THE ROLE OF ADHESION MOLECULE INTERACTIONS IN THE ENGRAFTMENT OF TRANSPLANTED HEPATOCYTES INTO HOST LIVER

by

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ABSTRACT

Orthotopic liver transplantation (OLT) remains the only proven treatment for end-stage liver disease. However the waiting list for OLT far exceeds the supply of donor organs. Hepatocyte transplantation may offer an alternative for these patients either as a bridge to OLT or replacing OLT altogether. Unfortunately efforts so far have failed to result in long term benefit despite initial promising results. The mechanisms regulating engraftment of transplanted hepatocytes into host liver, in particular the nature of their interaction with hepatic sinusoidal endothelial cells (HSEC), remain poorly understood. I have significantly improved the outcome of human hepatocyte isolation from liver tissue and shown that human hepatocytes express a range of adhesion molecules including β1-integrin. Rather than reducing adhesion, β1-integrin blockade significantly improves hepatocyte adhesion to HSEC under flow, increases transmigration across HSEC and leads to greater engraftment in a murine model of hepatocyte transplantation. Furthermore, I have shown that blocking β1-integrin on human hepatocytes leads to activation of the PKB/Akt signalling pathway, resulting in suppression of anoikis and improved viability, and promotion of cytoskeletal reorganisation that may lead to a more migratory phenotype. This work therefore demonstrates a possible target to improve hepatocyte engraftment and thus the outcome of hepatocyte transplantation.

DEDICATION

I dedicate this thesis to my wife Chen Li and children, Aiden and Alesha, who have been the source of my motivation and kept me going through many long days and nights.

Also to my parents, Chris and Jan, and my brother and sister, Paul and Stephanie, for your ongoing love and support.

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LIST OF ABREVIATIONS

AIH	Autoimmune hepatitis
ALCAM	Activated leukocyte cell adhesion molecule
ALD	Alcoholic liver disease
APC	Allophycocyanin
BAD	BCL-2-associated death promoter
BAX	BCL-2-associated X protein
BCL-2	B cell lymphoma-2
BEC	Biliary epithelial cells
BEM	Basal endothelial media
bFGF	Basic fibroblast growth factor
BIM	BCL-2-interacting mediator of cell death
BMSU	Biomedical services unit
BSA	Bovine serum albumin
CAM	Cell adhesion molecule
CD	Cluster of differentiation
cDNA	Complimentary DNA
CFSE	Carboxyfluorescein succinimidyl ester
COX	Cyclooxygenase
CRM	Colorectal metastases
DIABLO	Direct inhibitor of apoptosis binding protein with low pI
DISC	Death-inducing signalling complex
DMEM	Dulbecco’s Modified Eagle’s Medium
DNA	Deoxyribonucleic acid

DNA-PK	DNA-dependent protein kinase
DPPIV	Dipeptidyl peptidase IV
ECM	Extracellular matrix
EGTA	Ethylene glycol-bis (2-aminoethylether)-N,N,N,N-tetraacetic acid
ELISA	Enzyme linked Immunosorbent assay
FAC	Focal adhesion complex
FAH	Fumarylacetoacetate hydrolase
FAK	Focal adhesion kinase
FCS	Foetal calf serum
FITC	Fluoresceinisothiocyanate
FLP	Fibronectin-like polymer
FNH	Focal nodular hyperplasia
FRG	Fah-/-/Rag2-/-/Il2rg-/-
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
GGT	Gamma glutamyl transpeptidase
GSK3	Glycogen synthase kinase 3
HA	Hyaluronic acid
HBSS	Hank’s Balanced Salt Solution
HCC	Hepatocellular carcinoma
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HGF	Hepatocyte growth factor
HRP	Horseradish peroxidase
HSC	Hepatic stellate cells
HSEC	Human sinusoidal endothelial cells

ICAM	Intercellular adhesion molecule
IFN	Interferon
IgG	Immunoglobulin
ILK	Integrin-linked kinase
IVC	Inferior vena cava
MAA	Microaggregated albumin
mAb	Monoclonal antibody
MELD	Model for end-stage liver disease
MFI	Median fluorescence intensity
MMP	Matrix metalloproteinase
MPO	Myeloperoxidase
MSC	Mesenchymal stem cell
mTOR	Mammalian target of rapamycin
mTORC2	mTOR complex 2
NAC	N-acetylcysteine
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NCAM	Neural cell adhesion molecule
NK	Natural killer
NTBC	2-(2-nitro-4-trifluoromethylbenzoyl)-1, 3-cyclohexanedione
OLT	Orthotopic liver transplantation
PAGE	Polyacrylamide gel electrophoresis
PBC	Primary biliary cirrhosis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction

PDK-1	Phosphoinositide-dependent kinase-1
PE	Phycoerythrin
PECAM	Platelet endothelial cell adhesion molecule
PKB	Protein kinase B
PKC	Protein kinase C
PI-3K	Phosphoinositide 3-kinase
PMA	Phorbol 12-myristate 13-acetate
polyHEMA	Polyhydroxyethylmethacrylate
PP2A	Protein phosphatase 2A
PSC	Primary sclerosing cholangitis
PSG	Penicillin/streptomycin/glutamine solution
PTX	Pertussis toxin
PVDF	Polyvinylidene difluoride
RFP	Red fluorescent protein
RGD	Arginine-glycine-aspartic acid
RNA	Ribonucleic acid
RT-PCR	Real-time PCR
SCID	Severe combined immunodeficient
SDS	Sodium dodecyl sulphate
siRNA	Small interfering RNA
SMAC	Second mitochondria-derived activator of caspases
TBS	Tris-buffered saline
TBS/T	TBS/Tween
TEMED	Tetramethylethylenediamine
TGF	Transforming growth factor
TIMP	Tissue inhibitor of metalloproteinase

TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
TRAIL	TNF-related apoptosis inducing ligand
UKELD	United Kingdom end-stage liver disease
uPA	Urokinase type plasminogen activator
uPAR	Urokinase-type plasminogen activator receptor
VCAM	Vascular cellular adhesion molecule
VEGF	Vascular endothelial growth factor

CHAPTER 1: GENERAL INTRODUCTION

1.1 Introduction to the Liver and Liver Disease

1.1.1 The anatomy of the liver

The liver is the largest solid organ in the human body, located on the right side in the upper abdomen immediately beneath the diaphragm. It is unique in that it receives a dual blood supply. In the healthy individual, oxygenated blood arrives from the hepatic artery via the coeliac trunk, the first major branch of the descending aorta within the abdomen, and accounts for approximately 25% of the total blood supply to the liver. Approximately 75% of the blood supply arrives via the portal vein which is formed by the confluence of the splenic and superior mesenteric veins. The superior mesenteric vein and inferior mesenteric vein, which usually drains into the splenic vein, provide venous drainage for the small and large bowel. Hence the portal supply consists of deoxygenated but nutrient-rich blood. Venous drainage from the liver is via the left, middle and right hepatic veins which in turn drain into the inferior vena cava (IVC).

The liver is divided into right and left hemilivers by the middle hepatic vein, and can be further divided into sections and segments (Figure 1.1) (Lopez-Terrada et al. 2014). On entering the liver the hepatic artery and portal vein divide into separate branches supplying each segment before further dividing to supply individual lobules, travelling in portal tracts or triads consisting of a branch of the portal vein, a branch of the hepatic artery and a bile duct. Individual lobules form the functional units within the liver, and are arranged into polyhedral structures separated by fibrous septa and arranged around a central vein which drain into the hepatic veins. Portal triads lie within the septa at the corners of the lobules (Figure 1.2) (LeCluyse et al. 2012). Hepatocytes are arranged into cellular plates that radiate out from the central vein like spokes of a wheel.

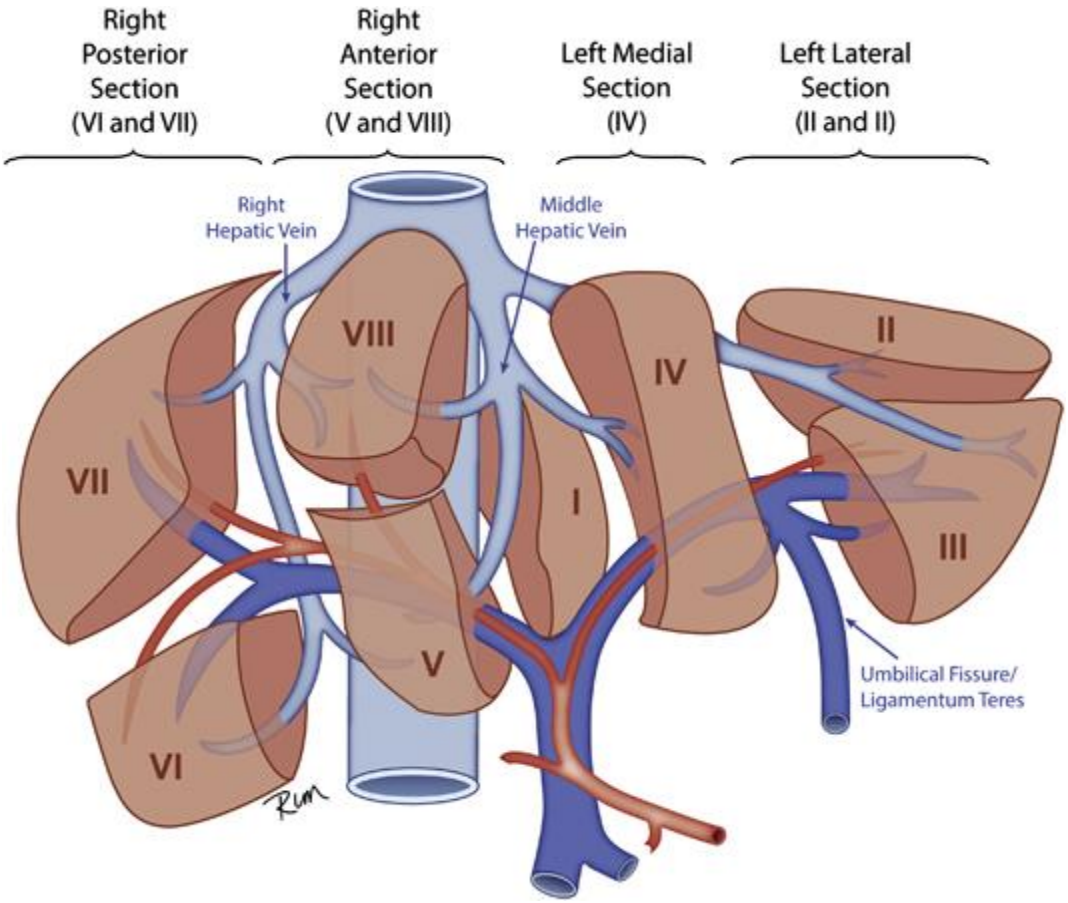


Figure 1.1 Blood supply and segmental anatomy of the human liver

The liver received blood from branches of the hepatic artery (red) and portal vein (dark blue). Bile ducts also run with these vessels (not shown). Venous drainage is via three main hepatic veins which empty into the IVC (light blue). Figure taken from the publication of Lopez-Terrada et al. 2014.

Bile canaliculi run between layers of hepatocytes within the hepatic plates and empty into bile ducts within the portal triad. Blood arriving via the portal triads enters hepatic sinusoids which lie between the hepatic plates and empty into the central vein. These coalesce forming the three main hepatic veins draining into the IVC.

1.1.2 The function of the liver

The liver performs a multitude of functions, the majority of which can be summarised into four categories: metabolism, synthesis, excretion, and storage. Metabolic functions include the metabolism of amino acids and carbohydrates as well as the regulation of blood glucose levels. The liver also metabolises ammonia via the urea cycle and metabolises endogenous and exogenous toxins and drugs. The liver synthesises numerous proteins, clotting factors, complement components, enzymes, hormones and vitamins, and excretes bile pigments, bile salts and cholesterol through the bile. It acts as a storage site for glycogen, vitamins (A, D and B12), iron and copper.

1.1.3 Liver disease and liver failure

The incidence of chronic liver disease is increasing worldwide largely due to the spread of Hepatitis B and C viruses as well as the increasing prevalence of alcoholic liver disease (ALD) and non-alcoholic fatty liver disease (NAFLD) particularly in developed countries (Williams 2006). There are also a number of other less common conditions such as autoimmune hepatitis (AIH), primary biliary cirrhosis (PBC), primary sclerosing cholangitis (PSC) and haemochromatosis that account for a smaller proportion of patients with chronic liver disease. All of these disorders may ultimately progress to cirrhosis and end stage liver failure when the liver is unable to carry out its normal functions. Cirrhosis is now one of the top 3 causes of death amongst those aged 35-65 in the UK (Office for National Statistics 2012).

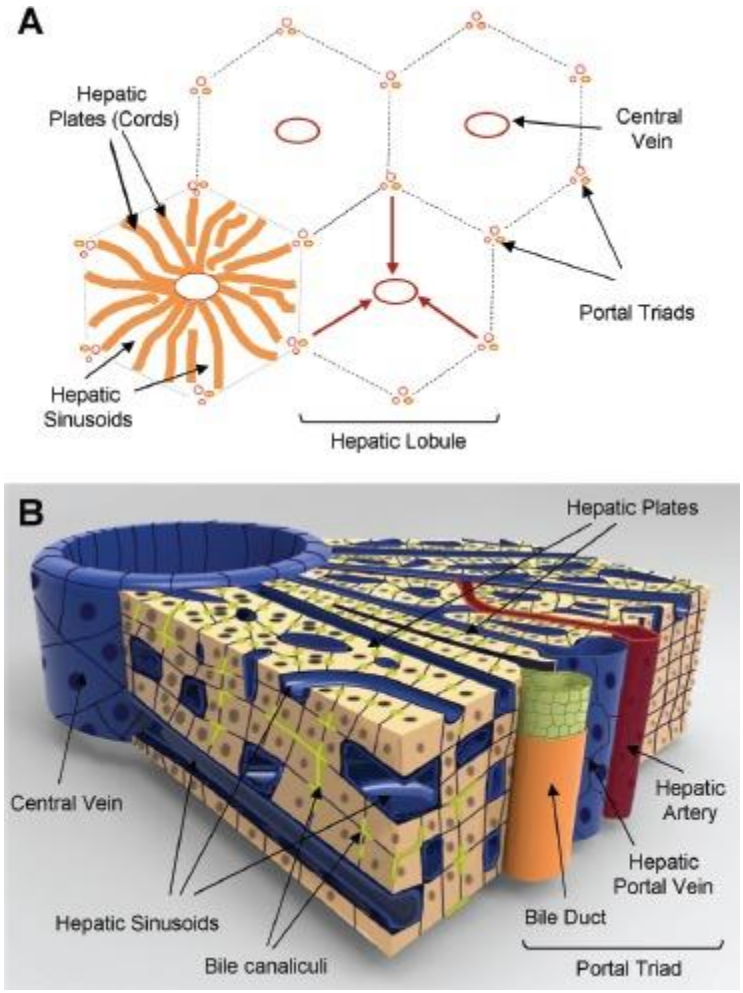


Figure 1.2 The microscopic structure of the liver lobule

The liver lobule is the functional unit of the liver. (A) In cross section the lobule is a polygon, usually hexagonal. Blood flows from the portal triads through the liver sinusoids towards the central vein at the centre of the lobule. (B) This image shows a close up view of part of a liver lobule between a portal triad and the central vein. Bile canaliculi run parallel but in the opposite direction to the blood flow carrying bile to the bile ducts situated within the portal triad. Figure taken from the publication of LeCluyse et al. 2012.

Despite advances in antiviral medication and other medical therapy, liver transplantation remains the only effective treatment for end stage liver disease.

Acute liver failure is less common but is associated with high mortality. Viral causes, in particular hepatitis E, and drug induced liver injury, often due to paracetamol overdose, predominate in the developing and developed world respectively. However, in many cases the cause is unknown (termed non-A non-B or seronegative acute liver failure) (Bernal et al. 2010). Acute liver failure can be classified according to the time interval from the onset of jaundice until the development of hepatic encephalopathy, and may be divided into hyperacute, acute and subacute (O'Grady et al. 1993). Hyperacute liver failure is more frequently associated with paracetamol overdose and hepatitis A or E and carries a more favourable prognosis. Acute and subacute liver failure, associated with hepatitis B and non-paracetamol drug toxicity or non-A non-B hepatitis respectively, carry a poorer prognosis without emergency liver transplantation.

1.2 Orthotopic Liver Transplantation and the Need for New Therapies

1.2.1 The problems with orthotopic liver transplantation

The first human liver transplant was performed in 1963 by Dr Thomas Starzl of Denver, Colorado, United States (STARZL et al. 1963). Since then, orthotopic liver transplantation (OLT) has come a long way with improvements in operative technique, superior immunosuppression regimes, improved preservation of donor organs, and better selection of recipients. Patient survival is now greater than 90% at 1 year and nearing 80% at 5 years (NHS Blood and Transplant 2011).

Liver transplantation is not without problems. Firstly, the operation is still a formidable and costly procedure with significant morbidity and mortality (Lee et al. 2004). It requires lifelong immunosuppression which in itself carries risks such as infection and malignancy. Further, due to the success of the technique and the increasing incidence of liver disease for which there is currently no other treatment, there is a growing demand for liver transplantation. Unfortunately, despite attempts to promote organ donation, this has not been matched by a similar increase in the availability of donor organs. Within the UK, the number of patients waiting on the active liver transplant list at 31 March 2011 was 510, an increase of 37% from 2010. However, there was only a 1% increase in deceased liver donors during the same period (NHS Blood and Transplant 2011). In light of this, there has been an interest in the use various alternatives to OLT in patients with acute and chronic liver failure as well as children with metabolic disorders.

1.2.2 Alternatives to orthotopic liver transplantation

Several alternative therapies for the treatment of liver failure are currently under development or are the subject of on-going clinical trials. These can be broadly grouped into three categories: extracorporeal artificial liver devices; bioartificial liver devices using hepatocytes; and hepatocyte transplantation (Sgroi et al. 2009). The objective of such treatments is to act as a bridge until such time that a suitable donor liver becomes available or to support the regeneration of the patient's own liver.

Hepatocyte cell transplantation has been suggested as an alternative to organ transplantation in patients with acute and chronic liver failure and is considered by some to be most appropriate in the management of patients with genetic or metabolic disorders

where a relatively small contribution to function could be curative and avoid the need for liver transplantation altogether (Gupta and Chowdhury 2002).

Following years of research with animal modelling and preclinical studies, a number of patients have undergone hepatocyte transplantation as part of clinical trials in acute and chronic liver failure as well as various forms of metabolic liver disease. The outcomes of these interventions have been mixed, and highlight the importance of careful patient selection as well as the problems facing hepatocyte transplantation. Of note though, the safety of hepatocyte transplantation has been demonstrated by the follow up of surviving patients, who have remained free of malignant or infectious disease, for several years (Fisher and Strom 2006). There is a growing experience of hepatocyte transplantation reported in the literature although treatment is limited by the availability of hepatocytes from unused donor tissue and the fact that any benefit of the procedure tends to be short lived with little effect on the clinical outcome and the majority of patients subsequently undergoing OLT (Hughes et al. 2012).

1.3 Hepatocyte Transplantation

1.3.1 Procurement of hepatocytes

The majority of hepatocytes used for human transplantation so far have been primary hepatocytes obtained from cadaveric livers which have been considered unsuitable for transplantation. This creates two problems: firstly the availability of cells for transplant is subject to the same shortage in donor tissue that exists for organ transplant; secondly, because livers made available for hepatocyte isolation have been rejected for organ transplant, the cells that are obtained from them are often not of the best quality (Lee et al. 2004). Other possible sources of primary hepatocytes which are likely to be of better

quality are unused tissue from split or reduced liver transplant, as well as whole organs for whom a suitable recipient is not identified (Strom et al. 1999). However these are relatively uncommon.

Autologous hepatocytes are ideal for transplant as long term immunosuppression should not be required. Combined with ex-vivo gene therapy, autologous hepatocyte transplantation has the potential to treat a wide range of metabolic disorders such as homozygous Familial Hypercholesterolemia (Raper et al. 1996). Clearly patients undergoing such a procedure would require liver resection in order to retrieve tissue from which to isolate the hepatocytes. However, this could be performed using minimal access surgery and would have a much lower morbidity than whole organ transplant.

