution alone for 30 minutes. 2 ml wash buffer was subsequently added to each tube which was spun down as previously.

2.4.2 Fibrinogen Flow Cytometry.

Cells were resuspended in 50 microlitres rabbit anti-Fibrinogen antibody (DakoCytomation) at 1:50, 1:100 or 1:200 dilution in blocking solution for 60 minutes at room temperature. 2 ml wash buffer was added to each tube which was vortexed and centrifuged as before. 50 microlitres swine anti- rabbit-

fluorescein isothiocyanate conjugate (SWAR-FITC; DakoCytomation) were added to each tube at 1:20 dilution in blocking agent and the tubes were incubated for a further 30 minutes. 2 ml wash buffer was added, and the cells spun down and resuspended in 200 microlitres of wash buffer ready for flow cytometry. Control primary antibody was an affinity-purified normal rabbit IgG fraction at the same dilutions as primary antibody (DakoCytomation).

The flow cytometer (Coulter EPICS XL-MCL; Coulter UK) was calibrated using control tubes prepared at the same time as the experimental samples. Excitation was by 488nm argon laser, and emission fluorescence was collected through a 525 (green), 575 (red) or 620 (deep red) nm narrow bandpass filter (+/- 5nm). In the case of FITC conjugated antibodies, the 525 nm filter set was used. Forward scatter and side scatter were set to exclude debris. Photomultiplier gain was adjusted to ensure data collection from all cells. Data were collected electronically. Tubes incubated with control rabbit antibody and secondary antibody were included in these tubes so as to estimate true background fluorescence. The gain on the flow cytometer was then finely adjusted, as were various gates, to discriminate between the negative and positive controls and collect specific data relating to different cell populations. Mixtures of both positive and negative controls were prepared and run through the cytometer to confirm that discrimination was satisfactory.

2.4.3 Results

Figure 2.5 shows the results expressed as relative fluorescence compared to control antibody, in cells with and without permeabilisation, at different levels of fixation. As can be seen, 0.5% formaldehyde with 0.1% Triton X provided the best permeabilisation and fluorescence signal, and the best signal to noise ratio was obtained with an antibody dilution of 1:200.

Figure 2.6 shows the individual cytograms obtained during the dilution curve of anti-fibrinogen antibody in permeabilised cells fixed with 0.5% formaldehyde. Although individual antibody dilutions must be determined empirically, all subsequent experiments with intracellular antigens utilised 0.5% paraformaldehyde in 10mM PBS as a fixative and 0.1% triton X as a permeabilisation agent.

2.4.4 Two colour flow cytometry for fetal hepatocytes.

Cultured fetal liver contains many different cells by phase contrast microscopy, and the histology of the liver at this developmental stage reveals that hepatocytes represent less than 50% of all cells. It was therefore necessary to develop a method to quantify hepatocytes unequivocally, so as to determine effects of various proliferation and maturation signals.

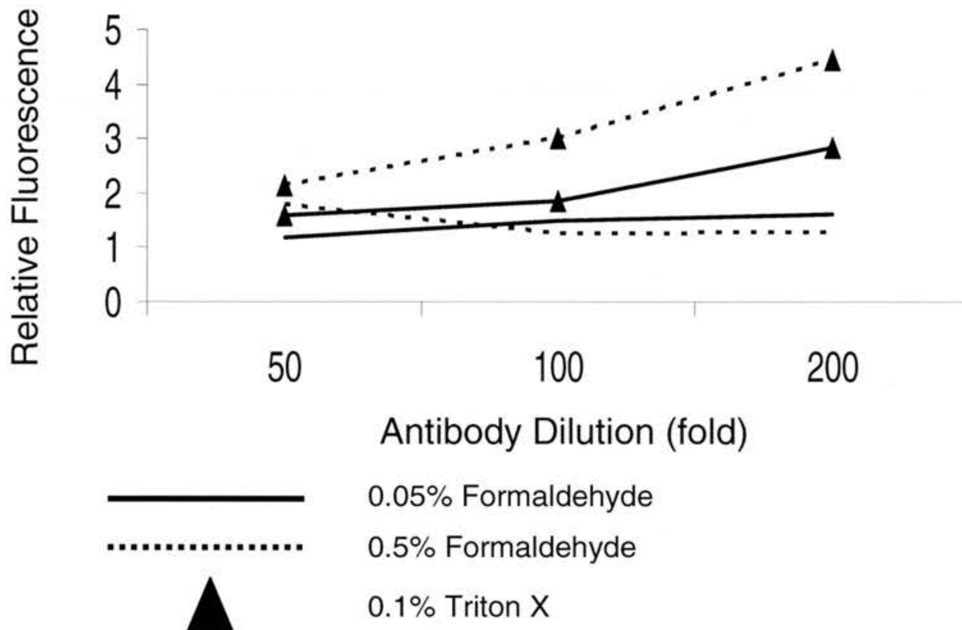
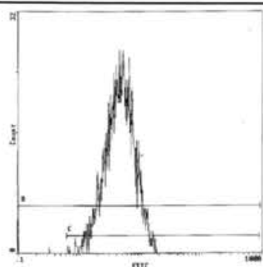
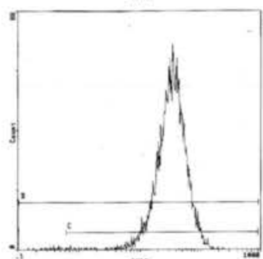


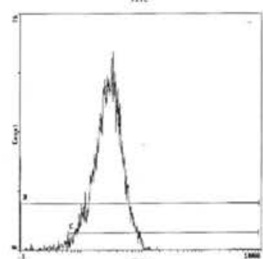
Figure 2.5. Fixation and permeabilisation for flow cytometry
Cell cultures were trypsinised, washed, and resuspended in 0.5ml fixative (0.05 or 0.5% formaldehyde) per tube and incubated at 4°C for 15 minutes. 2 ml wash buffer was added to each tube to neutralise fixative. Cells were spun down and permeabilised with either 0.1% triton X in blocking solution or blocking solution alone for 30 minutes. Cells were resuspended in 50 microlitres rabbit anti-Fibrinogen antibody at 1:50, 1:100 or 1:200 dilution in blocking solution for 60 minutes at room temperature. Cells were then washed with 2 ml wash buffer per tube, spun down and the supernatant discarded. 50 microlitres swine anti- rabbit-fluorescein isothiocyanate conjugate were added to each tube at 1:20 dilution in blocking agent, and the tubes were incubated for a further 30 minutes. 2 ml wash buffer was added, and the cells spun down and resuspended in 200 microlitres of wash buffer ready for flow cytometry. Control primary antibody was an affinity-purified normal rabbit IgG fraction at the same dilutions as primary antibody. The results were expressed as relative fluorescence compared to control antibody, in cells with and without permeabilisation, with the two fixatives.



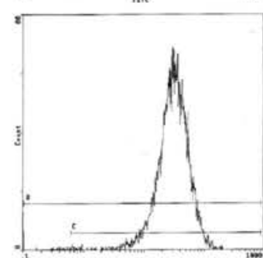
Normal Rabbit Serum control 1:50



Rabbit anti-Fibrinogen 1:50



Normal Rabbit Serum control 1:100



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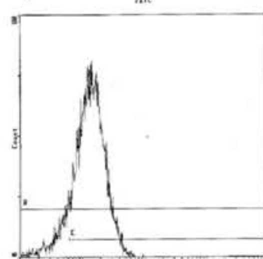
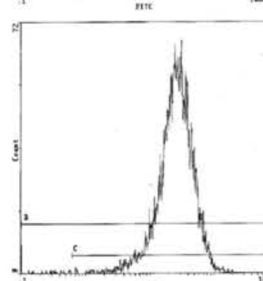


Normal Rabbit Serum control 1:200



Rabbit anti-Fibrinogen 1:200

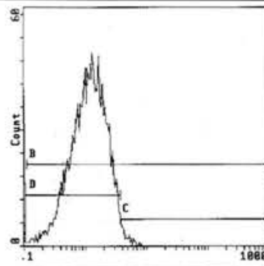
Figure 2.6. Cytograms for anti-fibrinogen flow cytometry in HepG2 cells.
Cells were fixed with 0.5% formaldehyde and permeabilised with 0.1% triton X. After washing, cells were incubated with rabbit anti-Fibrinogen antibody or normal rabbit serum at 1:50 to 1:200 dilution. After a wash step, cells were incubated with swine anti-rabbit FITC antibody at 1:20 dilution. Immunopositive cells were detected by flow cytometry with excitation at 488nm and detection at 525nm.

To quantify fetal hepatocytes, cytokeratin 18 monoclonal antibody was chosen in addition to the previously developed anti-fibrinogen technique. Two-colour flow cytometry would allow the identification and quantification of cytokeratin 18+ve/fibrinogen+ve hepatocytes, and would also allow the characterisation of any cytokeratin 18+ve/fibrinogen-ve epithelial cells, such as cells committed to biliary tract lineages, or primitive epithelial precursors.

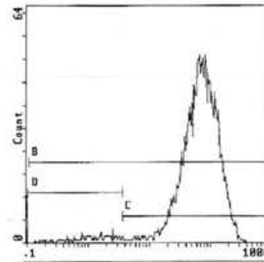
Cytokeratin 18 flow cytometry.

Cells in culture were fixed and permeabilised with the protocol already established above. The blocking serum used was 10% normal goat serum (Scottish National Blood Transfusion Service, Edinburgh) in flow cytometry buffer, and the primary antibody was mouse monoclonal anti-human cytokeratin 18 (DakoCytomation). The second antibody was Goat anti-mouse Phycoerythrin conjugate (DakoCytomation) at a dilution of 1:20 in cytometry buffer. Otherwise all other conditions were the same as for the fibrinogen flow cytometry. The flow cytometer was set to receive fluorescence via the 575nm narrow band pass filter, with excitation at 488nmm.

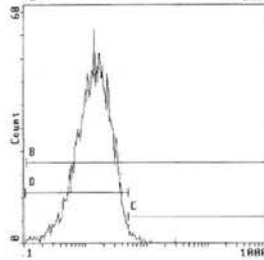
Serial dilutions of the primary antibody (1:25 to 1:100) were used to identify the dilution which gave the optimal specific signal with the lowest background. Figure 2.7 shows the cytograms obtained with anti-cytokeratin 18 and control antibody. The results showed that antibody at 1:100 dilution gave excellent results and this dilution was subsequently used.



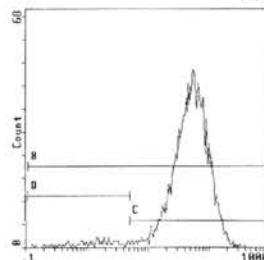
Mouse Immunoglobulin control 1:25



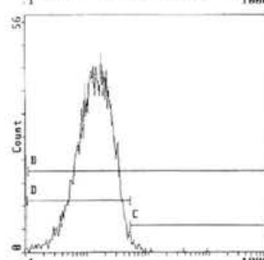
Mouse anti-cytokeratin 18 1:25



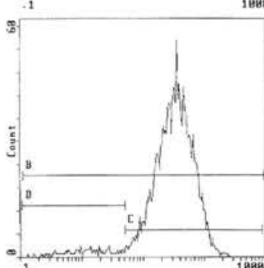
Mouse Immunoglobulin control 1:50



Mouse anti-cytokeratin 18 1:50



Mouse Immunoglobulin control 1:100



Mouse anti-cytokeratin 18 1:100

Figure 2.7. Cytograms for anti-cytokeratin 18 flow cytometry in HepG2 cells. Cells were fixed with 0.5% formaldehyde and permeabilised with 0.1% triton X. After washing, cells were incubated with mouse anti cytokeratin 18 antibody or mouse immunoglobulin at 1:25 to 1:100 dilution. After a wash step, cells were incubated with Goat anti mouse Phycoerythrin conjugate antibody at 1:20 dilution. Immunopositive cells were detected by flow cytometry with excitation at 488nm and detection at 575nm.

Having developed the methods for fibrinogen and cytokeratin 18 flow cytometry separately, the methods were then combined to deliver two-colour identification of four possible populations; dual positive hepatocytes, cytokeratin 18+ve epithelial cells (liver precursors), fibrinogen alone +ve cells (artefact) and dual negative cells. The protocol for this technique is described below.

Two colour flow cytometry.

Cultured cells were trypsinised and washed as previously described. Cells were then resuspended in 0.5% paraformaldehyde in 10mM PBS on ice, and incubated for 15 minutes at 4°C. 2 ml flow cytometry buffer was then added per tube, and cells spun down again.

Cells were then resuspended in 5% normal swine serum/5% normal goat serum in cytometry buffer (blocking buffer) containing 0.1% Triton X 100 and incubated at room temperature for 30 minutes. 2ml buffer were added per tube, and the cells were spun down as before. The supernatant was poured off, and blocking solution, primary antibodies (anti--cytokeratin 18 at 1:100 and anti-fibrinogen at 1:200) or control antibodies in blocking solution were added in a total volume of 50μl. Control antibodies were mouse immunoglobulin (MIG; DakoCytomation) and normal rabbit purified immunoglobulin fraction (NRIG; DakoCytomation). The control antibodies were made up so as to contain the

same immunoglobulin concentration as the primary antibodies, therefore MIG was used at a final dilution of 1:34, and the NRIG at 1:200.

The tubes were incubated at room temp for 60 minutes, and then 2 ml wash buffer was added and the cells were spun down as before. The supernatant was poured off, and the secondary antibodies, Goat anti-mouse phycoerythrin conjugate 1:10 in block and Swine anti-rabbit FITC conjugate 1:10 in block were mixed in equal volumes to give a solution with each antibody at 1:20. 50 microlitres were added to all tubes, and tubes were incubated at room temperature for 30 minutes in the dark. 2 ml wash buffer were added per tube, and cells were spun down as before. Cells were then resuspended in 250μl buffer on ice and kept in the dark until processed through the flow cytometer.

Forward scatter and side scatter were used to adjust the photomultipliers to get the cells to show 'on-screen'. Separate control tubes, made up in parallel to any experimental tubes, were used to calibrate the flow cytometer, to set the quadrant and linear gates to reject negative cells and count positive cells, and so ensure that colour balancing was correct. This was carried out using a preparation of cells, either unlabelled, single labelled or dual labelled, and by preparing various mixed samples so that cells were identified regardless of the background fluorescence on red or green channels (575 nm and 525 nm respectively). This procedure was carried out each time the flow cytometer was

used. On a daily basis, the flow cytometer was also calibrated and run through a quality control procedure with calibration microbeads, so as to ensure a satisfactory technical performance.

Having set the gates to count cells using the controls, the experimental samples were run through the cytometer without any further adjustment, so as to maintain the same exclusion and inclusion criteria to all cells in all tubes.

Figure 2.8 shows the cytograms for validation of the two-colour flow cytometry. The data show an excellent separation between positive and negative controls, and the double-labelled HepG2 cells are shown in the top right box, as would be expected for a cell population expressing both antigens.

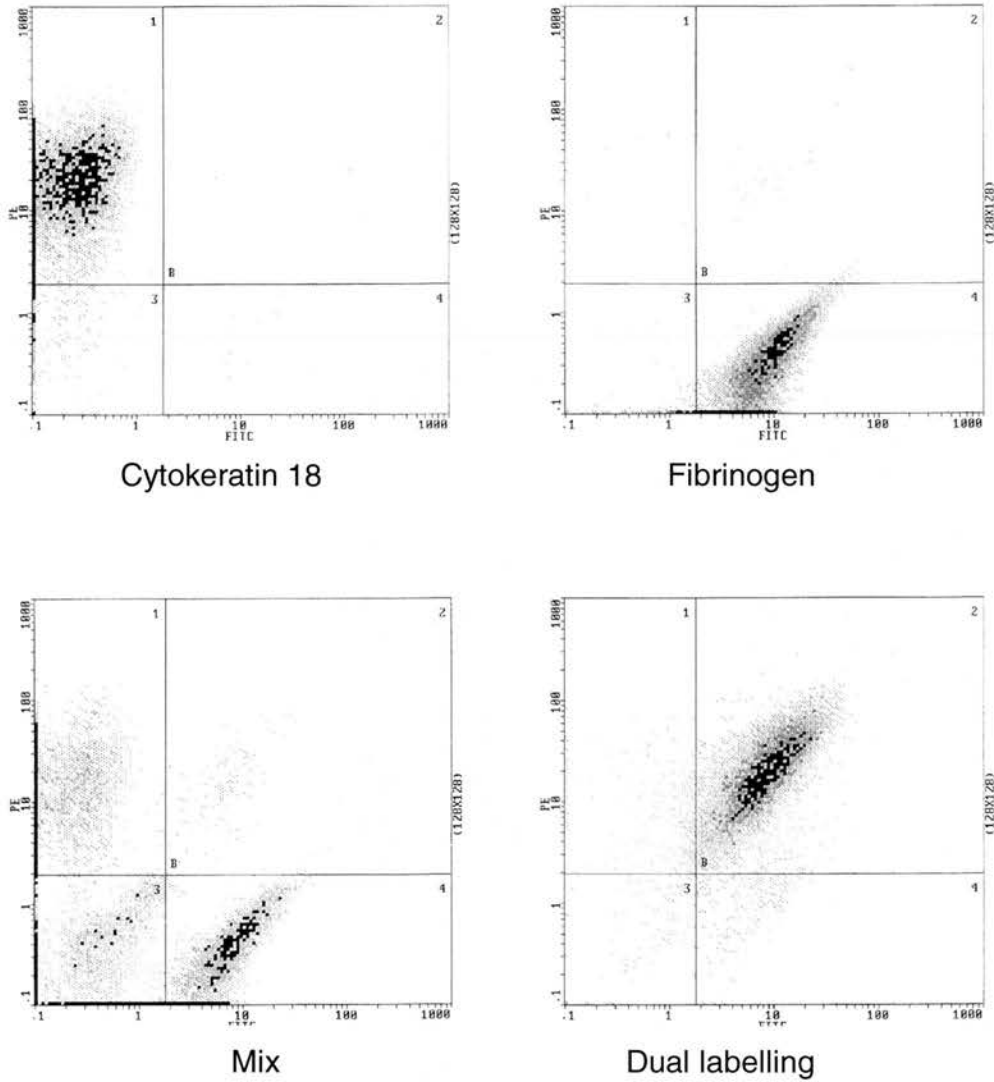


Figure 2.8. Dual labelling of HepG2 cells for Fibrinogen and cytokeratin 18. Cells were fixed with 0.5% formaldehyde and permeabilised with 0.1% triton X. After washing, cells were incubated with mouse anti-cytokeratin 18 antibody or mouse immunoglobulin at 1:100 dilution, in combination with rabbit anti-Fibrinogen antibody or normal rabbit immunoglobulin at 1:200 dilution. After a wash step, cells were incubated with Goat anti-mouse Phycoerythrin conjugate antibody at 1:20 dilution and Swine anti-rabbit FITC conjugate at 1:20 dilution. Immunopositive cells were detected by flow cytometry with excitation at 488nm and detection at 525 and 575nm. The panels represent cells labelled with anti-cytokeratin 18 and normal rabbit serum, anti-Fibrinogen and mouse immunoglobulin, or anti-cytokeratin 18 and anti-fibrinogen. The 'mix' panel represents a mixture of cells labelled with either cytokeratin 18, fibrinogen, or neither.

2.5 Enzyme Linked Immunosorbent Assays for liver proteins.

2.5.1 General Method.

All chemicals were analytical grade or better, and from Sigma, unless otherwise indicated. The general method is as recommended by DakoCytomation, suppliers of the various primary and secondary antibodies. For individual assays, incubations and antibody dilutions were determined empirically in the laboratory to give optimal sensitivity and reproducibility. Costar 96-well ELISA low protein binding plates were used throughout. The constituents of the various buffers and solutions required for the ELISA assays are depicted in Table 2.4.

Samples

Samples were stored frozen until the day of assay. Samples were then thawed and kept on wet ice prior to assay. Samples were centrifuged at 10,000g for 1 minute, diluted in wash/diluting buffer prior to use, from 1:10 to 1:50 as appropriate.

Plate coating

100µl of primary antibody was added to each well in coating buffer, covered, and incubated at 4°C overnight. The plate was then washed 4 times in wash buffer using an automated plate washer (Skatron plate washer; Skatron, Denmark).

Table 2.4 Solutions for ELISA Assays

Coating buffer

0.01M phosphate buffer, 0.15M NaCl, pH 7.2.

$NaH_2PO_4.H_2O$	0.35g
$Na_2HPO_4.2H_2O$	1.34g
NaCl	8.47g
H_2O	to 1 litre

Washing/dilution buffer

0.01M phosphate buffer, 0.05M NaCl, 0.1% Tween 20, pH 7.2

$NaH_2PO_4.H_2O$	0.35g
$Na_2HPO_4.2H_2O$	1.34g
NaCl	29.22g
Tween 20	1ml
H_2O	to 1 litre

Chromogenic substrate

Orthophenyline diamine tablet (2mg)	x 4 (DakoCytomation)
H_2O	12ml
Add 30% H_2O_2 after tablets dissolved	5µl

Reaction stop; 0.5M sulphuric acid

95-97% H_2SO_4	28ml
dH_2O	900ml

Adjust to 1litre with dH_2O

Standards and Samples

Standard or sample was diluted in wash buffer and 100µl of each were added to the microplate in triplicate. Each assay included blanks and a quality control in triplicate. Plates were then covered, incubated at room temperature for 2 hours, and washed again as described above.

100µl of peroxidase-conjugated antibody with the same specificity as the capture antibody was added to each well diluted in wash buffer, covered, and incubated at room temperature for 1 hour in the dark, before being washed again.
100µl of chromogen was subsequently added to each well, and the plate was incubated for 15 minutes at room temperature in the dark. The reaction was then stopped by adding 100µl of stop solution to each well. The absorption of each well at 490nm was then measured using an automated plate reader (Dynex) set to 490nm with a 630nm reference wavelength.

The data were processed using the onboard plate reader software and plotted as a sigmoid semi logarithmic plot of optical density against standard concentration. Results were derived directly by the plate reader. Means of the triplicate results were taken for individual data points.

2.5.2 Individual Assay Methods.

For the fibrinogen assay, the primary coating antibody was polyclonal rabbit anti-human antibody (DakoCytomation, Code A0080), diluted 1:10,000 in coating buffer. Standards were made up using a human fibrinogen standard (Sigma), which was made up to 8mg/ml in wash buffer and stored at -70°C in aliquots, so as to provide sufficient standard pool for this large series of assays. Doubling dilutions gave a standard curve from 800 to 3.12 ng/ml. A quality control sample (QC) was made using standard fibrinogen to give approximately 150ng/ml on the standard curve. This solution was stored similarly to the standard solution. The peroxidase-conjugated antibody was provided by DakoCytomation, and was identical to the primary antibody in all respects, except that the antibody had been labelled with horse radish peroxidase (Code P0445). It was used at 1:5,000 dilution. A sample standard curve for the Fibrinogen ELISA is shown in Figure 2.9.

Equivalent information for other ELISAs is shown in Table 2.5. All antibodies were obtained from DakoCytomation.

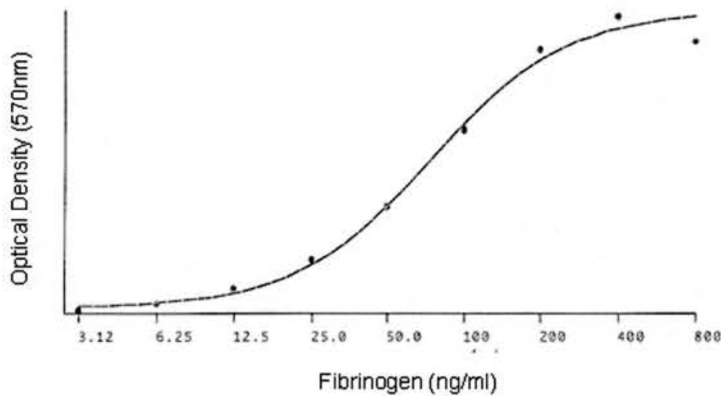


Figure 2.9. Fibrinogen ELISA standard curve Standard fibrinogen was made up in assay buffer at concentrations between 800 and 3.12ng/ml in doubling dilutions. Assay plates were coated with polyclonal rabbit anti-human Fibrinogen antibody, diluted 1:10,000 in coating buffer. Standard or sample was added to coated wells in triplicate and incubated for 2 hours. Peroxidase-conjugated rabbit anti-human fibrinogen antibody was added at 1:5,000 dilution and incubated from 1 hour in the dark. Chromogen (orthophenylene diamine solution. 0.67mg/ml/H_2O_2) was added and the reaction allowed to develop over 15 minutes in the dark. Stop solution (100 microlitres H_2SO_4) was added, and the absorption measured at 570nm.

Table 2.5. ELISA antibodies and standards.

Analyte	Capture Ab	Standards	QC	Peroxidase
Alpha-Fetoprotein	Ra-H* polyclonal 1:2,000 (A0008)	Doubling dilutions, 130-0.51ng/ml AFP (X0900)	AFP (X0900) 10ng/ml	Ra-H AFP-HRP 1:2,500 (P0128)
Prealbumin	Ra-H polyclonal 1:2,000 (A0002)	Doubling dilutions, 250-1.95ng/ml Sigma	Sigma prealbumin 40ng/ml	Ra-H 1:2,500 (PN22)
Alpha-1-antichymotrypsin	Ra-H polyclonal 1:1360 (A0022)	Doubling dilutions, 320-1.25 ng/ml protein calibration solution (X0908)	protein calibration solution (X0908) 50ng/ml	Ra-H 1:2,000 (PE870)
C-Reactive protein	Ra-H polyclonal 1:1,000 (A0073)	Doubling dilutions, 200-0.4ng/ml CRP calibration solution (X0923)	CRP calibration solution (X0923) 20ng/ml	Ra-H 1:4,000 (P0227)

*Ra-H; Rabbit anti human

2.6 Thiazylol blue assay for cell proliferation.

To estimate the viability of cells in culture, the conventional approach is to count the cells manually and determine viability in that way. However, where large numbers of samples are present, or small numbers of cells, this technique becomes too cumbersome and slow to provide accurate data. In these situations, a biochemical technique based on the formazan reaction may be used (Patel et al., 2004).

Various tetrazolium dyes may be converted by mitochondrial oxidases to insoluble formazan crystals which can be solubilised and their absorbance measured spectrophotometrically.

Cells are cultured in the conventional manner in 96 well plates in triplicate in a volume of 100µl medium. At the end of the experiment, 10µl of thiazylol blue tetrazolium (MTT) solution (5mg/ml made up in 10mM PBS, pH 7.4, sterile filtered with a 0.22µm filter) is added to each well, and the cells are incubated as before for 4 hours. 150µl SDS solution (10% Sodium dodecyl sulphate in 0.01M HCl pH 3.0) is added to each well, and the cultures are incubated overnight in the incubator as previously to dissolve the formazan crystals. The plate is then read on the Dynex plate reader at a wavelength of 570nM and a reference wavelength of 630nM. Blank wells without cells are subtracted from all wells, and the data expressed as a percentage of control. A calibration curve (Figure

2.10) overleaf demonstrates excellent linearity of the technique across a wide range of cell densities in culture.

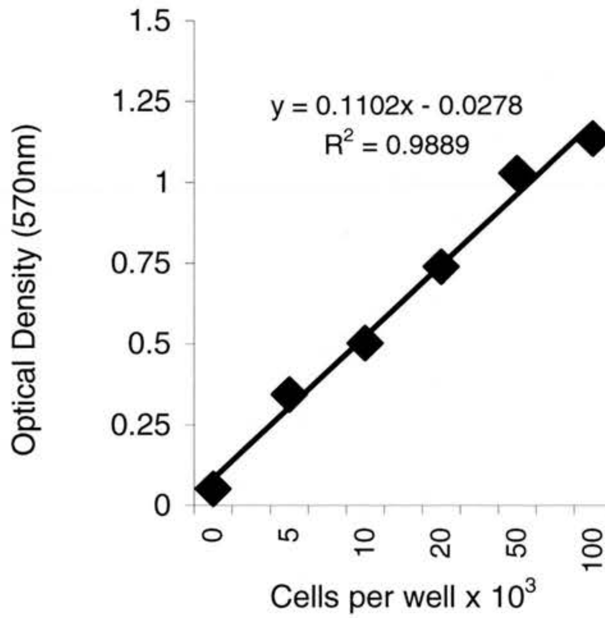


Figure 2.10 Thiazylol blue and cell density.
Cells were cultured in96 well plates in triplicate in a volume of 100µl medium. 10µl of thiazylol blue tetrazolium (MTT) solution (5mg/ml made up in 10mM PBS, pH 7.4) were added to each well, and the cells were incubated at 37°C for 4 hours. 150µl SDS solution (10% Sodium dodecyl sulphate in 0.01M HCl pH 3.0) were added to each well, and the cultures were incubated overnight to dissolve formazan crystals. Absorption minus reagent blank was measured at 570nm with a reference wavelength of 630nM.

Chapter 3.

Characterisation of human fetal liver in tissue culture.

3.1 Introduction.

The primary objective of this chapter was to develop a technique to culture human fetal liver tissue in vitro. This chapter details the development of the technique and characterisation required to permit subsequent detailed studies of human liver cells.

The preparation of human fetal liver cultures has been reported in historical studies (Gitlin and Biasucci, 1969). However, such studies did not attempt to maintain tissue in culture over time. Such techniques are therefore not applicable to the investigation of dynamic tissue processes, such as maturation and proliferation, as proposed in this thesis. To address the experimental questions posed in this work, it was necessary to develop such a technique whereby human fetal liver could be maintained in vitro for many days, and could be studied easily over this time period.