One way to optimise the availability of primary hepatocytes from discarded liver tissue would be to establish banks of cryopreserved hepatocytes. Indeed, the feasibility of setting up such a bank, and the subsequent successful treatment of patients using banked hepatocytes in a bioartificial liver device, has already been demonstrated (Baccarani et al. 2003;Baccarani et al. 2005). However, the use of cryopreservation is still associated with reduced viability and other groups have reported an impact on metabolic function. These and other problems associated with cryopreservation in relation to hepatocyte transplantation have been well described elsewhere (Terry et al. 2006). Further work is required to optimise the use of this technology if it is to provide a useful source of hepatocytes.

In view of the difficulties in obtaining primary hepatocytes from donor liver tissue, various other potential sources have been considered. Foetal hepatocytes from aborted foetuses have the benefit of being highly proliferative, which may facilitate liver repopulation (Lilja

et al. 1998), but further investigation will be required to demonstrate that these cells would express a fully differentiated phenotype after transplantation (Lee et al. 2004). Stem cells offer a further possible source of hepatocytes, and there has been much work looking at the potential of various types such as embryonic, mesenchymal, and induced pluripotent stem cells as well as liver oval cells (Oertel and Shafritz 2008). However, concerns remain about the possibility of malignancy as well as whether such derived `hepatocyte-like' cells are capable of taking on the full repertoire of hepatocyte functions (Strom and Fisher 2003;Thomas and Forbes 2009).

A further possibility is that of xenotransplantation. Animal studies have shown that it is possible to transplant hepatocytes from one species to another and for them to function and correct liver failure in the recipient (Nagata et al. 2003). Problems with this approach include the risk of transmission of zoonoses (Porcine Endogenous Retroviruses), immunogenicity and, not least, the regulatory hurdles that would have to be overcome in order to introduce such a technique into the clinical setting.

1.3.2 Anatomy of the liver and site of hepatocyte transplantation

When considering the different steps en route to hepatocyte engraftment it is pertinent to consider the anatomy of the liver and sites of cell entry.

Within the liver lobules, hepatocytes are arranged into cell plates separated by narrow sinusoidal vessels that are lined with specialised endothelium termed hepatic sinusoidal endothelial cells (HSEC). Hepatocytes lie in close proximity to the sinusoidal endothelium, separated only by the space of Disse, as well as bile canaliculi into which bile is secreted.

The portal vein carries nutrient-rich blood and accounts for approximately 75% of the blood supply in the healthy liver. The remaining 25% comes from the hepatic artery which carries oxygen-rich blood. On entering the liver, these vessels divide into segmental branches before further dividing to supply individual lobules travelling in portal tracts or triads consisting of a branch of the portal vein, a branch of the hepatic artery and a bile duct.

Blood entering the lobules via the portal tracts reaches the sinusoids, where the exchange of cells and substances between the blood and the liver parenchyma can occur, before draining into the central veins located at the centre of each lobule which in turn combine to form the hepatic veins draining into the IVC.

The specialised extracellular matrix and portal blood supply make the liver the optimum site for transplanted hepatocytes to engraft and survive and so the liver itself is thought to be the most desirable site for transplantation (Mazaris et al. 2005). This can be achieved by infusing hepatocytes directly into the portal vein or, using the spleen as a conduit, by injecting into the splenic pulp (Lee et al. 2004) following which hepatocytes will travel via the splenic vein into the portal vein and hence the liver. Intraportal infusion is a relatively straight forward technique, and the majority of the animal studies of hepatocyte transplantation, as well as procedures performed in humans where it can be achieved using a percutaneous approach, have made use of this technique.

Many ectopic sites for transplantation have been investigated including the kidney, lung, pancreas, spleen and peritoneal cavity (Gewartowska and Olszewski 2007). Several studies show that the spleen and liver are the optimum sites for hepatocyte transplantation (Gupta et al. 1994;Gupta et al. 1999b). The spleen, as well as acting as a conduit to the liver as

described above, has also been used as a site for transplant. The transplanted hepatocytes are trapped in the sinusoids and vascular spaces, which seems to be important for engraftment, and proliferate replacing up to 40% of the splenic pulp after a few months (Mazaris et al. 2005). The peritoneal cavity, in terms of its volume, also offers the potential for the transplantation of large numbers of cells. Animal studies have shown that transplanted hepatocytes are able to survive and function within the peritoneal cavity. However due to the anchorage-dependent nature of hepatocytes they need to be attached to some sort of extracellular matrix to survive (Demetriou et al. 1986a;Demetriou et al. 1986b).

Whilst most models of hepatocyte transplantation have used rodents, a few investigators have looked at larger animals such as pigs. In one such study hepatocytes were transplanted into other foregut structures including the stomach, pancreas, spleen, small bowel and mesentery with the latter two sites proving to be favourable (Sosef et al. 2007). However, the use of such techniques in the clinical setting would require more invasive techniques than percutaneous intraportal infusion and, even if they were to produce a substantial metabolically active `liver mass', the long term effects are unknown. Further investigation will be required in order to determine whether extra-hepatic sites will provide a feasible alternative for hepatocyte transplantation in humans.

1.4 Hepatocyte Engraftment

The successful engraftment of hepatocytes in the recipient liver is dependent on a multitude of different steps ranging from the method of isolation to the interaction with the liver endothelium and finally to the functional incorporation within the hepatic parenchyma. Figure 1.3 illustrates some of these important steps. It is useful to

systematically examine each of these stages, and the possible manipulations which may be undertaken to incrementally improve hepatocyte engraftment.

1.4.1 Hepatocyte isolation

The original technique of hepatocyte isolation was described in the 1960s for the isolation of rat hepatocytes using a combined mechanical and enzymatic digestion technique (Howard et al. 1967). This was modified to become the two-step collagenase perfusion technique which is widely used today (Seglen 1976). Perfusion with warmed buffers to remove any remaining blood or clots from the tissue and to warm the tissue to 37°C takes place via cannulae inserted into the exposed vessels on the cut surface of the liver. The liver is then perfused with a cation-chelating agent to remove calcium ions and disrupt desmosomal structures holding the cells together, prior to digestion with collagenase.

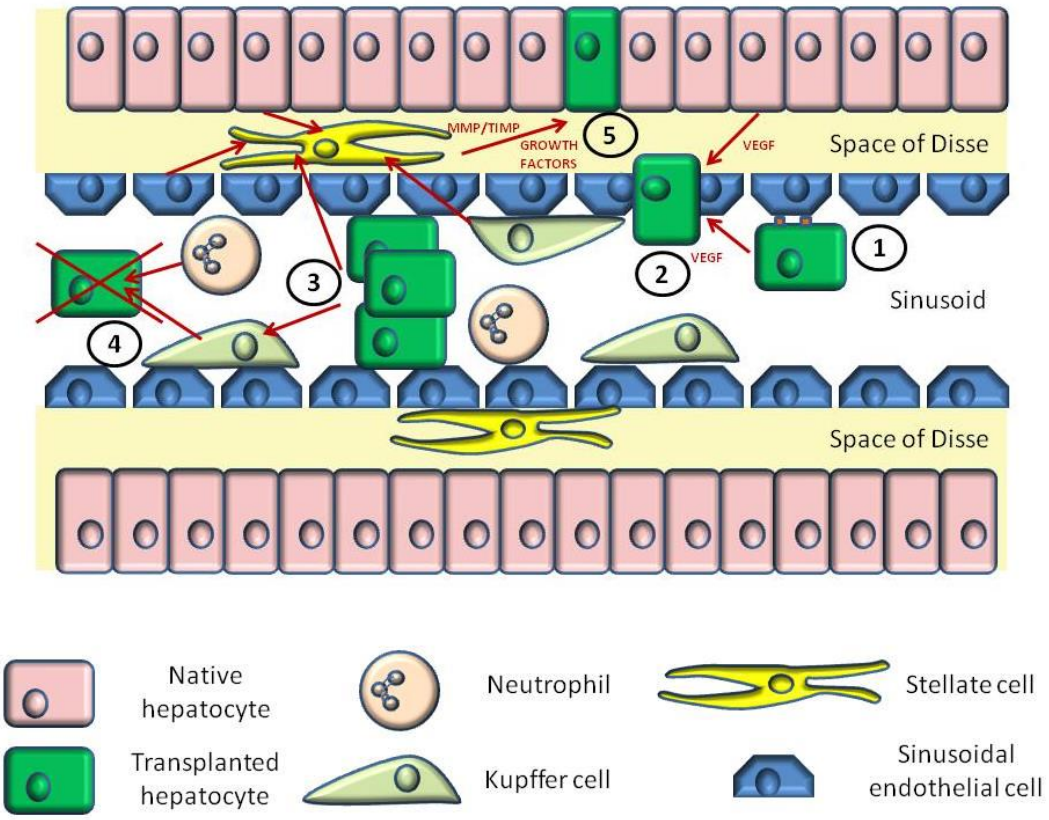


Figure 1.3 Steps involved during the engraftment of hepatocytes into liver during hepatocyte transplantation

(1) Transplanted hepatocytes delivered intravascularly adhere to sinusoidal endothelium. (2) Disruption of endothelium allows transplanted hepatocytes to pass out of the sinusoidal lumen. VEGF release from hepatocytes may be involved. (3) Occlusion of sinusoids by transplanted hepatocytes leads to ischaemia which may activate Kupffer and Stellate cells. (4) Kupffer cells and neutrophils activated during transplantation destroy a large proportion of transplanted hepatocytes. (5) Transplanted hepatocytes that make it across the endothelial barrier are integrated into liver plates. Stellate cells, activated by hepatocytes, HSEC, Kupffer Cells or ischaemia, may have a role in aiding this process through the release of various growth factors and matrix metalloproteinases.

Some groups add other enzymes such as DNase, dispase and hyaluronidase to the digestion buffer. Hepatocytes are then separated and purified by low speed centrifugation. A density gradient such as Percoll may be used to separate non-viable cells. The yield of isolated hepatocytes varies widely depending on how diseased the donor liver tissue is, as well as the exact techniques used for isolation. This wide variation in yield and viability highlights the need for further research to optimise the isolation technique. The use of collagenase and other such reagents in the isolation of hepatocytes may denude hepatocytes of vital adhesion molecules/receptors which may well impact on the ability of these cells to engraft. This has been demonstrated for other cell types. For example, the use of collagenase for liver-derived T cell isolation has little effect on the majority of cell surface markers but leads to a reduction in expression of CXCR3 (Shields et al. 1999). Incubation of peripheral blood mononuclear cells with collagenase and dispase results in reduced expression of CD3, CD4, CD8, αβ and γδ T cell receptors (Abuzakouk et al. 1996). The impact of different isolation methods on the surface receptor expression of hepatocytes, and their ability to engraft, has not been ascertained, nor has the time necessary for any potential recovery in adhesion molecule expression after isolation been determined.

1.4.2 Cell detachment and anoikis

When hepatocytes are isolated from donor liver tissue, and during the transplantation process, hepatocytes are detached from the normal extracellular matrix (ECM). This may cause them to undergo apoptosis due to the loss of the normal survival signals, a process known as anoikis. β1 integrin is known to be important in this process due to the loss of a β1 mediated survival signal, normally delivered through binding to RGD (Arg-Gly-Asp)

sites on ECM proteins, leading to activation of caspases and subsequent apoptosis (Boudreau et al. 1995;Zvibel et al. 2002).

Treating isolated hepatocytes with the agonistic β1 integrin antibody TS2/16 increases their survival (Kocken et al. 1997). This antibody is able to mimic a β1 integrin mediated signal, thus inhibiting caspase activation, preventing cell death. Furthermore, treatment with RGD-containing peptides can lead to a dose-dependent increase in survival with a 1.38-fold increase in cell numbers after 18 hours of culture compared to untreated hepatocytes (Pinkse et al. 2005). However another study found that pre-incubating cells prior to transplantation with collagen or fibronectin like polymer (FLP) (both RGD containing proteins) led to cell aggregation in vascular spaces (Kumaran et al. 2005) which would clearly be detrimental.

Whether anoikis will be a major problem in clinical practice will depend on the time frame between hepatocyte isolation and their subsequent transplantation, and how they are stored in the meantime. Cryopreservation will largely remove this problem, as cells will be frozen soon after isolation and only thawed when required for transplant. However, if cells are to be used soon after isolation or cryopreservation is not possible for some reason, then they will need to be attached to some form of ECM. Most assays used to measure the activity of isolated cells prior to transplant will also require them to be attached to a substrate. There is evidence that a differentiated phenotype can be maintained in culture for several days or longer using specialised culture medium (Pichard et al. 2006). Improved metabolic function, demonstrated by markers such as albumin production, glucose synthesis and cytochrome P450 function, can be achieved using more complex culture systems such as collagen sandwich/3d matrix (Wang et al. 2004;Wen et al. 2009)

or gel scaffolds (Wang et al. 2008). There is also evidence that some aspects of hepatocyte function may be better preserved in co-culture with other cell types such as stellate cells (Krause et al. 2009).

However, the effects of culture, the type of ECM used and the method used to detach the cells on the outcome of subsequent transplantation needs to be investigated. The effect on subsequent engraftment of treating cells as described above to increase survival is also unknown.

1.4.3 Impact of hepatic ischaemia and blood flow on hepatocyte transplantation

Following hepatocyte transplantation into the portal vein, either directly or via the spleen, cells are found in periportal areas and hepatic sinusoids. In periportal areas cells are surrounded by inflammatory infiltrates containing granulocytes, phagocytes and activated macrophages resulting in the removal of up to 80% transplanted cells within 24 hours (Gupta et al. 1999c). Therefore the sinusoids in the hepatic lobules provide the optimum site for engraftment. Due to the relatively large size of hepatocytes, leading to temporary occlusion of the liver sinusoids by cell emboli, a degree of hepatic ischaemia occurs during transplantation. In the normal liver gamma glutamyl transpeptidase (GGT) is expressed by biliary cells only. GGT is expressed by hepatocytes as a result of liver injury and can be used as a marker of hepatic ischaemia. GGT expression by host hepatocytes can be shown as early as 2 hours post transplantation. This increased expression can be reduced by the administration of vasodilators around the time of transplantation (Gupta et al. 2000). The use of vasodilators may also have an effect on the distribution of transplanted hepatocytes within the liver. The administration of nitroglycerine during the transplantation of cells

into rat models leads to an increase in transplanted cell entry into liver sinusoids with a beneficial effect on cell engraftment (Slehria et al. 2002).

Hepatic ischaemia has effects on various cell types within the liver, including Kupffer cells and stellate cells, and may therefore be important in the context of hepatocyte transplantation. As I will discuss later, the activation of stellate cells may be beneficial for hepatocyte engraftment while the activation of Kupffer cells is detrimental. Hepatic ischaemia may also induce changes in growth factor expression. Ischaemia-reperfusion/hypoxia has been shown to increase the production of vascular endothelial growth factor (VEGF) by hepatocytes (Archambault et al. 2001) and stellate cells (Ankoma-Sey et al. 2000), and increased VEGF expression is seen soon after hepatocyte transplantation as described later.

The use of vasodilators is an intervention that could readily be used to aid transplant of hepatocytes into humans given that there are several such drugs in regular clinical use already. Exactly which drugs to use, the appropriate dose, the timing of administration of the drug in relation to delivery of the hepatocytes and how to avoid potentially detrimental systemic side effects requires further animal and clinical study.

1.4.4 Adhesion to the sinusoidal endothelium during engraftment

The mechanisms regulating the recruitment of leucocytes into the liver have been extensively studied, and although such studies may inform the mechanism of hepatocyte recruitment, the process of hepatocyte engraftment is different since the transplanted cells must find a niche where they can survive and integrate. The recruitment of lymphocytes from the blood via sinusoidal endothelium to the liver parenchyma has been described

(Lalor and Adams 2002) and the molecular basis of this process is partly understood. Lymphocyte adhesion is dependent on ICAM-1, VCAM-1 and to a lesser extent VAP-1 with transmigration being mediated by ICAM-1, VAP-1 and chemokine mediated signals (Adams and Eksteen 2006;Lalor et al. 2002;Shetty et al. 2008).

Some detail of adhesive processes regulating the engraftment of transplanted hepatocytes has been described. Following hepatocyte transplantation in dipeptidyl peptidase IV negative (DPPIV-) rats (described later in section 1.5), histochemical staining for DPPIV with vinculin immunostaining demonstrates focal adhesion complexes (FAC) at sites of contact between transplanted cells and HSEC (Kumaran et al. 2005). Presumably in order for this to occur the movement of transplanted cells through the sinusoid must first be arrested with subsequent adhesion to the sinusoidal endothelium. This supports the idea that transplanted cells interact with HSEC and, whilst the movement of hepatocytes from the liver sinusoids into the parenchyma is not a physiological process, it is possible that transplanted hepatocytes are able to interact with the HSEC via specific pathways involving adhesion molecules and chemokine receptors similar to those described above for leukocytes. Alternatively, transplanted hepatocytes may simply become stuck via a passive process as a result of their size in relation to that of the sinusoid. This process has been well described in relation to the metastasis of cancer cells to the liver (Vidal-Vanaclocha 2008). However, even if the initial arrest of cell movement through the sinusoid is purely mechanical, there is evidence that specific interactions with the sinusoidal endothelium occur thereafter, and similarly such processes have been described in the context of tumour metastasis and migration. For example, proinflammatory cytokines such as IL-1β and TNF-α have been shown to up-regulate the expression of VCAM-1 on HSEC with a corresponding increase in adhesion of melanoma cells (Vidal-

Vanaclocha et al. 2000). The hepatic migration of metastatic breast cancer cells is dependent on the chemokine receptor CXCR4 (Muller et al. 2001) and the migration of hepatocellular carcinoma depends on the chemokine receptors CCR7 and CXCR4 (Schimanski et al. 2006a;Schimanski et al. 2006b). Several other metastatic tumours, such as melanoma and colorectal cancer, also express CXCR4 and in colorectal cancer the level of CXCR4 gene expression has been shown to be a statistically significant prognostic marker (Kim et al. 2006). CXCL12, the ligand for CXCR4 is highly expressed in the liver. Ligand-receptor interactions result in cell adhesion, proliferation and migration. Currently, little is known about how such mechanisms may affect hepatocyte recruitment, but there is evidence that integrin-mediated adhesion within the sinusoid may be important. Modification of the recipient liver to increase the adhesion of transplanted hepatocytes may increase engraftment. In one study portal infusion of collagen or FLP prior to transplant increased hepatocyte engraftment by 2.2 and 3.3 fold respectively compared to controls. FAC were observed more frequently in these rats than in those receiving hepatocytes alone (Kumaran et al. 2005). ECM components infused prior to transplant may bind within sinusoids providing extra binding sites for integrins, thus increasing adhesion of transplanted hepatocytes and therefore engraftment. When animals treated with prior infusion of FLP received hepatocytes that had been incubated with RGD peptide the number of transplanted cells per unit volume of liver decreased by 77% compared to animals receiving untreated hepatocytes. This is presumably due to the blocking of integrin binding sites by the RGD peptides. The treatment of hepatocytes with RGD peptides has been shown to significantly reduce binding to fibronectin in vitro although there is no effect on their binding to collagen I or collagen IV (Pinkse et al. 2005).

Interestingly there was no significant difference in engraftment in animals which had not received prior infusion of ECM components when receiving hepatocytes that had been incubated with RGD peptide, suggesting that there are other adhesion pathways involved. The decrease in hepatocyte engraftment in animals receiving RGD treated hepatocytes and infusion of FLP suggests that these other adhesion pathways are somehow disturbed by the infusion of ECM components (Kumaran et al. 2005).

Many of the potential targets for hepatocyte transplantation will be patients with established liver failure. The liver microenvironment and hence the mechanisms of hepatocyte adhesion in such patients may be altered with an effect on hepatocyte recruitment. For example, whilst collagen IV expression is increased in sinusoidal endothelium of patients with fulminant liver failure there is a loss of adhesion of hepatocytes to collagen coated plates when treated with serum from such patients. This loss of adhesion has been shown to be due a reduction in β1 integrin receptor activity; addition of a β1 integrin activating antibody (TS2/16) significantly increases adhesion (Newsome et al. 2004).

Further identification of the specific adhesion molecules and chemokine receptors involved in the adhesion of transplanted hepatocytes to and migration across sinusoidal endothelium, and how this process changes in different disease states, may allow treatments to be developed to increase the expression or activity of specific receptors and ligands on both hepatocytes and HSEC in order to increase adhesion and subsequent engraftment.

1.4.5 Disruption of the sinusoidal endothelium during engraftment

Having adhered to the sinusoidal endothelium, in order for transplanted cells to engraft into the liver they must first enter the space of Disse which separates liver plates from the sinusoids. This requires disruption of the sinusoidal endothelium (Gupta 2002) and has been demonstrated in animal models. At 2 hours post transplantation the sinusoidal endothelium appears normal. By 24 hours following transplantation, however, disruption of the sinusoidal endothelium and loss of the space of Disse occurs. At 8 hours following transplant VEGF activity can be shown in native hepatocytes adjacent to sinusoidal areas as well as some transplanted cells. VEGF activity then declines and is not seen after 24 hours post-transplant. Of note, no VEGF activity is seen in the liver of control animals, suggesting that the release of VEGF by both recipient and transplanted cells could be a factor in the disruption of sinusoidal endothelium (Gupta et al. 1999c). Increased VEGF expression under hypoxic conditions has been shown to increase the metastatic potential of melanoma cells, which may in part be due to an increased vascular permeability allowing extravasation into tissues (Rofstad and Danielsen 1999). Several studies have demonstrated the ability of VEGF to increase fenestration and permeability of HSEC (Funyu et al. 2001;Yokomori et al. 2003) as well as its role in regeneration of the sinusoidal endothelium after liver injury (Sato et al. 2001;Shimizu et al. 2001) supporting its possible involvement during hepatocyte transplantation.

Disruption of hepatic sinusoidal endothelium with the cytotoxic drug doxorubicin leads to improved engraftment of transplanted cells into rat liver. In control animals, intact endothelium covered >75% of the sinusoidal area in 95% of hepatic sinusoids, but in rats receiving a single dose of doxorubicin there was extensive loss of endothelium with 50-

75% of the sinusoidal area not covered by endothelium in >60% of sinusoids. This was associated with a several-fold increase in transplanted cells in the liver of rats receiving doxorubicin 24 hours prior to transplant compared to control rats (Kim et al. 2005). The use of cytotoxic drugs during hepatocyte transplantation into humans is unlikely to be acceptable as most of these drugs have potentially severe systemic side effects as well as being hepato-toxic. The sinusoidal endothelium provides an important barrier function regulating the transfer of molecules between the blood in the sinusoid and the space of Disse where they come into contact with hepatocytes, and is crucial in regulating the passage of other cell types into the liver. Widespread damage to the sinusoidal endothelium, even avoiding the use of cytotoxic drugs, is therefore likely to produce other unwanted effects. Indeed, there is evidence that damage to sinusoidal endothelium is a key step in certain types of liver injury (Arii and Imamura 2000;Knolle et al. 1996). Therefore manipulating the endothelial barrier to increase recruitment is a more sensible therapeutic strategy than removing it.

VEGF has already been the subject of various clinical trials, ranging from its use to improve coronary perfusion to its inhibition to restrict tumour growth (Ferrara and Alitalo 1999), and might offer a further treatment option in hepatocyte transplantation to increase the permeability of sinusoidal endothelium, thus allowing transplanted cells to engraft more readily. However, whilst an association between hepatocyte transplantation and increased local VEGF activity has been demonstrated, there is as yet no evidence of the effect on administering exogenous VEGF, or somehow stimulating its release, on hepatocyte engraftment. There is also the potential for a wide range of unwanted effects (Epstein et al. 2001) and so further experimentation in animal models is required to determine whether this is likely to be a safe treatment in humans.

1.4.6 Interactions of transplanted hepatocytes with hepatic stellate cells

Once through the sinusoidal endothelium, transplanted hepatocytes enter the space of Disse where hepatic stellate cells (HSC) are located. HSCs, which play a major role in the development of hepatic fibrosis (Safadi and Friedman 2002), can be activated by Kupffer cells, HSEC and hepatocytes (Pinzani and Marra 2001) through the release of various inflammatory mediators, such as VEGF (Liu et al. 2009). Activation leads to multiple events including HSC proliferation with increased desmin or α-smooth muscle actin expression.

Following hepatocyte transplantation into DPPIV- rats there is a significant increase in desmin positive HSC at 24 hours, 3 days and 7 days after treatment. There are few desmin positive HSC in control animals or early (6 hours) post-transplant. Further, co-localisation of desmin positive HSC and DPPIV positive transplanted hepatocytes at 3 days post transplantation shows that 64% of transplanted cells are in the immediate proximity of at least one desmin positive HSC, suggesting an interaction between the cells (Benten et al. 2005). Hepatic ischaemia may have a role in HSC activation during hepatocyte transplantation, as desmin positive HSC are seen within ischaemic GGT expressing areas. Similar findings are also observed following the injection of macroaggregated albumin (MAA) particles suggesting that mechanical occlusion of sinusoids leads to a degree of HSC activation. However, animals receiving nitroglycerin (a vasodilator) during transplantation, which reduces hepatic ischaemia and GGT expression, also develop a 2.7 fold increase in desmin positive HSC compared to control rats. Therefore ischaemia alone is not responsible for HSC activation and soluble factors are also likely to play an important role (Benten et al. 2005).

The activation of HSC during transplantation leads to increased production of several factors which may have a beneficial effect on hepatocyte engraftment. RT-PCR analysis of HSC-related genes has shown increased expression of hepatocyte growth factor (HGF), basic fibroblast growth factor (bFGF), VEGF and transforming growth factor (TGF) - β1 within 1 – 6 hours post-transplant. In addition there is increased expression of TGF β, platelet derived growth factor β and TNF α between 6 and 12 hours post-transplant. Values returned to near normal thereafter (Benten et al. 2005).

Following hepatocyte transplant there is also a marked upregulation of matrix metalloproteinases (MMP) and tissue inhibitors of metalloproteinase (TIMP) which are produced by HSC following liver injury (Benyon and Arthur 2001). There is markedly increased expression of MMP-13, the major interstitial collagenase in the rat, as well as MMP-3, which degrades several ECM components, at 12 hours post-transplant. Gelatinase A/basement membrane collagenase (MMP-2) and its activator protease, MMP-14, also increase moderately. TIMP-1 levels are raised following transplant and peak at 12 hours. However, whilst MMP expression returns rapidly to near normal levels, TIMP-1 expression remains elevated up to 72 hours post-transplant (Benten et al. 2005).

Hepatocyte transplantation activates cyclooxygenase (cox) pathways with the production of prostaglandin-endoperoxidase synthases 1 and 2 leading to the production of prostaglandins such as PGE2 which is a significant mediator of tissue inflammation. Blocking these pathways with drugs such as Naproxen and Celecoxib leads to an increase in hepatocyte engraftment. However, cox inhibition was not found to have an effect on neutrophil or Kupffer cell activity, nor on cytokine-chemokine responses. Rather, animals treated with these drugs showed an increase in desmin positive activated HSC, although

the mechanism of HSC activation here is not clear (Enami et al. 2009). The use of drugs such as Naproxen and Celecoxib could provide a possible treatment for human recipients. Further research into the mechanisms by which HSC activation occurs in this setting and the role of prostaglandin pathways may lead to other therapeutic targets. However, one must bear in mind that in diseased liver disease HSC may already be activated. Furthermore activated HSC are a key player in the development of liver fibrosis and cirrhosis (Li et al. 2008;Wu and Zern 2000). Clearly in the context of liver disease the effect of hepatocyte transplantation on HSC activation may therefore differ, and in the healthy liver the promotion of a profibrotic state may not be desirable.

1.4.7 Engraftment into liver parenchyma and attachment to extracellular matrix

Once the transplanted cells have crossed the sinusoidal endothelium into the space of Disse they must then become incorporated into the liver parenchyma. The upregulation of MMP and TIMP by activated HSC may have a role in facilitating entry into the liver parenchyma and subsequent remodelling.

The importance of hepatocyte attachment to extracellular matrix and the consequences for the initial survival of cells has already been discussed. Clearly it is also important that once within the recipient liver parenchyma the transplanted hepatocytes attach to the ECM in order to survive and spread. In vitro, up to 70% of seeded rat hepatocytes rapidly attach to wells coated with collagen I, collagen IV or fibronectin via β1 integrins. Antibody blocking studies suggest that α1 integrin is the dominant receptor involved (Pinkse et al. 2005). There is very little in the literature regarding the expression of adhesion molecules on primary hepatocytes, but one study suggests that the integrin repertoire is limited to

α1β1, α5β1 and α9β1 heterodimers (Couvelard et al. 1998) which is largely in keeping with the above observations.

The major ECM components in the liver include collagen, glycoproteins such as fibronectin, vitronectin, laminin and tenascin as well as proteoglycans such as hyaluronic acid (Bedossa and Paradis 2003). Collagens are the most frequent with types I, III and V confined mainly to the portal tract and central vein whereas type IV is associated with laminin and entactin forming a low-density basement membrane-like material along the sinusoidal wall (Martinez-Hernandez and Amenta 1993). Fibronectin appears to be particularly important in the space of Disse (Selden et al. 1999). The α5β1 integrin binds fibronectin and osteopontin, α1β1 binds collagen and laminin, and α9β1 binds VCAM, tenascin and osteopontin (Humphries et al. 2006). The relative composition of liver ECM, as well as the distribution of adhesion molecules and their receptors is known to alter in liver disease. For example, β1-integrin ligands VCAM-1 and matrix components together with the CD44 ligand hyaluronic acid are increased in diseased liver (Crosby et al. 2009).

Proper integration with host hepatocytes and restoration of normal polarity is critical for the maintenance of specialised function. Unlike the simple polarity of most epithelial cells, hepatocytes are organised in plates with several apical and basolateral poles. The apical poles form a continuous network of bile canaliculi and are separated from the basal domain or sinusoidal pole, abutting the space of Disse, by tight junctions (Decaens et al. 2008). Evidence from animal studies suggests that if transplanted cells survive the procedure and make the journey across the sinusoidal endothelium they do indeed become integrated into the host liver with restoration of polarisation. Transplanted hepatocytes enter liver plates and form hybrid gap junctions with adjacent host hepatocytes as well as

hybrid bile canaliculi within 7 days. Following the administration of fluorescent bile salt, excretion of bile is apparent throughout the liver including hybrid bile canaliculi formed by transplanted cells (Gupta et al. 1995). The actual process by which transplanted cells become incorporated into cell plates and regain polarity is not clear. Continued investigation of this final stage of hepatocyte engraftment may provide a further target for intervention.

1.4.8 Immune response and clearance of transplanted cells

A number of studies have demonstrated the role of inflammatory cells in the clearance of transplanted hepatocytes. Transplanted autologous, as well as allogeneic, hepatocytes appear to be recognised as non-self and are destroyed by granulocytes and mononuclear cells (Olszewski et al. 1998). Following transplantation there is an increase in cytokine and chemokine gene expression in the liver much of which is secreted by innate immune cells including macrophages and neutrophils. Depletion of these cells prior to hepatocyte transplant results in a reduction or normalisation of the expression of most of these genes (Krohn et al. 2009).

Kupffer cells are the resident macrophages of the liver and are likely to play a role in the early removal of transplanted hepatocytes. It is known that ischaemia-reperfusion in the liver is a potent activator of Kupffer cells (Serracino-Inglott et al. 2001). The function of Kupffer cells can be examined using techniques such as carbon uptake and 99mTc-sulphur colloid incorporation. Using such techniques it has been demonstrated that following infusion into the portal vein, transplanted hepatocytes quickly become surrounded by highly activated Kupffer cells (Joseph et al. 2002).

Kupffer cell function can be blocked by the use of substances such as gadolinium chloride (GdCl3). Following administration of GdCl3 prior to transplant there is a reduction of up to 40% both in the number of Kupffer cells containing carbon particles and in 99msulphur colloid accumulation. In animals treated with GdCl3 it has been found that the number of portal vein radicles containing transplanted cells increased from 34% to 66% at 72 hours and from 42% to 58% at 7 days compared to control animals. In addition, the number of transplanted cells per portal vein radicle, in periportal sinusoids and subsequently in the liver parenchyma was also greater in GdCl3 treated animals. This translated to a 2–3.8-fold increase in transplanted cells within the liver parenchyma at 1 day, and this increase remained constant at subsequent times after transplantation (Joseph et al. 2002;Krohn et al. 2009).

Similarly, neutrophil accumulation in the liver following hepatocyte transplant has been demonstrated using myeloperoxidase (MPO) staining. Depletion of neutrophils with anti-neutrophil antiserum prior to transplant results in a 3.2 fold increase in cell engraftment compared to untreated controls (Krohn et al. 2009).

The manipulation of the innate immune response in human recipients is perhaps one of the most difficult areas to address. The use of drugs such as GdCl3 is unlikely to be an option for patients. One of the benefits of hepatocyte transplantation is the possibility of avoiding life-long immunosuppressive drugs, although the use of such drugs at the time of transplantation may be sufficient to reduce initial clearance of transplanted cells, with subsequent withdrawal of immunosuppression once engraftment is established. Further research into the mechanisms of hepatic recruitment and activation of neutrophils and

Kupffer cells in the context of hepatocyte transplantation may allow the development of a more targeted intervention.

In addition to activation of the innate immune response, transplant of allogeneic hepatocytes leads to activation of the adaptive immune system. Animal studies suggest that this is mediated by CD4+ and CD8+ T cells, and that either of these can independently initiate an immune reaction (Bumgardner et al. 1999). The T cell response following hepatocyte transplantation does not appear to depend on the CD28/B7 and CD40/CD154 co-stimulatory pathways which are known to be important in the activation of T cells after other allogeneic transplants. In animal studies, blocking the CD28/B7 pathway did not lead to increased survival of hepatocellular allografts. Blockade of the CD40/CD154 system, or both together merely increased the time to rejection (Bumgardner and Orosz 2000) despite the fact that such treatments can result in indefinite survival of other allografts such as cardiac or skin (Larsen et al. 1996). It appears instead that ICAM-1/LFA-1 interactions are involved. Blocking this and the CD40/CD154 pathways together can result in the survival of transplanted hepatocytes in 100% of treated mice at 60 days (Wang et al. 2003). This could suggest a role for a NK cell mediated process as ICAM-1/LFA-1 interactions are crucial for NK adhesion to target cells with subsequent activation and polarisation of cytotlytic granules (Barber et al. 2004;Gross et al. 2010).

The differences in the adaptive immune response to transplanted hepatocytes compared to other allografts highlight an important area for further research. The development of immunosuppressive treatments targeting this unusual pathway may be critical to reduce the hepatocyte rejection although it is likely that manipulation of both the innate and adaptive

immune responses will be required to maximise the survival of allogeneic hepatocytes (Han et al. 2009).

1.5 Animal Models of Hepatocyte Transplantation

Experimental animals that have been humanized by engraftment of human tissue provide an opportunity for *in vivo* study of biological processes that would not otherwise be possible (Shultz et al. 2007). However, the study of human hepatocyte engraftment in particular has been limited by the lack of suitable animal models until relatively recently. As reviewed below there are differing requirements for animal models to facilitate human xeno-transplantation

In order for the donor hepatocytes to engraft and expand within the recipient liver an environment that is permissive to these processes must be provided. If cells are to be transplanted between species, some way of avoiding immune rejection is also required. These conditions are usually achieved by using an immunocompromised animal with a coexistent severe liver disease. Until recently most groups have used some form of alb-uPA mouse. In many studies, hepatocytes have been transplanted from one animal to another of the same species reducing the problem of immune rejection. However, in this situation a means of differentiating donor from recipient hepatocytes is required. Here, the DPPIV- rat has been widely used.

More recently, other strains of mice have been developed to get round the problems associated with the uPA mouse whilst allowing the transplantation of human hepatocytes. These include the model described by Krishnan et al (Krishnan et al. 2007) in which they successfully transplanted human hepatocytes into non-immunocompromised mice. This

group developed mice transgenic for the deleted form of human HGF (dHGF). Human albumin levels increased and peaked post-transplant demonstrating sustained engraftment in six out of nine transgenic mice. All six non-transgenic mice showed declining levels of human albumin post-transplant with no evidence of engraftment. Perhaps one of the most promising models, however, is the FRG mouse. These models are described in more detail below.

1.5.1 The uPA mouse

This transgenic mouse, originally developed to study bleeding disorders (Heckel et al. 1990), contains an additional copy of urokinase type plasminogen activator (uPA) genes under the control of an albumin promoter. This leads to hypofibrinogenaemia and death due to haemorrhage or liver failure.

The alb-uPA mouse has been combined with several types of immunocompromised mouse such as the severe combined immunodeficient (SCID) mouse, the recombination activating gene knockout mouse (Rag2-/-) which is depleted of mature B and T lymphocytes or the IL-2 receptor common γ-chain knockout mouse (Il2rg-/-) which has impaired B and T cell development and a complete lack of NK cell development (DiSanto et al. 1995). This allows for the study of treatment of liver failure in the absence of immune components.

Reasonable success rates in terms of engraftment levels have been reported following hepatocyte transplantation into these animals. However, uPA mice are problematic to work with because there is a narrow time window after birth for transplantation, they are difficult to breed and maintain and serial transplantation is not possible (Shafritz 2007). Other problems include damage to other organs, particularly the kidneys, which limits long

term survival. This is thought to be related to the production of human complement by human hepatocyte repopulated livers (Tateno et al. 2004).

1.5.2 The DPPIV- rat

Many of the mechanisms affecting hepatocyte engraftment which have been described in this chapter have been investigated using this model. These rats are a sub-strain of the Fischer 344 rat which is deficient in dipeptidyl peptidase IV (DPPIV). These have proved useful hosts for syngeneic transplantation of hepatocytes from DPPIV + rats. DPPIV expression is stable in the transplanted cells allowing them to be easily distinguished from the recipient tissue (Thompson et al. 1991).

1.5.3 FRG mice

Several groups have used strains of mice deficient in fumarylacetoacetate hydrolase (Fah). Fah is an enzyme involved in the tyrosine catabolism pathway. Its deficiency (type 1 tyrosinaemia in humans) leads to accumulation of toxic tyrosine metabolites leading to severe liver failure. The administration of 2-(2-nitro-4-trifluoromethylbenzoyl)-1, 3-cyclohexanedione (NTBC) inhibits an enzyme upstream in the tyrosine pathway and limits the production of these metabolites. Therefore the timing of liver injury can be manipulated and successful engraftment of functioning hepatocytes can be demonstrated by the ability to subsequently withdraw treatment with no weight loss or other ill effect for the mice.

Azuma et al (Azuma et al. 2007) attempted to transplant human hepatocytes into Fah-/-/Rag2-/-/Il2rg-/- (FRG) mice treated with NTBC but were not able to demonstrate engraftment in any mice unless they were pre-treated with uPA adenovirus. However, in

mice that had first received uPA adenovirus successful engraftment was achieved and in highly engrafted mice (>30% repopulation) their weight stabilised following withdrawal of NTBC after two rounds of treatment. This suggested that transplanted hepatocytes were functional and this was confirmed by measuring Fah enzyme activity. Furthermore it was demonstrated that up to four rounds of serial transplantation was feasible. Bissig et al (Bissig et al. 2007) went on to develop a similar model using FRG mice but avoiding the need for uPA expression and adenoviral vectors. Instead they administered intraperitoneal nafamostat mesilate, a protease inhibitor, to block the complement system. In addition mice were treated with liposomal clodronate which depletes Kupffer cells.

There are several advantages of the Fah model over others. Grompe et al (Grompe et al. 1995) showed that NTBC abolished neonatal lethality in Fah deficient mice and that the extent of liver disease can be controlled by administering and withdrawing NTBC. The mutation in Fah deficient mice is a deletion and so there is no possibility of reversion to wild-type by transgene inactivation (Grompe et al. 1993). Fah deficient mice are easier to care for, there is no time limit for transplantation and serial transplantation is possible. The use of antibodies to FAH, which is present in the donor human hepatocytes, provides a way to readily distinguish transplanted cells from the host murine hepatocytes. The disease caused by the knockout of the FAH gene is very similar to the human form of tyrosinaemia which is perhaps one of the likely targets for hepatocyte transplantation in humans. These mice could therefore prove much more useful as a model for hepatocyte transplantation and experiments using human hepatocytes and so I initially planned to use this model in this investigation.