Cell culture is an extremely well established technique which offers advantages in the experimental approach considered here. However, the loss of tissue

architecture, and of three-dimensional interactions of different cell types in dispersed cell culture, is a significant issue, particularly in a complex organ such as developing liver. In order to investigate factors regulating proliferation and maturation in fetal liver, it was necessary to develop a cell culture system suitable for developing liver, and then to characterise the cell cultures extensively, prior to embarking on the major experiments of the thesis.

3.2 Materials and Methods

3.2.1. Tissue assessment.

In order to begin cell culture experiments it was first necessary to assess the quality of the tissues obtained. In order to make a simple histological assessment, liver tissue was collected as previously described and placed directly in fixative (4% formaldehyde in PBS) overnight, then transferred to absolute ethanol and stored at 4°C, prior to paraffin embedding.

Tissues were dehydrated and embedded in paraffin wax using an automated Shandon VIP vacuum embedding processor (Shandon UK). The embedded tissue was then added to a sectioning grid, covered in hot wax, then allowed to cool prior to sectioning. Sections were cut at 3 microns on a Shandon 325 microtome. Sections were placed on coated slides (Superfrost slides, VWR, Glasgow, UK) prior to dewaxing.

Slides were dewaxed in baths of 100% xylene x 2, 100% ethanol, 90% ethanol, 80% ethanol, 70% ethanol and then water for 2 minutes each. Tissues were then stained with haematoxylin briefly, washed in distilled water, differentiated in acid alcohol (1% concentrated HCl in 70% ethanol), and 'blued' in Scott's tap water. After a wash in distilled water, sections were dipped in eosin and washed. Xylene and ethanol were obtained from BDH. Counterstains were supplied by Sigma.

After staining and washing, slides were assessed whilst wet under the microscope (Olympus BH2, Olympus UK) to confirm that there was satisfactory stain uptake. Slides were then dehydrated using a reversal of the dewaxing protocol above, but with different solvent baths so as to eliminate contamination. Slides were then mounted in Pertex (CellPath Powys, Wales), coverslipped (Chance Propper, UK) and photomicrographs were taken using an Olympus CK2 photomicroscope with Kodacolour ASA 200 film using a tungsten correction filter. Negatives were digitally scanned by the commercial developer (Pyramid Photography, Edinburgh).

3.2.2. Optimising tissue digestion.

The technique to prepare cell suspensions from tissue pieces was developed from prior work by the author in which fetal neuronal tissue was digested to yield single cell suspension (Currie et al., 1994). Fetal liver has a strikingly insubstantial nature, which suggested that a mild enzymatic digestion with

gentle mechanical agitation might be adequate to prepare single cells. Traditionally, collagenase has been used in the author's laboratory to disaggregate adult liver tissue, and so use of this enzyme preparation was retained.

Fetal liver tissue was collected with informed consent as part of ethically approved studies (Lothian Regional Ethics Committee Reference Numbers *LREC 2001/6/13* and *LREC 2000/6/57*) from therapeutic abortion of fetuses at13-18 weeks of gestation. The livers were removed on ice using an aseptic technique and placed in ice-cold sterile William's Medium on ice for transport to the laboratory.

The liver was dissected free of the biliary tree and large vessels, and the parenchymal tissue was then diced with a scalpel in a sterile glass Petri dish in a class II cabinet (Medical Air Technology, Manchester, UK). The tissue pieces were added to 20 ml ice cold Hank's Balanced Salt Solution without calcium or magnesium (HBSS-ve; Invitrogen) which was gently turned end over end to suspend the pieces and wash out blood cells. The pieces were allowed to settle and the supernatant was removed with a pastette (Corning) and discarded. This wash step was repeated 4 more times so that the supernatant became clear and free of red blood cells. Tissue pieces were then added to 10ml 0.1% Type II collagenase solution (Worthington), made up in warmed HBSS with calcium and magnesium (HBSS+ve; Invitrogen) to which was added 10mM HEPES, 0.1%

Human albumin, Penicillin/Streptomycin (100U/ml and 100micrograms/ml respectively) and incubated at 37°C for 15 minutes with gentle orbital shaking.

After tissue pieces were allowed to settle, the supernatant (Harvest 1) was added to 10ml ice cold WME/10% FCS to neutralize the collagenase. The suspension was then spun down for 6 minutes at 1200 rpm (320g) at 4°C (MSE Mistral 3000i, Thermo Scientific). Meanwhile, the tissue pieces were gently drawn up and ejected through a 1 ml pastette to break up larger chunks, and a further 10 ml of warm enzyme solution was added. The pieces were then incubated for a further 15 minutes exactly as before.

The second supernatant (Harvest 2) was taken off, and any remaining pieces were broken up by the pastette method. The supernatant was briefly added back to suspend cells freshly liberated from the tissue pieces. Large chunks were allowed to settle, and the supernatant was carefully removed. This was added to 10ml ice cold WME/10% FCS as before, and centrifuged under the same conditions.

After centrifugation, the supernatants from Harvests 1 and 2 were discarded, and the cells were gently resuspended in culture medium (see below) on ice. An aliquot of cell suspension was diluted in culture medium and added to an equal volume of 0.5% trypan blue solution in PBS (Sigma). Cell counting and viability

estimation was then carried out using a standard Neubauer haemocytometer as described below.

Initial work showed that the cell preparation might have been inadequately provided with glucose, and so the technique was modified by adding additional glucose to all media to provide a final concentration of 20mmol/l, rather than 5mmol/l. Viabilities of the cell preparations obtained were then compared.

3.2.3 Flow cytometry for fetal hepatocytes.

To demonstrate the presence of hepatocytes before and after cell culture, cells were subject to single label flow cytometry (Fibrinogen) as soon as the cell preparation was completed, and a portion of cells was plated and subject to further flow cytometry after culture (described below). To show conclusively that the cells were indeed hepatocytes, one culture was subject to two-colour flow cytometry, as described in chapter 2.

3.2.4 Phase contrast microscopy of cultured cells.

To provide morphological data in support of the results from flow cytometry, the appearance of the cells immediately after plating, and at 7 days in vitro, was recorded by phase contrast microscopy.

3.2.5. Optimisation of culture substrate and cell adhesion.

In order to maintain cells in satisfactory condition in vitro, it is necessary to provide an adhesive substrate to promote cell growth and function. To address the role of cell adhesion in the success of the fetal liver cultures, cell suspension was freshly prepared as previously described. Cells were then diluted to 2 x 10^7 cells/ml in warmed culture medium, comprising William's Medium Eagle, 10% fetal calf serum, 2 mmol L-glutamine, Penicillin/Streptomycin (100U/ml and 100micrograms/ml respectively) and Insulin-Transferrrin-Selenium concentrate (1ml per 100ml; Invitrogen). Two types of collagen were compared, as were different levels of fibronectin.

Collagen coating

Cells were plated out in 12 well plates which had previously been coated with type I collagen (Roche) or type IV collagen (Sigma). Coating was carried out by adding 180µl of collagen in 0.2% acetic acid at a concentration of 0.1mg/ml. This gave a coating of 5µg/cm^2. Wells were allowed to dry in the Class II cabinet so as to permit a thin film of collagen to form on the culture dishes. Collagen stock solutions were prepared by dissolving collagen powder in 2% glacial acetic acid and preparing 500µl aliquots which were stored at -70°C. To dilute for use, 4.5ml sterile tissue culture grade water was added to a stock vial.

Plating out in Fibronectin solution.

To each well at the time of plating was added 250 μl culture medium containing 0, 10, 20 or 40μg/ml of fibronectin (Sigma). 250 μl of cell suspension at a density of $2x10^7$ cells per ml were then added to give a final volume of 500μl, a total cell count of 5×10^6, and fibronectin concentrations of 0, 5, 10 or 20μg/ml. Each experimental condition was replicated in 6 identical wells, giving a total of 48 wells. Cells were maintained in 5%CO_2 at 37°C in a humidified atmosphere over a 7 day period.

Assessment of culture conditions for hepatocytes.

The culture supernatant was changed for fresh medium on day 1, 3, 6 and 7, with the culture supernatant being centrifuged at 10,000g for 1 minute, then frozen and stored at -20°C for later fibrinogen ELISA. In a parallel experiment, in which the same cell preparation and the same substrate modifications had been made, and in which the medium was changed at the same time, cell content of fibrinogen was determined as an index of adherent hepatocytes. To determine culture content, adherent cells in the culture dishes were scraped off in 500μl of culture medium and this homogenate was similarly stored at -20°C for later Fibrinogen ELISA.

In a further parallel experiment, cells were plated out at different densities on type I collagen coated wells and later trypsinised and counted so as to determine

the approximate numbers of cells able to persist in culture. These cells were photographed at day 0 and day 7 in vitro, so as to provide morphological information on the cells proliferating in the culture system.

3.2.6. Different cell types in liver cell culture

The fetal liver contains a large population of haematopoietic cells, in addition to the resident hepatocytes, biliary tract cells and stromal cells. In order to gain insight to the types of cells which might be interacting with the developing hepatocytes, it was elected to carry out flow cytometry using antibodies directed against a number of well characterized surface antigens.

Flow cytometry was carried out exactly as described for anti-cytokeratin 18 antibody (Chapter 2), except that the permeabilisation step was not required. The antibodies chosen and the dilutions used are shown in Table 3.1. These antibodies had been extensively characterized in the laboratory previously. All antibodies were obtained from DakoCytomation, excepting anti CD29, which was from Serotec UK.

3.2.7. Spectrum of secreted liver proteins

Having determined the appropriate culture conditions to maintain fetal hepatocytes in vitro for at least 7 days, culture supernatant was assessed by various specific ELISAs to determine the spectrum of proteins which were secreted by the cultured cells. In each case, ELISAs were carried out as

described in chapter 2. Culture supernatant was stored frozen at -20°C prior to assay. Samples were thawed and vortex-mixed, then centrifuged at 12,000g for 1 minute so as to precipitate any cells or other particulate material which might contaminate the assay. Samples were then diluted appropriately with assay buffer as previously described prior to assay, so as to ensure that the sample would fall in the dynamic range of the assay. Alpha-fetoprotein, fibrinogen, alpha-1-antitrypsin, C-reactive protein and prealbumin were determined by specific ELISA.

3.2.8. Urea synthesis

Urea synthesis by cultured cells is highly indicative of the presence of functional hepatocytes. To demonstrate urea synthesis, cells were cultured in 24 well plates as previously described. Culture medium was removed and stored for later assay. Wells were washed twice with warmed 0.5ml PBS per well. 300μl ureagenesis buffer (Table 2.1, chapter 2) were added per well and cells were then returned to the incubator for 4 hours. The incorporation of free ammonia was demonstrated by adding 1mM NH_4Cl to half the wells. All media and liquid supplements were from Invitrogen, and all biochemicals were from Sigma. After the incubation was complete, the supernatant was removed and stored at -20°C for later urea assay (chapter 2). Wells were washed twice with 0.5ml culture medium per well which was removed and discarded, then a further 0.5ml culture medium were added and cells were returned to the incubator.

Table 3.1 Antibodies for cell surface antigens.

Antibody	Dilution	Specificity
CD 3	1:50	T cells
CD 14	1:50	Mononuclear cells
CD29	1:50	β1-Integrin
CD34	1:50	Haematopoietic stem cells
CD45	1:50	Leukocyte common antigen
CD90	1:100	Thy-1 stem cell marker
CD235a	1:100	Erythrocyte lineage

The urea assay was carried out with and without urease by dividing the sample in two portions, so as to demonstrate the urea and the residual free ammonia present in culture supernatant.

3.3. Results

3.3.1. Histology of the developing liver.

The photomicrographs in Figure 3.1 demonstrate the integrity of the tissues and clearly show plates of cells which correspond to hepatocytes. At this gestational age, the ductules are still maturing from the primitive ductal plate. The histology shows no evidence of oedema or other cellular injury within hepatocytes, or any distortion of the architecture. Histological assessment reveals no adverse features or impediments to further cell preparation.

3.3.2. Optimising tissue digestion.

The method designed to prepare cell culture readily yielded single cell suspensions with little remaining tissue pieces. To assess the effects of preparation on the cells, viability was calculated as previously described. Table 3.2 shows that viability in the first 15 preparations was lower than expected, certainly below the 95% viability level which any satisfactory method should deliver. The poor viability was of concern, as it was likely to be those cells with the greatest metabolic requirements, the hepatocytes, which were undergoing cell death. The technique of Popovici et al (2001) described the use of DMEM for washing cells. Discussion with the authors of the paper revealed

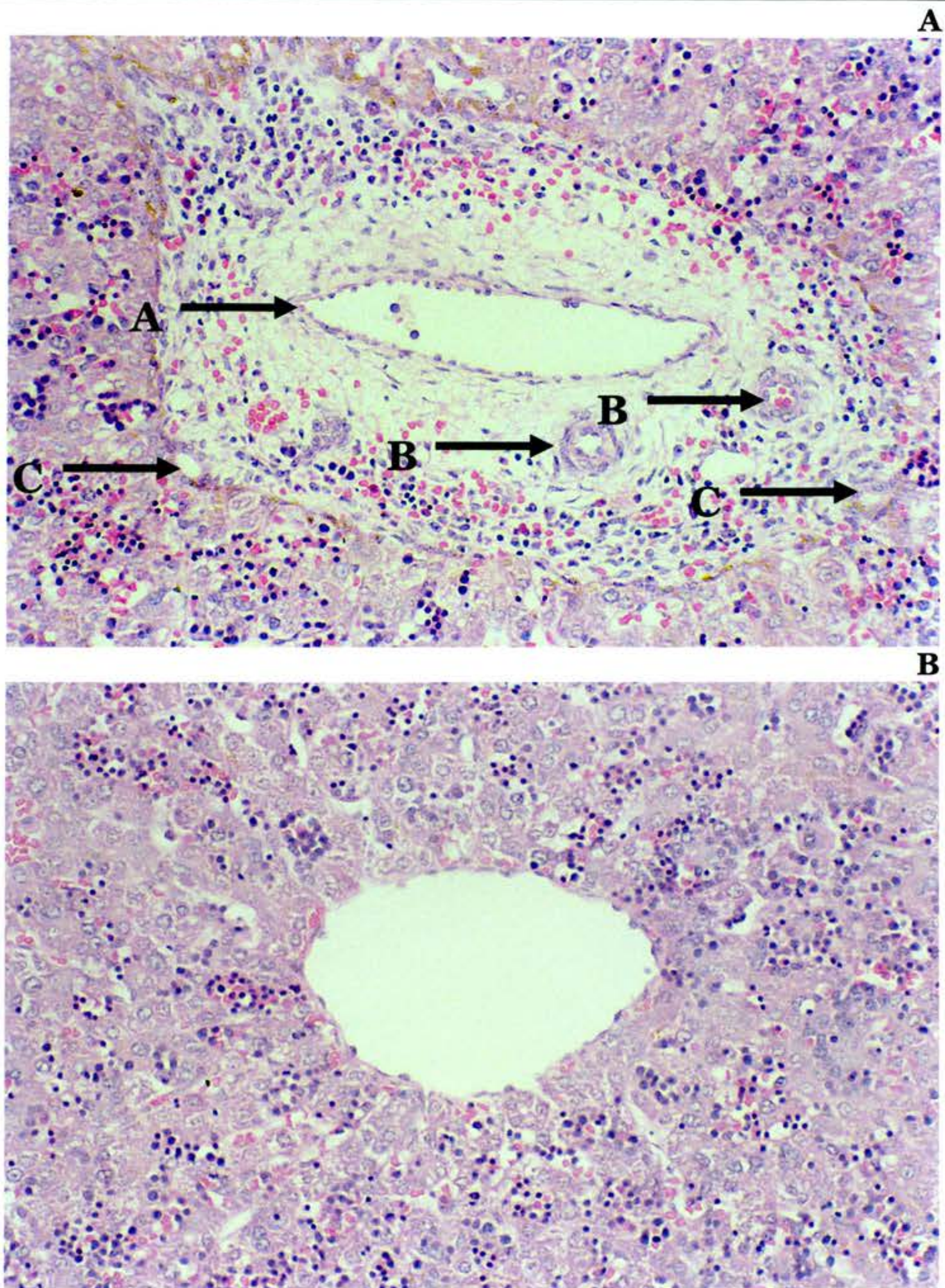


Figure 3.1. Photomicrographs of human fetal liver x 200. Micrograph **A** demonstrates a portal tract. At 'A', lies the portal vein. Within the surrounding mesenchyme, two hepatic arteries are shown at 'B', and biliary ductules can be seen at 'C' within the remodeling ductal plate. Micrograph **B** shows the tissues surrounding a central hepatic vein, the vascular outflow tract of the liver. Compared to the portal tract, there is no mesenchyme, no arterial component, and no biliary structures.

Table 3.2. The effect of glucose supplementation on cell viability.

Cultures (n)	Glucose	Viability
15	5mmol/l	91.3 +/- 1.3%
10	20mmol/l	95.4 +/- 1.2%*

*p<0.05, Student' s T test

that they used high glucose DMEM (20mmol/l) for washes. To assess the effect that additional glucose would have on cell viability, glucose supplementation was added to all cell preparation media to bring the concentration from 5 mmol/l to 20mmol/l. The effect of this modification on viability was significant (Table 3.2). Accordingly, glucose supplementation during culture preparation was routinely employed thereafter.

3.3.3 Flow cytometry for fetal hepatocytes.

Figure 3.2 demonstrates the proportions of hepatocytes determined by flow cytometry before and after cell culture. Approximately 60% of cells are hepatocytes by this single colour technique (Fibrinogen; n=8 preparations and n=3 cultures; mean +/- SE). Two-colour flow cytometry directed against fibrinogen and cytokeratin 18 was then used to confirm the presence of hepatocytes after 7 days culture in vitro. Figure 3.3A shows the cytogram for the control antibodies, and 3.3B the cytogram using both primary antibodies. The proportion of cells falling in the top right square is equal to the % of dual positive hepatocytes, which in this example is 50.3%. Those cells in the top left square are cytokeratin 18+ve, fibrinogen–ve (9.9%), and those cells in the lower left square are dual negative (39.5%). It is worthy of note that fewer than 1% of cells lie in the lower right square, meaning that essentially all cells which are fibrinogen +ve are cytokeratin 18+ve, whereas there is a not insignificant population of cells which are cytokeratin 18+ve and fibrinogen–ve. These data confirm using a two-colour technique that there are hepatocytes present after 7

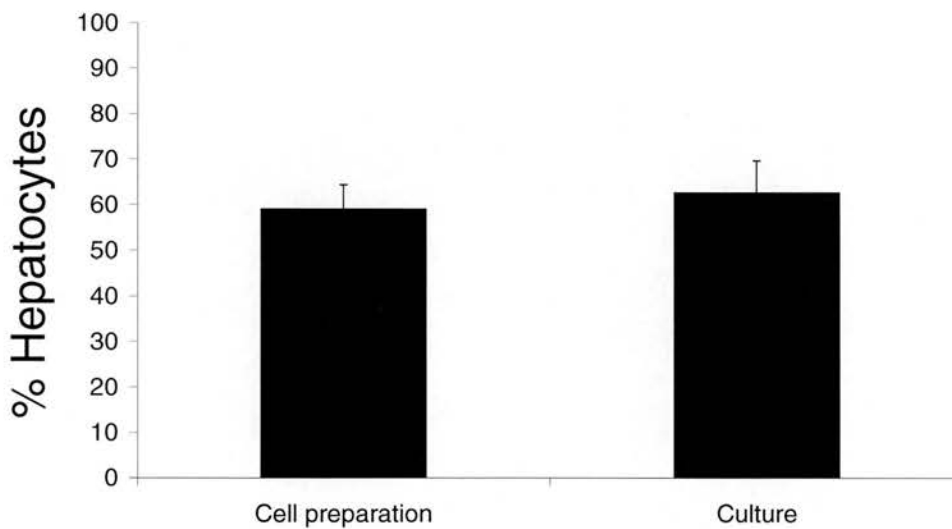
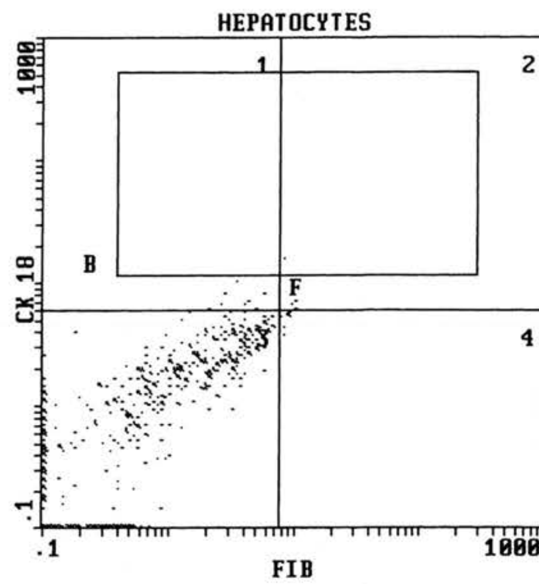


Figure 3.2. Proportions of hepatocytes in vitro.
Tissue pieces were incubated with collagenase twice, and the supernatants spun down to prepare a single cell preparation. The cells ('Cell Preparation') were then fixed with 0.5% formaldehyde and permeabilised with 0.1% triton X. Cells were labelled with rabbit anti-Fibrinogen and mouse anti-cytokeratin 18, and these antibodies were detected by goat anti-mouse-PE and swine anti-rabbit-FITC. Flow cytometry with excitation at 488nm and detection at 525nm and 575nm was used to determine the proportion of cells immmunopositive for both labels. Alternatively, the cell preparation was cultured for 7 days in vitro in culture flasks, then trypsinised and processed for flow cytometry exactly as before. n=8 preparations and 3 cultures; data are shown as means +/- SEM.

A



B

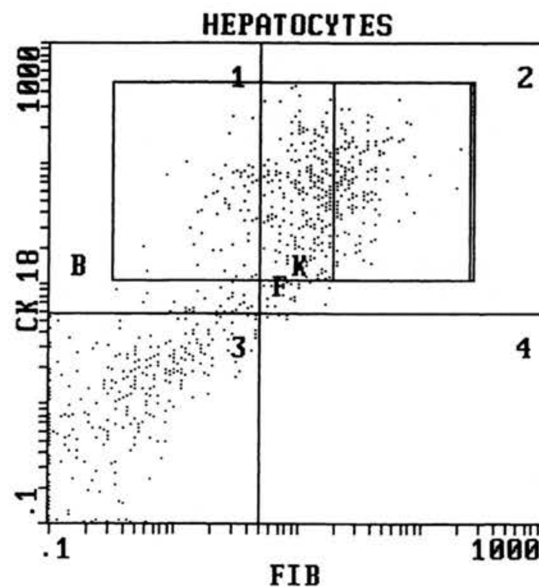


Figure 3.3. Two-colour cytograms demonstrate hepatocytes in vitro.
Cells were fixed with 0.5% formaldehyde and permeabilised with 0.1% triton X. After washing, cells were incubated with mouse anti-cytokeratin 18 antibody or mouse immunoglobulin at 1:100 dilution, in combination with rabbit anti-Fibrinogen antibody or normal rabbit immunoglobulin at 1:200 dilution. After a wash step, cells were incubated with Goat anti-mouse Phycoerythrin conjugate antibody at 1:20 dilution and Swine anti-rabbit FITC conjugate at 1:20 dilution. Immunopositive cells were detected by flow cytometry with excitation at 488nm and detection at 525 and 575nm. Panel A; control antibodies: Panel B; anti-cytokeratin 18 and anti-Fibrinogen antibodies.

days in vitro.

3.3.4 Phase contrast microscopy of cell cultures.

The appearance of the cultures after plating out, and at day 7 in vitro, is shown in Figure 3.4. Whilst cells initially show the rounded appearance expected of freshly prepared cells, the morphology is more complex by day 7 in vitro. Hepatocytes are shown well in Figure 3.4D, however, other cells with very different appearances are also present.

3.3.5. Optimisation of culture substrate and cell adhesion.

Figures 3.5A and 3.5B demonstrate the effects of different substrate coatings on the secretion of fibrinogen by the cultured cells, and figures 3.6A and 3.6B demonstrate the culture content of fibrinogen.

These results demonstrate first of all that there is a trend towards increasing fibrinogen secretion with time in vitro, with twice as much fibrinogen secretion in the 24 hours between day 6 and day 7, as compared with day 0 and day 1. Secondly, there is no obvious difference between type I and type IV collagen in terms of fibrinogen secretion. Finally, fibronectin seems to have little effect on fibrinogen secretion.

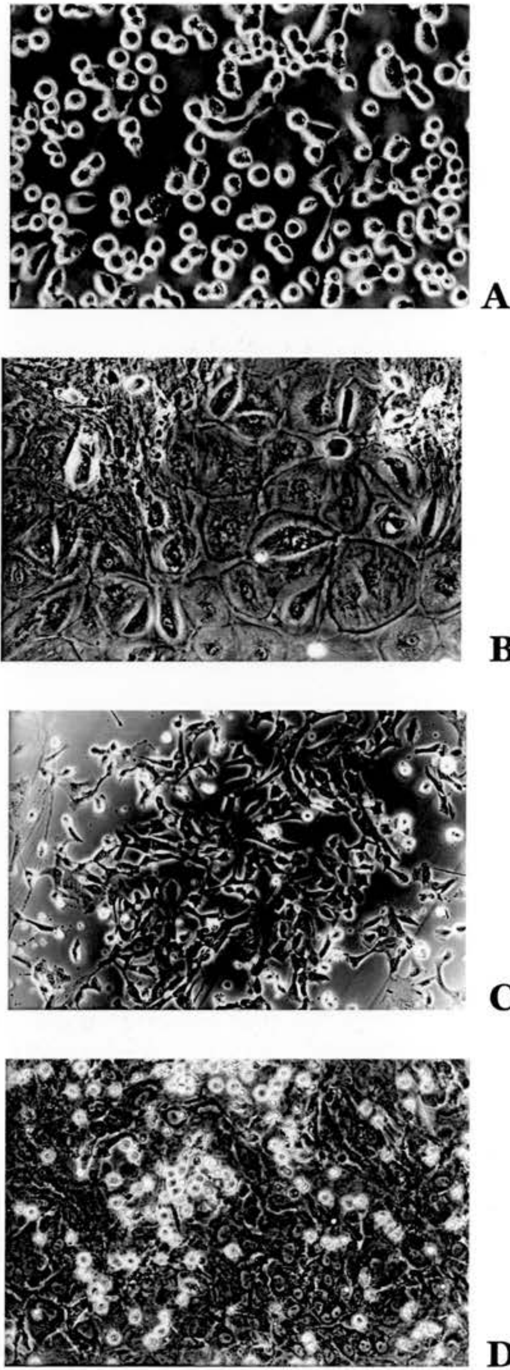


Figure 3.4. Photomicrographs of human fetal liver cells in culture.
Phase contrast photomicrographs show cells just after plating (A) or at one week in vitro (B,C,D). Several distinct morphologies are present: B; 'fried egg' cells, C; fusiform mesenchymal cells, D; hepatocytes with non-adherent cells in the foreground. x100, except B; x200.

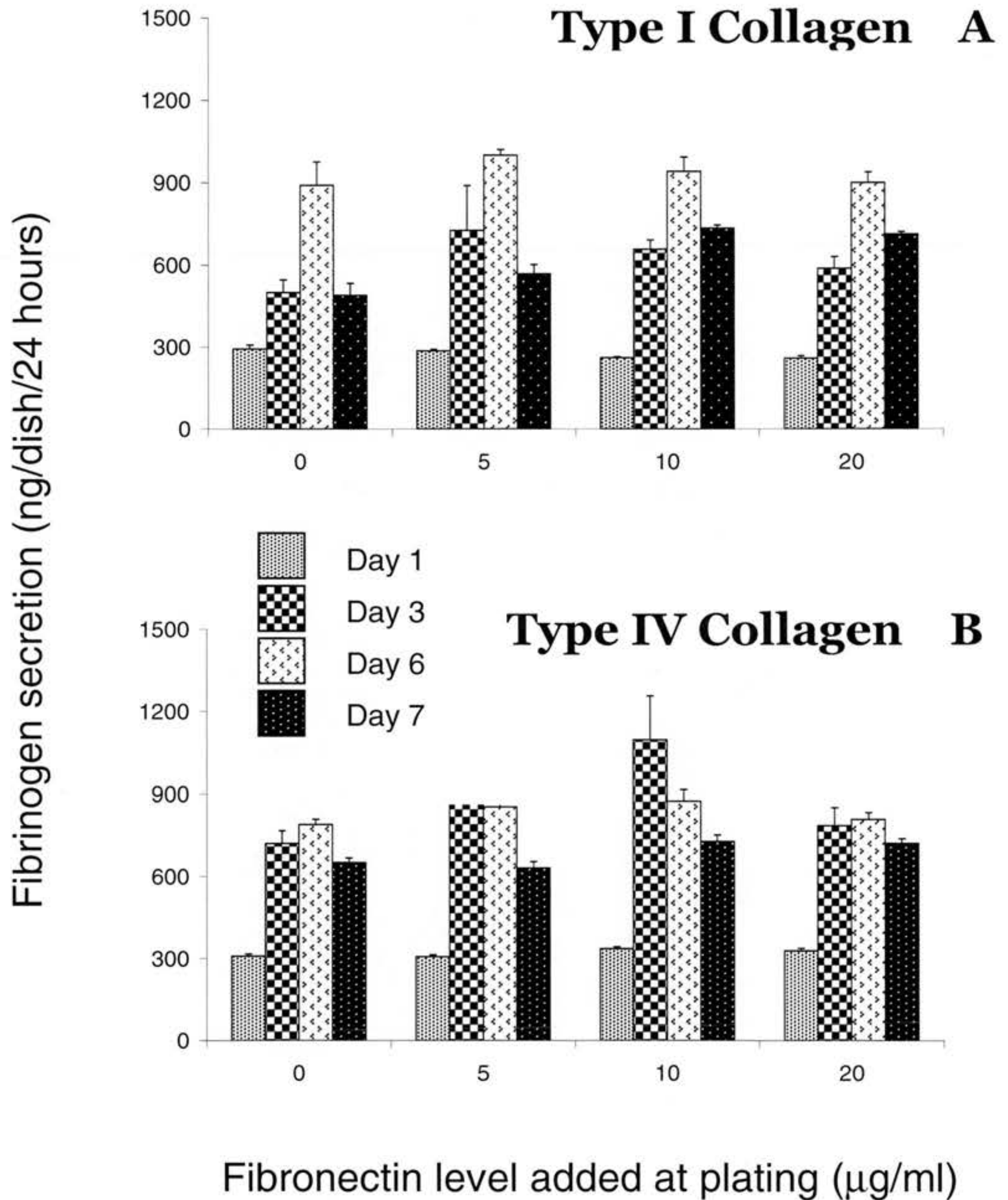


Figure 3.5. Time course of fibrinogen secretion. Cell cultures were prepared and plated in 12 well plates coated with type I (**A**) or type IV collagen (**B**). Coating was carried out by adding 180μl of collagen in 0.2% acetic acid at a concentration of 0.1mg/ml, to give a coating of 5μg/cm². At the time of plating, fibronectin was added to each culture well, to give final concentrations of 1 – 20 micrograms/ml. Culture medium was removed on days 1, 3 6 and 7 in vitro, and the medium was stored at -20oC until later Fibrinogen assay. Data shown are expressed as ng/dish/24 hours. n=6 wells for each treatment. Data are shown as mean +/- SE.