1.5.4 C57BL/6 mice

The C57BL/6 mouse is an inbred strain of laboratory mouse and is one of the most widely used backgrounds for genetically modified mice in the study of human disease (Mekada et al. 2009). It has been used extensively in the study of hepatocyte transplantation (Bumgardner et al. 1998;Ponder et al. 1991;Wu et al. 2008) and has been used as a background for the generation of FRG mice. The C57BL/6 mouse has been used with a carbon tetrachloride (CCl_4) liver injury model by our group and others for the study of various types of cell transplantation (Aldridge et al. 2012;Guo et al. 2002;Manuelpillai et al. 2012;Zhang et al. 2011). Wildtype C57BL/6 mice are relatively straight forward to care for and lack the complexity of genetic knockout mice. The CCl_4 liver injury model provides a model of liver fibrosis/cirrhosis thus allowing the investigation of cellular transplantation in the context of diseased liver. Furthermore, the use of CCl_4 provides a regeneration stimulus with a selective advantage for transplanted cells to proliferate/survive (Oertel and Shafritz 2008).

1.6 Outline of the Project

Liver failure represents a high burden of disease and the demand for OLT is increasingly greater than the available supply of donor organs. Alternatives to OLT are therefore urgently required. Hepatocyte transplantation is one such alternative but, despite promising results from early laboratory and clinical studies, the long term benefit remains limited. One reason for the lack of sustained clinical benefit appears to be the failure of engraftment of transplanted cells in sufficient quantity.

I am interested in the molecular mechanisms underlying the interaction between transplanted hepatocytes and hepatic sinusoidal endothelial cells within the liver sinusoids

and how such interactions modulate the engraftment of transplanted hepatocytes into the host liver.

The principle aims of my thesis were to:

1. Optimise a protocol to isolate primary human hepatocytes within the laboratory from normal and diseased liver tissue;
2. Characterise the adhesion molecule and chemokine receptor expression of primary human hepatocytes;
3. Investigate the role of adhesion molecules expressed by primary human hepatocytes in regulating adhesion to hepatic sinusoidal endothelium in vitro using static and flow-based adhesion assays;
4. Augment engraftment in vivo by manipulating mechanisms identified in vitro using a murine model of hepatocyte transplantation.

CHAPTER 2: METHODS AND MATERIALS

2.1 Isolation and Culture of Cells from Human Liver Tissue

2.1.1 Ethical approval

All human liver tissue was obtained from Queen Elizabeth Hospital Birmingham or Birmingham Children's Hospital with full approval of the Local Research Ethics Committee (reference number 06/Q702/61). Liver specimens were obtained from liver resections, whole explanted livers or donor liver which was either surplus to surgical requirements or unsuitable for transplantation. Written informed consent was obtained from all patients or their families.

2.1.2 Preparation of human tissue

Cells were isolated from explanted diseased livers from patients with ALD, PBC and PSC as well as a variety of other end-stage liver diseases. Cells were also isolated from tissue taken from patients undergoing resection of colorectal cancer liver metastases or benign disease such as recurrent cholangitis, focal nodular hyperplasia (FNH) and haemangiomas. Donor liver tissue which was surplus to surgical requirements or unsuitable for transplantation due to prolonged ischaemic times was also used.

Following explantation or resection, liver tissue was placed in a sterile bag on ice and transported to the laboratory for processing as soon as possible, usually within 1 hour. Exceptions to this were when liver tissue became available during late evening or at night, in which case specimens were kept sterile on ice and processed immediately the next morning. Another exception was in the case of donor liver tissue; in all cases this was initially brought back from the retrieval centre to Queen Elizabeth Hospital or Birmingham Children's Hospital where it was assessed by the transplant team. The liver was maintained in a sterile environment on ice at all times. If deemed unsuitable for

transplantation or surplus to requirements it was then transported to the laboratory for processing.

Liver wedges or slices were cut by a trained pathologist and stored in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen Life Sciences, Paisley, UK) at 4°C prior to use. Tissue for hepatocyte isolation was generally taken from segments II/III, V/VI, or VII/VIII in order provide a wedge of tissue with an intact Glisson's Capsule except for one cut surface. Tissue was only used for cell isolation if there was no indication of tumour or other suspicious lesion in that part of the liver after inspection by the pathologist in conjunction with available preoperative imaging.

2.1.3 Isolation and culture of primary human hepatocytes

All tissue culture, cell isolation and preparation of media were carried out in a class II biological safety cabinet using aseptic technique.

2.1.3.1 Preparation of hepatocyte culture medium

A supplemented serum-free medium based on that developed by Pichard et al. (Pichard et al. 2006) was prepared for the culture of hepatocytes after initial plating. This medium has been shown to maintain a hepatocyte phenotype for several weeks.

Briefly, 500ml Williams E was supplemented with the following (all from Sigma-Aldrich, Dorset, UK unless stated otherwise):

1. 0.02% w/v albumin from bovine serum fraction V
2. 2µl linoleic acid
3. 2µl linolenic acid

4. 110 units insulin (Invitrogen Life Sciences)
5. 2.75mg transferrin (Invitrogen Life Sciences)
6. 3.35µg selenium (Invitrogen Life Sciences)
7. 20µg dexamethasone
8. 10µg liver growth factor
9. 1.225mg cyclic-AMP
10. 5 international units prolactin
11. 30ng ethanolamine
12. 500µg glucagon
13. 25µg epidermal growth factor
14. 5 ml penicillin/streptomycin/glutamine solution (PSG, 10000 units/ml penicillin, 10mg/ml streptomycin, 200mM L-glutamine, Invitrogen Life Sciences)
15. 1.5ml fungizone solution (Invitrogen Life Sciences)

The mix of additives was prepared in advance, made up to 25 ml aliquots with Williams E medium and stored at -80°C until use.

2.1.3.2 Preparation of the liver wedge for perfusion

After cutting, the liver wedge was manually washed through via the exposed vessels on the cut surface with cold (4°C) DMEM using a 20ml syringe. This was to remove any remaining blood and to identify two suitable vessels for subsequent perfusion. The two vessels were chosen such as to be of sufficient size for cannulation and to provide

maximum perfusion of the wedge. The latter criterion was judged by observation and palpation of the wedge, as well as the ease of perfusion, while perfusing through all the available vessels of sufficient size. Any major vessels not to be used for perfusion were oversewn in order to minimise loss of fluids and maintain pressure within the wedge thus optimising perfusion. Two 18 or 20 gauge cannulae (Becton-Dickinson, Oxford, UK) were then secured in the chosen vessels and secured by means of a purse string suture placed prior to insertion of the cannula (Figure 2.1).

2.1.3.3 Preparation of perfusion buffers

Hepatocyte isolations were performed using a modified two-stage collagenase perfusion technique originally described in 1969 for the isolation of rat hepatocytes (Berry and Friend 1969). This has since been modified by a number of groups for the isolation of human cells (Dorko et al. 1994;Mitry et al. 2003;Pichard et al. 2006).

The following buffers were prepared in advance using Hank's Balanced Salt Solution without Calcium/Magnesium (HBSS, Invitrogen Life Sciences).

1. Wash buffer – 500ml HBSS + 10mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Sigma-Aldrich), pH 7.2.

2. Chelating buffer – 500ml HBSS + 10mM HEPES, 0.5mM ethylene glycol-bis(2-aminoethylether)-N,N,N,N-tetraacetic acid (EGTA) (Sigma-Aldrich), pH 7.2.

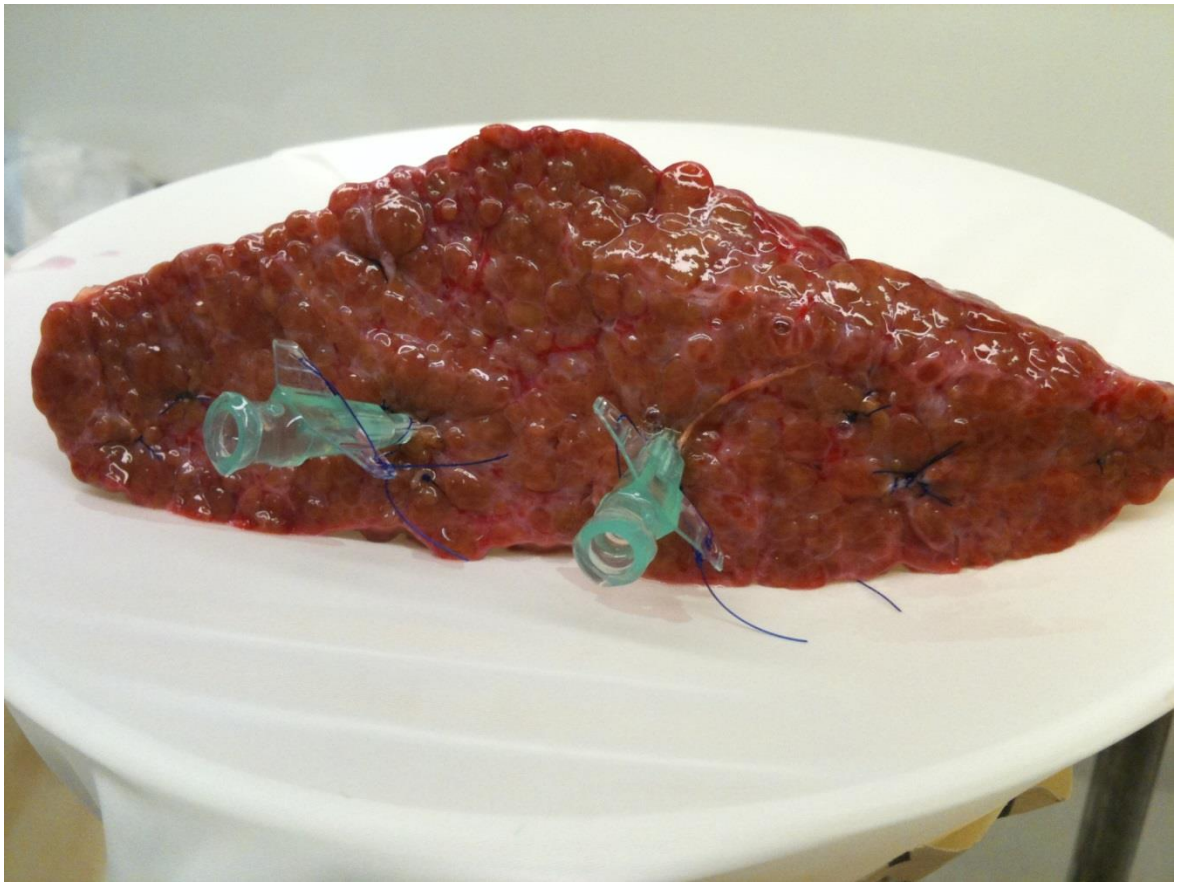


Figure 2.1 Preparation of a liver wedge for hepatocyte isolation

The photograph shows a liver wedge prepared for perfusion. The cut surface is shown with 2 cannulae inserted into separate vessels and held by means of a purse string suture. Several smaller vessels have been oversewn to prevent leakage of the perfusate.

Finally, an enzymatic dissociation buffer was prepared immediately prior to use once perfusion of the wash buffer had commenced. The following enzymes were used:

1. 0.5% w/v Collagenase A (from Clostridium histolyticum, Roche, West Sussex, UK)
2. 0.25% w/v Protease (Dispase® II neutral protease from Bacillus polymyxa, Roche)
3. 0.125% w/v Hyaluronidase (from bovine testes, Sigma-Aldrich)
4. 0.05% w/v DNase I (from bovine pancreas, Roche)

Aliquots of enzymes were removed from storage at -20°C and dissolved in HBSS. The solution was then sterile-filtered using a 0.22μm filter (Millex® Syringe-driven Filter Unit, Millipore, Watford, UK) and made up with HBSS to a final volume of 300ml. This was then supplemented with 5mM calcium chloride and 5mM magnesium chloride (both from Sigma-Aldrich).

All buffers were pre-warmed to 37°C in a water bath (Grant Instruments, Cambridgeshire, UK) prior to use.

2.1.3.4 Liver perfusion and isolation of cells

The liver was placed on a plastic funnel covered with a mesh, which in turn was supported over a collection vessel. Independent perfusion and waste circuits were set up using two sets of tubing and a peristaltic pump (Model IP 505DU, Watson-Marlow Ltd, Falmouth, UK). Initially one end of the perfusion tube was placed into the warmed buffer and led via the pump to a `y' connector dividing the tube into two. These two outlets were connected to the cannulae previously inserted into the liver wedge. One end of the waste tube was placed into the collection vessel and led via the pump to a waste vessel (Figure 2.2).

Perfusion commenced with 500ml wash buffer at a flow rate of 75ml/min in a non-recirculating fashion. This began the warming of the liver to the optimal temperature for enzymatic dissociation and ensured that any remaining blood remaining within the vasculature was washed out. Following this, the liver was perfused with 500ml EGTA chelating buffer (non-recirculating) in order to disrupt cell-cell and cell-extracellular matrix adhesions. The liver was then perfused with a further 500ml wash buffer in order to remove any remaining EGTA which would otherwise inhibit the action of the enzymes which require the presence of calcium and magnesium. The enzyme buffer was then placed into the collection vessel beneath the liver, having first removed any remaining fluid from the previous buffers. The waste tubing was clamped and the intake end of the perfusion tubing was placed in the collection vessel thus allowing the enzyme buffer to be recirculated through the liver. The liver was then perfused with the recirculating enzyme solution using a flow rate of 75ml/min until adequate digestion was achieved. During this time the appearance and texture of the liver was monitored closely. Perfusion was stopped once a digit could be inserted into the liver with minimal force.

Figure 2.2 Perfusion of a liver wedge

The photograph shows a liver wedge connected to the perfusion circuit inside a tissue culture hood. It is being perfused with wash buffer (HBSS + 10mM HEPES) contained in the bottle to the right. The clear tubing from the bottle leads to a pump which delivers the wash buffer to the liver wedge via the two cannulae. The liver is placed upon a sterile mesh through which the perfusate drains into the container below where it is pumped to waste via the red tube

Once perfusion was complete, the cannulae were removed and the liver was placed in cold (4°C) DMEM supplemented with 10% v/v heat inactivated foetal calf serum (FCS, Invitrogen Life Sciences) and 1% v/v PSG solution in a sterile glass dish. The liver was manually dissociated and the resulting suspension passed through a 250µm followed by 63µm sterile nylon mesh (John Staniar & Co, Manchester, UK). The cell suspension was then washed three times and the hepatocytes pelleted using low-speed centrifugation (50 × g for 5 minutes at 4°C) in supplemented DMEM.

Cell viability was determined by trypan blue dye (Sigma-Aldrich) exclusion. If the viability was low but the total cell yield was sufficiently high a density gradient centrifugation step was performed to improve the yield of viable cells. An isotonic stock solution of Percoll® (GE Healthcare, Buckinghamshire, UK) was prepared by adding 11ml 10× phosphate buffered saline (PBS) to 99ml Percoll®. A 30% isotonic Percoll® solution was then prepared by combining 15ml stock Percoll® with 35 ml supplemented DMEM containing the hepatocytes and centrifuged at 300 × g for 30 minutes at room temperature. Viable hepatocytes are pelleted at the bottom of the tube whilst non-viable cells form a layer at the surface and can be easily discarded.

2.1.3.5 Culture of primary human hepatocytes

Hepatocytes were plated in Williams E media (Sigma-Aldrich) supplemented with 10% v/v FCS and 1% v/v PSG. Cells were seeded on type I rat tail collagen coated 24 well plates (5×10^5 cells/well) or flasks and allowed to adhere for 3 hours. Seeding density was selected to ensure a confluent monolayer. After this period the media was changed to hepatocyte culture medium. Cells were maintained at 37°C in a humidified 5% CO_2 incubator. Media was replaced every 24 hours. For experiments, cells were removed from plates/flasks by treating with trypsin/EDTA (Trypsin, 0.05% (1×) with EDTA, Invitrogen

Life Sciences) for 2 minutes having removed the media. Once the cells had detached, the media was replaced and the cells washed in PBS at 2000 rpm for 5 minutes.

2.1.4 Isolation and culture of human hepatic sinusoidal endothelial cells

All tissue culture, cell isolation and preparation of media was carried out in a class II biological safety cabinet using aseptic technique.

2.1.4.1 Preparation of endothelial culture media

HSEC were cultured in basal endothelial media (BEM, Invitrogen Life Sciences) supplemented with 10% v/v heat inactivated human serum (PAA Laboratories GmbH, Pasching, Austria), 1% v/v PSG, 10ng/ml hepatocyte growth factor and 10ng/ml vascular endothelial growth factor (both from PeproTech EC Ltd, London, UK).

2.1.4.2 Isolation of human hepatic sinusoidal endothelial cells

Slices of liver tissue were placed on a sterile plate and finely chopped using a scalpel. The liver was then placed in a sterile beaker with 20ml PBS and left to digest at 37°C for 30-45 minutes depending on the texture of the liver. The resulting digest was then strained through a 63µm sterile nylon mesh (John Staniar & Co) mesh and washed four times at 2000 rpm for 5 minutes in PBS. After washing the cells were re-suspended in 24ml PBS. Percoll® density gradients were prepared as follows: an isotonic stock solution was prepared by adding 11ml 10× PBS to 99ml Percoll®; 33% Percoll® was prepared by adding 33ml stock to 67ml 1× PBS and 77% Percoll® was prepared by adding 77ml stock to 23ml 1× PBS. 3ml 77% Percoll® was layered beneath 3ml 33% Percoll® in each of 8 15ml conical bottom tubes. 3ml of the cell suspension was then layered onto each of the Percoll® gradients and centrifuged at 2000rpm for 25 minutes. The non-parenchymal cell layer was then removed and washed three times in PBS. The heterogeneous cell

suspension was then purified using immunomagnetic selection in order to remove contaminating biliary epithelial cells (BEC). Cells were resuspended in PBS and incubated with 10µg/ml mouse anti-human HEA 125 monoclonal antibody (mAb) (Progen Biotechnik, Heidelberg, Germany) for 30 minutes at 37°C. Following washing in PBS, cells were re-suspended in cold PBS and incubated with 10µl secondary antibody coated magnetic beads (Dynabeads® Sheep-anti Mouse IgG, Invitrogen Life Sciences) at 4°C for 30 minutes. BEC were then removed using magnetic selection. HSEC were then positively selected by incubating the remaining cell suspension with 10µl CD31 mAb-coated magnetic beads (Dynabeads® CD31 Endothelial Cell, Invitrogen Life Sciences) for 30 minutes at 4°C followed by magnetic selection.

2.1.4.3 Culture of human sinusoidal endothelial cells

After isolation, HSEC were cultured in supplemented BEM in 25cm^2 type I rat tail collagen coated flasks. Cells were maintained at 37°C in a humidified 5% CO_2 incubator and media was changed every 48 hours. Once the cells formed a confluent monolayer they were transferred to a 75cm^2 flask. The media was removed and the cells treated with a trypsin replacement enzyme (TrypLE™ Express, Invitrogen Life Sciences). Once the cells were released, media was replaced and the cell suspension washed in PBS at 2000 rpm for 5 minutes. The pellet was then re suspended in HSEC media and the cells seeded into a 75cm^2 flask. Cells were then maintained as before and passaged once they became confluent using the same procedure and splitting the resulting cell suspension 1:2 or 1:3 depending on the size of the pellet into further 75cm^2 flasks. Cells were used for experiments between passage 2 and passage 6.

2.2 Culture of Hepatoma Cell Lines

Huh7.5 cells were a kind gift from Professor Jane McKeating's group at the Centre for Liver Research, University of Birmingham, UK. HepG2 cells were purchased from ATCC (LGC Standards, Teddington, Middlesex, UK). Cells were maintained in DMEM supplemented with 10% v/v FCS, 1% v/v PSG and 1% v/v non-essential amino acids (Sigma-Aldrich) in uncoated 75cm^2 flasks and maintained in a humidified incubator at 37ºC in 5% CO_2. Cells were passaged once a confluent monolayer was formed. Media was removed and the cells were treated with TrypLE™ Express. Once the cells were released, media was replaced and the cell suspension washed in PBS at 2000 rpm for 5 minutes. The pellet was then re-suspended in supplemented DMEM and the cells split between 1:3 and 1:8 depending on requirements into further 75cm^2 flasks. All tissue culture was carried out in a class II biological safety cabinet using aseptic technique.