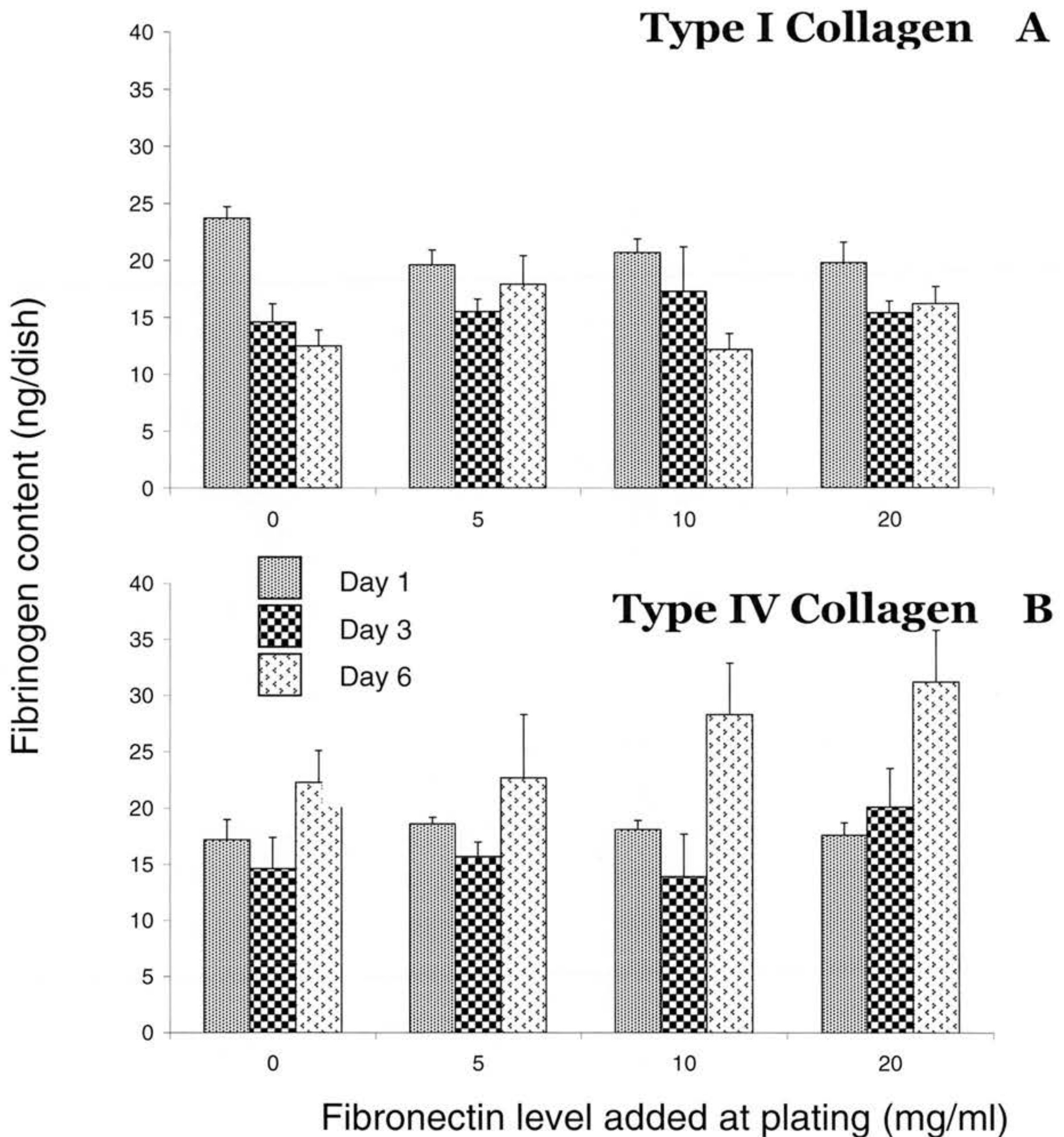


Figure 3.6. Time course of fibrinogen content. Cell cultures were prepared and plated in 12 well plates coated with type I (**A**) or type IV collagen (**B**) at5μg/cm². At the time of plating, fibronectin was added to each culture well, to give final concentrations of 1 – 20 micrograms/ml. Cell cultures were washed on days 1, 3 and 6 in vitro, and cultures were scraped from the substrate and the material stored at -20oC until later Fibrinogen assay. Data shown are expressed as ng/dish. n=2 wells for each treatment. Data are shown as mean +/- SE.

Figure 3.6 reveals the very small amounts of fibrinogen which represent intracellular stores. In each case, the culture content is about 5% of the secreted amount, or less. Nonetheless, some general upward trend is visible in the culture content of fibrinogen in those cells plated on type IV collagen by day 6 of culture. In this experiment, there were insufficient wells to permit the preparation of day 7 culture homogenate.

Figure 3.7 shows the number of cells present in the culture dishes (plated on type I collagen) as a function of the number of cells added to the well in the first instance. There is obviously a linear relationship, but what is more striking is the very small number of cells which are adherent from a large number of cells plated. From 20 million cells plated, approximately 0.5 million were present in culture 1 week later. This may simply reflect the very large number of non-anchorage dependent haematopoietic cells present in the original cell preparation. Certainly, when changing the medium at day 3, it was clear there were a great many floating cells. Despite the reduction in cell number, the data describing protein secretion support the hypothesis that hepatocyte function was not deteriorating over time.

Given the lack of any obvious difference between the substrates, or between fibronectin levels, and the very much increased cost and complexity associated with type IV collagen or fibronectin, it was decided to continue with type I collagen as the preferred culture substrate without fibronectin. Certainly, the

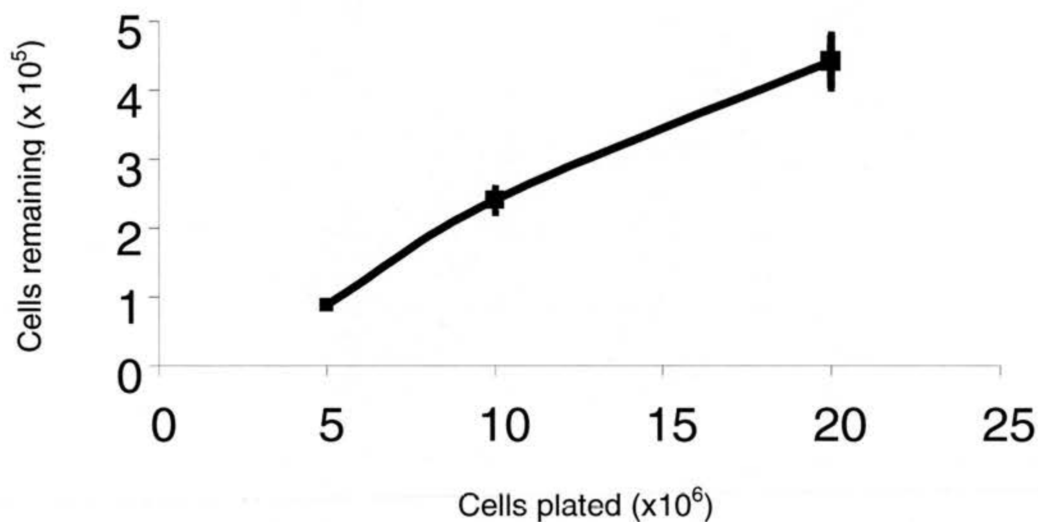


Figure 3.7 The relationship between cells plated and residual cell number. Cell cultures were prepared and plated on type I collagen at 5μg/cm². Cultures were plated at 5 – 20 million cells per well in 12 well plates. Cultures were maintained for 7 days in vitro, and then cultures were trypsinised and counted using a trypan blue technique. n=5 wells at each point. Data are shown as mean, +/- SE.

upward trend in fibrinogen secretion over 1 week in vitro seen in all culture conditions was most reassuring, despite the small number of cells adherent to the culture dishes.

3.3.6. Different cell types in liver cell culture.

To understand the cell types which are present in culture alongside fetal hepatocytes, flow cytometry was carried out against a range of surface antigens present in freshly prepared cell suspension. Figure 3.8 demonstrates the relative abundance of cells bearing these antigens (note; y axis: log scale). Whilst some cells are plainly absent, mononuclear cells (CD14), epithelial cells (CD29; β1-integrin), leucocytes (CD45), red cells lineages (CD235a; Glycophorin A) and haematopoietic stem cells (CD34, CD 90) are present. Clearly, many haematopoietic lineages are present, presenting the possibility that this model could also be used to investigate haematopoiesis.

3.3.8. Spectrum of secreted liver proteins

Alpha-fetoprotein, fibrinogen, alpha-1-antitrypsin, C-reactive protein and prealbumin in culture supernatant were determined by specific ELISA. Figure 3.9 shows the quantities of these proteins detected (n=6 wells for each data point). It is noteworthy that CRP and prealbumin were undetectable.

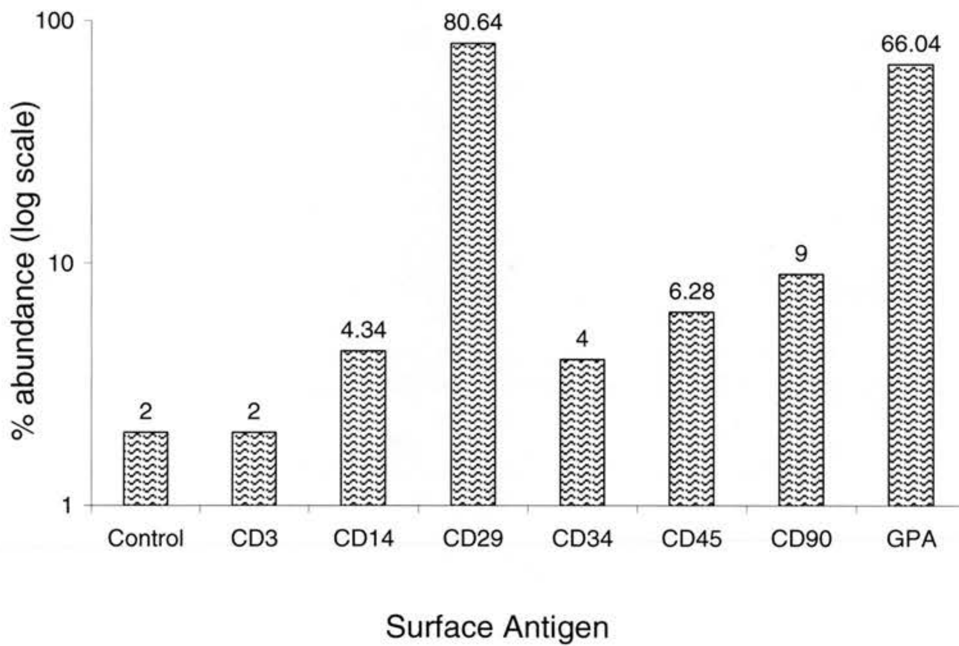


Figure 3.8. Different cell types in fetal liver cell culture.
Cells were prepared as a unicellular suspension by collagenase digestion of fresh tissue. The cell preparation was washed in flow cytometry buffer, and incubated with mouse monoclonal antibodies at 1:50 dilution in blocking solution directed against a selection of well-characterised surface epitopes. After a wash step, cells were incubated with Goat anti-mouse Phycoerythrin conjugate antibody at 1:20 dilution. Immunopositive cells were detected by flow cytometry with excitation at 488nm and detection at 575nm. The immmunopositive fraction is shown. N=1 experiment.

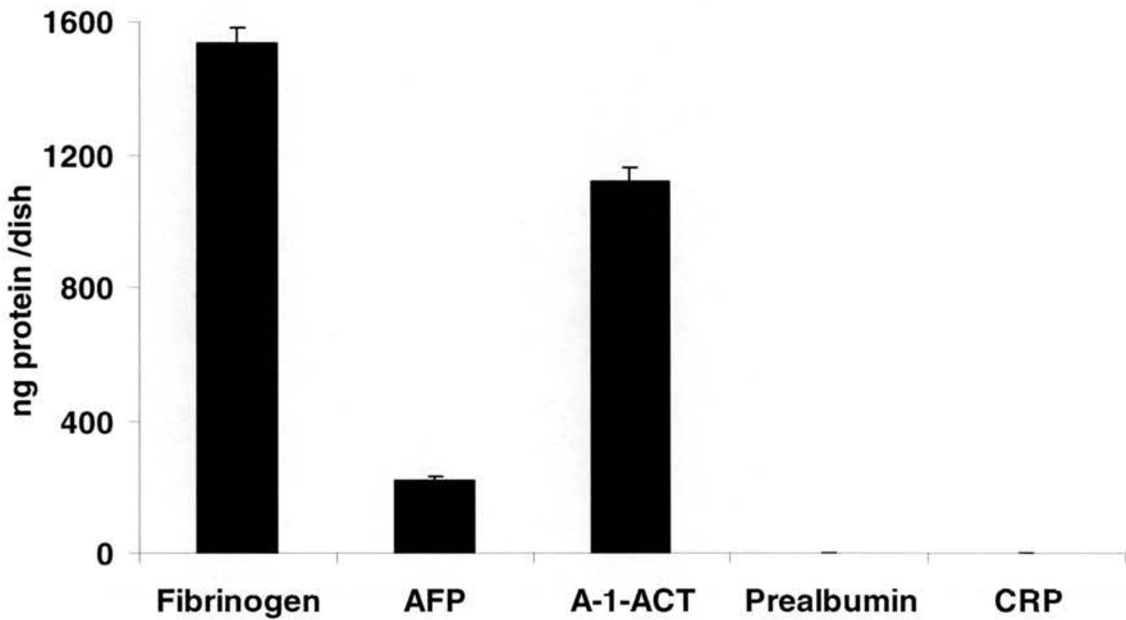


Figure 3.9. Secretion of different hepatocellular proteins by cultured liver cells. Cell cultures were prepared from fresh tissue and plated on type I collagen at 5µg/cm². Cultures were plated at 5 million cells per well in 24 well plates. Cultures were maintained for 7 days in vitro, with a medium change on day 3. Culture supernatant was removed on day 7 and stored for later protein assay at -20°C. Data are shown as mean, +/- SE. n=6 wells for each data point.

3.3.9. Ureagenesis in cultured liver cells.

Figure 3.9 shows the amounts of urea synthesised under basal conditions and in response to NH_4Cl loading (n=6 wells for each data point, +/- SEM). The data clearly show that urea is synthesised at a basal level which is increased in response to addition of ammonia, confirming ureagenesis. This response was significant, and was observed at two different times in vitro.

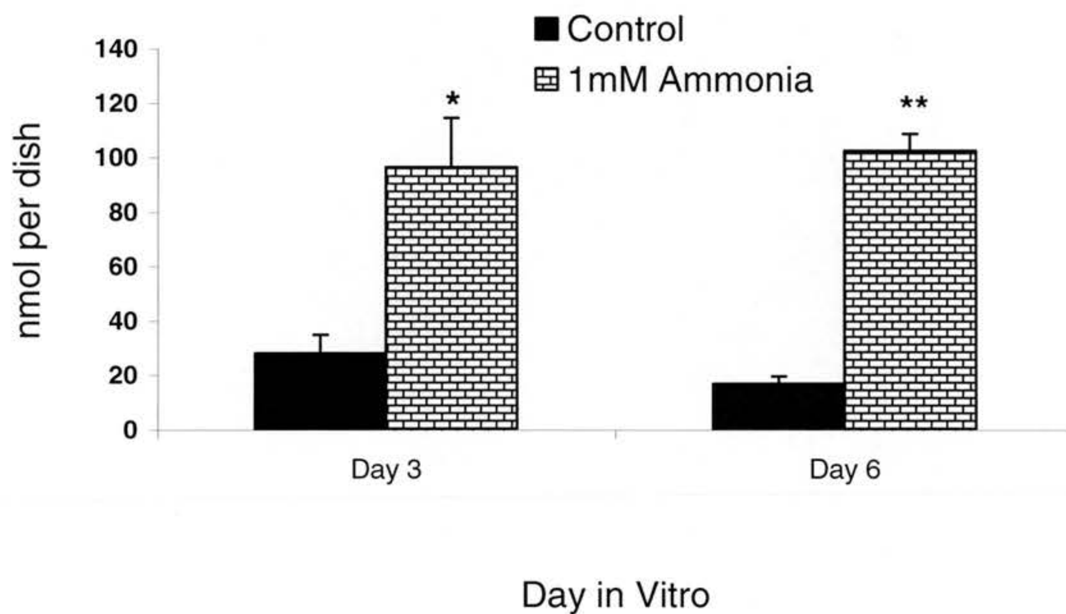


Figure 3.10. Urea synthesis in vitro. Cell cultures were prepared from fresh tissue and plated on type I collagen at 5μg/cm². Cultures were plated at 5 million cells per well in 24 well plates. Cultures were maintained for 7 days in vitro, with a medium change on day 3. On day 3 and day 6, medium was removed, the cells were washed with HBSS, and fresh HBSS containing glucose, HEPES, BSA and pyruvate, +/- 1 mM ammonium chloride, was added. Cells were incubated for 4 hours. The HBSS was removed and stored at -20°C for later assay. Medium was replaced and the cells returned to the culture incubator. Data are shown as mean, +/- SE. n=6 wells for each data point. *$p<0.05$; **$p<0.01$ compared to control, t-test.

3.4 Discussion.

The purpose of this section of the thesis was to develop and characterize the cell cultures so as to establish a satisfactory model for the study of developing human hepatocytes. The data in this chapter convincingly demonstrate that human fetal hepatocytes can survive in the culture system, and indeed can secrete appropriate hepatocellular proteins and incorporate ammonia as expected of living hepatocytes. Furthermore, subsidiary experiments in this chapter demonstrated the existence of many other cell types in this culture system, including haematopoietic cells and some cells with surface markers of stem cells. This model therefore represents an exciting prospect to investigate hepatocytes, and to examine the dynamic interactions between different lineages in the developing human liver.

Human fetal liver cultures have been prepared historically using collagenase digestion of minced tissue (Guguen-Guillouzo et al., 1984), dispase enzymatic dispersion (Tokiwa et al, 1987) or a combination of several different enzymes (Salas-Prato et al., 1985). More recently, collagenase/dispase (Popovici et al., 2001) or collagenase alone (Malhi et al., 2002) was reported in the preparation of purified fetal human hepatocyte cultures, as was mechanical disruption alone (Muench et al., 2002). Most authors report excellent viability with these various techniques, implying that the specific method of tissue digestion has little bearing on the subsequent outcome. For the purpose of the studies described in this thesis, techniques of enzyme digestion of liver tissue (O'Riordain et al.,

1995) and fetal tissues in general (Currie et al., 1994) were adapted to suit fetal liver cultures. The data described in this chapter suggest that the enzyme incubation is satisfactory, and indeed the cell yield for these 2nd trimester livers compares most favourably with other published work (Malhi et al., 2002, Muench et al., 2002,).

Despite good cell yield, viability was initially problematic in this work. Discussion with Dr. R. Popovici determined that the use of DMEM with increased, rather than normal, glucose as a wash buffer was a potentially significant difference between protocols (Popovici et al., 2001, personal communication). Glucose supplementation of the collagenase solution to a level of 20mmol/litre, as found in high glucose DMEM, restored viability to an satisfactory level. It is likely that fetal hepatocytes, which exist in a comparatively hypoxic environment compared to adult liver cells, are more dependent on glycolysis than adult cells. The presence of increased glucose levels during cell preparation would permit increased glycolysis during relative anoxia, thereby preventing cell death.

Having optimized the preparation of single cell suspensions, the presence of hepatocytes was confirmed by flow cytometry in the fresh cell preparation and in cultured cells. However, optimal culture conditions, and hence appropriate culture substrate, were central to the further success of the culture system. Fibronectin, laminin, type IV collagen and β1-integrins have been implicated in

the binding of adult human hepatocytes to extracellular matrix (Newsome et al., 2004; Popovici et al., 2001). Histochemical studies have shown that the cytoplasm of neonatal human hepatocytes is immmunopositive for fibronectin, and the matrix surrounding the hepatocytes shows a pericellular fibronectin reaction product, consistent with the expected distribution of this adhesive glycoprotein (Rescan et al., 1989). Fibronectin immunoperoxidase studies of 1st trimester human fetal liver show scattered immmunopositive hepatocytes and reaction product lining the sinusoids (Terrace J, 2005, personal communication). It is therefore likely that fetal hepatocytes are able to synthesise and secrete fibronectin. In the present study, no credible effect of fibronectin supplementation was observed on functional status, in terms of fibrinogen secretion, whether cells were plated on type I or type IV collagen. This implies that fibronectin is unimportant to hepatocyte adhesion and function. By contrast, it is also reasonable to suggest that sufficient fibronectin was present as a result of endogenous hepatocellular synthesis in vitro, obviating the effects of added fibronectin. Whilst no attempt was made to assess whether liver cultures secreted fibronectin, the simultaneous production of large quantities of other hepatocellular proteins would support this view.

β1-integrin (CD 29) is expressed by proliferating liver progenitors (Tanimizu et al., 2004), and is complexed with α5- integrin to form a binding site for the RGD motif of fibronectin, and with α6-integrin to form the laminin receptor; both

these transmembrane heterodimers link the cytoskeleton and intracellular kinase pathways with extracellular collagens, bridging via laminin or fibronectin (Wierzbicka-Patynowski and Schwarzbauer, 2003). Published evidence suggests that such ligand interaction with β1-integrin signals to prevent hepatocyte apoptosis, (Newsome et al., 2004), actions which are likely mediated via a MAP kinase-dependent mechanism (Zhang et al., 2002). Antibodies to β1-integrin prevent hepatocyte adhesion to fibronectin, laminin, type I and type IV collagen, however, the same antibodies reduce apoptotic cell death (Pinkse et al., 2004).

β1-integrin was detected on the cell surface of approximately 80% of cultured cells in the present work. These initial experiments were not designed to show which integrin heterodimers were expressed, nor whether hepatocytes in particular bear CD29. However, it seems very likely that if 50% or more of cultured cells are hepatocytes, as demonstrated by two-colour flow cytometry, then most or perhaps all express β1-integrin. This would be entirely consistent with the known epithelial distribution of β1-integrin. It remains a strong possibility that the haematopoietic cells in the cultures also express β1-integrin, thus accounting for the large proportion of cells which were positive for this antigen. In fact, knockout studies have shown that is an absolute requirement for haematopoietic cells to colonise the liver in fetal life, strongly supporting the view that at least some of these cells were haematopoietic in nature (Potocnik et al., 2000). This is further corroborated by the data from the present work

showing that the stem cell antigens CD34 and CD90 were detected in the developing liver.

Type IV collagen has been identified within the periportal mesenchyme and in close relation to the ductal plate as it arises at week 9 of gestation in humans, although prior to this stage, laminin appears to be the primary extracellular matrix protein, fading significantly as type IV collagen appears (Quondamatteo et al., 1999). Type I collagen is found in the perisinusoidal space, along with fibronectin, laminin, and type IV collagen (Amenta and Harrison, 1997). Type IV collagen, the physiological basement membrane collagen, was expected to confer a benefit on cell function as compared to Type I in vitro. However, the present study showed no obvious difference in Fibrinogen secretion between the two. It is clear that early in development, there is no type IV collagen present in the liver, and so the concept of a formalised basement membrane may be inappropriate at this stage of liver development. If hepatocytes are not dependent for survival on attachment to a basement membrane, but instead rely on anchorage mediated by adhesive glycoproteins such as laminin and fibronectin, it is perhaps unsurprising that there was no great difference between the two collagens. Certainly over the 7 day period of study, no detriment accrued to either group of cells. On this basis, it was elected to continue with type I collagen.

In order to provide robust evidence that the cultures contained viable hepatocytes, effort was made to quantify urea synthesis and measure specific hepatocellular protein secretion. In developing human liver, previous authors have determined that the full complement of urea cycle enzymes is present as early as week 9 of development (Karsai and Elodi, 1982), and that urea cycle activity rises to near adult levels by week 36 (Mukarram Ali Baig et al., 1992). No authors have examined urea synthesis in a culture model of human fetal hepatocytes, and so no data are available to compare the level of function in these cultures. It is notable that very large amounts of ammonia are taken up by cultured cells, corresponding to the large amounts of urea which are synthesised. It is also notable that urea is present in culture supernatant, even when no ammonia has been added; suggesting that endogenous urea synthesis from amino acids is taking place. Such data are most reassuring, as is the observation that urea synthesis does not deteriorate between day 3 and day 6 in vitro, confirming that culture viability remains satisfactory during the study period.

Protein synthesis by cultured human hepatocytes has been demonstrated extensively in adult liver (O'Riordan et al., 1995), and, to a lesser degree, in fetal liver (Gitlin and Biasucci, 1969; Gitlin et al., 1972; Gulbis et al., 1998). However, whilst adult tissue culture systems have been developed to carry out interventional experiments on cultured material, this is much less the case with human fetal liver material. Presently, only Popovici et al. (2001) has studied

liver protein secretion in culture in response to treatment with an external agent. The literature is scant in this regard.

In the present work, secretion of AFP, Fibrinogen and α-1-antichymotrypsin was detected, however, we did not detect prealbumin release from cultured cells. Only one previous report of prealbumin secretion from a short-term culture of human fetal liver exists in the literature, using a technique very different to the present work (Gitlin and Biasucci, 1969). Interestingly, a number of authors have noted that prealbumin immunostaining in the liver is weak or absent, as is prealbumin mRNA by in situ techniques, in second trimester human fetal liver (Jacobsson, 1989; Gray et al., 1985). These data suggest that liver production of prealbumin in fetal life is low. It may also be that the culture conditions did not favour prealbumin release, or that the amount of protein was extremely modest.

C-Reactive Protein (CRP) is an acute phase protein, and is only detected in quantity during an acute phase response. In fact, the total amount in the plasma volume of a healthy adult is approximately 10 mg, giving a plasma concentration of less than 2 μg/ml. In cord blood from second trimester fetuses without inflammatory disease, the median CRP concentration is approximately 40ng/ml (Yoon et al., 2003), which is 50 times less than the adult. Although the assay used to detect CRP in this study extended as low as 10ng/ml, it is possible that fetal liver CRP secretion in vitro, in the absence of an acute phase stimulant, was

too low to detect reliably. It may yet be possible to investigate CRP, and prealbumin, secretion from these cultures, however, a different approach with higher cell densities or acute phase stimulants might be required. Nonetheless, these data confirm the synthesis of an acute phase protein (fibrinogen), a house-keeping protein (AFP), and a negative acute phase protein (α-1-ACT), confirming the validity of this preparation as a valuable model in the study of developing liver.

Chapter 4.

Maturation signals in the fetal liver.

4.1 Introduction.

In animal studies, a range of hormones are implicated in the maturation of fetal liver. Glucocorticoids in the fetal circulation increase markedly during late gestation (Norman et al., 1985) in parallel with glucocorticoid receptor expression (Speirs et al., 2004), which may enhance ureagenesis in the liver (Ulbright and Snodgrass, 1993). Maturation of the urea cycle is an absolute requirement for perinatal survival (Schofield et al., 1999) and for perinatal gluconeogenesis (Hanson and Reshef, 1997). Thyroid hormones increase albumin mRNA and protein synthesis in a dose-dependent manner whilst AFP mRNA and protein is co-ordinately decreased (Anteby et al.,1993). Whilst there are few data linking maturational effects of sex steroids in fetal liver, there is a strong gestational trend in both oestrogen and gestagen levels. These are present in extremely high concentrations and are conducted directly to the fetal liver by the umbilical veins and may act to mediate liver maturation.