2.3 Assessment of Hepatocyte Function

Albumin and urea synthesis by primary human hepatocytes were used as basic markers of hepatocyte function in culture.

2.3.1 Albumin synthesis

Albumin synthesis by primary human hepatocytes in culture was confirmed by analysing samples of human hepatocyte culture supernatant using a sandwich ELISA kit (Abnova, CA, USA) according to the manufacturer's instructions. This assay employs a quantitative sandwich enzyme immunoassay technique that measures human albumin using a 96-well microplate that has been pre-coated with a polyclonal antibody specific for human albumin. Albumin in standards and samples is sandwiched by the immobilized polyclonal antibody and a biotinylated polyclonal antibody specific for human albumin, which is

recognized by a streptavidin-peroxidase conjugate. All unbound material is then washed away and a peroxidase enzyme substrate is added. The colour development is stopped and the intensity of the colour is measured at 450nm. Samples were analysed in triplicate and the albumin concentration was calculated with reference to a standard curve generated using known concentrations of human albumin.

2.3.2 Urea synthesis

Urea synthesis by primary human hepatocytes in culture was confirmed by analysing samples of human hepatocyte culture supernatant using a QuantiChrom™ Urea Assay Kit, (Bioassay Systems, Hayward, CA, USA) according to the manufacturer's instructions. This kit uses a colorimetric quantitative urea determination method adapted from the method described by Jung et al (Jung et al. 1975). A chromogenic reagent forms a coloured complex specifically with urea and the intensity of the colour, measured at 520 nm, is directly proportional to the urea concentration in the sample. Tissue culture supernatant samples were analysed in triplicate and urea concentration was determined with reference to a standard concentration using the following formula:

$$\frac{OD\ sample - OD\ blank}{OD\ standard - OD\ blank} \times n \times [STD]\ (mg/dl)$$

OD sample, OD blank and OD standard are the optical density values of the sample, water and standard respectively; n is the dilution factor; [STD] is 50 mg/dl.

2.4 Flow Cytometry

2.4.1 Labelling of hepatocytes/hepatoma cell lines with antibodies for flow cytometry

Cells were removed from flasks using TrypLE™ Express (cell lines) or trypsin/EDTA (primary hepatocytes) as previously described. Cells were then washed in PBS at 900g for 5 minutes and re-suspended at 1×10^6 cells/ml in flow cytometry buffer (PBS supplemented with 2% v/v FCS). 100µl (1×10^5 cells) were then transferred to FACS tubes (Becton-Dickinson) and incubated with antibodies targeting adhesion molecules and chemokine receptors. Isotype matched control antibodies were also used to determine background staining levels. All antibodies were used at optimal concentrations recommended in the manufacturer's datasheet and incubations were carried out for 30 minutes in the dark at 4°C. Details of primary, secondary and isotype matched control antibodies used for flow cytometry are shown in Table 2.1. The cells were then washed twice (2000 rpm, 5 minutes) to remove unbound antibody. For antibodies that were not available directly conjugated to a fluorescent marker, the cells were then re-suspended in flow cytometry buffer and incubated with appropriate conjugated secondary antibody under the same conditions as above. After washing, cells were re-suspended in 500µl flow cytometry buffer for analysis. Data was collected using a CyAn-ADP (Dako Cytomation, UK) and analysed using Summit software (Dako Cytomation).

Antibody	Clone	Host	Conjugation*	Company
α1-integrin	TS2/7	Mouse	RPE	Serotec
α2-integrin	HAS3	Mouse	PE	R&D systems
α3-integrin	IA3	Mouse	PE	R&D systems
α4-integrin	7.2R	Mouse	PE	R&D systems
α5-integrin	SAM-1	Mouse	Alexa Fluor 488	eBioscience
α6-integrin	450-30A	Mouse	RPE	Serotec
α7-integrin	Polyclonal	Rabbit	Unconjugated	Abcam
α8-integrin	481709	Mouse	PE	R&D systems
α9β1-integrin	Y9A2	Mouse	Unconjugated	Serotec
α10-integrin	Polyclonal	Rabbit	Unconjugated	Millipore
α11-integrin	Polyclonal	Rabbit	Unconjugated	Millipore
αIIb-integrin	M148	Mouse	PE	Abcam
αL-integrin	B-B15	Mouse	PE	Abcam
αM-integrin	44aacb	Mouse	PE	Abcam
αV-integrin	13C2	Mouse	PE	Abcam
αVβ3-integrin	23C6	Mouse	PE	R&D systems
αVβ5-integrin	P5H9	Mouse	PE	R&D systems
αVβ6-integrin	10D5	Mouse	Unconjugated	Millipore
β1-integrin	P5D2	Mouse	PE	R&D systems
β2-integrin	212701	Mouse	PE	R&D systems
β3-integrin	256809	Mouse	PE	R&D systems
β4-integrin	422325	Mouse	Unconjugated	R&D systems
β7-integrin	FIB504	Rat	PE	Abcam
CCR1	53504	Mouse	PE	R&D systems
CCR2	48607	Mouse	PE	R&D systems
CCR3	61828	Rat	PE	R&D systems
CCR4	205410	Mouse	PE	R&D systems
CCR5	45531	Mouse	PE	R&D systems

CCR6	53103	Mouse	PE	R&D systems
CCR7	150503	Mouse	PE	R&D systems
CCR8	191704	Rat	PE	R&D systems
CCR9	112509	Mouse	PE	R&D systems
CCR10	314305	Rat	PE	R&D systems
CXCR1	42705	Mouse	PE	R&D systems
CXCR2	48311	Mouse	PE	R&D systems
CXCR3	49801	Mouse	PE	R&D systems
CXCR4	44717	Mouse	PE	R&D systems
CXCR5	51505	Mouse	PE	R&D systems
CXCR6	56811	Mouse	PE	R&D systems
Goat anti Mouse IgG H+L	Polyclonal	Goat	PE	R&D systems
Goat anti Rabbit IgG	Polyclonal	Goat	FITC	Abcam
Mouse IgG1	11711	Mouse	PE	R&D systems
Mouse IgG2a	20102	Mouse	PE	R&D systems
Mouse IgG2b	133303	Mouse	PE	R&D systems
Rat IgG2a	54447	Rat	PE	R&D systems
Rat IgG2b	141945	Rat	PE	R&D systems
Rabbit polyclonal IgG	Polyclonal	Rabbit	Unconjugated	Abcam

Table 2.1 Antibodies used for flow cytometry

The table lists the various primary, secondary and isotype matched control antibodies used for flow cytometry. *RPE - R-phycoerythrin, PE – phycoerythrin, FITC - fluorescein isothiocyanate.

2.4.2 Labelling of hepatocytes/hepatoma cell lines with a viability marker for flow cytometry

A far red viability marker (LIVE/DEAD® Fixable Far Red Dead Cell Stain Kit, Invitrogen Life Sciences) was used to exclude non-viable cells during flow cytometry. This assay uses a dye which reacts with amines on the surface of viable cells and, due to the passage of the dye across the compromised membrane, internal as well as surface amines on necrotic cells. This results in bright staining of non-viable cells and relatively dim staining of viable cells.

Cells were removed from flasks using TrypLE™ Express (cell lines) or trypsin/EDTA (primary hepatocytes) as previously described. Cells were then washed in PBS at 2000 rpm for 5 minutes and re-suspended at 1×10^6 cells/ml in PBS. The fluorescent dye was prepared according to the manufacturer's instructions. The dye was then added to the cell suspension (1µl dye per 1ml cell suspension) and incubated on ice for 30 minutes in the dark. The cells were then washed in PBS (2000 rpm for 5 minutes) and then re-suspended in flow cytometry buffer for further staining as previously described.

2.5 RNA Extraction from Cells

RNA was extracted from cells using a Quiagen RNeasy kit (Quiagen Ltd., Crawley, UK) according to the manufacturer's protocol. RNA purity was assessed using a Nanodrop spectrophotometer (Implen Geneflow). Purity was assessed using 260/ 280 absorbance ratio to give an indication of protein contamination, and samples with a value less than 1.8 were not used. RNA was stored at -80°C until use.

2.6 Preparation of Cell Lysates

Following experimental treatment, cells were washed twice in PBS for 5 minutes at 2000rpm, resuspended in 200µl cell lysis buffer (CelLytic MT supplemented with Protease Inhibitor Cocktail, both from Sigma Aldrich) and incubated at 4°C with agitation for 15 minutes. The lysate was then centrifuged at 15,000g for 10 minutes at 4°C and the supernatant transferred to a cooled micro tube (Eppendorf, Cambridge, UK). Lysates were stored at -80°C until use.

2.7 Protein Determination Assay

The protein concentration of cell lysates was determined using an assay based on the Sigma Total Protein Kit Micro Lowry, Onishi & Barr Modification (Sigma Aldrich). A 1mg/ml bovine serum albumin (BSA; Sigma Aldrich) solution in PBS was made and the following standard concentrations were produced using serial dilution: 1000µg/ml, 800 µg/ml, 600 µg/ml, 400 µg/ml, 200 µg/ml, 0 µg/ml (PBS alone). 20µl BSA standards or lysate was added per well in a 96 well plate. 220µl Biuret reagent (Sigma Aldrich) was added per well and incubated for 10 minutes at room temperature. 10µl Folin & Ciocalteau's Reagent was then added and incubated for 30 minutes at room temperature. The sample plate was then read at 750nm on a plate reader. A calibration curve was plotted using the BSA standards and used to determine the protein concentration of the lysates.

2.8 Adhesion Molecule Gene Expression by Human Hepatocytes and Hepatocyte Cell Lines

2.8.1 Preparation of cDNA

RNA was extracted from cells as described above and cDNA was synthesised using an i-script cDNA Synthesis Kit (Bio-Rad, Hertfordshire, UK) according to the manufacturer's protocols. Briefly, 4µl of 5x iScript reaction mix and 1µl iScript reverse transcriptase was added to a mixture to nuclease free water and RNA (0.25µg/µl) resulting in a reaction volume of 20µl in PCR tubes (Thermoscientific). The complete reaction mixture was incubated in a PCR machine (Geneflow TC512) for 5 minutes at 25ºC, 30 minutes at 42ºC and 5 minutes at 85ºC. The cDNA was then stored at -20ºC until use. Control samples were prepared using water substituted for Reverse Transcriptase.

2.8.2 18s PCR to test for RNA contamination of cDNA samples

Reverse transcription was carried out using 18S rRNA primers (forward GTAACCCGTTGAACCCCATT; reverse CCATCCAATCGGTAGTAGCG) to assess integrity of cDNA and to identify any potential RNA contamination. Hyperladder IV DNA ladder (Bioline, London, UK) was used as amplicon length marker. 1µl of cDNA or control samples was added to 1µl of ready mixed primers (Sigma), 3µl nuclease free water and 5µl of Biomix Red 2× PCR reaction mix (Bioline) in a PCR tube. 7µl of oil was layered over the samples to prevent loss of sample due to evaporation and the samples were subjected to PCR using the thermal profile shown in Table 2.2. The PCR product for 18S rRNA was visualised on a 2% agarose gel stained with ethidium bromide (10mg/ml, Sigma) using standard protocols. Briefly, 1.4g agarose was dissolved in 70ml 1×Tris-Borate

electrophoresis buffer (TBE) prepared using the following reagents (all from Sigma-Aldrich) as 10×TBE in 1000ml nanopure water:

:

1. 0.89M Trisma base
2. 0.89M Boric acid
3. 0.5M EDTA disodium salt.

2µl ethidium bromide was added and the gel allowed to set in a tray. Samples were loaded alongside a lane of Hyperladder IV to indicate the size of the bands and run in electrophoresis buffer at 135V for 1 hour. The gel was removed and photographed on a Genegenius Bioimaging System Imaging machine.

2.8.3 Real time PCR

Samples were loaded into a TaqMan Custom Array plate (Life Technologies Ltd, Paisley, UK) according to the manufacturer's instructions and subjected to RT-PCR using an Applied Biosystems 7900HT Fast Real-Time PCR System (Life Technologies Ltd) and the data analysed using SDS software (Life Technologies Ltd).

	Temperature (^{O}C)	Time (s)
Initialisation	95	225
Denaturing	95	45
Annealing	55	45
Extension	72	45

Table 2.2 Thermal profile for 18s PCR

18s PCR was carried out using the thermal profile detailed in the table (30 cycles) to check cDNA integrity and exclude RNA contamination.

2.9 Static Adhesion Assay

2.9.1 Preparation of frozen liver tissue sections

Tissue blocks were prepared from various types of liver tissue as detailed in 2.1.2. The blocks were then snap-frozen in liquid nitrogen and stored at -80°C prior to sectioning. 10µm thick sections were cut using a cryostat and placed onto glass microscope slices coated with 0.01% v/v poly-L-lysine (Sigma Aldrich) prepared in distilled water. Sections were then fixed in acetone for 5 minutes and stored at -20°C prior to use.

2.9.2 Stamper-Woodruff assay

The adhesion of human hepatocytes and hepatoma cell lines to liver tissue was investigated using a static adhesion assay based on a technique originally described to study leukocyte adhesion (Stamper, Jr. and Woodruff 1976). This technique has been modified in our lab to study the adhesion of various cell types to liver tissue.

10 µm cryosections cut from human liver tissue were fixed in acetone for 5 minutes, air dried and wrapped in foil. Sections were stored at -80ºC until required for adhesion assays. Frozen liver tissue sections were removed from storage and allowed to warm to room temperature for 30 minutes before fixing in acetone for 5 minutes. The slides were then labelled and the section marked circumferentially with a wax pen before being moistened with PBS. The slides were then rested on glass rods on a tray lined with paper moistened with distilled water. Cells were removed from culture, washed in PBS (2000 rpm, 5 minutes) and resuspended in media. Function-blocking antibodies or isotype matched controls (detailed in Table 2.3) were added and incubated for 30 minutes at 37°C. Following incubation the cells were washed again in PBS (2000 rpm, 5 minutes) and resuspended in PBS at 1×10^6/ml. 100µl cell suspension (1×10^5 cells) was added per

section and incubated statically at room temperature for 30 minutes. The sections were then washed gently with PBS to remove non-adherent cells and fixed in acetone for 5 minutes before washing twice with PBS for 5 minutes. The sections were then stained with Mayers Haematoxylin (VWR International, Lutterworth, UK) for 30s and rinsed in tap water for 5 minutes. Glass cover slips (Leica Microsystems, Milton Keynes, UK) were then mounted using Immu-Mount (Thermo Shandon, Runcorn, UK) and left to dry overnight. Sections were then viewed using a light microscope and the number of adherent cells in 10 random fields of view was counted. The total number of adherent cells was then expressed as the mean number of cells per high power field of view.

2.10 Fluorometric ECM Component Adhesion Assay

Huh 7.5 cells were prepared as for the Modified Stamper-Woodruff assay. Binding to individual ECM components other than hyaluronic acid and VCAM was then assessed using an ECM Cell Adhesion Array Kit (Merck Millipore, Massachusetts, USA) according to the manufacturer's instructions. Briefly, 1×10^5 cells were added to pre-manufactured wells coated with ECM components that were included in the kit and incubated for 1 hour at 37ºC. Wells were then washed to remove unbound cells before adding a lysis/dye buffer and incubating at room temperature until lysis was observed (around 20 minutes). The labelled cell lysates were then transferred to a new 96 well plate and analysed on a plate reader using 485/528nm filters on a plate reader. Analysis of binding to hyaluronic acid and VCAM binding was performed in an identical manner using 96 well plates pre-coated in the laboratory with hyaluronic acid (2.5mg/ml; Sigma Aldrich) or VCAM-1 (10µg/ml; R&D Systems).

Antibody	Clone	Host	Concentration	Company
β1-integrin	P5D2	Mouse	10µg/ml	R&D Systems
α1-integrin	0.T.63	Mouse	10µg/ml	Abcam
αV-integrin	272-17E6	Mouse	10µg/ml	Abcam
Mouse IgG1 isotype control	11711	Mouse	10µg/ml	R&D Systems
Mouse IgG2a isotype control	ICIGG2A	Mouse	10µg/ml	Abcam

Table 2.3 Function blocking antibodies used in static and flow adhesion assays.

2.11 Flow Adhesion Assay

2.11.1 Preparation of microslides

Plastic multi-channel slides designed for the observation of cells under flow (μ-Slide VI; Ibidi GmbH, Martinsried, Germany) were coated with type I rat tail collagen for 2 hours and then washed with copious PBS. HSEC were removed from culture using TrypLE™ Express, washed in PBS and resuspended in 400-600μl HSEC media depending on confluency. 30μl cell suspension was added to each of the 6 channels of the slide and the slide placed at 37°C in a humidified 5% CO_2 incubator for 2 hours to allow the cells to adhere. The media was then topped up as required and the cells maintained in the incubator until use.

2.11.2 Stimulation of HSEC

After 24 hours of culture, and 24 hours prior to the experiment, HSEC were treated with pro-inflammatory cytokines. HSEC media was aspirated from each of the channels in the slide and replaced with new media supplemented with TNFα and IFNγ (PeproTech) at a concentration of 10ng/ml.

2.11.3 Preparation of hepatocytes

Primary human hepatocytes or Huh 7.5 cells were removed from culture as described above and incubated with blocking antibodies or isotype matched control (Table 2.3) in for 1 hour at 37°C. Cells were then washed and were resuspended in endothelial media supplemented with PSG and 0.1% v/v BSA immediately prior to the assay.

2.11.4 Adhesion assay

After treatment of the endothelium and/or hepatocytes the slide was placed on a microscope stage (Olympus IX50; Olympus, Southend-on-Sea, UK) within an enclosure

regulated at 37°C. One end of the channel to be examined was connected to a syringe driver (Harvard PHD 2000; Harvard Apparatus, Massachusetts, USA) and the other to an electronic 2-way valve (Lee Products, Gerrards Cross, UK) using proprietary flow assay adaptors (μ-slide VI flow kit; Ibidi) extended with silicone tubing (Fisher Scientific, Loughborough, UK). The syringe driver was set to reproduce a physiological shear stress similar to that found in the liver sinusoids of 0.5dynes/cm^2 and the valve allowed switching between the cell suspension and wash buffer. Wash buffer consisted of unsupplemented endothelial media.

2.12 Hepatocyte Migration across HSEC

A HSEC monolayer was prepared within a microslide and stimulated with pro-inflammatory cytokines as described for the flow adhesion assay. Huh 7.5 were incubated with an anti-β1-integrin blocking antibody or isotype matched control as previously described and flowed across the HSEC in an identical manner to the flow adhesion assay. After five minutes the flow was stopped and the cells were allowed to settle onto the HSEC monolayer. The microslide was then transferred to a JuLi Smart Fluorescent Live Cell Imager (Bulldog Bio Inc., NY, USA) at 37°C in a humidified 5% CO_2 incubator. A representative field of view was selected and the imager was set up to record an image of the field of view once every 2 minutes for 4 hours. The images were then combined as a movie and analysed using ImageJ (Schneider et al. 2012) and the number of Huh7.5 seen migrating into/through the HSEC layer was assessed by counting the absolute number of migrating cells in a single field of view.

2.13 Measurement of Hepatocyte Caspase 3 Activity by Cleaved Caspase 3 ELISA

Cells were incubated in suspension with anti-β1-integrin blocking antibodies or isotype matched control for 1 hour at 37 °C. Cell lysates were prepared and the protein concentration determined as previously described. The protein concentration of the lysates was normalised to 1mg/ml and the assay performed according to the manufacturer's instructions using a Pathscan® Cleaved Caspase-3 Sandwich ELISA Kit (Cell Signalling Technology, Danvers, USA). Briefly, 100µl lysate was mixed with 100µl sample diluent and 100µl of the diluted lysate was added per well. The plate was then incubated overnight at 4°C. After washing, 100µl biotinylated caspase 3 rabbit detection antibody was added to each well and incubated for 1 hour at 37°C. Following further washing, 100µl horseradish peroxidase (HRP)-linked secondary antibody was added to each well and the plate incubated for 30 minutes at 37°C. The plate was washed again and 100µl TMB substrate was added to each well and the plate incubated for 10 minutes at 37°C before adding 100µl STOP solution to each well. The 450nm absorbance was then read on a plate reader.