In order to address maturational endocrine signals in human fetal liver, the following experiments set out to examine several hormonal stimuli. The work assessed the changing functions of liver cells in response to specific endocrine influences, and to delineate proliferative and morphological responses.

4.2 Materials and methods.

Human fetal liver was collected under aseptic conditions, and a liver cell suspension was prepared as described in chapter 3. Cells were made up to specific densities as required for individual experiments, and plated on type I collagen-coated dishes. Cells were exposed subsequently to various added endocrine agents, and protein secretion and cellular proliferation was assessed as described below.

Stock solutions

Dexamethasone (MWt. 392.5) was dissolved in absolute ethanol and made up to a concentration of 0.392mg/ml (1 mmol/l). Cortisol (MWt. 362) was similarly dissolved and prepared at 3.62mg/ml (10 mmol/l). Progesterone (MWt. 314.5) was made up to 0.314mg/ml (1 mmol/l), and oestradiol was prepared at a concentration of 2.72 mg/ml (MWT. 272; 10 mmol/l). Working solutions were made by adding 1 part in 1000 to culture medium, then carrying out serial 10-fold dilutions. Control solution contained ethanol at 1:1000 dilution. All steroid stock solutions were kept at -20°C.

Glucagon (MWt. 3485) was made up in EBSS to 0.348 mg/ml (100μmol/l). Working solution was prepared by adding the appropriate volume to culture medium and carrying out serial dilutions as described. Stock was stored at 4°C for up to 1 month.

Tri-iodothyronine ('T3'; MWt. 651) was dissolved in 0.1M NaOH to a concentration of 0.651 mg/ml (1 mmol/l). This was further diluted 10-fold with water to provide the final stock solution at 100μmol/l. Working solutions were made by carrying out a 1000-fold dilution, and then further 10-fold dilutions. Experiments showed that this small amount of added base was buffered successfully by culture medium, leading to no change in pH, and was not therefore neutralised. Control medium was prepared using the same amount of added base. Stock was stored at 4°C for up to 1 week.

4.2.1. Effects of endocrine agents on fibrinogen secretion.

Cells were diluted to a density of 1×10^7/ml in warmed culture medium. 50 μl containing 5×10^5 liver cells were added to each well of a 96-well plate previously coated with type I collagen at 5 μg/cm² (16 μl of collagen solution per well, 100μg/ml). To each well were added a further 50 μl culture medium containing either control medium, cortisol, dexamethasone, oestradiol, progesterone, insulin, glucagon or tri-iodothyronine at concentrations encompassing physiological levels. In each case, four different concentrations of agent were prepared by serial 10-fold dilution. Each treatment was applied in triplicate. Culture medium was changed on day 3, and removed on day 7 for storage at -20°C and subsequent fibrinogen ELISA. This experiment was repeated in 3 separate wells for each treatment (n = 3 wells, +/- SEM).

4.2.2. Effects of endocrine agents on liver cell proliferation.

Cell cultures were set up as described in 4.2.1 above. Cells were maintained in culture until day 7, at which point the thiazylol blue technique was employed to determine the relative cell content of each culture well, as described in chapter 2, (section 2.5). The mean absorbance of each triplicate treatment was calculated, as was the standard error of this mean (n = 3 wells, +/- SEM).

4.2.3. Effects of Dexamethasone on fibrinogen secretion.

Having carried out an initial screening experiment in sections 4.2.1 and 4.2.2, a more detailed assessment of the response to glucocorticoid was carried out. Cells were plated as described above, and dexamethasone was added to triplicate wells as before. A serial dilution of control medium was carried out to match vehicle concentration precisely. Medium was collected between days 3 – 7 for fibrinogen ELISA. This experiment was performed in triplicate wells from which the mean was taken for each culture. This was repeated in each of 7 separate liver preparations (n = 7 cultures, +/- SEM).

4.2.4. Effects of Dexamethasone on liver cell proliferation.

Cultures were prepared and treated with dexamethasone or control as described in 4.2.3. On day 7, cultures were processed for thiazylol blue assay as previously described. Treatments were in triplicate, from which the mean was calculated for each culture. This experiment was repeated in 7 separate cultures (n=7).

4.2.5. Effects of Dexamethasone on urea synthesis.

Cells were made up to $1x10^7$/ml in culture medium, and 500μl were added to each well of a 24 well plate. In half the wells, 5μl control medium was added. In the remainder, 5 μl dexamethasone solution was added to give a final concentration of $1x10^{-7}$mol/l. Urea synthesis was determined in each well using the technique described in section 3.2.7. Each treatment was carried out in duplicate, and this experiment was repeated in 5 separate cell cultures (n=5) on day 7 in vitro. In addition to urea synthesis, the effects of dexamethasone on ammonia uptake and incorporation of ammonia into non-urea metabolites was determined.

4.2.6. Effects of Dexamethasone on cellular morphology.

The effect of glucocorticoid on morphology was assessed by adding dexamethasone at a final concentration of 100μmol/l to cultures of liver cells growing on type I collagen-coated slides. Cells were photographed at day 7 in vitro under phase contrast conditions.

In order to appreciate the histological maturation of liver cells cultured on chamber slides, cultures were processed for immunocytochemistry directed against cytokeratin 18 and fibrinogen, such that hepatocytes could be identified unequivocally. The technique for immunocytochemistry was identical to that described for cytokeratin 18 and fibrinogen flow cytometry, except that slides

were washed in a bath of PBS rather than subject to centrifugation between steps. Slides were mounted in Faramount (DakoCytomation), coverslipped, and examined with a fluorescence microscope (Leica, UK) using Openlab image acquisition, with a filterset appropriate to 525nm and 575 nm fluorescence maxima.

4.2.7. Statistics.

Analyses in which more than 2 groups were compared were first assessed by Analysis of variance using the SPSS package. Pairwise comparisons were carried out using a t-test where permitted by ANOVA. The significance level was set at 0.05.

4.3. Results.

4.3.1. Effects of endocrine agents on fibrinogen secretion.

Figure 4.1 shows the responses of cell cultures in terms of fibrinogen secretion after 7 days incubation, with each dose of the six compounds, compared to a matched control. Dexamethasone and cortisol demonstrate similar dose-response curves, but neither progesterone, oestradiol, T3 nor glucagon demonstrated a convincing dose-response in terms of fibrinogen secretion.

4.3.2. Effects of endocrine agents on liver cell proliferation.

The thiazylol blue technique was used to determine whether the various agents had any growth promoting activity on the cultured cells, quite separately from the effects on secretory function. The data suggested that none of the agents had an effect on cell proliferation (Figure 4.2).

4.3.3 Effects of Dexamethasone on fibrinogen secretion.

Figure 4.3 shows the marked effect of glucocorticoid on fibrinogen secretion by these cells in comparison to paired vehicle controls. ANOVA revealed dexamethasone to have a significant overall effect ($p = 0.001$). Dexamethasone had a significant stimulatory effect ($p<0.05$) on fibrinogen secretion compared to paired controls at 10 - 1000nmol/l. This was in stark contrast to the effects of vehicle, which showed no effect at any of the dilutions applied in this experiment (ethanol; 1:1000 to 1:10,000,000).

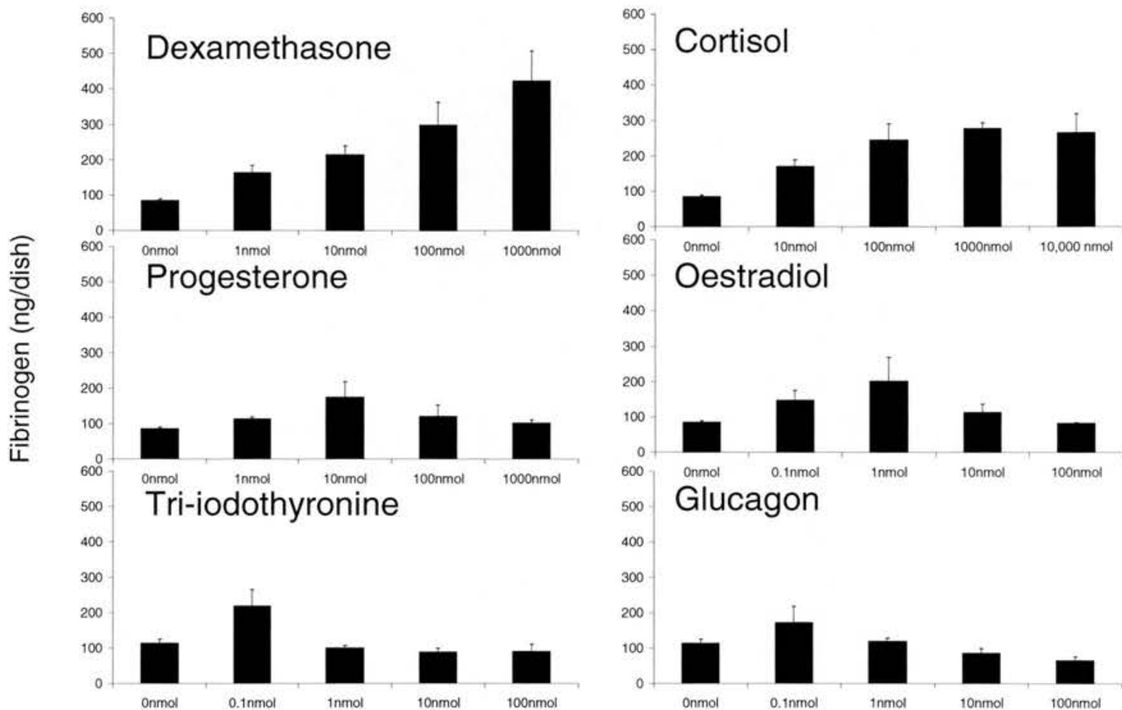


Figure 4.1. The dose-response of fibrinogen secretion in fetal liver cultures. Cultures were prepared from fresh tissue and plated in 96 well plates at $5x10^5$ cells per well on type I collagen. Hormone supplements were added, and the cultures were maintained in vitro for 7 days, with a medium change on day 3. Culture supernatant was removed for later assay on day 7 and stored at -20°C. (n=3 wells. Mean +/- SEM).

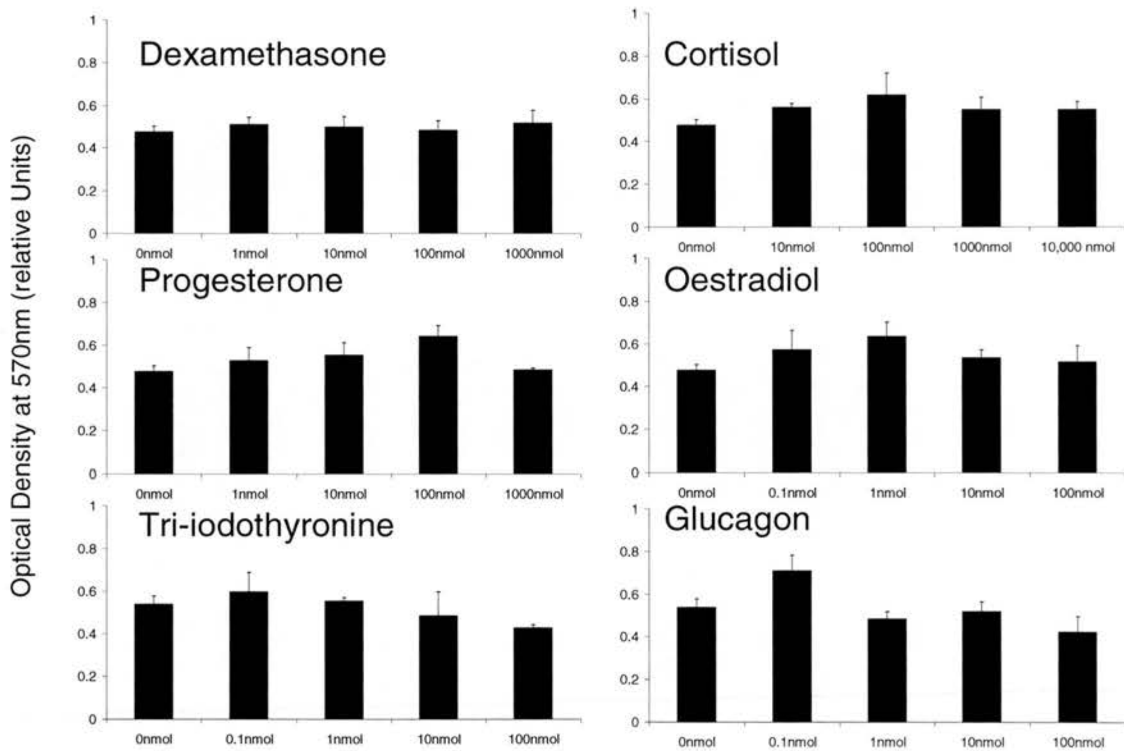


Figure 4.2. The dose-response of cell proliferation in fetal liver cultures. Cultures were prepared from fresh tissue and plated in 96 well plates at $5x10^5$ cells per well on type I collagen. Hormone supplements were added, and the cultures were maintained in vitro for 7 days, with a medium change on day 3. MTT solution was added to the cultures on day 7, incubated for 4 hours, and then acid SDS solution was added to solubilise the formazan crystals. After an overnight incubation, the absorption at 570nm was read. n=3 wells. Mean +/- SEM.

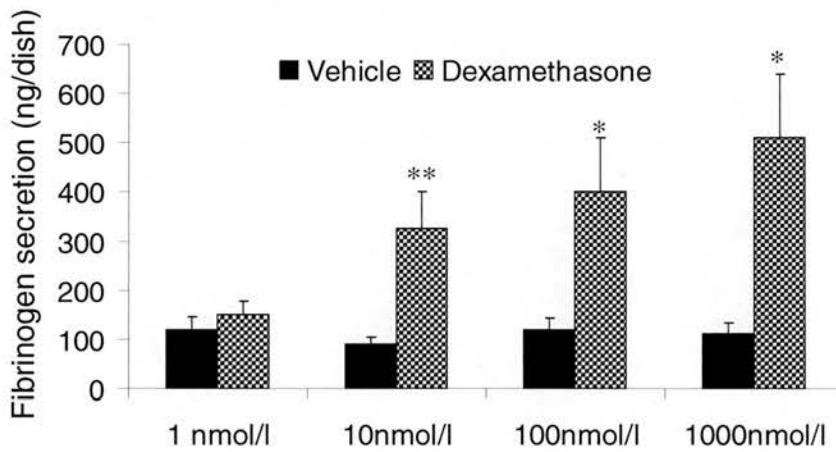


Figure 4.3. The dose-response of fibrinogen secretion to dexamethasone. Cell cultures were prepared from fresh tissue and plated on type I collagen at 5μg/cm². Cultures were plated at 0.5 million cells per well in 96 well plates. T o each well was added dexamethasone in vehicle or vehicle alone in triplicate. Cultures were maintained for 7 days in vitro, with a medium change on day 3. On day 7, culture supernatant was removed and stored at -20°C for later assay. n=7 cultures. Mean +/- SEM. * $p<0.05$ or ** $p<0.01$ compared to paired vehicle control.

4.3.4 Effects of Dexamethasone on liver cell proliferation.

Figure 4.4 shows that compared to control, there was no effect of dexamethasone or vehicle on cell proliferation.

4.3.5 Effects of Dexamethasone on urea synthesis.

Figure 4.5 shows the effects of dexamethasone and time in vitro on urea synthesis, ammonia uptake, and incorporation of ammonia into non-urea metabolites (organification). The data show that neither time in vitro nor dexamethasone had any effect on urea synthesis. However, uptake of ammonia increased with time in vitro, an effect which was significant only in the absence of dexamethasone. The data also demonstrate that the incorporation of ammonia into metabolites other than urea (that is, ammonia uptake which is in excess of urea synthesis) is significantly increased with time in vitro in the absence of dexamethasone. When dexamethasone was present, the increase in ammonia organification was less marked, and did not reach statistical significance in this series of experiments.

4.3.6 Effects of Dexamethasone on cellular morphology.

Figure 4.6 shows that at day 7, cells cultured in the absence of dexamethasone adopt a spindle-shaped morphology, reminiscent of mesenchymally-derived cells rather than mature epithelioid hepatocytes. Cells cultured in the presence of dexamethasone show a flatter pavement of polygonal cells, more in keeping with a mature, epithelial phenotype.

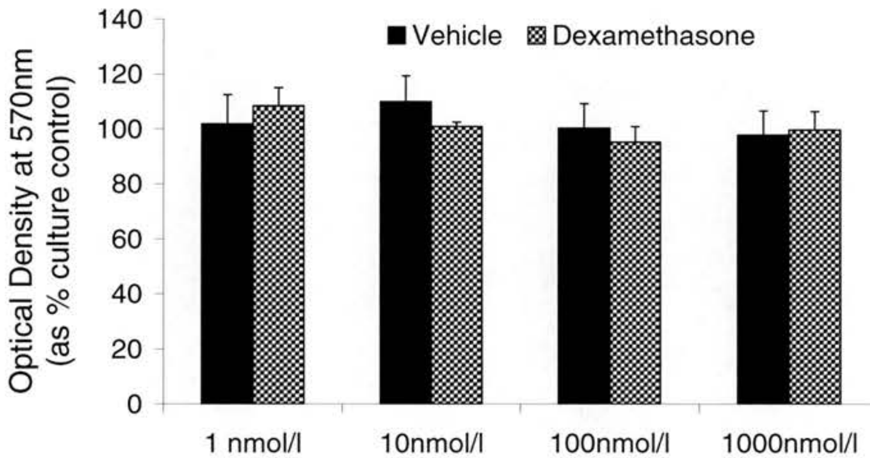


Figure 4.4. The dose-response of proliferation to dexamethasone.
Cultures were prepared from fresh tissue and plated in 96 well plates at $5x10^5$ cells per well on type I collagen. Dexamethasone in vehicle or vehicle alone was added to each well in triplicate. The cultures were maintained in vitro for 7 days, with a medium change on day 3. MTT solution was added to the cultures on day 7, incubated for 4 hours, and then acid SDS solution was added to solubilise the formazan crystals. After an overnight incubation, the absorption at 570nm was read. n=7 independent cultures. Mean +/- SEM.

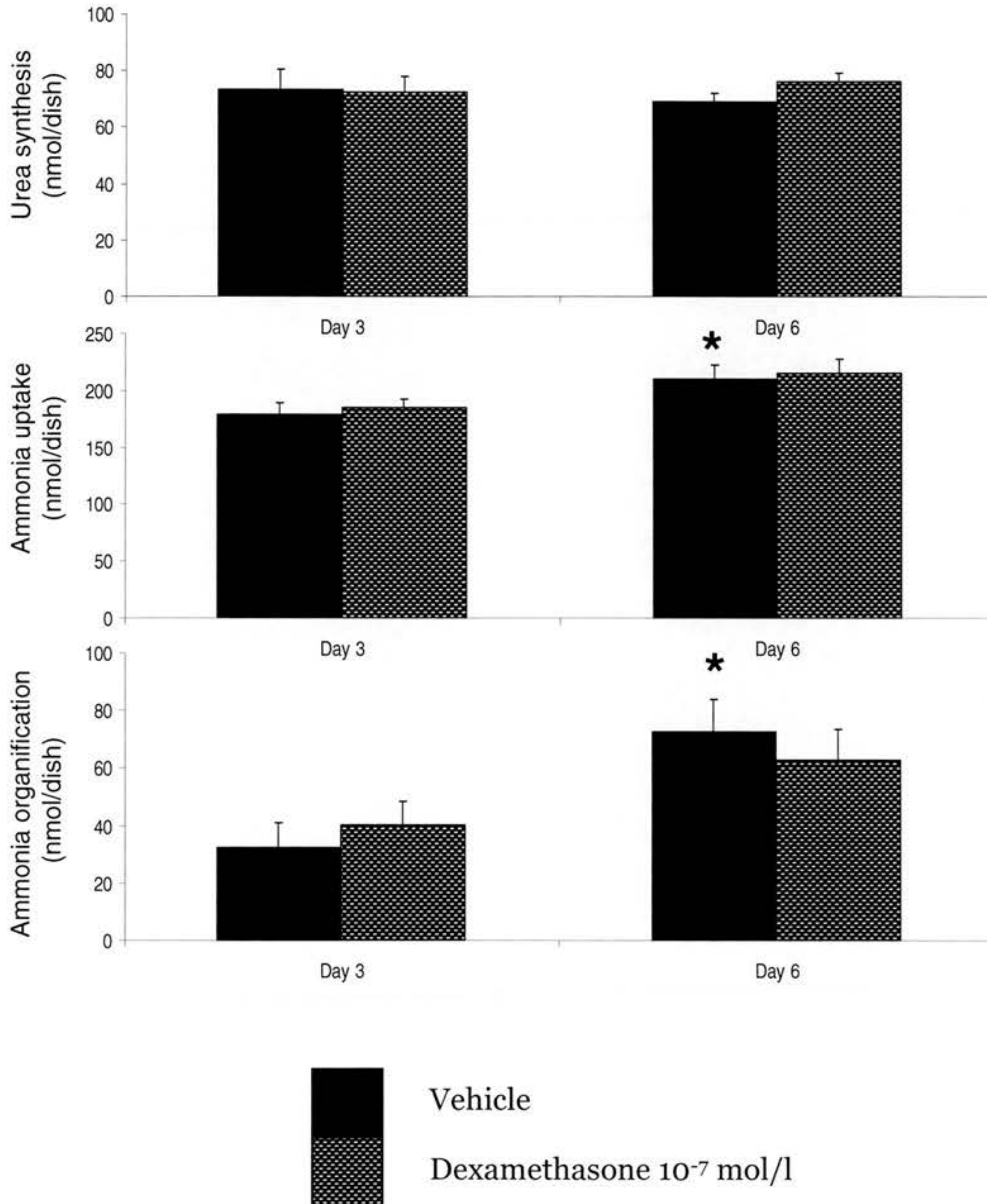


Figure 4.5. Nitrogen metabolism in fetal liver cultures. Cell cultures were prepared from fresh tissue and plated on type I collagen at 5μg/cm². Cultures were plated at 5 million cells per well in 24 well plates. Cultures were maintained for 7 days in vitro, with a medium change on day 3. On day 3 and day 6, medium was removed, the cells were washed with HBSS, and fresh HBSS containing glucose, HEPES, albumin, pyruvate and 1mM ammonium chloride was added. Cells were incubated for 4 hours. The HBSS was removed and stored at -20°C for later assay or urea and ammonia. Medium was replaced and the cells returned to the culture incubator. Data are shown as mean, +/- SE. n=5 cultures. * $p<0.05$ compared to day 3 matched control.

In order to assess the morphology of specifically identified hepatocytes in vitro in the presence and absence of dexamethasone, two-colour immunocytochemistry was carried out, staining for cytokeratin 18 and fibrinogen. Figure 4.6 shows the spindle-shaped morphology of cells expressing cytokeratin 18 (orange) and faintly staining for fibrinogen (green). This contrasts with the flatter, epithelioid cells seen which stain more strongly for fibrinogen in the presence of dexamethasone. These data, although subjective, emphasise the morphological, and hence structural, maturation observed in the presence of glucocorticoids.

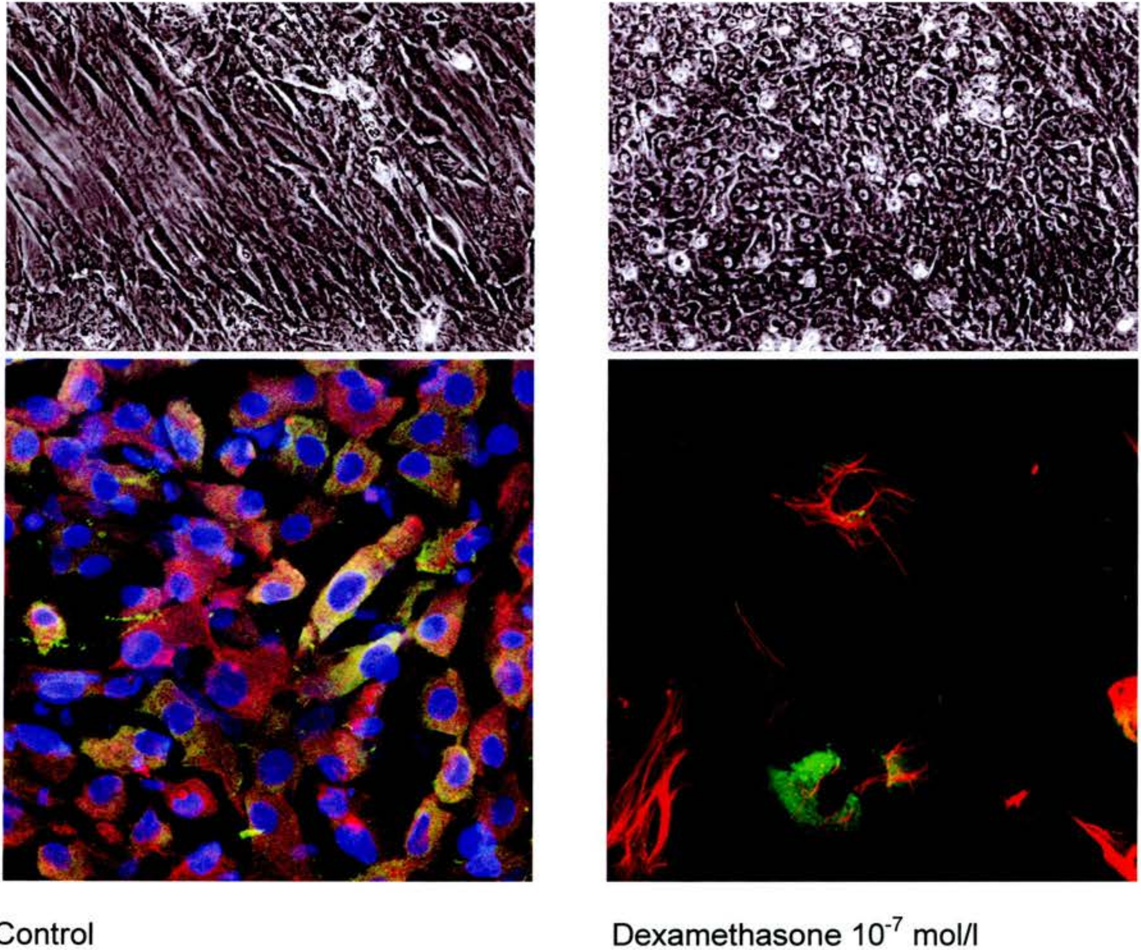


Figure 4.6. Culture morphology in response to glucocorticoid. Cell cultures were prepared from fresh tissue and plated on type I collagen at 5μg/cm². Cultures were plated at 8x10⁴ cells per well in chambered slides. Cultures were maintained for 7 days in vitro, with a medium change on day 3, in the presence and absence of glucocorticoid. Cells were then rinsed and fixed in 0.5% formaldehyde. After a blocking step, cells were incubated with mouse anti-cytokeratin 18 antibody at 1:100 and rabbit anti-Fibrinogen antibody at 1:200 dilution. After a wash step, cells were incubated with Goat anti-mouse Phycoerythrin conjugate antibody at 1:20 dilution and Swine anti-rabbit FITC conjugate at 1:20 dilution. Cultures were rinsed, mounted in Faramount and coverslipped. Cultures were photographed under phase contrast and immunofluorescence conditions at 525 and 575nm. Phase contrast micrographs were obtained at x100, immunofluorescence at x400.

Section 4.4. Discussion.

This chapter investigated which endocrine substances might affect maturation and proliferation in fetal liver during development. The data showed that glucocorticoids alone influenced fibrinogen secretion from the cultured cells. This effect was not mediated by cell proliferation; rather, it appeared to be the result of increased secretion per cell. Immunofluorescence studies showed that glucocorticoids enhanced the phenotypic maturation of hepatocytes to favour a mature, epithelioid, morphology with increased cytoplasmic fibrinogen expression.

The most striking finding in this study was of the dramatic effect of glucocorticoids to stimulate fibrinogen secretion from cultured human fetal hepatocytes. Although there are no published data from fetal tissues, it is relevant that patients with Cushing's syndrome, in which glucocorticoid excess is the central pathological feature, demonstrate markedly elevated plasma fibrinogen concentrations, a finding which was highly significant compared to controls (Tauchmanova et al., 2002). Such linkage may be indirect. However, in the hepatoma cell line HepG2, dexamethasone is reported to have a modest enhancing effect on fibrinogen secretion when cells are previously stimulated by Il-6 (Kasza et al., 1994). Dexamethasone also stimulates fibrinogen secretion by isolated rat hepatocytes (Crane and Miller, 1977). Moreover, glucocorticoid response elements, which positively regulate gene transcription, are present in the upstream regulatory sequences of human β- and γ- fibrinogen precursors

(Huber et al., 1990; Asselta et al., 1998). Whilst this does not conclusively demonstrate that intact, hetero-hexameric ($(\alpha\beta\gamma)_2$) fibrinogen synthesis can be increased by glucocorticoids, increased expression of any of the fibrinogen precursors, α-, β-, or γ-, leads to increased synthesis of all precursors and increased synthesis and release of mature fibrinogen (Redman and Xia, 2001). Together with the present results, these data confirm for the first time that fibrinogen secretion from human liver can be strongly enhanced by glucocorticoids.