2.14 Assessment of AKT Phosphorylation in Hepatocytes by Western Blotting

2.14.1 Incubation of cells and preparation of lysates

Hepatocytes were removed from the culture flask using trypsin/EDTA as previously described and incubated with anti-β1-integrin blocking antibody or isotype matched control for 1 hour at 37°C. Cell lysates were then prepared using CelLytic MT

supplemented with Protease Inhibitor Cocktail as described above. Protein concentration was determined as described above and the concentration corrected to 2mg/ml.

2.14.2 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and gel transfer

An 11% SDS-PAGE gel was made as follows:

1. 3.7ml nanopure water
2. 3.7ml 30% (w/v) degassed acrylamide/bis-acrylamide solution (Sigma Aldrich)
3. 2.5ml gel buffer*
4. 10% w/v SDS (Sigma Aldrich)

* Resolving gel buffer – 1.5M Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl; Sigma Aldrich), pH 8.8; stacking gel buffer – 0.5M Tris-HCl, pH 6.8.

A solution of 10% w/v ammonium persulfate (APS; Sigma Aldrich) in water was prepared. 60µl APS and 60µl tetramethylethylenediamine (TEMED; Sigma Aldrich) were added to the resolving and stacking gel buffers immediately prior to pouring between gel plates.

A 10× running buffer was made by dissolving the following in 1l nanopure water:

1. 30.3g Tris-HCl
2. 144g glycine (Sigma Aldrich)
3. 10g SDS

A transfer buffer was made by dissolving the following in 2l nanopure water:

1. 28.8g glycine
2. 6.0g Tris-HCl
3. 400ml methanol
4. 1g SDS

A 5× SDS loading buffer was prepared as follows:

1. 200mM Tris-HCL solution pH6.8
2. 20% v/v glycerol (Sigma Aldrich)
3. 10% w/v SDS
4. 0.05% w/v bromophenol blue (Sigma Aldrich)

The gels were placed into PAGE tank with 1× running buffer. 5µl 5× loading buffer was added to 20µl of each sample as well as 10µl each of prestained molecular weight markers and biotinylated protein marker (both from Cell Signalling Technology) in an Eppendorf and heated for 2 minutes before centrifuging a high speed for 1 minute. 20µl of each sample (or 10µl of marker) was then loaded per well and electrophoresis commenced at 200V for 30 minutes or until the blue dye was near the bottom of the gel. Gels were then transferred to a polyvinylidene difluoride (PVDF) membrane in transfer buffer at 100V for 1 hour.

2.14.3 Western blotting

Western blotting was performed based on manufacturer's instructions provided with the antibodies from Cell Signalling Technology. A 10×Tris-buffered saline with Tween-20

(TBS/T) solution was prepared as 0.2M Tris base, 1.36M sodium chloride (NaCl), 1.0% Tween-20 in 1l nanopure water as follows:

1. 24.2g Tris (Sigma Aldrich)
2. 80g Sodium Chloride (Sigma Aldrich)
3. pH adjusted to 7.6 with HC

Following transfer, the PVDF membrane was washed with 25ml 1× TBS/T for 5 minutes at room temperature. The membrane was then incubated with 10ml blocking buffer (1× TBS/T with 5% w/v non-fat dry milk; Marvel, Premier Foods, Hertfordshire, UK) for 2 hours at room temperature and then washed 3 times for 5 minutes each with 15ml 1× TBS/T. The membrane was next incubated with rabbit anti-human phospho-Akt (Ser473) antibody (Cell Signalling Technology) at a 1:1000 dilution in 10ml 1× TBS/T with 5% w/v BSA overnight at 4ºC with gentle agitation. The membrane was further washed 3 times with 15ml 1× TBS/T and incubated with HRP-conjugated anti-rabbit IgG secondary antibody (1:2000 dilution) and HRP-conjugated anti-biotin antibody (1:1000 dilution)(both from Cell Signalling Technology) in 10ml blocking buffer for 1 hour at room temperature with gentle agitation. Finally the membrane was washed three times as before and incubated with 10ml LumiGLO Substrate (Cell Signalling Technology) for 1 minute at room temperature with gentle agitation. Excess LumiGLO Substrate was drained and the membrane covered with plastic wrap and exposed to x-ray film (Kodak, Rochester, NY, USA) for 5 minutes before developing using a Kodak X-Omat 1000 processor. Images were scanned as grey-scale and saved as a TIFF file. The gel analysis function in ImageJ was then used to perform densitometric analysis of bands.

2.15 Transfection of Huh 7.5 cells with GFP tagged vinculin and RFP tagged actin

Plasmids containing green fluorescent protein (GFP) tagged vinculin and red fluorescent protein (RFP) tagged actin were prepared within our laboratory and were a generous gift from Dr Christopher Weston. 24 hours prior to transfection, Huh 7.5 cells were seeded into uncoated 6 well plates to achieve approximately 80% confluence on the day of transfection (30×10^4 cells in 2ml media without penicillin/streptomycin per well). The following day Huh 7.5 cells were transfected using Lipofectamine 2000 and Opti-MEM media (Life Technologies Ltd) according to the manufacturer's instructions. Cells were used for experimental purposes 48 hours after transfection.

2.16 Live Cell Confocal Microscopy to Examine Cytoskeletal Rearrangement in Hepatocytes

Huh 7.5 cells transfected with GFP tagged vinculin and RFP tagged actin were removed from the culture flask as previously described and incubated with anti-β1-integrin blocking antibody or isotype matched control. Ibidi μ-slides were prepared as for the flow adhesion assays. Huh7.5 cells were then flowed across the HSEC for 5 minutes and the flow was then stopped. The slide was then transferred to a confocal microscope with a heated (37ºC)/5% CO_2 stage (Zeiss LSM 780 Zen confocal) and z-stack images were obtained using a Plan-ACHROMAT 100x oil immersion objective lens.

2.17 Murine Models to Study Engraftment of Transplanted Hepatocytes

2.17.1 C57BL/6 mice

2.17.1.1 Maintenance of C57BL/6 mice

C57BL/6 mice were purchased (Taconic, New York, USA) and housed at the University of Birmingham Biomedical Services Unit (BMSU). Mice were maintained under standard animal welfare conditions. Mice were used for experimental work at the age of 6-10 weeks.

2.17.1.2 Carbon tetrachloride liver injury model

Carbon tetrachloride (CCl_4) was diluted 1:4 in mineral oil (both from Sigma Aldrich). 6-10 week old C57BL/6 mice received twice-weekly intraperitoneal injections (1µl/g equivalent to 250µl/kg CCl_4). Animals were monitored for any signs of ill health or distress and sacrificed using a schedule 1 method if required.

2.17.1.3 Short term engraftment studies

Primary human hepatocytes were incubated with blocking antibodies or IgG control as required before labelling with carboxyfluorescein succinimidyl ester (CFSE, Sigma Aldrich). Cells were washed twice at 2000rpm for 5 minutes in PBS/0.1% (w/v) BSA and resuspended in 2ml. A 10µM CFSE in PBS/0.1% BSA labelling solution was prepared and 2ml were added to the cell suspension to give a final labelling concentration of 5µM CFSE. The solution was mixed gently and incubated at 37°C for 10 minutes in the dark. Following incubation, an excess of ice cold culture medium was added and the cells were washed three times at 2000 rpm for 5 minutes in cold medium. The cell suspension was rested on ice in the dark for 5 minutes prior to the final wash in order to let un-

incorporated CFSE diffuse out of the cells. The cells were then resuspended at 1×10^7 cells/ml in PBS and kept on ice in the dark until use.

Wild type or CCl_4–treated C57BL/6 mice were anaesthetised in an anaesthetic chamber using 5% isoflurane with 1-2l/min oxygen. The mice then received 0.05-0.1mg/kg buprenorphine by subcutaneous injection, the abdomen was shaved and the mouse placed on the operating table on a warming mat. Anaesthesia was maintained using a face mask with 1.5-3% isoflurane as required and 1-2l/min oxygen. Depth of anaesthesia was monitored by observation of respiratory rate and confirming a lack of response to stimulation, in particular the absence of the pedal withdrawal reflex.

Surgery was performed using sterile technique. The skin was prepared using Povidone-Iodine and the mouse covered except for the operative site with a sterile drape. A midline laparotomy was performed, the bowel retracted and the portal vein identified. 1×10^6 cells (100µl of the cell suspension) were injected into the portal vein using a 30G needle (BD Microlance 3). Bleeding from the vein was arrested with pressure using a cotton bud. The mouse was kept under anaesthesia for 15 minutes from the time of injection and then culled by cervical dislocation. The liver was immediately dissected, snap frozen in liquid nitrogen and stored at -80°C until processing. The liver was then cryosectioned (5µm slices) and the number of fluorescent hepatocytes per field of view was counted in 40 random fields of view spread across multiple sections taken from different parts of the liver.

The short term engraftment studies were intended as preliminary studies that would lead on to longer-term engraftment models to be carried out in future work. These experiments were designed to investigate a novel mechanism to improve hepatocyte engraftment and

there is little published literature in this area on which to base a likely or desired effect size and thus undertake a power calculation. Furthermore, the experimental technique is technically challenging and represents a major insult for the mice undergoing surgery, with some inevitable loss of animals before completion of the procedure during preliminary work to refine the surgical technique of portal vein injection using saline only. Because of this, and the fact that these procedures represented preliminary work only, it was decided in consultation with the Veterinary Surgeon and senior staff within the animal facility to initially perform 4 procedures on wild type mice and a further 4 procedures on CCl_4 treated mice before reviewing the results and undertaking further procedures as necessary. All but one of these experiments were successful, resulting in the animals surviving for 15 minutes until culling and subsequent demonstration of early hepatocyte engraftment within their livers. One animal in the wild type group did not survive the 15 minute period following portal vein injection and was therefore excluded. However, the data from the remaining 3 animals revealed a significant effect and so no further procedures were peformed. We felt that this approach was adequate for preliminary work and adhered to the 3 Rs (Replacement, Reduction, Refinement) approach to animal research.

2.17.2 FRG mice

2.17.2.1 Acquisition and breeding of FRG mice

Two male FRG mice were kindly provided by Markus Grompe's laboratory, Oregon Health and Science University, Oregon, USA. Female Rag2/Il2rg double knockout mice were purchased (Taconic). The male FRG mice were bred with the female Rag2/Il2rg mice by the BMSU, University of Birmingham. Further breeding using homozygous and

heterozygous FRG mice was performed to establish a colony of homozygous triple knockout FRG mice.

2.17.2.2 Genotyping of FRG mice

DNA was extracted from mouse ear clippings. Tissue was added to 100µl 50mM NaOH and heated to 95°C for 40 minutes with brief agitation after 20 minutes. 8µl 1M Tris-HCl (pH 8.0) was then added and the mixture agitated.

The presence or absence of the FAH, RAG-2 or Il2rg genes was detected by polymerase chain reaction (PCR) amplification. Details of the primers and PCR protocols used are shown in Table 2.4. The PCR products were then subjected to 2% agarose gel electrophoresis at 124V for 40 minutes. Hyperladder IV (Bioline) was used as a marker.

2.17.2.3 Maintenance of FRG mice

FRG mice were maintained in the BMSU under standard animal welfare conditions and allowed a normal diet with drinking water supplemented with NTBC. Supplemented drinking water was prepared based on instructions provided by the Grompe laboratory. A 2mg/ml NTBC stock solution was prepared by dissolving 3g sodium bicarbonate (Sigma Aldrich) into 30 ml nanopure (Barnstead Nanopure Diamond, Barnstead International, Dubuque, USA) water by heating to 42°C with constant stirring for 2 hours. The sodium bicarbonate solution was added to 220ml nanopure water with 500mg NTBC powder (Swedish Orphan Biovitrum, Stockholm, Sweden) and heated to 50°C for 3 hours with constant stirring. The solution was then sterile filtered and stored refrigerated and protected from light for up to 3 months.

To make NTBC drinking water, 250µl red food colouring (Sainsbury's Supermarkets Ltd, London, UK) was added to 1l nanopure water and autoclaved. The red food colouring was

added in order to differentiate the NTBC supplemented water from normal drinking water. The autoclaved water was refrigerated overnight before adding 8ml of the 2mg/ml NTBC stock solution. The NTBC drinking water was then delivered to the BMSU where it was stored refrigerated and protected from light for up to 2 weeks.

2.18 Statistical Analysis

A two-tailed Student's t-test (paired or unpaired as appropriate) was used to analyse data when comparing numerical variables between two groups unless otherwise stated. All statistical analysis was performed using the GraphPad Prism software version 5. Data were considered statistically significant when $p<0.05$. Levels of significance were expressed as follows: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$.

	FAH	RAG-2	Il2rg
Primers	Mutant forward: GGATTGGGAAGACAATAGCAGGC Common Reverse: TTGCCTCTGAACATAATGCCAAC Wild type forward: TGAGAGGAGGGTACTGGCAGCTAC	Mutant forward: GTTGTGCCCAGTCATAGCCG Common Reverse: ATTTCAATCGTGTTGTCCCC Wild type forward: TAAGAGTGGACCTTCCCCTG	Mutant reverse: CCTGCGTGCAATCCATCTTGTTCAAT Common forward: CTGCTCAGAATGCCTCCAATTCC Wild type reverse: GATCCAGATTGCCAAGGTGAGTAG
Reaction Mix	12.5µl 2× Biomix Red 0.3µl each primer 2.9µl water 4µl DNA	12.5µl 2× Biomix Red 1.0µl each primer 2.5µl water 2µl DNA	12.5µl 2× Biomix Red 0.16µl common forward 0.12µl mutant reverse 0.24µl wild type reverse 3.98µl water 3µl DNA
PCR	3' at 94°C + 35×(30" at 94°C, 30" at 60°C, 1; at 72°C) + 7' at 72°C	5' at 94°C + 35(45" at 94°C, 45" at 58°C, 1' at 72°C) + 5' at 72°C	3' at 94°C + 35×(30" at 94°C, 30" at 60°C, 1' at 72°C) + 7' at 72°C

Table 2.4 Genotyping protocols for FRG mice

CHAPTER 3: OPTIMISATION OF HEPATOCYTE ISOLATION FROM NORMAL AND DISEASED LIVERS

3.1 Introduction

Hepatocytes are the major parenchymal cells in the liver and account for 70-80% of the total liver cell mass. As well as being the major structural cells of the liver, hepatocytes also form the functional units for liver metabolism and regeneration (Si-Tayeb et al. 2010). It is becoming widely accepted that the majority of hepatocyte cell lines, while providing a valuable resource for preliminary studies, lack many important aspects of primary cell function. Further, it is unlikely that such cells would be used in the therapeutic setting. Therefore the procurement of high quality viable hepatocytes is essential for a range of biomedical and pharmaceutical studies. The use of primary human hepatocytes has gained increasing interest for drug development and drug toxicity studies (Li 2007) as well as the elucidation of underlying biochemical pathways in liver disease. Cell therapy is an expanding area of research and the potential for primary human hepatocytes to support liver function through hepatocyte transplantation shows promise in laboratory and early clinical studies. However, the isolation of human liver cells requires a carefully planned logistical network of surgeons, pathologists, technicians and biologists (Knobeloch et al. 2012). Furthermore, human liver tissue is a limited resource. Most studies of hepatocyte isolation describe the use of surplus donor tissue made available through liver transplant programmes or normal tissue removed during resection of liver tumours (Alexandrova et al. 2005;Hughes et al. 2006;Kawahara et al. 2010;Li 2007;Vondran et al. 2008). However, the majority of liver tissue available for research is from explanted diseased liver which yields much fewer viable cells. A means of improving the outcome of hepatocyte isolation from diseased liver would allow better use to be made of available liver tissue increasing the pool of primary hepatocytes for research purposes.

The standard method of human hepatocyte isolation is based on a two-step collagenase digestion technique which was first described in 1969 by Berry and Friend for the isolation of rat hepatocytes (Berry and Friend 1969). Several groups have since modified this technique for the isolation of human and other animal hepatocytes.

The procedure involves the initial perfusion of the liver tissue with a warm buffer containing EGTA in order to loosen cell-cell junctions and to bring the liver to the optimum temperature for enzymatic digestion. The second step is to perfuse the liver with an enzymatic solution containing collagenase to digest the liver parenchyma and release a single cell suspension. Typically several other enzymes are added to this digestion buffer. Hyaluronidase is a polysaccharidase that breaks down hyaluronic acid which is found in high concentration in connective tissues and within the liver where it is synthesised mainly by hepatic stellate cells. Hepatocytes are then be separated by low speed centrifugation which may be followed by a density gradient step to enrich for viable cells.

3.1.1 Liberase

Standard collagenase preparations are prepared from bacteria and consist of a mixture of collagenases with contaminating proteases, esterases and other enzymes with a somewhat variable composition that differs from lot to lot. Liberase is a relatively new enzyme initially developed to improve the outcome of pancreatic islet isolation (Brandhorst et al. 1999;Linetsky et al. 1995;Linetsky et al. 1997) and comprises purified high specific activity collagenases (clostridial collagenase I and collagenase II) blended with high specific activity neutral protease (dispase or thermolysin) in an optimal ratio. Liberase blends are also xeno-free which is important for downstream applications. Little has been published on the use of Liberase for human hepatocyte isolation, but it has been shown to improve the viability of isolated porcine hepatocytes compared to standard collagenase

(Donini et al. 2001). Here I have investigated the use of Liberase in place of standard collagenase in the human hepatocyte isolation protocol in order to improve the outcome of human hepatocyte isolation.

3.1.2 N-acetyl cysteine

N-acetylcysteine (NAC) is an antioxidant which acts through the replenishment of hepatic glutathione stores as well as having direct antioxidant properties (Atkinson 2002) and appears to have hepatoprotective effects through a number of mechanisms (Zwingmann and Bilodeau 2006). Animal data suggests that NAC may have a protective effect against liver ischaemia/reperfusion injury (Dunne et al. 1994;Fukuzawa et al. 1995;Fusai et al. 2005) and the use of NAC has recently been shown to improve the viability of human hepatocytes isolated from steatotic liver (Sagias et al. 2010).

Therefore in this chapter I firstly set out optimise human hepatocyte isolation from normal and diseased liver tissue for use in subsequent experiments. The second aim was to investigate the effects of the use of Liberase and NAC on the outcome of human hepatocyte isolation.

3.2 Results

I initially re-established the standard human hepatocyte isolation procedure used previously in our laboratory using the collagenase digestion technique to isolate primary human hepatocytes. I then attempted to combine the use of Liberase and NAC in order to improve the outcome of human hepatocyte isolation.

3.2.1 Isolation of human hepatocytes from normal and diseased liver

Human hepatocyte isolation was initially performed using a standard protocol which had been previously employed in our laboratory (Bhogal et al. 2011). Subsequently, isolations

were performed using the modified protocol with the addition of NAC and the substitution of Liberase for standard collagenase.

Hepatocytes were isolated from a variety of different liver diseases including ALD, PBC and PSC. Hepatocytes were also isolated from resections for colorectal metastases (CRM) and various sources of normal liver tissue including hepatic resections for benign disease, surplus tissue from paediatric transplants (where a large liver may be `cut down' to a more suitable size) and other unused donor tissue. The latter was mostly obtained from organs rejected for transplant due to prolonged ischaemic times and/or extensive steatosis. When preparing the tissue from CRM resections care was taken to keep well away from the tumour when taking the wedge for hepatocyte isolation. Indeed, if there was any doubt as to the location of tumours following review of imaging and inspection of the resection then no wedge was taken for hepatocyte isolation. The wedges obtained from CRM resections therefore consisted of `normal' tissue; however as the vast majority of these patients had received chemotherapy prior to resection, this group will be considered separately in the subsequent analysis and will be described as `resected'. All other normal tissue will be described as `normal'.

Human hepatocytes were isolated from 65 livers altogether with an overall success rate, defined as maintenance of a cells as a confluent monolayer in culture for 48 hours, of 62%. Median viability was 60% (range 0-100%) with a median total cell yield of 35×10^6 cells (range 0-638 $\times 10^6$ cells). The outcome of hepatocyte isolation by liver type is summarised in Table 3.1.