The effects of dexamethasone noted here might occur only with non-physiological concentrations of glucocorticoid, as plasma levels of cortisol in the fetus may be much lower than the range in adult humans, which is 100-700 nmol/l. Published evidence suggests, however, that fetal cortisol levels in the last few weeks of gestation are relatively high, with levels recorded at parturition of around 1000nmol/l (Goldkrand et al., 1976). This level is well above the apparent maximally effective concentration of cortisol in this work which was approximately 100nmol/l. Similarly, the levels of dexamethasone which had effects on fibrinogen secretion in this study are the same as those in cord blood samples from dexamethasone-treated premature human neonates (Kream et al., 1983). The glucocorticoid effects that have been observed in the present study, therefore, tie in well with previous work, suggesting that this is not an effect

secondary to supra-physiological doses of steroid but is in fact a physiological response of developing hepatocytes to glucocorticoid.

Gestational glucocorticoid is clearly required to bring about organ maturation in preparation for birth (Cole et al., 1995; Hanson and Reshef, 1997). Exposure to glucocorticoids may, however, have deleterious effects. Exogenous glucocorticoid during fetal life leads to reduced body weight and liver weight at birth (Quinlivan et al., 1998) and adverse biochemical and physiological changes in adult life, namely, impaired glucose tolerance, hyperinsulinaemia, persistently elevated glucocorticoid receptor expression (Nyirendra et al., 1998) and increased blood pressure (Levitt et al., 1996). This constellation, which has been termed the 'metabolic syndrome', has been likened to the complications of Cushing's syndrome (Seckl and Walker, 2001). The physiological relevance of these findings has been emphasised by experiments in which 11 β-hydroxysteroid dehydrogenase type II (11β-HSD II), the placental and fetal liver enzyme which metabolises glucocorticoids, has been itself inactivated with a potent inhibitor, carbenoxolone, administered to the mother. Such experimental treatment leads to hypertension, hyperglycaemia and hyperinsulinaemia in offspring which persists into adulthood (Lindsay et al., 1996a; Lindsay et al., 1996b). In human studies, hyperfibrinogenaemia is a super-added risk factor as part of the metabolic syndrome, the role of which as a major risk for myocardial infarction has been confirmed in a prospective study

of 10,500 men (Scarabin et al., 1998). The developmental aetiology of adult hyperfibrinogenaemia is unclear, however, gene knockout techniques have shown glucocorticoid action in the fetal liver has a significant role (Morton et al., 2001). The data presented in this study strongly support the hypothesis that fetal glucocorticoid could have a major role in the aetiology of hyperfibrinogenaemia, and might therefore contribute to cardiovascular risk in adult life.

No attempt was made to determine whether fibrinogen secretion might continue at a high level after glucocorticoid was removed in the present work. However, data exist to suggest that DNA demethylation, mediated by the glucocorticoid receptor in fetal liver-specific sequences, could underpin lasting effects of glucocorticoids, long after steroid has been withdrawn (Thomassin et al., 2001). This mechanism is an attractive explanation for observed persisting changes into adult life secondary to fetal glucocorticoid exposure.

The difference between the effects of cortisol and dexamethasone in terms of fibrinogen release were somewhat surprising. Whilst dexamethasone at the highest concentration (1 micromol/l) had a fivefold stimulatory effect on fibrinogen, the highest dose of cortisol (10 micromol/l) had a threefold enhancing effect. Notwithstanding that the efficacy of dexamethasone to bind the glucocorticoid receptor is approximately 7 times that of cortisol (Baxter and Rousseau, 1979), both agonists fully occupy the glucocorticoid receptor at these

biologically equivalent concentrations. It is therefore not clear why dexamethasone should be a more efficacious ligand to increase fibrinogen release.

On the other hand, dexamethasone is not a substrate for 11β-HSD II, the endogenous liver enzyme which inactivates cortisol. Work by Hirasawa et al. (1999) has shown weak immunostaining for 11β-HSD II in hepatocytes at week 8 and week 20 of gestation in human fetal liver. Stewart et al. (1994) detected 11β-HSD II mRNA and low levels of enzyme activity in fetal human liver from mid gestation. Notably, they found no 11β-HSD I mRNA or enzyme activity in any fetal tissues. Whilst 11β-HSD II was not localised as part of this thesis, it remains an attractive possibility that the inactivation of cortisol by 11β-HSD II explains the relative ineffectiveness of cortisol compared to dexamethasone in stimulating fibrinogen release shown here. In this regard, the data show that the maximal efficacy of cortisol was observed at 100nmol/l, and it may be that any levels in excess of this concentration were metabolised by 11β-HSD II.

In this work, considerable emphasis was placed on the effect of hormones to cause liver cell proliferation, as the implications of altered protein secretion were different if cell proliferation was also taking place. As glucocorticoids alone had an effect on protein secretion, and the initial study showed no effect of any agent

on cell proliferation, only glucocorticoids were selected for further investigation in this regard.

The literature concerning the role of corticosteroids in hepatocyte proliferation is a little contradictory. In whole animal models, published data suggest that there is no effect on liver cell replication. For example, methylprednisolone administration pre-operatively to rats undergoing partial hepatectomy caused no difference in hepatocyte Ki-67 labelling, cyclin D1 expression, or mitotic index in liver tissues examined during the post-operative phase. In fact, post-operative liver function tests suggested reduced liver injury in the steroid-treated group (Glanemann et al., 2004). However, dexamethasone pre-treatment of rats which subsequently underwent partial hepatectomy caused delay in hepatocellular DNA replication by 6 hours (Nagy et al., 2000), and glucocorticoid treatment in a rat cold ischaemia/liver transplant model caused impaired hepatocyte proliferation after transplantation as detected by reduced BrdU uptake, and lead to increased hepatocellular necrosis (Debonera et al., 2003). None of these studies was able to identify specific mechanisms of glucocorticoid action, however, data from HepG2 cells show that dexamethasone causes induction of cyclin-dependent kinase inhibitor p21WAF1/CIP1, thus abolishing Cdk2 phosphorylation and preventing cell cycle progression (Park et al., 2001). Glucocorticoid receptor activation was linked to p53 phosphorylation, and downregulation of p53 by antisense techniques prevented dexamethasone-induced p21 expression (Urban et al., 2003).

Data from the present work clearly shows no decrement in cell numbers after corticosteroid treatment, even after dexamethasone at 1 micromol/l for 7 days, when compared to control. Together, these data support the hypothesis that glucocorticoids act neither to inhibit nor stimulate proliferation in human fetal liver cells. The explanation for the stimulation of fibrinogen secretion by dexamethasone must lie with increased fibrinogen synthesis and release in each cell.

In this chapter, it was expected that glucocorticoids would stimulate the production of urea from ammonia. Published studies show that glucocorticoids promote synthesis of mRNA and subsequent enzyme activity for carbamoyl phosphate synthestase I (Lamers et al., 1984), arginiosuccinate synthetase (Bourgeois et al, 1997), argininosuccinate lyase (Husson et al., 1990) and arginase (Nebes and Morris, 1988), four out of five enzymes of the urea cycle. Ornithine transcarbamylase mRNA was not increased by steroid, however, enzyme activity increased, potentially by a protein stabilization mechanism (Ulbright and Snodgrass, 1993). Thus, the synthesis and/or activity of the five enzymes of the urea cycle is increased by glucocorticoid treatment (Husson et al., 1985).

Despite these observations, no effect was noted of dexamethasone on urea synthesis in these mid-trimester liver cultures. This was somewhat surprising, given the body of literature which describes the effects of glucocorticoids on the

urea cycle. It may be that the quantity of urea synthesized, or ammonia taken up, is not a reliable marker of urea cycle enzyme activity in these cultures. In general, enzyme activity is best measured by dynamic studies of maximal enzyme velocity (Vmax). This would have required detailed studies investigating the rate of urea synthesis in the presence of different quantities of ammonia. The present work utilised the quantity of urea as an end point after a 4 hour incubation. This is very likely to represent a dynamic equilibrium. In fact, the quantity of urea present at equilibrium simply provides an index of the relative thermodynamic favourability of the forward and back reactions, which is little affected by the quantities of enzymes present. Detailed studies of urea cycle enzyme activities may be prudent before asserting that glucocorticoids exert no effect on urea synthesis in fetal liver cells in vitro.

The potential exists for other mechanisms to inhibit urea synthesis in vitro. For example, insulin has previously been reported to inhibit markedly the stimulant effect of glucocorticoids in cultured hepatocytes. In fact, insulin causes marked downregulation of all urea cycle enzymes, excepting ornithine transcarbamylase, in fetal rat liver cultures (Husson et al., 1985). The culture system developed for these studies required insulin as a growth-supporting supplement. Full characterization of the cell cultures with and without insulin was not possible. It is well recognized that primary culture of fetal cells in particular, but also of many primary cell types in general, often requires insulin as a pleiotropic factor to support growth. Therefore, these cells were grown in insulin-supplemented

medium to ensure cell survival. It may be that the absence of a dexamethasone effect in these studies was secondary to the presence of high levels of insulin.

Whether or not dexamethasone had any effect, it was noted that only two thirds of added ammonia were taken up within a 4 hour period. This was thought to be a rather small fraction of the total, implying urea cycle immaturity. Previous authors have noted that the rate limiting enzyme, CPSI, is present in fetal liver cells at only $1/50^{th}$ the level of adult cells, with $1/10^{th}$ the synthetic capacity during development (Lamers et al., 1984). Despite this, urea synthesis is demonstrable in vitro in preparations of human fetal liver by week 9, although urea synthesis in vivo seems to occur only in the second half of gestation (Karsai and Elodi, 1982). It is possible that there were insufficient quantities of L-aspartate present in the cultures. This amino acid is a necessary requirement for ureagenesis which is consumed in the pathway. However, adult human hepatocytes grown in the same medium and subject to the same protocol metabolise all added ammonia to urea (J. Black, personal communication), refuting this explanation. Taken together, it is likely that the urea cycle activity reflects physiological immaturity, and that any enhancing effect of glucocorticoids was abrogated by the significant quantities of insulin present. Further work could allow these hypotheses to be explored.

Despite the unremarkable effects of dexamethasone on urea synthesis, it was noted that over time in vitro, less ammonia was left in the supernatant. That is

to say that ammonia incorporation increased with time, although urea synthesis appeared static. This effect was significant when dexamethasone was omitted from the culture medium. Ammonia may be incorporated in the liver via the glutamate dehydrogenase reaction as well as the urea cycle. Glutamate dehydrogenase, a mitochondrial enzyme found only in hepatocytes, catalyses the reaction in which ammonia and α-ketoglutarate combine to form glutamate. This is a key step towards the synthesis of non-essential amino acids, and is complemented by further ammonia uptake in the reaction to form glutamine from glutamate and ammonia. These data support the hypothesis that the metabolic pathways in developing liver are maturing in vitro, so as to favour the synthesis of various amino acids as would normally be observed in adult liver.

In this series of experiments, the classical flattened morphology of mature hepatocytes was evident only in the presence of glucocorticoids. Previously, the role of hepatic transcription factors in the maintenance of hepatocyte morphology has been examined at the molecular level, and it is clear that the mature phenotype is critically dependent on the expression of the transcription factor HNF4α and the subsequent cell surface expression of E-cadherin (Parviz et al., 2003). For example, dedifferentiated, fusiform, hepatoma cells can be driven to express mature hepatocyte morphology after transfection with an HNF4α vector, which leads to surface expression of E-cadherin (Spath and Weiss, 1998). Molecular ablation of the HNF4α transcription factor with Cre-

recombinase techniques in whole animals results in an abnormal developing liver, populated with small rounded hepatocytes, without intercellular junctions, and which express extremely low levels of hepatocyte-specific enzyme systems (Parviz et al., 2003).

Whilst the role of HNF4α is in little doubt, it is not clear from the present work how glucocorticoids might bring about the mature morphology noted. There are no data in human liver as to the relationship between HNF4α and glucocorticoids. Surprisingly, there is only one publication demonstrating glucocorticoid binding activity in the fetal human liver (Ballard and Ballard, 1974), but no studies demonstrating immunolocalisation of glucocorticoid receptor in liver (Condon et al., 1998). However, DNA footprinting techniques have shown that the upstream regulatory region of the HNF4α promoter sequence contains an enhancer element which is activated by glucocorticoids and liver enriched transcription factors (Bailly et al., 2001). As previously stated, the glucocorticoid receptor has not been mapped in developing human liver, but the marked effects of corticosteroids on fibrinogen secretion as noted here strongly support the presence of GR in developing hepatocytes. Therefore, it is likely that glucocorticoids in this work are binding to glucocorticoid receptor and enhancing HNF4α expression, with resultant phenotypic maturation. Confirmation of this hypothesis awaits further study.

In this study, only corticosteroids showed a credible dose-response relationship between hormone concentration and fibrinogen release. In cell culture studies of HepG2 cells, oestradiol was previously noted to have no effect on fibrinogen release (Niessen et al., 1995). By contrast, human fetal liver cultures respond to estradiol levels between 0.1nmo/l and 0.1 micromol/l, as used in the present study, by synthesising increased quantities of cholesterol in a dose-responsive manner (Carr and Simpson, 1984). The lack of a clear dose relationship between oestradiol concentration and fibrinogen secretion, in the presence of oestradiol levels clearly shown to be effective in other studies, suggests that oestradiol has no specific effect on fibrinogen secretion from human fetal liver. This is supported by the absence of oestradiol receptors detected in fetal liver of rat and monkey (Hochner-Celnikier et al, 1986). However, the results of Carr and Simpson (1984) suggest that the human fetal liver may well possess oestradiol receptors. Without further detailed experiments, any role for oestradiol remains obscure.

Progesterone levels in the current experiments span the levels seen in physiological menstrual cycles (10-60 nmol/l) and in term pregnancy (1-5 micromol/l). Fibrinogen secretion has not previously been investigated in response to progesterone, however, cholesterol synthesis in fetal human hepatocytes may be increased by progesterone when present at levels greater than 1 micromol/l (Carr and Simpson (1984). It is reasonable to suppose that the fetal liver is insensitive to circulating progesterone. Therefore, the present

observation, that progesterone may have caused a small increase in fibrinogen release, only at a level of 10 nmol/l, the lowest physiological level in women, remain speculative.

Several other hormones, in addition to the steroid hormones above, were investigated to determine whether there were stimulatory effects on hepatocyte function. Glucagon has a physiological role to stimulate gluconeogenesis in liver. It is also a stress hormone, is elevated as part of the metabolic response to adverse physiological conditions, and may have a role in the acute phase response of the liver. In normal humans, glucagon infusion results in acutely increased fibrinogen synthesis and plasma levels (Tessari et al., 1997), an observation which might explain pathophysiological hyperfibrinogenaemia in type II diabetic patients (Barazzoni et al,. 2000). However, in cultured rat hepatocytes, glucagon had no effect on fibrinogen secretion (Zupke et al., 1998).

Detailed studies of fetal liver confirm that glucagon receptor levels are much lower than adult levels (Berthoud et al., 1992). In fetal sheep, data have been presented to suggest that there is marked glucagon resistance during fetal life (Devasker et al., 1984), which is supported by observations of markedly impaired adenylate cyclase activation in response to glucagon (Vinicor et al., 1976).

It is likely that the absence of any observed effect of glucagon on cultured hepatocytes in the present study is due to the marked insensitivity of fetal liver cells to glucagon. In support of this, the concentrations of glucagon employed here were more than sufficient to elicit a physiological response (Vinicor et al., 1976). Although there are no studies of human fetal liver and responses to glucagon in the literature, low receptor expression likely plays a significant role.

Tri-iodothyronine (T3) was investigated in the present work as fetal cells may require T3 as a permissive or trophic agent to support normal function. The data here suggested that T3 neither caused cell proliferation, nor had any effect on protein secretion. The absence of an effect on proliferation was noteworthy; in adult rats, T3 supplementation in vivo causes hepatocyte proliferation, a response which has been used in whole animals to produce massive proliferation in transplanted hepatocytes (Selden et al., 2003) and to enable viral transduction of replicating hepatocytes in situ, paving the way for liver gene therapy (Forbes et al., 2000).

With regard to hepatocyte function, many studies have demonstrated the significant role of thyroid hormones to support normal liver function. For example, T3 is central to bile acid synthesis (Ellis et al., 1998) and glycogen synthesis (Betley et al., 1993) in rat and human hepatocytes. In humans, hypothyroidism may present with coma and hyperammonaemia, mimicking

hepatic failure, and suggesting a supportive role for thyroid hormones in ureagenesis (Thobe et al., 2000).

In cell culture studies, T3 causes a dose-responsive decrease in AFP and α-1-antitrypsin secretion, in contrast to a stimulation of α-1-acid glycoprotein (Kobayashi and Horiuchi, 1995). T3 also mediates the maturational change from fetal liver AFP secretion to neonatal albumin synthesis and release in fetal mouse hepatocytes in vitro (Anteby et al., 1993). In support of the absence of any effect as seen in the present study, other workers have shown that T3 has a significant effect on hepatocyte protein synthesis in vitro, but had no effect on protein secretion from cultured primary rat hepatocytes (Gallo et al., 1987). The present study was designed to assess any simple effects of T3 on fetal human hepatocyte function, and no effect was observed on proliferation or fibrinogen release. It remains possible that detailed study might reveal significant effects of T3, for example on AFP release, metabolic rate, or urea production. However, within the confines of the present work, no effects were observed.

This study set out to assess which of several hormones present during fetal life might have significant maturational effects on human fetal liver cells. Fibrinogen secretion and cell morphology were chosen as indices of maturation. Glucocorticoids had a potent maturational effect in terms of protein synthesis, observations which were confirmed with two separate glucocorticoid agents.

These data were corroborated by the effect of glucocorticoid to bring about a more mature epithelial phenotype in cultured cells. This chapter has provided good evidence that glucocorticoids are implicated in the functional and phenotypic maturation of fetal human liver cells.

Chapter 5.

Cytokines, growth factors, and proliferation in fetal liver.

5.1 Introduction.

Prior to ductal plate formation, developing hepatoblasts and haematopoietic cells form the liver parenchyma, with only portal and central venous channels interrupting these two compartments. The liver cell complement must therefore be derived by proliferation of hepatoblasts, or from a stem cell compartment which gives rise to hepatoblasts, or both. Although the role of growth factors and cytokines in liver cell proliferation has been investigated extensively in adult liver, the data in the developing liver are scant, and the data from fetal human liver even more so. Understanding the proliferation signals in developing liver is important, as such insight also allows understanding of liver repair and liver cancer.

During fetal life, bile ducts contain strong staining for both TGF-α and EGF receptor (Terada et al., 1994), and published data show a generalised proliferative response to EGF in cultured human liver cells (Malhi et al., 2002). Hepatocyte growth factor, and its receptor c-met, have been localised in fetal liver. c-met is expressed in the ductal plate and in the developing bile ducts, as well as in hepatocytes, but only at low levels (Terada et al., 1998). HGF may be

important in the proliferation of biliary ductules during development (Pagan et al., 1999), whereas KGF may cause significant proliferation in hepatocytes (Housley et al., 1994).

Ultimately, the growth factors and cytokines implicated in fetal liver development are not adequately defined, and the true mechanisms which subserve hepatoblast and cholangiocyte proliferation in developing liver are not known. In order to clarify which growth factor pathways may be implicated in the developing human liver, this chapter sought to examine the effects of different growth factors and cytokines on cellular proliferation in developing liver cells.

5.2 Materials and Methods.

Cell cultures were prepared as previously described. Cells were diluted as required and on plated type I collagen-coated dishes as appropriate to individual experiments. Cells were then exposed to various growth factors and cytokines, and cellular proliferation was assessed as described below.

Growth Factors and Cytokines.

All growth factors and cytokines were obtained from Peprotech, UK.

Epidermal Growth Factor (EGF) was prepared in sterile water to a concentration of 1 mg/ml, divided into 20 aliquots of 50 μl and stored at -70°C. Keratinocyte

Growth Factor (KGF), Hepatocyte Growth Factor (HGF), Interleukin-6 (IL-6) and Tumour Necrosis Factor α (TNF) were similarly prepared and stored.

For experimental purposes, peptides were diluted in culture medium to give a final working concentration when added to cells as follows; EGF; 25ng/ml: KGF; 20ng/ml: HGF; 10ng/ml: IL-6; 20ng/ml: TNF; 1ng/ml. Peptides, once thawed, were discarded after single use.

5.2.1 [3H] Thymidine incorporation in cultured fetal liver cells.

Cells were diluted in culture medium to a density of $2x10^7$ cells per ml, and 25μl were added to each of triplicate wells in a 96-well plate ($5x10^5$ cells per well). To each well were added a further 25 μl culture medium containing growth factor or cytokine. The total volume was made up to 100μl, and cells were cultured in a humidified CO_2 incubator as previously. After 24 or 48 hours, tritiated thymidine (Amersham, Berks) was added at an activity of 25μCi per ml in a volume of 20μl to all wells using an Eppendorf multichannel micropipette (Sigma).

72 hours after plating, plates were gently agitated to resuspend particulate matter and dead cells, and the medium was removed by gentle suction. 200μl of warm PBS without calcium/magnesium was added per well, then the plate was gently agitated and the medium removed as before. This wash step was

repeated, and 100μl of warm trypsin solution per well were added. Cultures were returned to the incubator for 10 minutes or more, and cell detachment was confirmed visually at this stage. An automated cell harvester (Skatron type 7020 semiautomated cell harvester, Lier, Norway) was used to transfer the contents of each well onto individual filter mats (Skatron). Filter mats were then thoroughly dried. Filter discs, each one corresponding to a single well, were punched out into individual counting vials (Pony vial, Packard), to which were added 5 ml Aquasafe 300 Plus scintillation fluid (Zinssen Analytical, Berkshire). Vials were counted automatically on a Hewlett Packard Tricarb 1900 LA liquid scintillation counter (Canberra Packard UK). Specific activities were calculated automatically by the scintillation counter and these data were used for calculating proliferation indices.

In each culture, means of triplicate wells were calculated. These experiments were repeated and means calculated (n= 3 cultures for the 24 hour incorporation, n=2 cultures for the 48 hour incorporation). Data were plotted as means +/- SE for the different growth factors and cytokines.

5.2.2. Cell proliferation as determined by manual counting.

Initial data suggested a potentially toxic effect of tritiated thymidine on cultured cells. To verify and complement the proliferation indices calculated from the

experiments described above, further studies were designed which would allow manual cell counts to be performed.

Cell cultures were prepared in 24-well plates to provide sufficient cells to allow cell counting. 250μl of freshly prepared primary cell suspension in culture medium at a density of 2×10^7 cells/ml were added (5×10^5 cells per well) to each well previously coated with type I collagen. A further 250μl culture medium was added containing no additive (control) or peptide to provide the appropriate working dilution. Cells were cultured in a humidified CO_2 incubator. On day 3, culture medium was removed and replaced with 250μl fresh culture medium, after which a further 250μl culture medium containing no additives (control) or peptide was added as before. Each experimental group was carried out in duplicate wells and each experiment was repeated in 4 separate cultures (n=4 cultures).

On day 7, culture medium was removed from the wells, and 500μl of warmed PBS without calcium or magnesium was added to each well. This was removed after gentle agitation and placed in a polystyrene tube on ice (LP3), with one tube per well. This wash step was repeated, and the supernatant retained once more. 100μl of trypsin solution was added to each well, and cells were returned to the incubator for 10 minutes. After trypsinisation, 200 μl chilled buffer (Flow cytometry buffer, as described in chapter 3, section 3.2.4) were added to each

well, and the cell suspension was removed into the relevant polystyrene tube. Tubes were then centrifuged at 340g for 5 minutes at 4°C. The supernatant was discarded, and cells were resuspended in 200ml chilled PBS/BSA. A portion was removed and made up with an equal volume of trypan blue, and viable cell counts were carried out (mean of two separate counts for each well), as described in chapter 2, section 2.2. Means were taken between the two replicates in each culture, and means +/- SE were calculated for the 4 separate cultures (n=4 cultures). The effects of growth factors and cytokines were expressed as a percentage of the matched control wells.

5.2.3 Immunophenotyping of proliferating cells.

Cell cultures were prepared as described in the preceding section, and treated with growth factors or cytokines in the same manner. On day 7 in vitro, cells were trypsinised, spun down as before, and then washed in 2 ml PBS. Cells were spun down again and fixed by incubating with 1 ml 0.5% paraformaldehyde in 10mM PBS on ice for 15 minutes in the refrigerator (4°C). These fixed cells were then processed for two-colour flow cytometry as described in chapter 3. The proportions of cells expressing cytokeratin 18 but not fibrinogen, cytokeratin 18 and fibrinogen, the total of cytokeratin 18-expressing cells, or cells negative for both markers, were calculated for each treatment. The means of two wells for each treatment were taken, and the experiment was repeated in 4 separate cultures.

Results were calculated by multiplying each phenotype proportion by the total number of cells in the well. The quantities of cells in each of the four groups were then expressed as a percentage of the appropriate cell phenotype in control cultures (n=4 cultures). Data were analysed by ANOVA, followed by post-hoc testing where permitted.

5.2.4 Immunocytochemistry of developing liver.

To gain insight to the cell types detected by flow cytometry, liver tissues were fixed in formaldehyde, embedded, sectioned and dewaxed as previously described in section 3.2.1. Antigen retrieval was carried out by boiling in citrate buffer, 0.1M, pH 6.0, for 3x5 minutes in a microwave. After cooling to room temperature, slides were placed in 0.05M Tris buffered saline (TBS) , then incubated with 5% normal serum as appropriate (Blood Transfusion Service, Edinburgh) in TBS for 30 minutes. This blocking buffer was carefully dabbed off, and primary antibody or control $IgG_1$was applied to tissue sections diluted in blocking buffer for 1 hour at room temperature in a humidified chamber. Slides were then rinsed with TBS, and incubated in a rocker bath of TBS for 10 minutes. The second antibody (HRP conjugate) was then added, diluted in blocking buffer for an hour under the same conditions as previously. Slides were rinsed and washed as before. To develop the slides, Di-amino Benzidine (DAB; DakoCytomation) solution was added as per the manufacturer's instructions for up to five minutes, or until the colour reaction had taken place. The reaction was stopped, and the slides were counterstained with haematoxylin, dehydrated

and mounted in Pertex (CellPath, Powys, UK) as previously described. Mouse monoclonal anti-cytokeratin 18 and cytokeratin 19 antibodies were used to assess the epithelial lineages in the liver. Rabbit anti-Ki67 was used to examine proliferation in various compartments, whereas mouse monoclonal anti-EGF receptor was used to document the distribution of this important pathway in the epithelial compartment. Rabbit anti-mouse HRP conjugate or Goat anti-rabbit HRP conjugate was used as the second antibody in each case. All antibodies were obtained from DakoCytomation.

Slides were then photographed on an Olympus CK2 photomicroscope with Kodacolour ASA 200 film using a tungsten correction filter as described in chapter 3.

5.2.5. Statistics.

Analyses in which more than 2 groups were compared were first assessed by Analysis of variance using the SPSS package. Pairwise comparisons were carried out using a t-test where permitted by ANOVA. The significance level was set at 0.05.

5.3 Results

5.3.1 [^{3}H] Thymidine incorporation in cultured fetal liver cells.

Figure 5.1.A shows the incorporation of tritiated thymidine over a 24 hour period in fetal liver cultures incubated in the presence of control medium, or with a range of growth factors or cytokines. Figure 5.1.B shows a parallel study in which thymidine incorporation was carried out for 48 hours. It is clear from simple inspection that there is no stimulatory effect of any of the growth factors tested, over either 24 or 48 hours. However, the data from the second experiment show markedly less thymidine incorporation than the 24 hour experiment. This suggested a toxic effect of [^{3}H] Thymidine on cultured liver cells.

5.3.2 Cell proliferation as determined by manual counting.

In control cultures, there were 5.54 +/- 2.0 x 10^4 cells per well after 7 days in vitro (n=4 separate cultures). There was considerable variation in control cell number between different cell preparations; however, the responses within preparations were consistent. To derive maximum information from the data, cell counts in response to the growth factors or cytokines were therefore expressed as a percentage of the culture control. Figure 5.2 shows these data represented graphically. EGF alone significantly stimulated cell proliferation to 180.0+/-34.4% compared to matched control (100%).

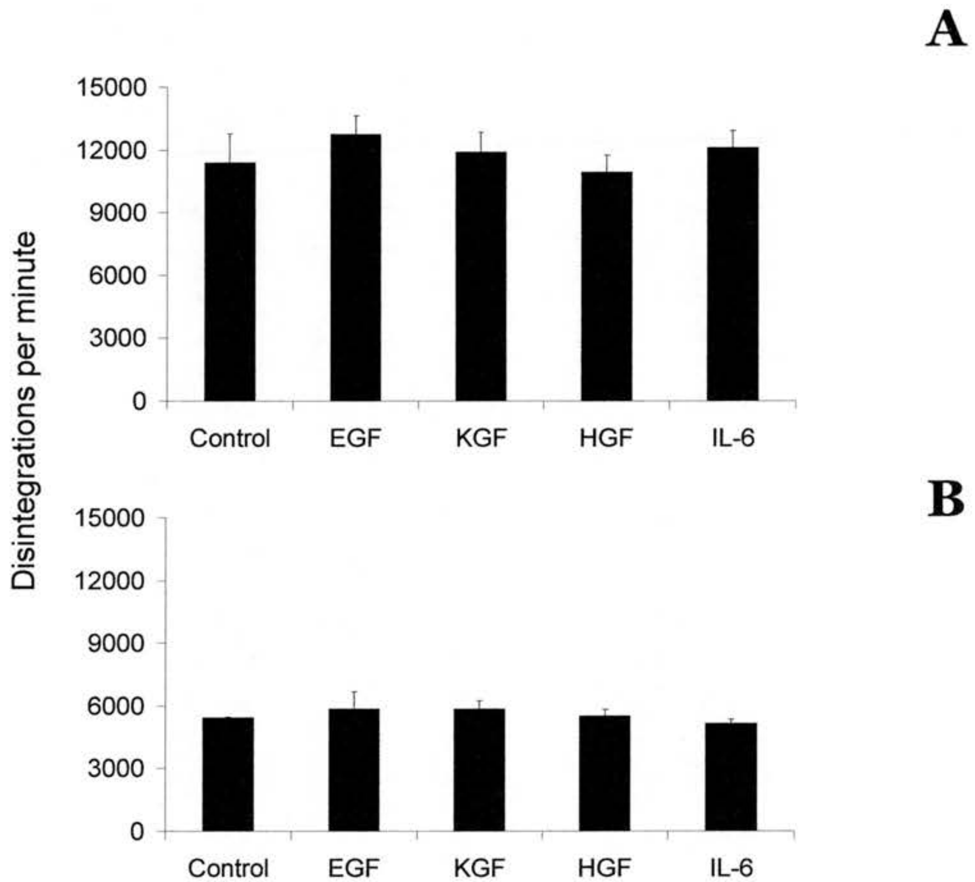


Figure 5.1. The effects of growth factors on liver cell proliferation determined by tritiated thymidine. Cell cultures were prepared from fresh tissue and plated on type I collagen at 5μg/cm². Cultures were plated at 0.5 million cells per well in 96 well plates. T o each well was added growth factor or cytokine in triplicate, and 0.5μCi tritiated thymidine was added either 24 or 48 hours after plating. Cells were washed and trypsinised at 72 hours, and an automated cell harvester was used to transfer cells onto individual filter mats. Mats were placed in scintillation vials, to which was added 5ml scintillation fluid. Cultures underwent scintillation counting (Packard Tricarb). Panel A; 24 hour label (n=3 cultures, +/- SEM) and Panel B; 48 hour label (n=2 cultures, +/- SEM).

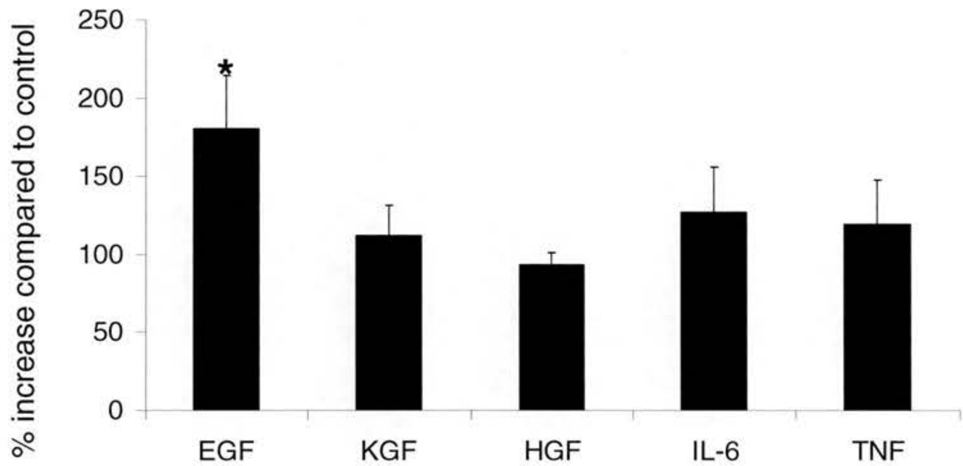


Figure 5.2. Proliferative responses to growth factors and cytokines by cell counting. Cell cultures were prepared from fresh tissue and plated on type I collagen at 5μg/cm². Cultures were plated at 5 million cells per well in 24 well plates. Cultures were maintained for 7 days in vitro, with a medium change on day 3. Cultures were trypsinised on day 7 and cell counting was carried out using a haemocytometer in cells stained with trypan blue. EGF was significantly different from paired control, $p<0.05$. n=4 cultures, data shown +/- SEM.

5.3.3 Immunophenotyping proliferating cells.

In order to determine which cell population was responding to EGF, immunophenotyping was carried out by dual-label flow cytometry directed against cytokeratin 18, a liver epithelial marker, and Fibrinogen, a hepatocyte marker.

In control cultures, 26.25+/-7.8 % of all cells were cytokeratin 18 +ve alone, whereas 27.13+/-9.5% were dual positive hepatocytes (cytokeratin 18+ve; Fibrinogen +ve). A total of 56.75+/-8.5% of cells were therefore cytokeratin 18+ve epithelial cells, and 46.0+/-9.5% were dual negative cells (n=4 cultures, means +/-SEM). 2.3+/-0.4% of cells were fibrinogen +ve alone, which represents the background detection of light signals not related to epithelial cells.

In order to demonstrate the magnitude of the proliferative response, the number of cells within each phenotype was calculated by multiplying the number of cells in each well by the % for each phenotype. This number was expressed as a % of the relevant control figure for each culture. Figure 5.3A shows the responses of the cell population which was positive for cytokeratin 18 and negative for fibrinogen (epithelial cells). Figure 5.3B shows the population positive for cytokeratin 18 and fibrinogen (hepatocytes), whereas figure 5.3.C shows the entire cytokeratin 18+ ve population (A+B). Figure 5.3D shows the responses of non-parenchymal cells, positive for neither cytokeratin 18 nor fibrinogen, to the

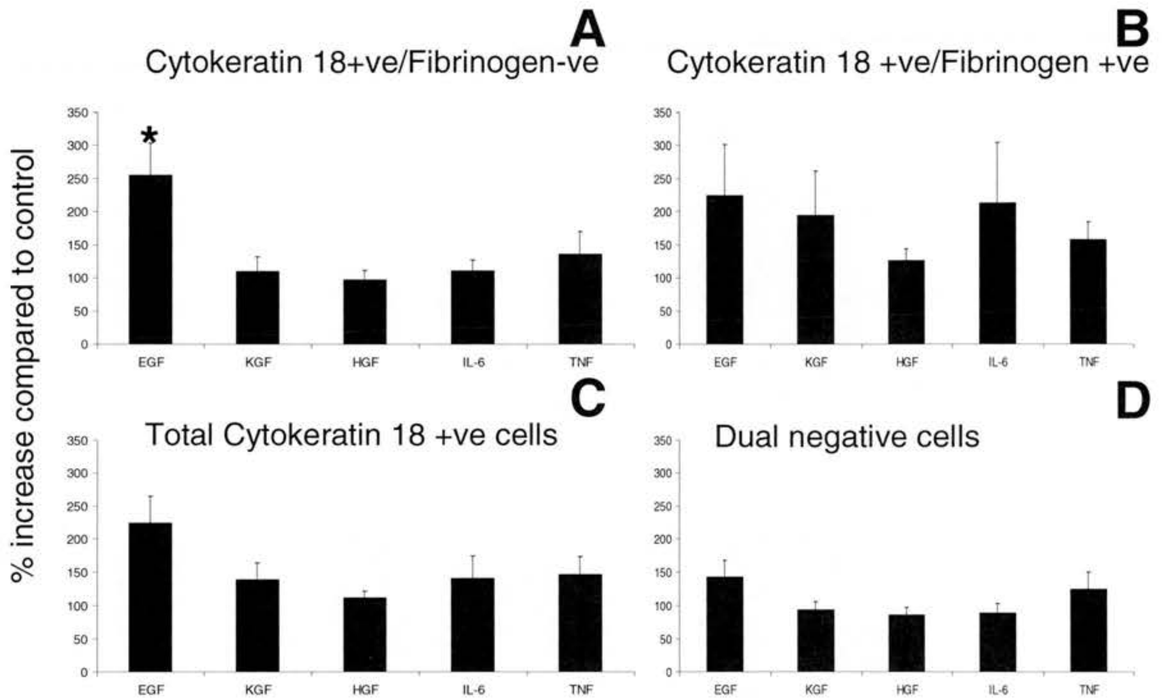


Figure 5.3. The effects of growth factors on proliferation of liver cell subsets. Cell cultures were prepared from fresh tissue and plated on type I collagen at 5μg/cm². Cultures were plated at 5 million cells per well in 24 well plates, to which were added the various growth factors. Cultures were maintained for 7 days in vitro, with a medium change on day 3. Cultures were trypsinised on day 7, placed in polystyrene tubes, fixed with 0.5% formaldehyde and permeabilised with 0.1% triton X. After washing, cells were incubated with mouse anti-cytokeratin 18 antibody at 1:100 dilution, in combination with rabbit anti-Fibrinogen antibody at 1:200 dilution. After a wash step, cells were incubated with Goat anti-mouse Phycoerythrin conjugate antibody at 1:20 dilution and Swine anti-rabbit FITC conjugate at 1:20 dilution. Immunopositive cells were detected by flow cytometry with excitation at 488nm and detection at 525 and 575nm. Data show that EGF selectively stimulated the proliferation of cytokeratin 18+/Fibrinogen –ve cells (ANOVA =0.001; $p<0.05$). The effects of growth factors on other cell types were not significant.

various growth factors and cytokines. These data show that only EGF caused a significant expansion of cells (ANOVA; $p = 0.001$; Pairwise comparisons; $p<0.05$), and that these cells were strictly within the epithelial compartment (cytokeratin 18+ve), but were not hepatocytes (Fibrinogen –ve). In fact there was no significant effect on dual labelled hepatocytes (cytokeratin 18+ve/Fibrinogen+ve; Figure 5.3.B) or on non-parenchymal cells (dual negative; Figure 5.3.C).

5.3.4 Immunocytochemistry of developing liver.

Figure 5.4 shows the results from the immunocytochemistry studies. Cytokeratin 18 reaction product was present in the epithelial compartment alone. The cells which were labelled corresponded to hepatocytes and to biliary tract cells. By contrast, cytokeratin 19 labelled only the ductal plate and developing biliary ductules. Interestingly, these data confirmed that biliary tract and ductal plate were positive for cytokeratins 18 and 19. Ki-67 labelling showed that cell proliferation occurred in all compartments within the developing liver, although the ductal plate seemed to show no staining. EGF receptor was present on the ductal plate, biliary ductules and the hepatocytes.

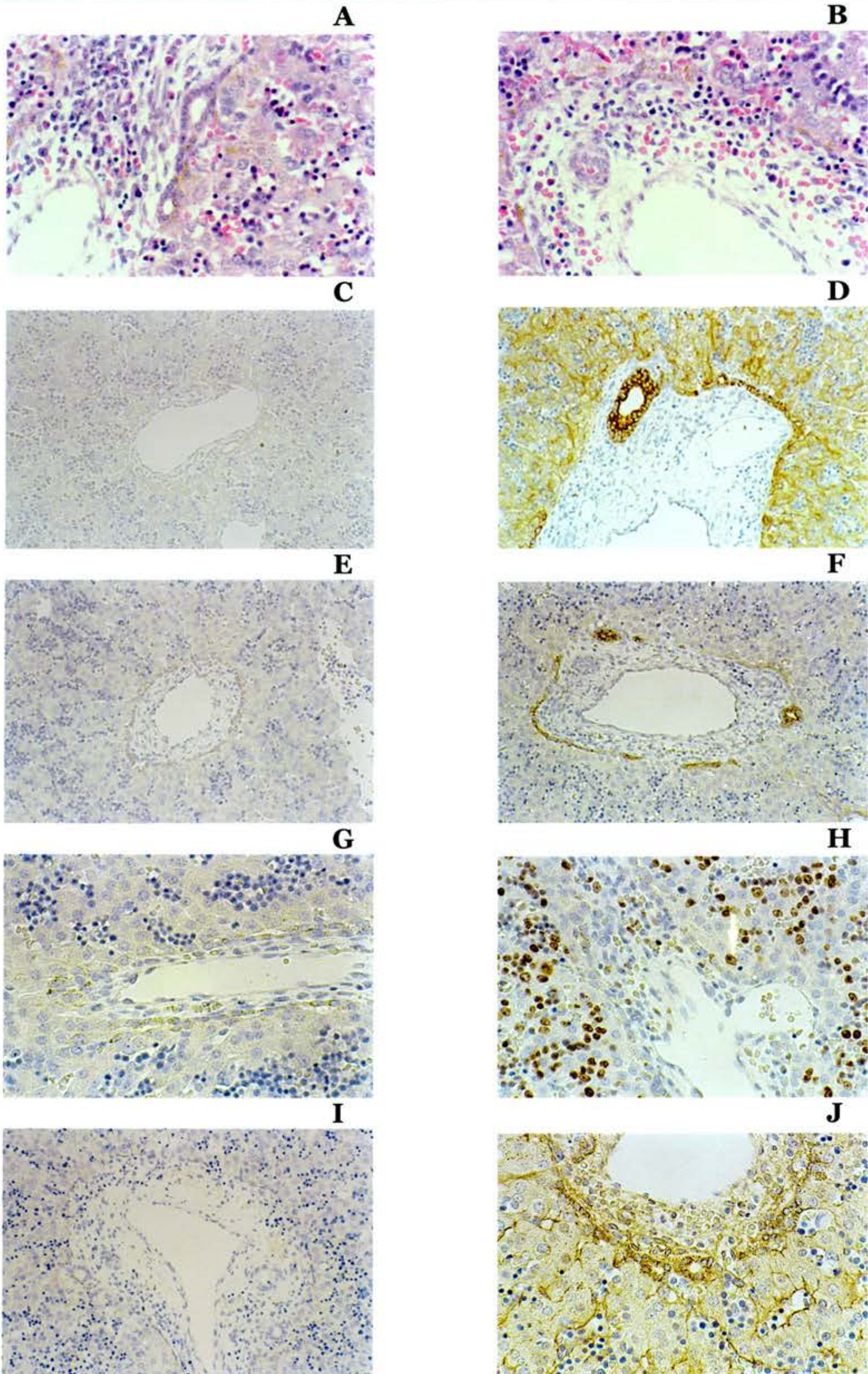

A
B
C
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Figure 5.4. Immunocytochemistry in the second trimester fetal liver.

A and B; Haematoxylin and eosin stained sections (x400) are provided for reference. Multiple biliary ductules can be seen in A along the ductal plate. In B, intraportal haematopoiesis is evident from the nucleated red cells present. Hepatocytes are faintly eosinophilic cell outside the portal tract. Haematopoiesis is also evident within the liver sinusoids.

C and D; Mouse immunoglobulin (IgG1) control and anti-cytokeratin 18 (1:100) x 200. The cytokeratin 18 antibody stains all epithelial structures (biliary tract and hepatocytes) whereas the control slide is not stained.

E and F; Mouse immunoglobulin (IgG1) control and anti-cytokeratin 19 (1:25) x 200. The cytokeratin 19 antibody stains only biliary structures (biliary ductules and ductal plate). The control is negative.

G and H; Rabbit immunoglobulin control and Rabbit anti-Ki-67 (1:50) x 400. The nuclear labelling indicates cells within the replicative phase. The arrow clearly shows a labelled hepatocyte nucleus. Many other proliferating cell types are present. No labelling is observed in control sections.

I and J; Mouse immunoglobulin (IgG1) control and anti-EGF receptor (1:100) x 200. EGFR immunostain is observed on hepatocytes, bile ducts and ductal plate. Whilst the haematopoietic fraction in the sinusoids appears negative, there may be some staining in the periportal mesenchyme.

5.4 Discussion.

These experiments set out to investigate which growth factors might have a role in supporting proliferation in the developing liver. The data showed that EGF alone was effective in stimulating proliferation. This proliferative effect was restricted to the epithelial compartment, in particular, cells which were positive for cytokeratin 18, but negative for fibrinogen. Immunocytochemistry suggested that the cells responding to EGF are within the epithelial compartment, namely the ductal plate, the developing biliary ductules or the hepatocytes. These data support the hypothesis that EGF is a potent mitogen for primitive liver cells during human liver development.

To investigate the pathway that mediates proliferation of fetal liver cells during development, cultures were incubated with a number of different growth factors in the presence of tritiated thymidine. The results showed that cells did not respond to growth factors with a proliferative response. This was a surprising finding, and the concern that insufficient time had been allowed for labelling to take place was addressed by doubling the incubation time from 24 hours to 48 hours. This revealed a paradoxical reduction in the labelling of cultured cells, and no effect of growth factors. This response, which was consistent with the first set of observations, introduced the possibility that tritiated thymidine might have a deleterious effect on cultured cells.

Tritiated thymidine has been extensively utilised to assess proliferative responses in cell cultures, including the proliferative response to EGF in fetal (Fabregat et al., 1992) and neonatal rat hepatocytes (Ragan et al., 1997). Tritiated thymidine has been used to label human fetal hepatocytes proliferating in vitro in response to EGF (Malhi et al, 2002). This paper showed a stimulatory effect of EGF, however, the effect was only observed in fetal human liver cells passaged 10 times, and not seen in primary cultures. The present study showed the same result as Mahli; no proliferation was detected in response to EGF in primary cultures as detected by tritiated thymidine. The data presented here are entirely consistent with a toxic effect of tritiated thymidine, and the data of Mahli et al. can be interpreted in the same way.

It is not clear why a stimulatory EGF effect might appear after 10 passages of cultured cells, however, long term culture may allow changes in differentiation status which may reduce the toxicity of tritiated thymidine, or increase resistance to apoptosis. Ultimately, Mahli et al. did not determine the cell type responding to EGF in the serially passaged cells.

Given the potential for a toxic effect of tritiated thymidine as noted above, the hypothesis that growth factors could cause proliferation in cultured liver cells had not been appropriately addressed by the initial experiments of this chapter. The experiment was redesigned such that cultures were subject to cell counting

after exposure to growth factors and cytokines. These data revealed that EGF alone caused a significant increase in cell counts in vitro.

EGF has previously been reported to cause proliferation in human fetal hepatocytes. EGF treatment resulted in an increase cell numbers at 7 days, as determined by cell counting, of approximately 180% compared to control, and an increased gamma-glutamyl transferase activity (Rehman et al., 2004). However, these authors used unpurified hepatocytes to prepare their cultures, and did not clarify which cell types were in fact undergoing proliferation in response to EGF. Furthermore, they did not distinguish whether changes in enzyme activity were related to maturation of a subset of cells, or whether an increase in the number of cells expressing the enzymes were responsible.

Gamma-glutamyl transferase activity is usually associated with the biliary tract. Therefore, increased gamma-glutamyl transferase activity may in fact relate to proliferation of cholangiocytes rather than hepatocytes. Previous work in mouse hepatoblasts by Rogler (1997) described gamma-glutamyl transferase activity in association with a ductular phenotype, in cells lacking albumin expression. Therefore, despite the assertions of the authors, the data, as presented by Rehman et al (2004), do not definitively support the hypothesis that hepatocytes *per se* proliferate in response to EGF. Ultimately, it is not possible to say what the cell of origin might be which gives rise to the very primitive cytokeratin 18+/fibrinogen- progeny in this study, however, it seems likely to be an

epithelial progenitor bearing the EGF receptor. Immunocytochemistry in this study showed that all of the epithelial cells are candidates, however, evidence of proliferation in vivo was noted only in the hepatocellular fraction. There are strong data to support both a biliary and a hepatocellular origin for the primitive epithelial cells observed in this study.

The data from these experiments support the hypothesis that EGF-induced proliferation of a primitive fraction of liver epithelial cells, positive for cytokeratin 18 and negative for fibrinogen. Is not clear whether this might represent primitive hepatocytes or cholangiocytes undergoing proliferation, or a fraction more primitive still. In rat liver injured by administration of 2-acetylaminofluorene, biliary ductules proliferate, invading the hepatic parenchyma, and give rise to both hepatocytes and cholangiocytes. These proliferating ductules are positive for cytokeratins 7,8,18 and 19, but are negative for markers of hepatocellular differentiation, such as P450 enzymes (Alison et al., 1995). These authors do not provide evidence as to whether cells were positive or negative for fibrinogen, however, it seems likely that these proliferating progenitors were negative. Data from isolated biliary epithelial cells in adult human liver show that the phenotype is cytokeratin 7,8,18 and 19 positive (Liu et al., 2004). No extensive immunophenotyping of the proliferating cytokeratin 18+ fibrinogen- cells was possible in the present study, as there were limitations imposed by the flow cytometer as to how many labels could be utilised in one pass. Interestingly, proliferating ductular cells in injured

livers have been described as "oval cells", given their striking morphology (Alison et al, 1996; Petersen et al, 1998;). These cells express the haematopoietic marker Thy-1, and are thought to represent a progenitor compartment, giving rise to hepatocytes and cholangiocytes. In this study, no Thy-1 labelling was carried out, however, it remains a fascinating possibility that cytokeratin 18+/Fibrinogen- cells are in fact oval cells.

In support of a physiological role for EGF in development, immunocytochemistry has shown the presence of TGFα, a potent, physiological EGF receptor agonist, in the fetal human liver, specifically within hepatocytes (Yasui et al., 1992). By contrast, the most intense immunostaining for EGF receptor was in the bile ducts (Terada et al., 1994). It is notable that both agonist and receptor were also present in the same cell types, that is to say that cholangiocytes were positive for EGFR and TGFα, as were hepatocytes (Terada et al, 1994), raising the possibility of an autocrine signalling loop. The data in the present study agree with these findings.

In conclusion, studies were carried out to determine the responses of fetal liver cells to growth factors and cytokines. Whilst initial studies suggested that none of the experimental agents had stimulatory effects on liver cells, a second experiment confirmed that EGF alone caused proliferation of fetal liver cells. Two-colour flow cytometry was developed in this thesis, and then used to

determine which fraction responded to EGF. The data showed that cells that were cytokeratin 18+ and fibrinogen – proliferated in response to EGF. Neither maturing hepatocytes, which were cytokeratin 18+ and fibrinogen +, nor non-epithelial cells, which were dual negative, showed a significant response. Immunocytochemistry showed only one population of cytokeratin 18+ cells which corresponded to liver epithelium. These data show that EGF acts on the liver epithelium to stimulate proliferation in developing human liver.

Chapter 6.

Cytokines, growth factors, glucocorticoid, and fetal hepatocyte function.

6.1 Introduction.

In developing liver, specific regulation of cellular proliferation, differentiation and functional capacity is critical to the acquisition of sufficient liver mass with adequate biochemical competence at term. The parallel demands of proliferation and biochemical maturation may result in separate compartments within the developing liver, each dedicated to proliferation or maturation. Despite the clinical and scientific importance of functional status and liver cell proliferation in humans, no data describe this relationship in the literature.

It is not clear whether proliferating hepatocytes are, in general, functionally competent. In the fetal rat, hepatocytes isolated late in gestation correspond to two fractions; one proliferating and one quiescent (Gruppuso et al., 1997). Extensive studies by this group resulted in the surprising finding that the differentiation status, as determined by immunostaining for carbamoyl phosphate synthase I and alpha-fetoprotein, was independent of proliferative status, as detected by proliferating cell nuclear antigen (PCNA) staining (Gruppuso et al., 1999). However, to support this assertion, these authors

directly equated immunostaining for a particular antigen with maturity or immaturity, and made no assessment of functional capacity. This is a very important distinction, as the presence or absence of particular enzymes or proteins has no bearing on the functional status of liver cells (Van Beers et al., 1998; Mukarram Ali Baig et al., 1992).

In order to understand how the functional capacity of liver cells might change during development, and the interaction between proliferation and maturation signals, liver cell cultures were exposed to cytokines, growth factors and glucocorticoids which have previously been shown to exert significant proliferative or maturational effects on liver cells. Hepatocyte function was then assessed by measuring protein secretion and urea synthesis over one week in vitro.

6.2 Materials and Methods.

Cell cultures were prepared and plated on type I collagen-coated 24-well plates as previously described. Cells were then exposed to various growth factors and cytokines, +/- dexamethasone 10^{-7}mol/l, then protein secretion and urea synthesis was assessed as described below. Growth factors and cytokines were used at the same concentrations as described in the previous chapter. Each experiment included paired groups of 12 wells, one set including dexamethasone 10^{-7}mol/l, the other set including vehicle (ethanol; 1:10,000 dilution).

6.2.1.

The effects of growth factors, cytokines, and glucocorticoid, on urea synthesis. Medium was changed on day 3 in vitro. Culture medium containing the relevant peptide +/- steroid, was then replaced. On day 7, cultures were washed with 2 changes of 500 microlitres of warm HBSS. Cells were then incubated with 1mM ammonium chloride for 4 hours as described in chapter 3. The medium was removed and stored at -20°C for later assay.

All treatments were carried out in duplicate, and the experiment was repeated in 5 separate cultures. Samples were divided into 2 portions, which were assayed in the presence and absence of urease as previously described in chapter 3. In order to assess the effects of the various treatments, urea released by the cells into the medium, and residual ammonia, was determined. The ratio between urea released and ammonia removed was calculated and expressed as a

percentage. A figure of 100% implied that all ammonia taken up was released as urea. Conversely, a figure less than 100% implied that some ammonia had been incorporated into anabolic pathways, whereas, a figure greater than 100% suggested that catabolic processes were funnelling nitrogen into urea synthesis. Finally, these data were expressed as a percentage of paired control wells.

6.2.2.

The effects of growth factors, cytokines, and glucocorticoid, on protein secretion. Medium was changed on day 3 in vitro and was replaced with fresh medium containing the relevant peptide, with and without steroid. Cultures were maintained until day 7, when supernatant was removed and stored at -20°C for later assay. All treatments were carried out in duplicate, and the experiment was repeated in up to eight separate tissue preparations. Protein assays for AFP, α-1-ACT, Fibrinogen, CRP and TBPA were carried out as previously described. Protein secretion was expressed as a percentage of the matched culture control.

6.2.3 Statistics.

Data were plotted as means (+/- S.E.). Statistical analysis utilised univariate analysis of variance plus post-hoc pairwise t-test, as permitted by univariate ANOVA.

6.3 Results

6.3.1.

The effects of growth factors, cytokines, and glucocorticoid, on urea synthesis.

Figure 6.1. shows the effects of the various growth factors and cytokines, with and without dexamethasone, on urea synthesis in vitro. Importantly, steroid alone had no significant effect on urea synthesis, or ammonia uptake or ratio between the two (ANOVA; $p = 0.439$, 0.797 and 0.809 respectively). However, ANOVA showed that growth factors (ANOVA $p=0.005$) and the interaction between steroid and growth factors on urea synthesis (ANOVA $p = 0.036$) were significant. Pairwise comparisons showed that, in the absence of dexamethasone, a small but significant enhancement of urea synthesis was observed in those cultures treated with EGF or HGF. By contrast, urea synthesis was significantly reduced in response to KGF, but only when dexamethasone was present.

Figure 6.2 shows that there were no significant effects of any treatment on ammonia uptake by cultured cells (ANOVA; growth factor effect: $p=0.169$. steroid and growth factor interaction; $p = 0.224$).

In Figure 6.3, EGF and HGF caused a significant increase in the ratio of urea synthesis to ammonia uptake in the absence of dexamethasone, implying a catabolic funnelling of nitrogen from cellular stores towards urea (ANOVA; growth factor effect: $p=0.001$. Steroid and growth factor interaction; $p = 0.019$).