Liver Type	Weight of Wedge (g)	Perfusion Time (minutes)	Total Cell Yield (×106)	Viability (%)	Success Rate (%)
Normal (n=17)	132 (56-437)	4 (1-10)	45 (5-638)	78 (0-100)	76
ALD (n=15)	72 (50-486)	6 (3-11)	24 (0-148)	25 (0-95)	47
Biliary cirrhosis (n=11)	83 (47-117)	5 (3-16)	21(12-283)	86 (5-100)	73
Resected (n=10)	86 (38-123)	3 (2-3)	99 (18-438)	59 (0-93)	70
Other (n=12)	78 (46-154)	6 (3-14)	36 (0-185)	19 (0-83)	42
Total (n=65)	85 (38-486)	5 (1-16)	35 (0-638)	60 (0-100)	62

Table 3.1 Outcome of human hepatocyte isolation from normal and diseased liver tissue.

This table summarises the yield, viability and success rate obtained after hepatocyte isolation from various different types of liver tissue. Data expressed as median (range).

3.2.2 Morphology of primary human hepatocytes in culture

Primary human hepatocytes isolated from normal and diseased liver had a similar appearance to that previously reported by us and others (Bhogal et al. 2011;Laba et al. 2005). Figure 3.1 shows the morphology of hepatocytes isolated from normal liver tissue at different time points after isolation. On initial plating cells appeared rounded and phase bright, but during the next 24 hours, cells gradually flattened out forming a confluent monolayer and demonstrating a typical cuboid or polygonal appearance with many cells being binucleate. This morphology was maintained in culture for at least 1 week.

3.2.3 Comparison of hepatocyte isolation using the original and new protocols

Human hepatocytes were isolated from 30 consecutive liver specimens using the original protocol. Hepatocytes were then isolated from a further 30 consecutive liver specimens in an identical manner except for the addition of NAC to the EGTA-containing buffer and the substitution of Liberase for standard collagenase (new protocol).

The underlying disease of the tissues used for cell isolation is shown in Table 3.2 and included PBC or PSC, ALD, tissue removed during colorectal metastasis resection and normal or donor tissue. A number of baseline factors related to the liver tissue used for isolation in the two groups are shown in Table 3.3.

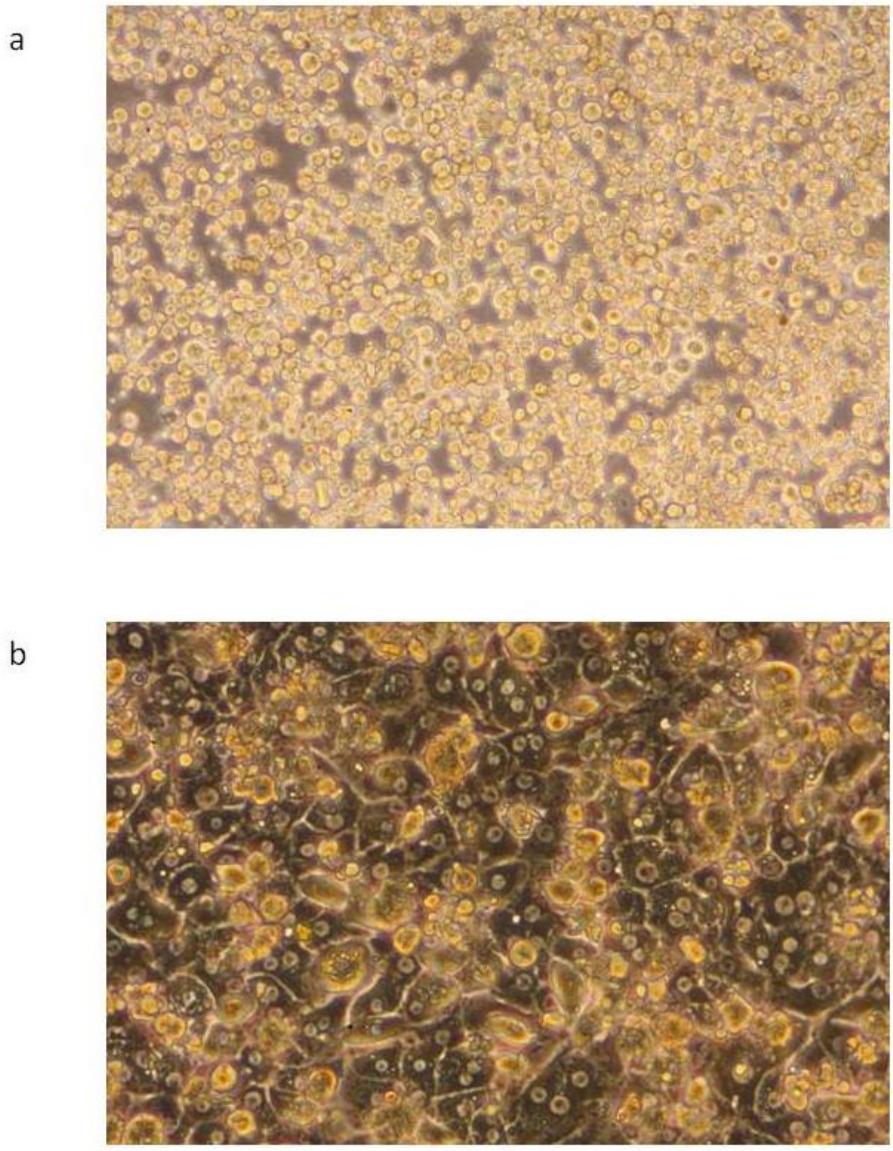


Figure 3.1 Morphology of human hepatocytes isolated from liver tissue.

Representative example of primary human hepatocytes isolated from normal donor liver tissue showing typical morphological changes: (a) 1 hour after plating the cells are rounded and phase bright; (b) after 48 hours a confluent monolayer has formed. The hepatocytes have flattened out becoming phase dark. The typical cuboid/polygonal appearance is seen with many cells possessing two nuclei.

These include patient age and gender, Model for End-Stage Liver Disease (MELD) and United Kingdom End-Stage Liver Disease (UKELD) scores, delay from the time of liver explant/resection (or, for donor/normal tissue, from the time that the liver arrived at Queen Elizabeth Hospital, Birmingham) until commencement of perfusion and the weight of the wedge used for isolation. Of note there was no significant difference in either the disease category or any of the variables between the two groups, with the exception of time delay which was significantly longer in the original protocol group (median 4 hours vs. 2 hours, p=0.007).

3.2.3.1 The effect of protocol modification on the perfusion time and Percoll use

Although the isolation procedure itself remained unchanged other than the modifications being studied, the duration of perfusion (enzymatic digestion) and use of Percoll density gradient centrifugation varied depending on the nature of the particular liver specimen and the initial cell yield and viability as described above. I therefore investigated the effect of the new protocol on these factors and the results are summarised in Table 3.4. The new protocol necessitated a slightly longer perfusion time than the original protocol (median 4.5 vs. 3.0 minutes, p=0.028). However, there was no difference in the frequency with which the Percoll step was required (p=0.792). Further, where Percoll was used, the initial cell yield prior to Percoll was similar with the new protocol compared to the original protocol (median 256×10^6 vs. 275×10^6, p=0.728) as was viability (median 19% vs. 18%, p=0.839), suggesting that the same criteria were applied for its use.

Disease	Original protocol	New Protocol
Biliary cirrhosis (primary biliary cirrhosis or primary sclerosing cholangitis	5	7
Alcoholic liver disease	6	8
Resected tissue	6	4
Normal	2	1
Donor tissue	8	5
Other	3	5

Table 3.2 Source of liver tissue for human hepatocyte isolation.

Liver tissue from a similar variety of diseases was used for hepatocyte isolation in each of the study groups with no significant difference in the range of diseases or number of samples between each group (p=0.77).

Factor	Original protocol	New Protocol	p-value
Age (Years)	57.5 (38.0 - 65.8)	56.5 (45.0 - 62.0)	0.997
Gender (Male)[#]	18 (60.0%)	17 (56.7%)	0.600
UKELD	56.5 (50.3 - 58.8)	54.5 (49.3 - 59.8)	0.494
MELD	18.0 (12.8 - 21.3)	14.5 (9.3 - 19.0)	0.254
Delay (Hours)	4.0 (2.0 - 19.0)	2.0 (1.0 - 3.0)	0.007*
Weight Of Wedge (g)	83.5 (58.3 - 125.1)	88.7 (67.5 - 107.3)	0.502

Table 3.3 Baseline factors of tissue used for human hepatocyte isolation.

Data are displayed as median (quartiles) with p-values from Mann-Whitney Tests to compare protocols, unless stated otherwise. Analyses were also repeated parametrically, where applicable, and returned comparable results. [#]Data displayed as n (%) and p-values from Fisher's Exact Tests. *Significant at $p<0.05$.

Factor	Original Protocol	New Protocol	p-value#
Perfusion Time (Min)	3.0 (2.5 - 6.1)	4.5 (4.0 - 6.0)	0.028*
Percoll Used[#]	13 (43.3%)	11 (36.7%)	0.792
Cell yield ($\times 10^6$)before Percoll	275 (77.5-474.8)	256 (115.0-317.5)	0.728
Viability before Percoll	18% (11.3%-32.5%)	19% (13.0%-26.0%)	0.839

Table 3.4 The effect of the isolation protocol on perfusion time and Percoll use.

Data for perfusion time are displayed as median (quartiles) with p-values from Mann-Whitney Tests to compare protocols, unless stated otherwise. Analyses were also repeated parametrically and returned comparable results. [#]Data displayed as n (%) and p-values from Fisher's Exact Tests. *Significant at $p<0.05$

3.2.3.2 The effect of protocol modification on the outcome of hepatocyte isolation

The next stage was to compare a number of key outcomes between the two protocols. For continuous variables, comparisons between the groups were performed using Mann-Whitney tests, with Fisher's Exact test used for binary outcomes. In addition to this, the analyses were repeated using general linear models or binary logistic regression, as appropriate, in order to adjust the p-values to account for the fact that the delay differed significantly between protocols. These results are summarised in Table 3.5. Initial viability and cell yield (before Percoll) and final viability and cell yield (following Percoll where it was used) are reported. No effect was found on the initial or final cell yield. However, the new protocol resulted in significant increases in both initial (p=0.007) and final (p=0.043) viability, with the median values rising from 10% to 25% and 48% to 70% respectively. In addition to this, the final viable cell yield/g tissue showed a significant increase (p=0.003), with the median rising from $7.3x10^4$ in the original protocol to $28.3x10^4$ in the new protocol. Success rates also increased significantly (p=0.037), from 12/30 (40.0%) under the original protocol, to 21/30 (70.0%) with the new protocol. After adjustment to account for the difference in the average delay between the protocols, the effect of the protocol on final viability ceased to be significant (p=0.063). However, all other variables found to be significant in univariate analysis remained so after adjustment for the delay.

The effect of protocol modification on hepatocyte isolation from liver tissue of different disease types is summarised in Table 3.6. There was an improvement in outcome for all liver tissue types but the greatest benefit was seen with ALD and resected liver tissue. For ALD liver, the median final viable cell yield increased to $9.83\ x10^4$ with the new protocol

from 0.26 $x10^4$ with the old protocol (p=0.04). The success rate also significantly improved to 63% using the new protocol whereas no isolations from ALD tissue performed using the old protocol resulting in maintenance of a confluent monolayer in culture for 48 hours (p=0.03). For resected tissue, the median final viable cell yield increased to 61.88 $x10^4$ with the new protocol from 9.48 $x10^4$ with the old protocol (p=0.02). The success rate increased from 33% to 100% although this did not reach statistical significance.

Factor	Original Protocol	New Protocol	Unadjusted p-value[#]	Adjusted p-value[§]
Initial Yield/g Tissue	105.1 (28.2 - 400.8)	125.0 (41.5 - 277.6)	0.775	0.872
Initial Viability	10.0% (0.0% - 31.3%)	25.0% (14.5% - 56.3%)	0.007*	0.015*
Final Cell Yield ($x10^6$)	29.0 (12.4 - 104)	42.5 (15.9 - 119.4)	0.478	0.236
Yield Cells/ g Tissue ($x10^4$)	27.5 (18.9 - 134.1)	48.5 (16.2 - 114.4)	0.654	0.289
Final Viability	48.0% (0.0% - 80.0%)	70.0% (23.2% - 83.1%)	0.043*	0.063
Final Viable Cell Yield /g Tissue ($x10^4$)	7.3 (0.0 - 20.5)	28.3 (8.2 - 70.2)	0.003*	0.001*
Success	12 (40.0%)	21 (70.0%)	0.037*	0.014*

Table 3.5 The effect of protocol modification on initial (before Percoll) and final (after Percoll) cell yield and viability and overall success.

Data are displayed as median (quartiles) for continuous variables, and n (%) for binary outcomes. [#]p-value from a Mann-Whitney or Fisher's Exact test for continuous and binary outcomes respectively. [§]p-value from a General Linear Model or Binary Logistic Regression, for continuous and binary outcomes respectively, after adjustment for the effect of the time delay. *Significant at $p<0.05$

Disease Type	Final Viable Cell Yield/g Tissue (x104)		Success	
	Old Protocol	New Protocol	Old Protocol	New Protocol
ALD	0.26 (0.00-0.90)	9.83* (3.51-31.61)	0%	63%*
Biliary Cirrhosis	10.59 (5.48-20.34)	29.12 (17.23-85.78)	60%	71%
Resected	9.48 (0.00-20.39)	61.88* (52.26-97.54)	33%	100%
Normal/Donor	11.52 (4.88-24.76)	16.57 (9.31-38.55)	60%	83%

Table 3.6 The effect of the isolation protocol on the final viable cell yield and success rate of hepatocyte isolations from different types of liver tissue.

Data are displayed as median (quartiles) for continuous variables. *Significant at $p<0.05$.

3.2.3.3 The function of hepatocytes isolated using the original and new protocols

Albumin and urea concentration were analysed in tissue culture supernatants from normal, PBC/PSC and ALD hepatocytes at days 1, 3, 5 and 7 in order to evaluate metabolic function of isolated hepatocytes. Albumin synthesis by hepatocytes isolated from normal, PBC/PSC or ALD liver tissue using the modified protocol was maintained for at least 1 week in culture (Figure 3.2). Underlying liver disease was found to have a significant effect on albumin synthesis ($p<0.001$, Table 3.7) with synthesis by normal hepatocytes significantly higher than both ALD and PBC/PSC (both $p<0.001$). There was no significant difference between the albumin measurements of ALD and PBC/PSC hepatocytes ($p=0.187$).

I next compared albumin synthesis by hepatocytes isolated using the new and original protocols (Table 3.8). There was no significant difference in albumin values for ALD tissue ($p=0.053$). However, for the other two tissue types, significant changes were detected. For hepatocytes isolated from normal tissue, the geometric mean albumin measurements increased from 220 under the old protocol to 379 with the new protocol ($p<0.001$). For PBC/PSC, on the other hand, the new protocol caused a significant reduction in albumin, from 226 under the old protocol, to 207 under the new one ($p=0.012$).

Urea synthesis by hepatocytes isolated from normal, PBC/PSC or ALD liver tissue using the modified protocol was maintained for at least 1 week in culture (Figure 3.3). Tissue type was found to have a significant effect on urea measurements ($p=0.002$, Table 3.9). The average ALD urea measurements were significantly lower than both normal ($p=0.006$) and PBC/PSC ($p=0.002$). There was no significant difference between the urea

measurements of normal and PBC/PSC hepatocytes (p=0.778). I next compared urea synthesis by hepatocytes isolated using the new and original protocols (Table 3.10). The results showed no significant difference in urea values between the two protocols for ALD tissue types (p=0.159). However, significant increases were brought about by the new protocol for both normal and PBC/PSC tissue (p=0.001 and 0.002 respectively).

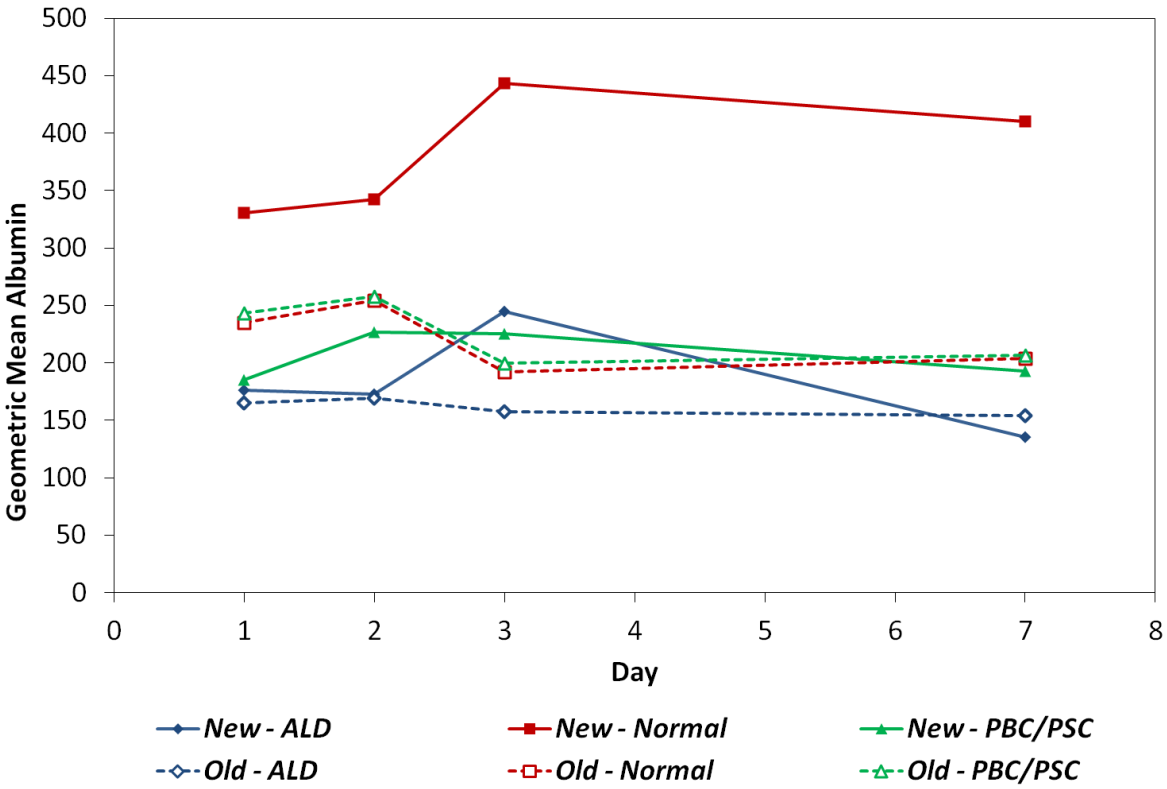


Figure 3.2 Albumin synthesis by primary human hepatocytes isolated from normal and diseased liver.

Albumin concentration in tissue culture supernatants was used as a measure of albumin synthesis by human hepatocytes in culture. Solid lines represent new protocol; broken lines represent original protocol. Data are displayed as the geometric mean of 3 samples taken at days 1, 2, 3 and 7 of culture.

Tissue Type	Geometric Mean (95% CI)	Tukey's HSD p-Value vs. ALD	Normal	PBC/PSC
ALD	176 (159-195)	-	<0.001*	0.187
Normal	368 (326-415)	<0.001*	-	<0.001*
PBC/PSC	199 (180-221)	0.187	<0.001*	-

Table 3.7 Albumin concentration in tissue culture supernatants from normal and diseased liver isolated using the new protocol.

Albumin concentration was used to compare albumin synthesis by human hepatocytes isolated from different types of liver tissue using the new protocol. Hepatocytes isolated from normal tissue synthesised significantly more albumin than those isolated from ALD or PBC/PSC tissue. *Significant at $p<0.05$.

	Geometric Mean (95% CI)		
Tissue Type	Original Protocol	New Protocol	p-value
ALD	161 (150-173)	178 (166-191)	0.053
Normal	220 (208-233)	379 (355-404)	<0.001**
PBC/PSC	226 (216-235)	207 (198-216)	0.012**

Table 3.8 Albumin concentration in tissue culture supernatants isolated using the new and original protocols.