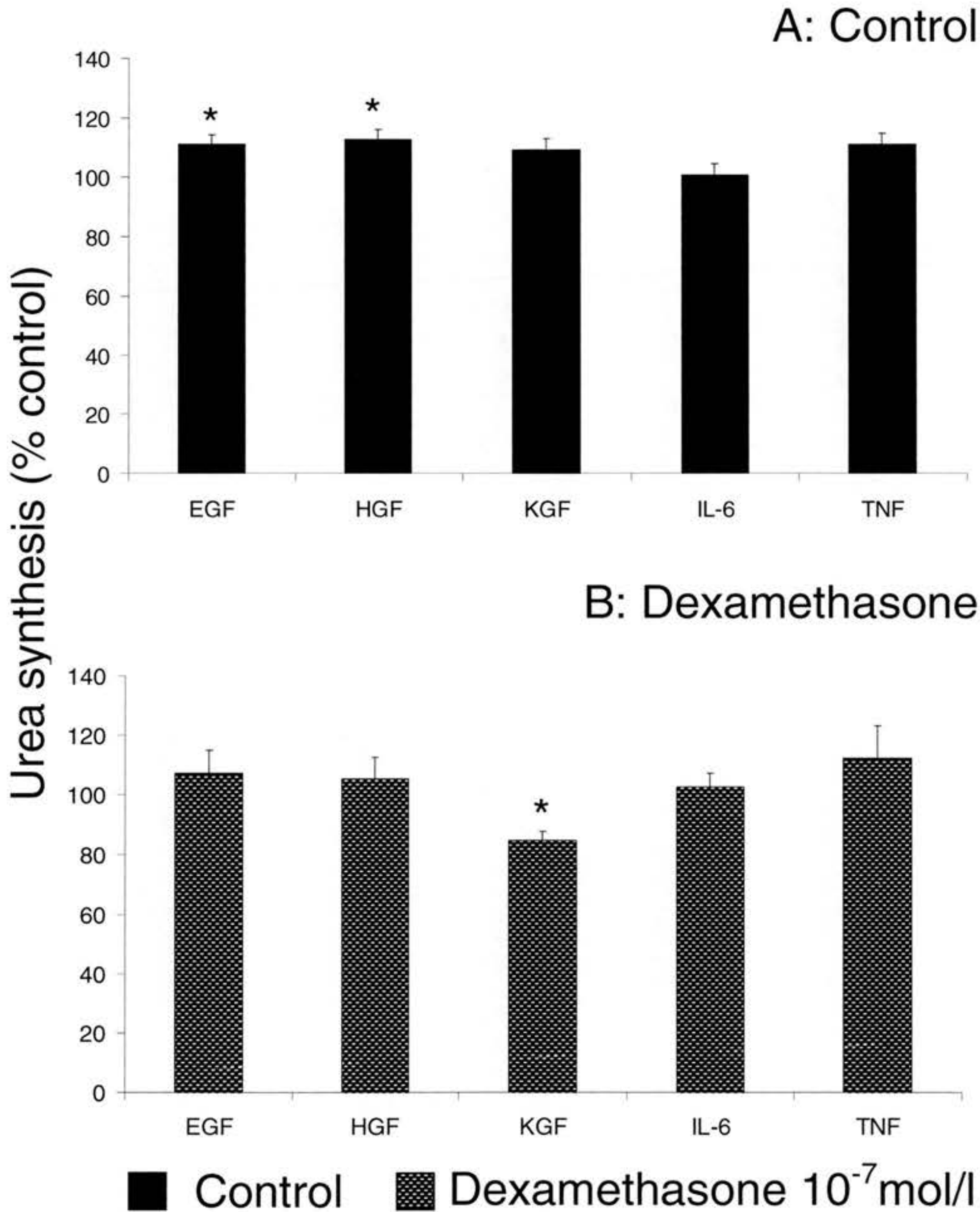

Figure 6.1. Urea synthesis in response to growth factors +/- steroid.
Cultures were plated at 5 million cells per well in 24 well plates and maintained for 7 days in vitro with a medium change on day 3. Growth factors +/- dexamethasone 10^{-7}M were added to cultures in duplicate. On day 7, the cells were washed with HBSS, and fresh HBSS containing 1mM ammonium chloride was added for 4 hours. HBSS was removed and stored at -20°C for later assay. Data are shown as mean, +/- SE. n=5 cultures for each data point. ANOVA with post hoc t-test showed * $p<0.05$ compared to matched control.

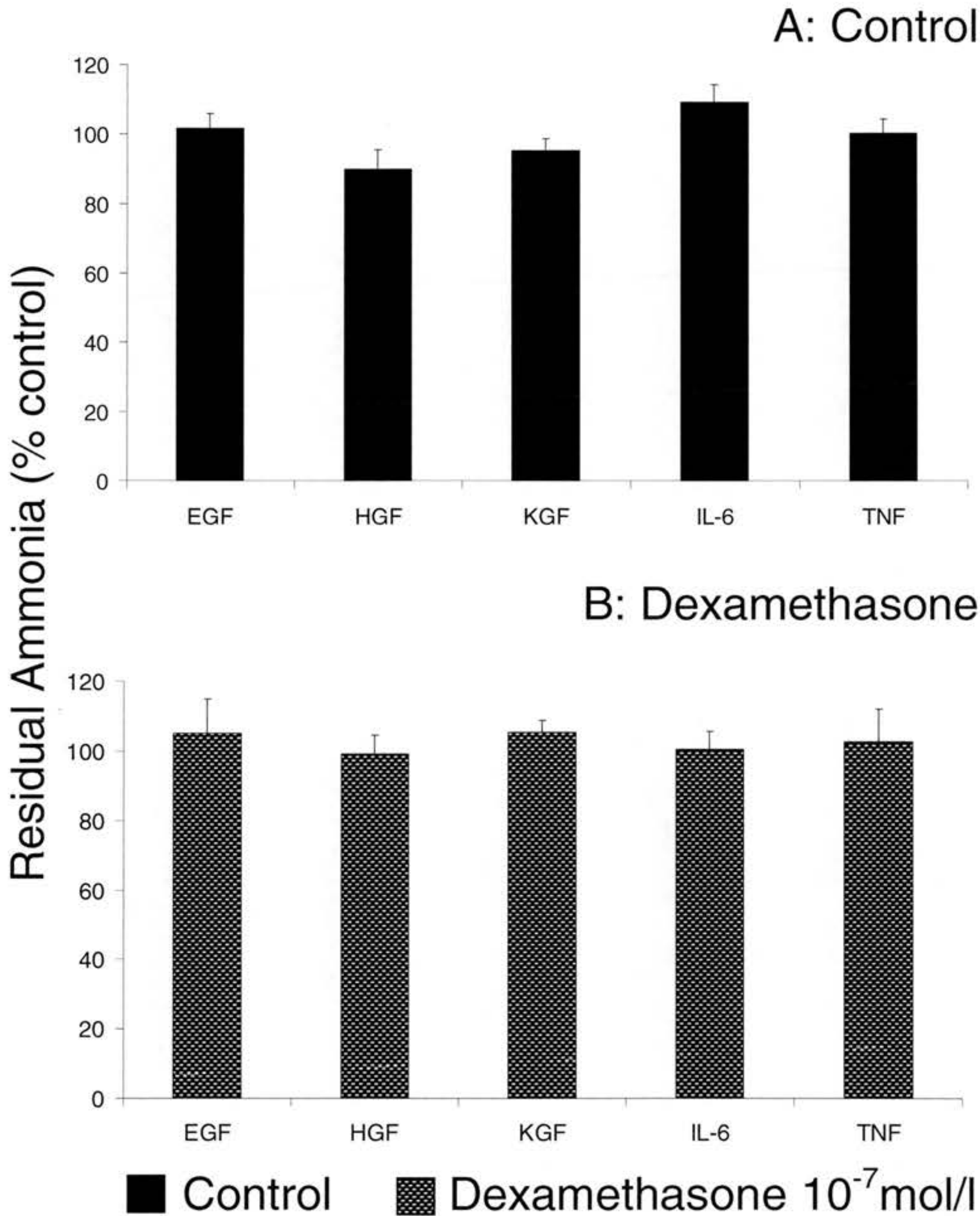

Figure 6.2. Residual ammonia in response to growth factors +/- steroid. Cultures were plated at 5 million cells per well in 24 well plates and maintained for 7 days in vitro with a medium change on day 3. Growth factors +/- dexamethasone 10^{-7}M were added to cultures in duplicate. On day 7, the cells were washed with HBSS, and fresh HBSS containing 1mM ammonium chloride was added for 4 hours. HBSS was removed and stored at -20°C for later assay. Data are shown as mean, +/- SE. n=5 cultures for each data point. ANOVA showed that no treatment was significantly different from control.

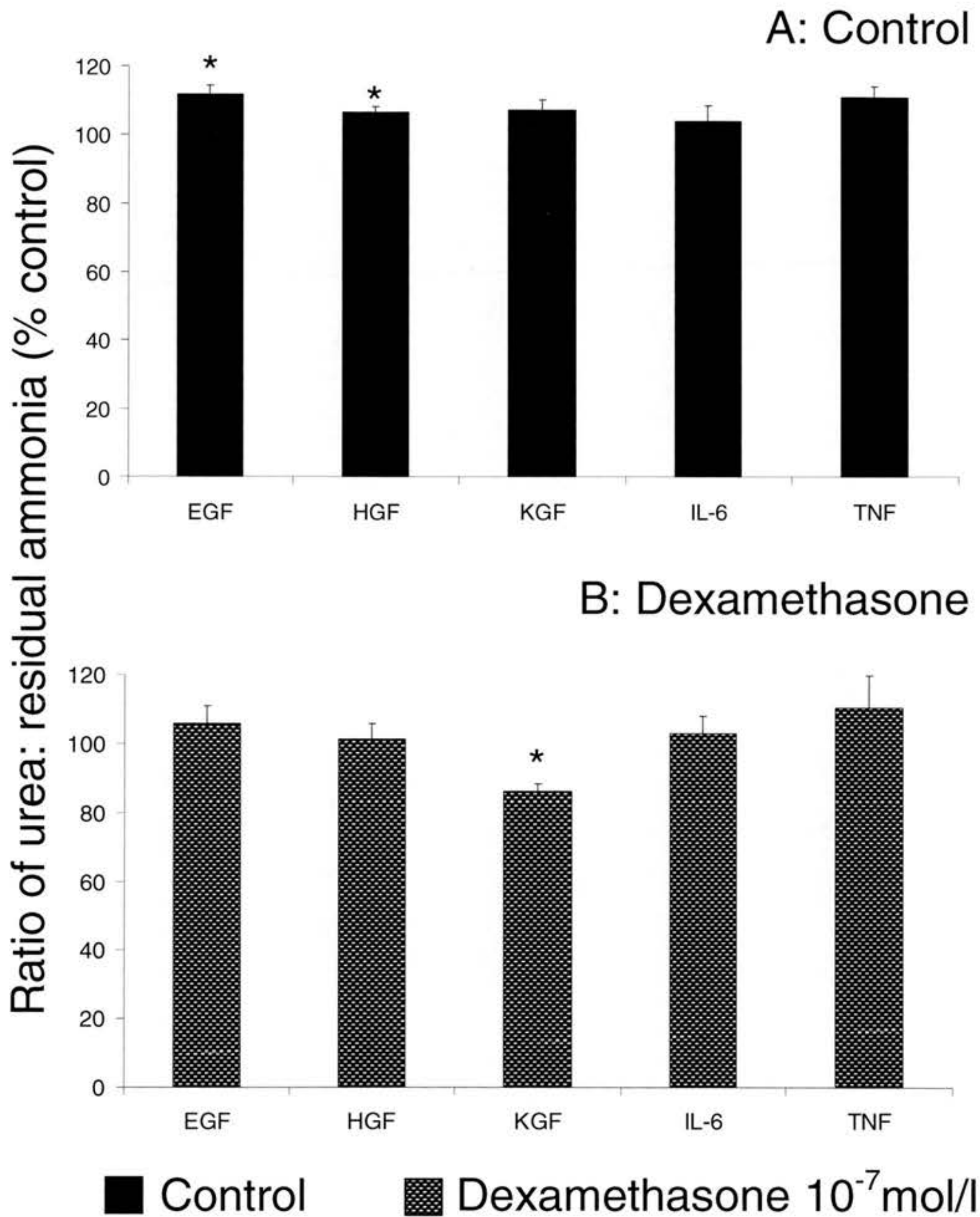

Figure 6.3. Ratio of urea synthesis to ammonia uptake in response to growth factors +/- steroid. Cultures were plated at 5 million cells per well in 24 well plates and maintained for 7 days in vitro with a medium change on day 3. Growth factors +/- dexamethasone 10^{-7}M were added to cultures in duplicate. On day 7, the cells were washed with HBSS, and fresh HBSS containing 1mM ammonium chloride was added for 4 hours. HBSS was removed and stored at -20°C for later assay. Data are shown as mean, +/- SE. n=5 cultures for each data point. ANOVA with post hoc t-test showed * $p<0.05$ compared to matched control.

In the presence of dexamethasone, this effect was abolished. However, KGF caused a significant reduction in the ratio of urea synthesis to ammonia uptake. This suggests that KGF favoured the channelling of ammonia into anabolic pathways such as glutamate synthesis rather than urea.

6.3.2.

The effects of growth factors, cytokines, and glucocorticoid, on protein secretion. CRP and TBPA levels were below the limit of detection in all cases.

Figure 6.4 demonstrates the effects of glucocorticoid on the secretion of AFP, fibrinogen and α-1-ACT. In each case, dexamethasone significantly enhanced protein secretion.

Figure 6.5 demonstrates that there were no significant effects of growth factors or cytokines on AFP secretion by fetal liver cultures (ANOVA; growth factor effect: $p=0.135$. Steroid and growth factor interaction; $p = 0.267$).

Figure 6.6 shows that there were no significant effects of the various treatments on fibrinogen secretion in the absence of dexamethasone. However, EGF and TNF both significantly inhibited fibrinogen secretion when glucocorticoid was present (ANOVA; $p = 0.004$ and 0.017 respectively).

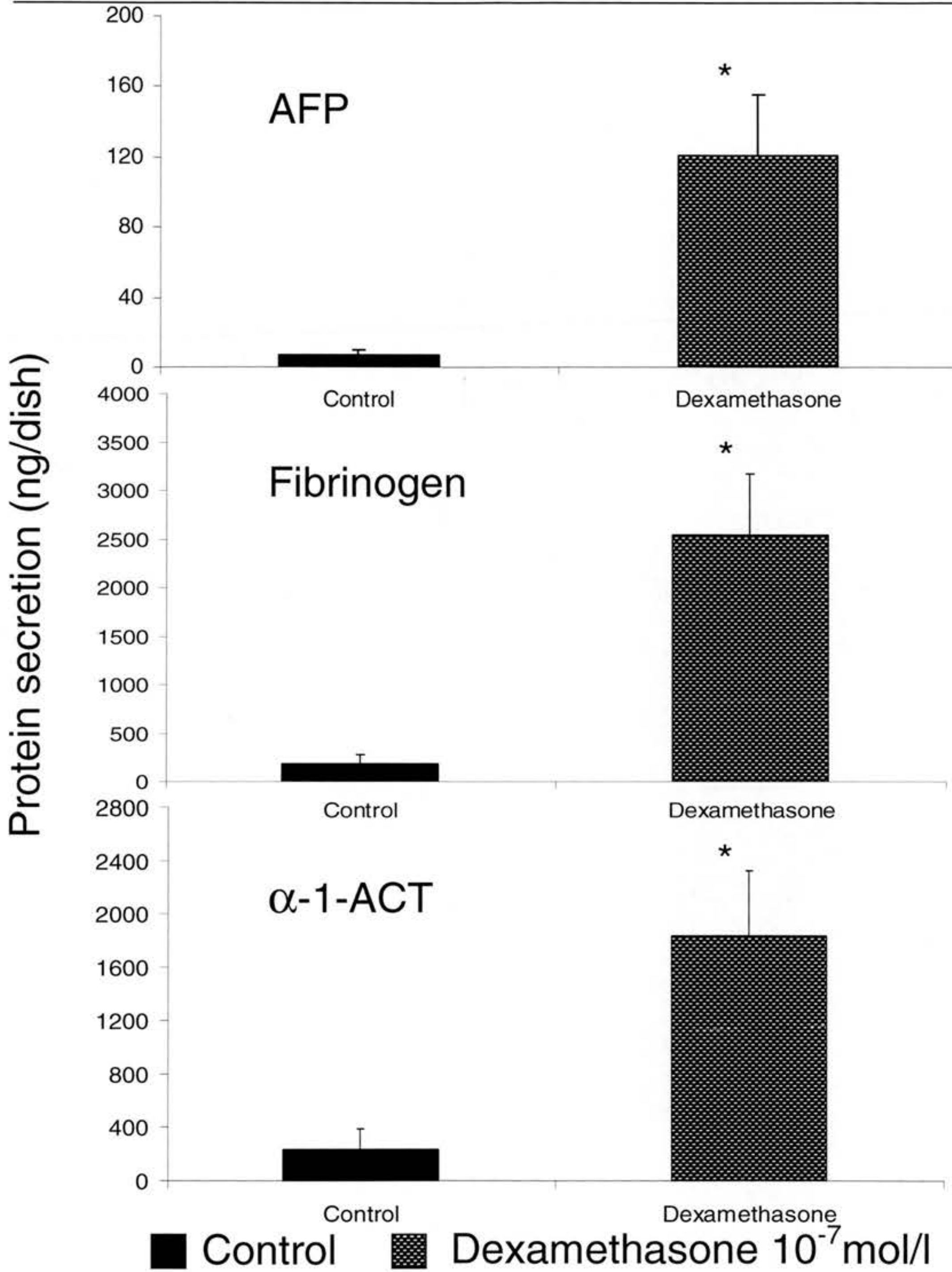

Figure 6.4. Protein secretion in vitro in the presence and absence of dexamethasone. Cultures were plated at 5 million cells per well in 24 well plates and maintained for 7 days in vitro with a medium change on day 3. Dexamethasone 10^{-7}M or vehicle was added to wells in duplicate. On day 7, the medium was removed and stored at -20°C for later assay. Data are shown as mean, +/- SE. n=4 cultures for each data point. ANOVA with post hoc t-test showed * $p<0.05$ compared to matched control.

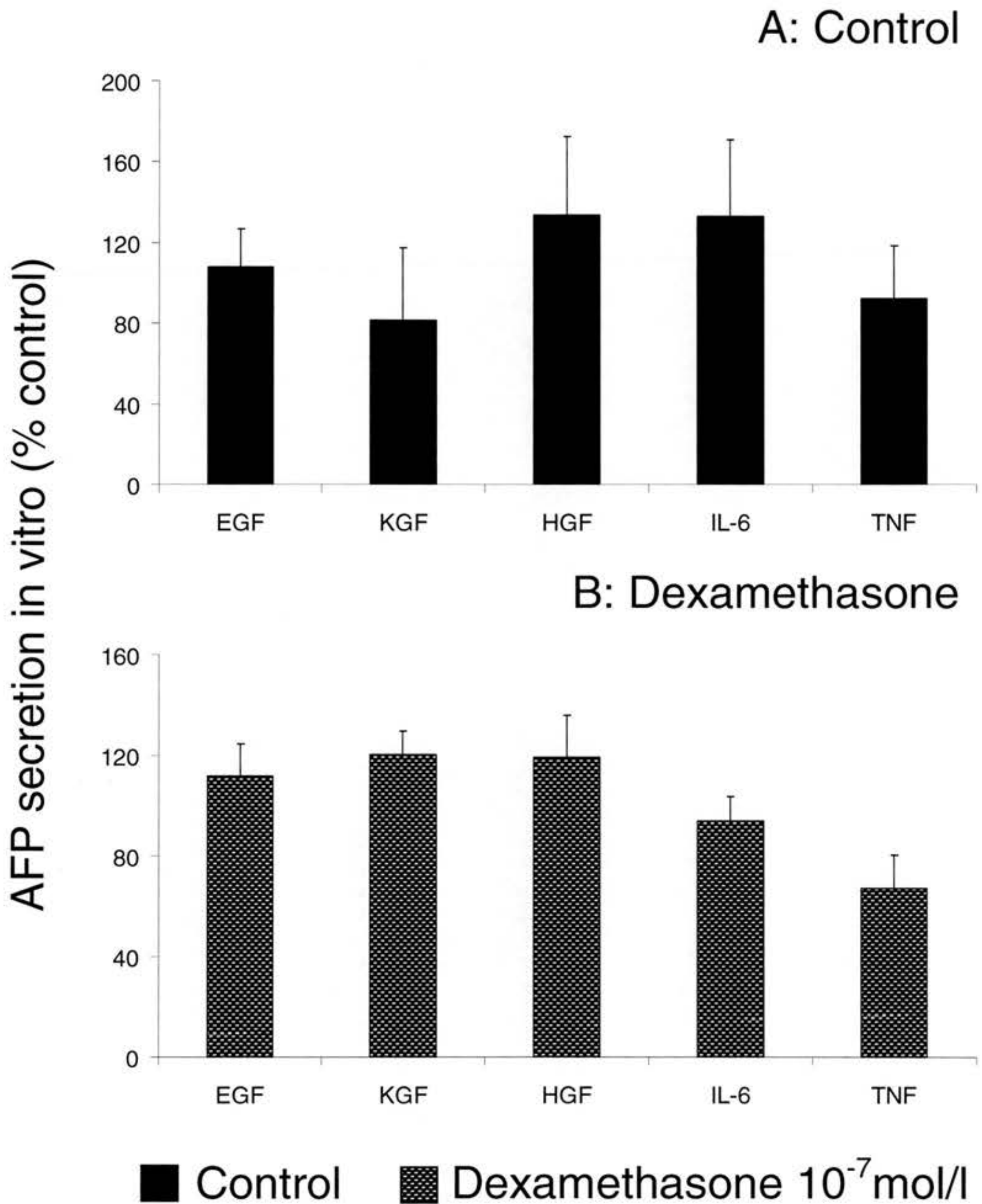

Figure 6.5. AFP secretion in response to growth factors +/-steroid.
Cultures were plated at 5 million cells per well in 24 well plates and maintained for 7 days in vitro with a medium change on day 3. Growth factors and Dexamethasone 10^{-7}M or vehicle were added to wells in duplicate. On day 7, the medium was removed and stored at -20°C for later assay. Data are shown as mean, +/- SE. n=4 cultures for each data point. ANOVA showed no significant effects.

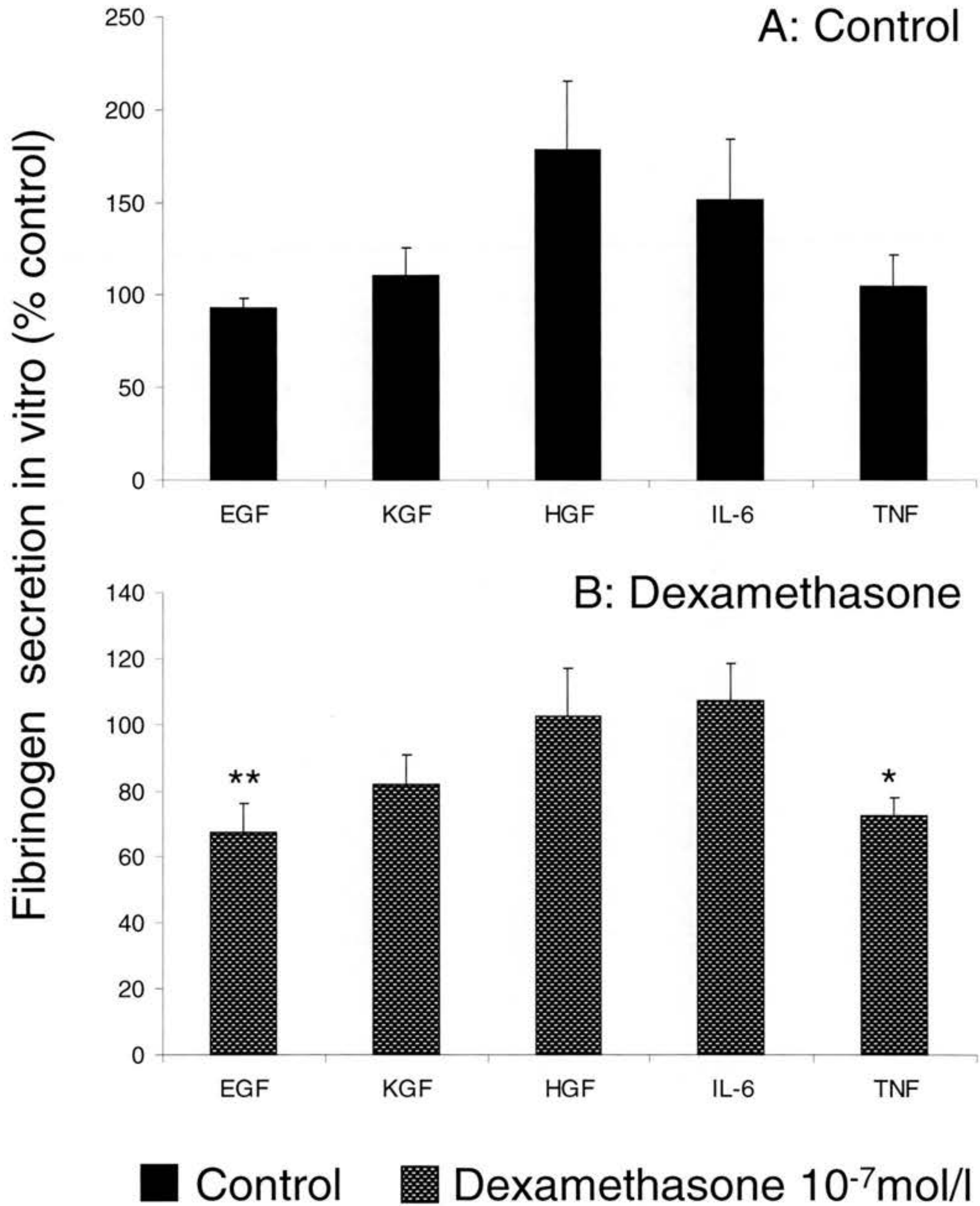

Figure 6.6. Fibrinogen secretion in response to growth factors +/- steroid. Cultures were plated at 5 million cells per well in 24 well plates and maintained for 7 days in vitro with a medium change on day 3. Growth factors and Dexamethasone 10^{-7}M or vehicle were added to wells in duplicate. On day 7, the medium was removed and stored at -20°C for later assay. Data are shown as mean, +/- SE. n=4 cultures for each data point. ANOVA with post hoc t-test showed* $p<0.05$ and ** $p <0.01$ compared to matched control.

Figure 6.7 demonstrates that in the absence of dexamethasone, there were no significant effects on α-1-ACT. By contrast, EGF significantly inhibited α-1-ACT secretion in the presence of this steroid (ANOVA; $p = 0.019$).

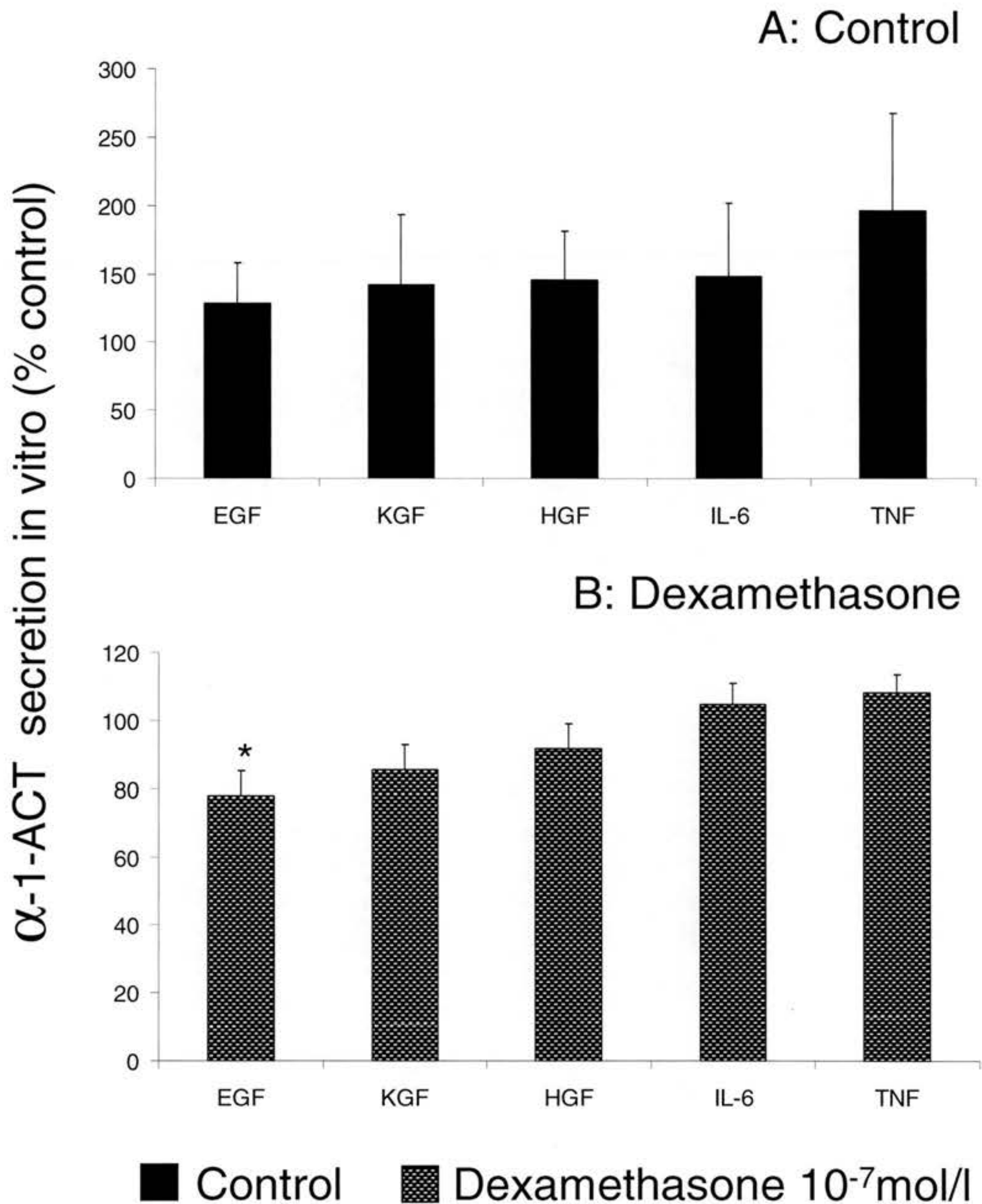

Figure 6.7. α-1-ACT secretion in response to growth factors +/- steroid. Cultures were plated at 5 million cells per well in 24 well plates and maintained for 7 days in vitro with a medium change on day 3. Growth factors and Dexamethasone 10^{-7}M or vehicle were added to wells in duplicate. On day 7, the medium was removed and stored at -20°C for later assay. Data are shown as mean, +/- SE. n=4 cultures for each data point. ANOVA with post hoc t-test showed* $p<0.05$ compared to matched control.

6.4 Discussion.

In this chapter, the interactions of dexamethasone and growth factors on urea synthesis and protein secretion were investigated. Overall, the data show that dexamethasone exerted a general enhancing effect on protein secretion. However, growth factors had highly selective effects which were different in each case. In contrast to dexamethasone, growth factors had effects on both protein secretion and nitrogen metabolism, and the presence of these effects was dependent on glucocorticoid.

Glucocorticoid had no effect on ammonia metabolism in the experiments carried out in chapter 4 of this thesis, and this result was confirmed in the present chapter. However, ammonia metabolism in fetal liver cells was modulated by growth factors, and these responses were, in turn, moderated by glucocorticoid. In the absence of dexamethasone, EGF and HGF significantly stimulated urea synthesis by approximately 10%, had no significant effect on ammonia uptake, but significantly increased the ratio of urea synthesis to ammonia uptake. These data indicate that the source of increased urea nitrogen in response to EGF and HGF is not ammonia, and implies that amino acids are being increasingly metabolised in response to these growth factors. However, the data do not show precisely which mechanism underlies this relatively subtle effect.

The time allowed during the incubations with added ammonia (4 hours) permits enzyme-catalysed steps to proceed towards equilibrium, independently of

quantitative limitations in enzyme activity, but dependent on the various equilibrium constants of the cycle. In fact, the relatively small increases in urea synthesis may represent a large increase in the amount or activity of urea cycle enzymes present. Alternatively, this may indicate that post-translational processing of specific enzymes is enhanced by certain growth factors, leading to a greater proportion of cellular nitrogen being metabolised to urea. Maturation in vivo is likely to involve at least three mechanisms, with increased quantities of enzyme possessing increased specific activity, and a change in the equilibrium constant in favour of urea synthesis. Finally, these same observations could be simply explained by increased amino acid breakdown, in the absence of any increase in enzyme activities.

Ultimately, mature adult human hepatocytes in vitro metabolise all added ammonia to urea in this model (J. Black; personal communication), and so the not insignificant amount of residual ammonia is a likely corollary of immaturity in the human fetal urea cycle. Clearly, elucidation of the true mechanisms subserving the modest effects of EGF and HGF on urea synthesis would require substantial further studies.

When glucocorticoid was present, the effects of EGF and HGF were abolished. By contrast, KGF, which had had no effect in the absence of dexamethasone, caused a significant reduction in urea synthesis with no change in ammonia uptake. These data indicate that ammonia nitrogen was being channelled

towards anabolic pathways, most likely the synthesis of glutamate from ammonium and α-ketoglutarate, as a precursor of non-essential amino acids. The observation that glucocorticoids may modify the responses of developing hepatocytes to growth factors was unexpected. Surprisingly, there are no data in the literature which demonstrate immunostaining for glucocorticoid receptor in the human fetal liver, although glucocorticoid receptors have been detected there by radioisotope binding studies (Ballard and Ballard, 1974). The effects of glucocorticoids in human tissues are dependent on the activity of an intracellular glucocorticoid-inactivating enzyme, 11 β-hydroxysteroid dehydrogenase type II (11β-HSD II), which has a physiological role to reduce the effects of glucocorticoid exposure in developing liver in animals (Lindsay et al., 1996a,b). In humans, 11β-HSD II mRNA (Stewart et al., 1994) and 11β-HSD II immunoreaction product (Hirasawa et al., 1999) have been detected in developing liver in early to mid gestation. As dexamethasone, in contrast to cortisol, is not substrate for 11β-HSD II, it is likely that the responses of the developing liver to growth factors are modulated by glucocorticoid, and the activity of glucocorticoid in turn is determined by the level of 11β-HSD II. As the current studies used dexamethasone, no insight is provided as to the potential role of 11β-HSD II in moderating glucocorticoid effects. However, it is now clear that the role of this enzyme is much more complex than previously suspected, as these data show that glucocorticoid activity modulates growth factor responses.

Whilst the published data relating to the effects of growth factors and cytokines on ammonia metabolism is exceedingly scant, the literature which describes their effects on hepatocellular protein secretion is considerable. In the current chapter, EGF selectively inhibited fibrinogen and α-1-antichymotrypsinogen secretion from human fetal liver cells, but had no effect on AFP. Notably, effects were only observed in the presence of glucocorticoid. EGF treatment of murine hepatocytes has previously been shown to inhibit the secretion of acute phase response proteins in vitro (Rokita and Szuba, 1991). More recent studies show a complex interrelationship between EGF-mediated inhibitory and IL-6-mediated stimulatory pathways in HepG2 cells. EGF brought about activation of Src kinases and STAT pathways, with activation of Erk1 and Erk2. These factors interacted with downstream signaling by STAT3, resulting in stimulation or inhibition of IL-6-mediated acute phase protein release (Wang et al., 1999). These studies feature tumour cell data, and may not reflect accurately the physiology of developing primary human hepatocytes. In cell culture of fetal human hepatocytes, EGFR protein was detected in physical combination with STAT3 protein, suggesting a signaling link after receptor activation (Runge et al., 1999). Available data, although complex, support an inhibitory effect of EGF on acute phase proteins, as shown here.

Although the current results show an effect of EGF, they do not determine whether there is a biological role for EGF in the human liver during

development. In the human fetus, TGFα and the EGFR show a strong immunoreactive signal in developing bile ducts, contrasting with a less marked immunostain for TGFα, and weak stain for the receptor, in hepatocytes (Terada et al., 1994). With maturation, TGFα and EGFR signals diminished further in hepatocytes, however, the signals in bile ducts remained similar to those seen in development. In contrast to the results shown here, no functional data were presented by these authors. However, the expression of both agonist and receptor on bile ducts implies an autocrine loop to support biliary proliferation. As noted in chapter 5, the identification of an EGF-mediated CK18+ve/Fibrinogen-ve fraction in these experiments is consistent with an EGF effect occurring in the biliary compartment. What cannot be ignored, however, is the observation that hepatocellular protein secretion is inhibited by EGF, whereas urea synthesis is stimulated. Regardless of any proliferative effects on different cell compartments, EGF has potent and specific effects on developing human hepatocytes, effects which are dependent on glucocorticoids.

TNF (and IL-6) are key mediators of the acute phase response in mammals. Secretion of TNF by macrophages, for example, leads to the re-prioritisation of liver protein synthesis and secretion, one of the defining features of the acute phase response. Data suggests that TNF may act as a trigger, rather than a primary initiator, in this regard. In humans, TNF infusion results in an early IL-6 rise, followed by a later rise in acute phase reactants (Stam et al., 2000a; Stam

et al., 2000b). These acute phase reactants include fibrinogen, C-reactive protein, serum amyloid A and complement C3, the levels of which are massively increased during the acute phase response. By contrast, albumin and transferrin levels fall, as is expected for negative acute phase reactants. Whilst TNF may act as a trigger, IL-6 is critical to the induction of the acute phase response, and data from rat and human hepatocyte culture experiments show that IL-6 causes an acute phase response in hepatocytes in vitro (Wigmore et al., 1997) Castell et al., 1990; Bader et al., 1992). Critically, IL-6, rather than TNF, is both necessary and sufficient to elicit the acute phase response (Saad et al., 1995).

In the present work, an inhibitory effect of TNF was noted on fibrinogen secretion. This appears at odds with the documented role of TNF as stimulatory to the acute phase response. However, this observation is not without precedent. Adult human hepatocytes, proven to respond to IL-6 with an acute phase response in vitro, showed no response to TNF treatment over a 20 hour period (Castell et al., 1990). Whilst fibrinogen secretion was stimulated by IL-6 in primary cultures of human hepatocytes and in a hepatoma cell line PLC/PRF/5, TNF was inhibitory to fibrinogen secretion in both cell types (Gabay et al., 1995). Paradoxically, this paper showed that TNF was synergistic in stimulating Fibrinogen when added with IL-6 (Gabay et al., 1995). . Other studies, utilizing rat and human cell cultures in parallel, have confirmed that TNF alone does not cause an acute phase response in liver cells in vitro (Bader et al., 1992).

The present experiments confirmed previous studies showing that TNF was inhibitory to fibrinogen secretion, although previous data relate to adult human liver cells and cell lines rather than fetal human liver cells (Gabay et al., 1995). The mechanism by which TNF might cause inhibition of fibrinogen secretion is not known. The presence of a tissue response to TNF, as shown by the inhibitory effects on fibrinogen, strongly suggests the presence of TNF receptor in the fetal human liver. TNF receptors 1 and 2 are expressed ubiquitously in developing tissues, and TNF mRNA and protein are detected in the fetal mouse liver by RT-PCR and immunocytochemistry respectively (Doi et al., 1999; Kamiya and Gonzalez 2004; Kutteh et al., 1991). Disruption of the TNF signaling pathway in fetal development, for example by knockout of the Rel A subunit of NFκB which transduces the nuclear TNF signal, causes death in utero at day 14-15 of development in mice. This fetal death is characterized by massive hepatocyte apoptosis (Beg et al., 1995), which is prevented in Rel A -/- TNF -/- double knockout animals (Doi et al., 1999). The TNF signaling mechanism mediated by NFκB is therefore critically important in protecting fetuses from the pro-apoptotic effects of TNF during development. The absence of cell death caused by TNF, as seen in the previous chapter, in conjunction with the inhibitory TNF response seen here, suggests that the TNF cellular signaling mechanism is functionally intact in the mid-trimester fetal human liver. These data reveal how TNF could have an effect, but do not clarify the intracellular mechanism subserving an inhibitory response.

STAT3, which binds to the IL-6RE, and NFκB, has been shown to compete for upstream binding sites in acute phase protein promoter regions (Zhang and Fuller, 1997; 2000). Specifically, NFκB shows greater affinity for an overlapping NFκB/IL-6RE sequence than does STAT3, can displace STAT3, and inhibits fibrinogen gene transcription. Furthermore, these rat studies are complemented by human sequence data showing that such NFκB/IL-6RE overlapping regions are present in both the α– and γ-fibrinogen promoter regions. These data show that TNF, acting through NFκB, could inhibit fibrinogen gene transcription and so inhibit fibrinogen secretion. Such a model supposes that some level of STAT3-stimulated fibrinogen gene transcription is ongoing in these cell cultures. No attempt was made to measure such activity, however, STAT3 activity may be stimulated by many signaling pathways, including glucocorticoid receptor (Zhang et al., 1997). Inhibitory effects of TNF were only seen in the presence of glucocorticoids in this study. This suggests that inhibitory TNF effects require a background of enhanced protein transcription, in this case, mediated by glucocorticoid.

Surprisingly, IL-6 had no effect on protein secretion in this study. In particular, the acute phase proteins fibrinogen, and C-reactive protein were expected to demonstrate a positive response to IL-6. In humans in vivo, data suggest that IL-6 is the final common mediator of the acute phase response (Stam et al., 2000a; 2000b) and a previous study in human fetal liver cells suggests that IL-6

stimulates CRP release (Bauer et al., 1991). To bring about its effect, IL-6 binds to a specific 80kD receptor subunit, which confers specificity to the particular IL-6/LIF family member, and a gp130 subunit, which mediates the signalling pathway. In human fetal liver, immunocytochemistry has demonstrated the specific IL-6R receptor subunit on hepatocytes, and the corresponding mRNA is detected in liver by RT-PCR (Dame and Juul, 2000). In addition, human fetal liver has been documented to produce IL-6 in vitro (Sennikov et al., 2001) and to express the gp130 protein. In fetal rat liver cultures, IL-6 showed no effect on liver cells, until soluble IL-6 receptor was added (Kamiya et al 1999), at which point, cellular responses were restored to normal. There are no data which describe the status of soluble IL-6 receptor during human or rat development, and the absence of this moiety from the present studies may well explain the lack of effect of IL-6 on fetal liver cells.

This lack of effect seen begs the question as to what might be the physiological role for IL-6 during normal development. IL-6 knockout animals show decreased liver weight compared to wild-type animals (Wallenius et al., 2001) and gp130 knockout mice demonstrated abnormal liver maturation (Kamiya et al., 1999), observations which support the hypothesis that IL-6 treatment should affect fetal human hepatocytes. On the other hand, the fetal acute phase response is relatively quiescent, as attested to by the largely undetectable levels of CRP in human fetal blood, even after parturition (Thompson et al., 1993). This may reflect a developmental down-regulation of the acute phase response.

The nature of such a down-regulation in not clear. However, it is now recognized that SOCS (suppressors of cytokine signaling), in particular SOCS3, down regulate IL-6 signaling, and that this is relevant in physiological regulation (Lang et al., 2003). Although the activity of this pathway in fetal development is unknown, SOCS3 activation may explain the lack of a fetal acute phase response in vivo (Thompson et al., 1993), and in vitro, as seen here.

Keratinocyte growth factor has been recognised as a potent inducer of hepatocyte proliferation (Housley et al., 1994) and differentiation (Krakowski et al., 1999). Over-expression of KGF in mouse liver, using an apolipoprotein E promoter during development, exerts a widespread and complex range of effects. In particular, the developing liver is enlarged, with prominent biliary hyperplasia. There are also grossly dilated collecting ducts within the renal cortex and medulla (Nguyen et al., 1996). The KGF receptor is a tyrosine kinase, which is largely expressed by epithelial cells, in contrast to KGF which is secreted in the main by mesenchyme (Rubin et al., 1995). Administration of KGF to rats in vivo results in proliferation of hepatocytes and increased systemic albumin levels (Housley et al., 1994). In human hepatoma cells, KGF receptor mRNA is detected, however, addition of KGF had no effect. In parallel, human primary hepatocytes did not express the KGF receptor mRNA, neither did they respond to KGF (Asada et al., 2003). The data shown in the present chapter are in support of previous experiments, showing that, despite positive findings in the rat, KGF has no effects on protein secretion from human hepatocytes (Strain

et al., 1994). However, KGF appeared to have a modest effect on urea synthesis, channeling ammonia nitrogen into synthesis of non-essential acids. Clearly, this effect requires detailed further study as KGF may be important in the acquisition of mature liver function.

In contrast to the effects of EGF and TNF, HGF showed no effect on protein secretion in this study. By definition, cells capable of protein secretion are undergoing maturation, appropriate to function in post-natal life. During development, c-met, the HGF receptor, is expressed by immature hepatocytes, and undergoes a dramatic downregulation in maturing cells (Spijkers et al., 2001), perhaps abrogating any effect on protein secretion. On the other hand, HGF was identified as capable of increasing albumin, transferrin and alpha-2-macroglobulin after burn injury in adult rats, confirming the ability of HGF to support the negative acute phase reactants in mature liver (Jeschke et al., 2000). As previously noted, it may not be possible to extend observations in fetal rat to fetal human liver cells, and the present work supports the hypothesis that HGF has no effect on protein secretion from fetal human liver cells.

In summary, these studies have demonstrated complex actions of glucocorticoid, growth factors and cytokines on fetal hepatocyte urea synthesis and protein secretion. Urea synthesis was easily demonstrable, and treatment with glucocorticoids had no effect on any index of ammonia metabolism. However, specific growth factors were mildly stimulatory or inhibitory to urea synthesis,

and interacted with dexamethasone in this regard. By contrast, a robust stimulatory response to glucocorticoid was observed in terms of protein secretion, although the acute phase response appeared inactive in mid trimester liver. Nonetheless, selective inhibitory effects of experimental peptides strongly support specific, receptor-mediated mechanisms. Again, the data supported an interaction between growth factors and glucocorticoid to bring about these effects.

These data show that the fetal liver behaves in a manner quite different to the mature organ, which may reflect the differing developmental requirements placed on the liver in mid-trimester compared to the demands in mature animals. Furthermore, the data reveal a complex interaction between growth factors and glucocorticoids, and potentially with 11β-HSD II, which has not previously been described in the literature.

Chapter 7.

General Discussion.

7.1 Introduction.

The shortfall in liver grafts for human liver transplant has lead to new initiatives in liver transplantation research, in particular directed towards cell-based strategies for liver replacement and support. Whilst early work demonstrated that adult human liver cells had little capacity for replication or maintenance of differentiated function over time in vitro, animal studies suggested that fetal cells in general possess a superior capacity to survive, divide and differentiate in the laboratory. These studies were directed to explore the pathways which might be active in stimulating human fetal liver cells to undergo proliferation and maturation in vitro. This may provide a starting point to develop novel cell-based therapies in human liver disease, with a hope to reduce the significant mortality of patients awaiting liver transplantation.

7.2 Maturation in human fetal liver.

Maturation of liver function prior to parturition is central to survival of the parturient phase. In particular, adequate hepatic glycogen stores and coagulation pathway proteins are necessary to prevent fetal and neonatal loss. Previous studies indicate that glucocorticoids are central to fetal maturation in

preparation for birth. Glucocorticoids enhance secretion of surfactant from type II pneumocytes (Grier and Halliday, 2004), promote maturation of the adrenal medulla and adrenaline synthesis (Kennedy and Ziegler, 2000) and markedly enhance glycogen synthesis (Plas and Nunez, 1976; Schwartz et al., 1975), all of which are absolute requirements for the survival of parturition.

In the present study, glucocorticoids massively enhanced secretion of certain liver proteins (AFP, Fibrinogen, α-1-ACT), but had no effect on others (CRP, prealbumin). Fibrinogen is a key player in neonatal haemostasis, such that null/null mice genetically modified so as not to express fibrinogen die from overwhelming haemorrhage during the first two weeks of life (Camerer et al., 2004). Similarly, humans affected by the genetic condition afibrinogenaemia have a very high incidence of fatal haemorrhage which may occur anytime during fetal or postnatal life. (Neerman-Arbez et al., 2003). Clearly, adequate haemostasis in humans is significantly dependent on plasma fibrinogen levels.

This thesis shows for the first time in human fetal liver that glucocorticoids can bring about a maturational response in fibrinogen secretion. This observation is important both in the pursuit of novel therapies in liver disease and in paediatric practice. The medical management of preterm babies, in which spontaneous intracranial haemorrhage is a devastating complication, may be positively influenced by the observations in this thesis. It is noteworthy that intracranial

haemorrhage is a significant cause of death in those children suffering from afibrinogenaemia noted above. It is possible that low plasma fibrinogen levels may somehow predispose to spontaneous intracranial haemorrhage. A natural extrapolation from the data presented here suggests that glucocorticoid therapy for preterm neonates may reduce the incidence of intracranial haemorrhage by favourably altering the coagulation pathway. Whilst the mechanism of such an effect has not yet been demonstrated in humans, such an association has already been observed (Crowley, 1995).

The favourable effect of glucocorticoids on haemostasis proteins is clearly very important for the treatment of patients with liver disease. An artificial liver device would ideally provide coagulation factors as well as the necessary detoxification mechanisms for seriously ill patients with liver failure. Haemostatic failure with massive haemorrhage is a major cause of death of such patients with liver failure. This thesis suggests that the haemostatic function of a cell-based, artificial liver support device could be markedly improved by glucocorticoid treatment of the liver cells.

Glucocorticoid also produced a morphological maturation in cultured liver cells, with cultures adopting a mature epithelial phenotype and increased fibrinogen immunofluorescence after glucocorticoid exposure. Such morphological maturation may be associated with the functional polarisation of hepatocytes observed in mature liver, with a sinusoidal aspect and an oppositely disposed

face applied to a basement membrane. Such morphological changes and polarisation have been observed in dedifferentiated hepatoma cells transfected with a constitutively expressed HNF4α construct, the phenotypic effects of which have been shown to results from E-cadherin expression (Parviz et al., 2003; Spath and Weiss, 1998). It is likely that the morphological effects of glucocorticoid as seen here are mediated via transcriptional control of this factor (Bailly et al., 2001).

At odds with a maturational role for glucocorticoids was the observation that AFP secretion was markedly enhanced in the presence of dexamethasone. In human development, AFP levels decline during fetal life, such that term levels are 10% of those present at 32 weeks (Bader et al, 2004). A maturation signal should theoretically result in a fall in AFP secretion, not an increase as observed here. However, the observations of Bader et al. relate to liver late in gestation, not mid trimester liver as utilised here. It may be that the responses of liver cells to certain signals are different at different points in development, perhaps due to the interplay of different factors. These studied have shown that the responses of cells to growth factors and cytokines are different in the presence and absence of glucocorticoid, and a subtle interplay of trimester-dependent factors may differentially regulate AFP secretion. Tri-iodothyronine has been shown to differentially regulate AFP secretion in a manner consistent with physiological maturation in rats (Anteby et al.,1993). No effect of T3 was seen on fibrinogen

secretion in chapter 4, and so further studies were not undertaken. It may be that had the focus been on AFP, not fibrinogen, then a specific effect would have been observed. The role of hormones in regulating AFP during development awaits further study.

Taking further the point that glucocorticoids did not bring about 'true' maturation of AFP secretion, steroid had no demonstrable effect on urea synthesis. In part this was due to the approach taken in these studies, which was largely a qualitative assessment of urea cycle function. No dynamic studies were carried out, which, as indicated in the relevant chapter, would be essential to demonstrate any functional effects. Nonetheless, it was notable that the concentration of residual ammonia was significant, at approximately 300 μmol/l after a 4-hour incubation, under the conditions documented. By contrast, adult hepatocytes maintained in the same conditions metabolise essentially all added ammonia. There is clearly scope for further studies on the maturation of the urea cycle, and it may be that the answer lies with growth factors or cytokines. The experiments reported here show that KGF appeared to direct ammonia towards anabolic pathways, with a consequent reduction in urea output for the same ammonia uptake. This result establishes the potential for growth factor modulation of urea synthesis. In addition, there may be a factor, as yet unreported, which can better support ammonia detoxification in the fetal human liver than those investigated here.

The studies in this thesis demonstrate that glucocorticoids can exert dramatic effects on developing hepatocytes in vitro. However, a further level of complexity is introduced by the presence of the hydroxysteroid dehydrogenase enzymes which can inactivate (11 βHSD II) or reactivate (11 βHSD I) endogenous glucocorticoids. In fetal humans, low levels of 11 βHSD II mRNA, protein and enzyme activity are detected in the second trimester with no evidence of 11 βHSD I activity (Hirasawa et al., 1999; Stewart et al., 1994). These observations indicate that endogenous cortisol could have direct effects similar to those shown in this thesis. It was noted in chapter 4 that the maximal concentration of cortisol (10,000nmol/l) had effects similar to 100nmol, in support of the hypothesis that active 11 βHSD II is destroying cortisol above this concentration. By contrast, the effects of dexamethasone, which is a poor substrate for 11 βHSD II, increased with each rise in concentration. These data suggest the possibility that the overall glucocorticoid activity in fetal liver is regulated by 11 βHSD II activity, rather than glucocorticoid concentration.

By contrast to the many effects of glucocorticoid observed in these studies, growth factors either had no maturational effects, or had only very subtle effects to promote an immature state. Chapter 5 showed that EGF, KGF and IL-6 appeared to have a positive effect on the proportion of hepatocytes in culture compared to control, implying a maturational effect. However, the effect was not significant owing to considerable variation in responses between different

cultures. It may be that repetition of these studies, looking specifically at individual factors, may yield significant results in this regard. With the present data, it must be concluded that there were no effects of cytokines or growth factors on phenotypic maturation of hepatocytes. In subsequent functional studies of growth factors, it was clear that there were no obvious maturational effects of any growth factors on liver cells.

It is likely that glucocorticoids exert a significant maturational effect on developing fetal liver, in particular with regard to protein synthesis and secretion. More importantly, these studies show that there are likely to be many subtle interactions between different agents acting on the liver, and it is probable that a complex interplay is responsible for liver maturation in vivo, rather than any overriding single maturation signal.

7.3 Cellular proliferation in developing human liver

In contrast to the striking effects of glucocorticoid on protein secretion, there was no effect of this agent on cell proliferation or survival. Data from chapter 3 show that there was no discernable effect on the number of cells in vitro after one week of exposure to different levels of glucocorticoid. These data underscore the observations above, namely that steroid increases the secretion of fibrinogen per cell. They also provide powerful evidence that glucocorticoid is not a proliferation signal in developing liver.

Although glucocorticoids had no effect on cell number, EGF provided a powerful proliferative signal to developing liver cells. Two-colour flow cytometry showed that EGF stimulated the proliferation of cytokeratin 18+ve/fibrinogen-ve cells, which corresponded to an epithelial phenotype. The response in this compartment was striking, with cell counts showing an increase of 250% compared to controls. This proliferation was associated with a small reduction in Fibrinogen and α-1-ACT secretion, with a very modest increase in urea synthesis, providing dexamethasone was absent. These data lend credence to the hypothesis that EGF stimulates the proliferation of presumptive biliary tract cells, or of primitive epithelial cells which are not differentiated hepatocytes.

Whilst the role of the EGF/TGFα pathway has been investigated extensively with regard to liver tumour progression, fewer data exist to describe the role of this pathway in liver development. Overexpression of TGFα in transgenic mice expressing TGFα under the control of a metallothionein promoter resulted in a doubling of liver size, as a result of increased cell number, without obvious morphological abnormality (Sandgren et al., 1990). These authors did not report at which point in development the TGFα effect took place, but available data in humans reveals the presence of EGFR in hepatocytes and cholangiocytes throughout development (Terada et al., 1994). The proliferative effect of EGF as reported in this thesis, acting predominantly in the epithelial compartment in second trimester liver, may reflect the stage of development, in which

hepatocytes and cholangiocytes are present in culture and able to respond independently to EGF. In fact, the data in this thesis strongly support this view.

Presumably, the increased liver size with normal morphology in animals transgenic for TGFα reflects the activity of this pathway from the earliest stages in development, before the lineage separation of hepatocytes and cholangiocytes. This suggests that TGFα is a mitogen for hepatoblasts, which could be explored in developing human liver in first trimester cells. No such data exist in the literature.

The observation that EGF may stimulate the specific expansion of the cholangiocyte lineage is novel, but as stated in chapter 5, all available data in human fetal studies support this interpretation, despite the assertions of authors that hepatocyte expansion is the mode of action. Previous data from Terada et al. (1994) indicates that in fetal human liver, EGFR immunostaining is present at the greatest level in biliary epithelium. The data from chapter 5 certainly show a high level of EGFR signal in this compartment, but also support the presence of EGFR in the parenchymal hepatocytes. Further data from TGFα/c-myc transgenic mice show that cholangiolar proliferation may be conspicuous in tumours arising in the livers of these animals (Sandgren et al., 1993). It is not unreasonable to say that the EGF/TGFα pathway acts predominantly on

cholangiocytes in second trimester human liver, however, more detailed studies would be required to confirm this hypothesis.

Of course, the possibility exists that the phenotype of the progeny may be different compared to the cells of origin; in particular these proliferating cells may be less differentiated and so fail to express markers such as fibrinogen. Whilst data in the literature are scant, Alison's group have already shown in rat liver that cellular proliferation in response to injury occurs in the biliary compartment, and that these cells then differentiate into mature hepatocytes (Golding et al., 1995). It may be the case that removal of the proliferation signal allows the epithelial cells to differentiate, perhaps into hepatocytes or cholangiocytes. This experiment was not attempted here, but would clearly be an important avenue to pursue.

Knockout studies of the EGF/TGFα pathway have rarely touched on the developing liver. In part, the redundancy in ligands (EGF and TGFα) may minimise the effect of a single knockout. EGFR knockout results in fatality within the first few days of life, with stunted growth, gut and pulmonary immaturity contributing to death (Miettinen et al., 1995). Interestingly, no mention was made of liver in this paper, however, the mice shown in colour photographs did not appear jaundiced, nor was any mention made of subsequent jaundice prior to death. This implies sufficient development of the

biliary tree, at odds with the assertions made above. Of course, there may be species differences between rodents and humans which renders the EGFR less important in rodent development. In the fetal rat and mouse liver, EGFR is absent until late in gestation (Hortsch et al., 1983), which contrasts sharply with the human (Terada et al., 1994).

An interesting observation was made by Miettinen et al. (1995) in their studies of EGFR null mice. These authors noted that EGFR null mice died from epithelial immaturity, and went on to state that lung immaturity, resembling neonatal respiratory distress, was the main cause of death. This was accompanied by a necrotising enterocolitis-like disorder. Both of these pathologies are associated with pre-term delivery in humans, and both are reduced by corticosteroids in human studies. This implies a role for both EGF and glucocorticoids in fetal maturation, quite aside from any role in cellular proliferation.

7.4 Suggestions for further research

The observations in this thesis have revealed potential avenues for further research. These are summarised as follows.

1) A role for thyroid hormones in re-prioritisation of liver protein synthesis during development.

2) A role for oncostatin M in liver cell maturation.

3) Urea cycle regulation in fetal liver development.

4) Sequential proliferation and maturation signals in fetal human liver.

5) EGF-induced proliferation in developing human liver; cell compartment of origin and differentiation after proliferation.

6) Studies of first trimester liver and the effects of growth factors on proliferation.

Clearly, many studies might be pursued in this new area of research. The above project outlines tie in well with this thesis, and will be very important in the search for new treatments for liver dysfunction.

7.5 Summary and conclusions.

This thesis set out to examine maturation and proliferation signals in the developing human liver. A wide range of putative signalling molecules were investigated, and interactions between these agents were also assessed. The data show conclusively that glucocorticoid positively enhances hepatocyte

maturation, but that other factors are likely involved in true, physiological maturation with reprioritisation of protein synthesis as would be observed in vitro. With regard to proliferation, EGF was the sole agent capable of inducing proliferation in the cultured cells. Whilst the data indicate that this proliferation occurred in the biliary compartment, published data leave room to speculate that removal of the proliferation signal, or the action of other signals, may induce the differentiation of hepatocytes from the proliferating compartment. From a functional standpoint, urea synthesis and protein secretion were influenced very subtly by growth factors, and differentially by glucocorticoid. Taken together, these studies have identified, for the first time in fetal human liver, dynamic responses to maturation and proliferation signals, and have shown which factors might be useful in the development of novel, cell-based liver therapies to treat human liver disease.

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