Albumin synthesis by human hepatocytes isolated using the original and new protocols was compared. Albumin synthesis by hepatocytes isolated from normal tissue was significantly improved under the new protocol. There was also a trend towards an improvement in albumin synthesis by hepatocytes isolated from ALD tissue although those isolated from PBC/PSC tissue showed a small but significant decrease in albumin synthesis under the new protocol. **Significant after Bonferroni Correction for 3 comparisons ($p<0.0167$).

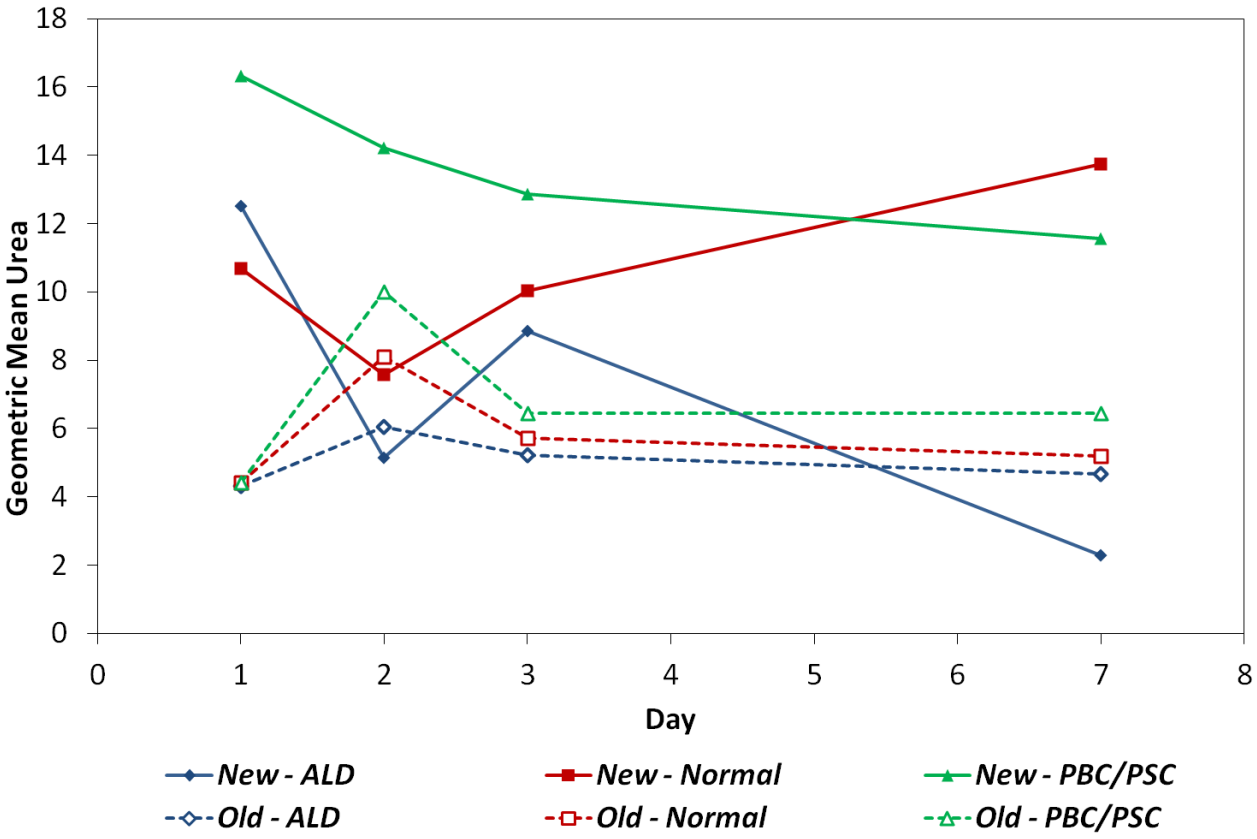


Figure 3.3 Urea synthesis by primary human hepatocytes isolated from normal and diseased liver.

Urea concentration in tissue culture supernatants was used as a measure of urea synthesis by human hepatocytes in culture. Solid lines represent new protocol; broken lines represent original protocol. Data are displayed as the geometric mean of 3 samples taken at days 1, 2, 3 and 7 of culture.

Tissue Type	Geometric Mean (95% CI)	Tukey's HSD p-Value vs. ALD	Normal	PBC/PSC
ALD	5.6 (4.3-7.2)	-	0.006*	0.002*
Normal	10.7 (8.3-13.8)	0.006*	-	0.778
PBC/PSC	12.0 (9.3-15.4)	0.002*	0.778	-

Table 3.9 Urea concentration in tissue culture supernatants from normal and diseased liver isolated using the new protocol.

Urea concentration was used to compare urea synthesis by human hepatocytes isolated from different types of liver tissue using the new protocol. Hepatocytes isolated from normal or PBC/PSC tissue synthesised significantly more urea than those isolated from ALD tissue. *Significant at $p<0.05$.

	Geometric Mean (95% CI)		
Tissue Type	Original Protocol	New Protocol	p-value
ALD	5.0 (4.1-6.1)	6.0 (5.0-7.3)	0.159
Normal	5.7 (4.7-6.7)	10.3 (8.8-12.1)	0.001**
PBC/PSC	6.5 (5.2-8.2)	13.6 (10.8-17.2)	0.002**

Table 3.10 Urea concentration in tissue culture supernatants isolated using the new and original protocols.

Urea synthesis by human hepatocytes isolated using the original and new protocols was compared. There was no significant difference for hepatocytes isolated from ALD tissue, but urea synthesis by hepatocytes isolated from normal and PBC/PSC tissue was significantly increased using the new protocol. **Significant after Bonferroni Correction for 3 comparisons ($p<0.0167$).

3.3 Discussion

Our group previously published a study of hepatocyte isolation from over 100 liver specimens of which 54% were from cirrhotic, end-stage liver diseases showed for the first time that viable functioning hepatocytes may be routinely isolated from diseased liver (Bhogal et al. 2011). In that series, our overall success rate was 51% with a median viability of 40%. Whilst we successfully isolated cells from all types of liver disease, we showed that ALD livers produced the poorest results and that cells isolated from ALD livers had inferior metabolic function in terms of albumin and urea synthesis. Further, we showed that time delay between hepatectomy/explant and the commencement of perfusion influenced the likelihood of success.

Whilst the results from our previous series were encouraging, the viability achieved fell short of that achieved by groups using more favourable tissue and in nearly half the cases the outcome was not successful. I therefore set about improving the viability and cell yield focussing on the use of Liberase and NAC as other studies in the literature suggested they may be of benefit. Donini et al (Donini et al. 2001) investigated the outcome of hepatocyte isolation from 14 porcine livers randomly assigned to standard collagenase or Liberase. Mean cell viability in that study was extremely high even without the use of Liberase (90%), most probably due to the fact that livers were retrieved from normal healthy pigs and perfused in situ with a cold preservation solution before immediate progression to hepatocyte isolation. Liberase significantly increased mean viability to 95% although there was no effect on cell yield. Sagias et al (Sagias et al. 2010) isolated hepatocytes from 10 severely steatotic (>60%) livers with two specimens from each liver being randomly assigned to standard collagenase digestion or the same digestion technique with the addition of NAC. Use of NAC significantly increased mean viability from 66%

to 81% as well as the mean viable cell yield from 1.10×10^6 cells/g tissue to 2.59×10^6 cells/g tissue.

I decided to combine the use of both Liberase and NAC at the same time in an attempt to optimise the impact on hepatocyte isolation. As liver tissue is in high demand within our laboratory for the isolation of various other cell types, I was unable to randomise tissue from the same liver to each of the two protocols. However, my approach has enabled me to produce the largest series to date investigating the use of Liberase and NAC and, to the best of my knowledge, the only series to use both in combination. Furthermore, I know of no other study describing the use of these reagents to isolate human hepatocytes from a wide range of diseased and cirrhotic livers. My data confirm that the use of Liberase and NAC in combination significantly improves the outcome of human hepatocyte isolation from normal and diseased liver in terms of both viable cell yield and overall success rate.

The time delay until the start of the isolation procedure was significantly shorter in the new protocol group and this is likely to reflect improvements that have been made to the arrangements for collecting liver specimens from the hospital and their subsequent processing. These improvements occurred during the period when I performed the first few isolations included in the study; the median delay to processing for the second half of the old protocol group (specimens 16-30) was 2 hours, the same as for the new protocol group. Despite this there was no trend to improved outcomes during the first 30 patients (old protocol), suggesting that an improvement in time delay alone was not responsible for the improved outcome with the new protocol. This is further demonstrated by the fact that even after adjusting for this difference in time delay between the two groups the outcomes were still significantly improved. Similarly, the lack of any trend towards improving

outcomes within the first 30 isolations suggests that learning curve was not an important factor in the observed improvements in the success of hepatocyte isolation.

The enzyme perfusion time required for digestion with the new protocol was slightly longer than with the old protocol. Perfusion time is not pre-determined, rather perfusion continues until adequate tissue digestion is achieved. As such it is a function of both the nature of the liver tissue being digested and the specific enzyme cocktail being used. Although there was no significant difference in the range of liver disease types used between the two groups overall, there was possibly more cirrhotic liver in the new protocol group (slightly more biliary cirrhosis and ALD liver). I also suspect that Liberase has a gentler action than standard crude collagenase preparations and may require a longer time to act. Indeed, other authors have reported longer digestion times for other tissues such as pancreas (Georges et al. 2002) and ovary (Dolmans et al. 2006) when using Liberase compared to standard collagenase.

The decision to use Percoll is somewhat subjective and in my experience always results in a loss of cells over and above what would be expected given the initial cell viability and yield. Therefore I only use this step when there are a large number of cells with very low viability. The fact that Percoll use was similar between the two groups suggests I was consistent in the application of criteria for its use and that differential use of Percoll does not account for the improvement seen with the new protocol.

The fact that I have shown an improvement in overall success rate, which I defined as the maintenance of cell adhesion and morphology for 48 hours, as well as viable cell yield is important. Many experimental protocols require cells to be plated down and maintained in culture for a period of time and so an increased yield of viable cells which then fail to

attach in culture would be of limited benefit. Further, hepatocytes are known to undergo anoikis or detachment-related cell death (Pinkse et al. 2004;Rosner et al. 2002), and so failure to attach will quickly render isolated hepatocytes useless. Even if used immediately for cell transplantation, it seems likely that such cells would be less able to survive and engraft (Zvibel et al. 2002). It has been shown that establishment of a confluent monolayer is critical for maintenance of the differentiated hepatocyte phenotype (Greuet et al. 1997) and so I used the presence of absence of a confluent monolayer, rather than attempting to quantify adhesion, in order to determine success. A 48 hour culture period was a pragmatic choice that would be likely to provide sufficient time for downstream experiments or use of the hepatocytes for transplantation without the loss of phenotype associated with prolonged culture.

It is interesting that the new protocol did not result in an increase in total cell yield. The previous studies of NAC or Liberase in hepatocyte isolation also reported no increase in absolute cell yield (Donini et al. 2001) or only reported an increase in viable cell yield (Sagias et al. 2010). It is therefore likely that the beneficial effects of both these agents are exerted mainly through an improvement in viability, and indeed, it is difficult to see how the antioxidant and hepatoprotective actions of NAC might improve absolute cell yield. Obtaining a high cell yield depends on sufficient tissue digestion, and I continue enzyme perfusion until the liver can be easily manually dissociated rather than setting a fixed perfusion time for all types of liver. I believe that this approach already maximises the absolute cell yield obtained, but that the continued exposure of the hepatocytes to enzymes during this time is detrimental. Therefore it is likely that an improved enzyme preparation that is less damaging to individual cells will improve final viability and viable cell yield but not the overall number of cells obtained.

In this study I confirmed our previous findings that hepatocytes isolated from ALD liver generally have poorer metabolic function compared to those isolated from normal or PBC/PSC liver. This may be due to the fact that many ALD patients had advanced cirrhosis or it may reflect toxicity effects secondary to alcohol. The metabolic function of hepatocytes isolated using the new protocol was similar to that of hepatocytes isolated using the original protocol for ALD hepatocytes and generally improved for normal and PBC/PSC hepatocytes. Whilst PBC/PSC hepatocytes showed a lower average albumin synthesis, this result is perhaps explained by the higher albumin values for PBC/PSC hepatocytes isolated using the original protocol during the first 48 hours of culture; by day 7 the values for both groups were similar. There are of course many other aspects of hepatocyte function that I have not investigated and I have not attempted to show maintenance of the complete hepatocyte phenotype in culture. However, these results are encouraging as they suggest that the improved overall success rate and viable cell yield do not occur at the expense of metabolic function. It is also of note that the greatest improvement in metabolic function was seen in hepatocytes isolated from normal liver tissue, and it is these that are most likely to be used for clinical purposes and are preferred for laboratory investigation.

In conclusion, I have shown that the combined use of NAC and Liberase for the isolation of human hepatocytes from normal and diseased liver results in a higher success rate and viable cell yield. Furthermore, metabolic function is maintained for up to 1 week in culture and is improved compared to hepatocytes isolated without NAC and Liberase. The routine use of NAC and Liberase should greatly increase the availability of primary human hepatocytes both for research and clinical applications.

CHAPTER 4: EXPRESSION OF ADHESION MOLECULES AND CHEMOKINE RECEPTORS BY PRIMARY HUMAN HEPATOCYTES AND HEPATOCYTE CELL LINES

4.1 Introduction

During hepatocyte transplantation, the transplanted cells enter the liver sinusoids from where they must cross the sinusoidal endothelium in order to engraft within the liver parenchyma. Part of this process may be purely mechanical, whereby the relatively large hepatocytes become lodged within the small diameter sinusoids. However, there is evidence from animal models that a specific interaction between the transplanted hepatocytes and the sinusoidal endothelial cells occurs. Therefore understanding the mechanisms involved in the adhesion of hepatocytes to HSEC may uncover a potential target for manipulation in order to improve engraftment.

4.1.1 Adhesion Molecules

Cell adhesion molecules (CAMs) are proteins present on the surface of cells that are able to interact with ligands on the surface of other cells or with extracellular matrix proteins thereby regulating cellular adhesion. Most CAMs are transmembrane proteins with cytoplasmic, transmembrane and extracellular domains. The cytoplasmic domain interacts with the cell cytoskeleton and the extracellular domain binds to other cell adhesion molecules or extracellular matrix. There are several families of CAMs including integrins, cadherins, selectins, and the immunoglobulin superfamily cell adhesion molecules (Albelda and Buck 1990). Integrins are a large family of receptors which are particularly important in mediating cell-ECM interactions and will be discussed further. Cadherins are widely expressed molecules mainly located in adherens junctions that form calcium-dependent homophilic cell-cell interactions and are crucial for tissue morphogenesis during development (Halbleib and Nelson 2006). They may be further divided into several subgroups. The classical cadherins include E-cadherin (found in epithelial tissue), N-cadherin (found on neurons) and P-cadherin (found in the placenta). The other subgroups

include the desmosomal cadherins, protocadherins and atypical cadherins (Nollet et al. 2000). Selectins are carbohydrate-binding molecules found on endothelial cells (E-selectin), leukocytes (L-selectin) and platelets (P-selectin) which are involved in cellular trafficking in the immune system (Ley 2003). Adhesion molecules of the immunoglobulin superfamily include neural cell adhesion molecule (NCAM), activated leukocyte cell adhesion molecule (ALCAM), intercellular cell adhesion molecule-1 (ICAM-1), platelet endothelial cell adhesion molecule (PECAM) and vascular cell adhesion molecule 1(VCAM-1) (Wai et al. 2012). They are characterised by the presence of immunoglobulin-like domains and mediate calcium-independent adhesion through binding to other immunoglobulin-like domains of the same structure on adjacent cells (homophilic adhesion) or through interaction with integrins and carbohydrates (heterophilic adhesion) (Barclay 2003).

I decided to concentrate on investigating integrin expression as integrins form a large group of adhesion molecules which are expressed on many cell types. Previous studies reported that hepatocytes express a small number of functional integrins. Animal experimentation suggests that integrins may have a role in hepatocyte engraftment into the liver during transplantation and it has previously been shown that recruitment of lymphocytes to the liver is dependent on molecules such as VCAM-1 and ICAM-1 which are expressed by HSEC and which bind integrins. In addition I also decided to investigate chemokine receptor expression. Chemokines are small 8-12kd cytokines that bind to GPR couple receptors to trigger migraton responses and activation of integrin mediated adhesion through intergrin clustering and conformational activation. They play a crucial role in the recruitment of leucocytes to tissues including the liver as well as metastasis of tumour cells.

4.1.1.1 Integrins

The integrins are a superfamily of adhesion molecules which are expressed on almost every cell type and mediate cell-cell and cell-matrix interactions (Hogg and Leitinger 2001;Sonnenberg 1993). Integrins are αβ heterodimeric type 1 transmembrane receptors. In humans there are 18α and 8β subunits which combine to form 24 different heterodimers (Humphries et al. 2006;Takada et al. 2007). Integrins may become activated through clustering on the cell surface, which increases the avidity of molecular interactions, or through conformational changes leading to increased affinity for the ligand (Campbell and Humphries 2011).

The α- and β-integrin subunits have distinct domain structures, with the extracellular domains of each subunit contributing to the ligand binding site. The sequence arginine-glycine-aspartic acid (RGD) has been identified as a general integrin-binding motif, but different integrins also have specific protein ligands. These may be extracellular matrix proteins such as collagen or fibronectin, or other adhesion molecules such as VCAM-1 or ICAM-1 (Humphries et al. 2006;Takada et al. 2007).

Integrin binding not only supports cell adhesion but is also crucial for embryonic development, tissue repair, host defence and haemostasis (Harburger and Calderwood 2009). The linkage between the short cytoplasmic integrin domains and the intracellular cytoskeleton allows transmission of force across the plasma membrane (Calderwood et al. 2000;Evans and Calderwood 2007). Integrins are able to transmit chemical signals from outside into the cell (termed outside-in signalling) and are also able to regulate their affinity for extracellular ligands via conformational alterations in response to signals impinging upon the cytoplasmic tails (termed inside-out signalling) (Calderwood 2004;Hynes 2002;Miranti and Brugge 2002).

4.1.1.2 Chemokine receptors

Chemokines are a group of at least 46 small (8-14kDa) structurally related molecules expressed by a variety of cell types. Their name derives from their ability to induce chemotaxis (chemotactic cytokines) and they play a pivotal role in inflammatory processes and immunity (Zlotnik et al. 2006;Zlotnik and Yoshie 2000). Chemokines may be divided into two major groups based on the presence (CXC chemokines) or absence (CC chemokines) of an amino acid between two N-terminal cysteine residues. Two further classes of chemokines have also been described: CX3C has 3 amino acids between the first two N terminal cysteine residues and C chemokines which lack cysteine residues one and three (Zlotnik and Yoshie 2000). Chemokines function through binding to G-protein coupled receptors that have seven transmembrane spanning domains. Binding of chemokines to chemokine receptors leads to dissociation of the coupled heterotrimeric G proteins into α and $\beta\gamma$ subunits. These G protein subunits act as second messengers leading to calcium influx and activation of protein kinases. Effects include the activation of chemotaxis through actin-dependent processes, upregulation of adhesion molecules and a wide range of other functions (Horuk 2001). Chemokine receptors are named in a similar manner to chemokines according to the type of chemokine they bind.

Thus in this chapter I investigate the surface expression of integrins and chemokine receptors by primary human hepatocytes.

4.2 Results

4.2.1 Optimisation of Methods for Flow Cytometric Analysis of Primary Human Hepatocytes and Hepatocyte Cell Lines

In order to optimise my protocols for flow cytometric analysis of human hepatocytes and hepatocyte cell lines I initially looked at the surface expression of a small panel of common integrin subunits that I felt were likely to be expressed. This included integrins that have been previously reported to be expressed by hepatocytes, which are expressed by other epithelial cell types or have been shown to be important in the adhesion of other non-leukocyte cells in the liver. This included the following integrins: α1, α2, α3, α4, α5, α6, α9 β1, αVβ3, αVβ5, β1, β2, β3, β4. Antibodies to individual integrin subunits were used when available. Figure 4.1 shows representative side scatter/forward scatter plots of Huh 7.5 cells with high surface expression of β1 integrin detected using a PE-conjugated antibody.

For the analysis of primary human hepatocytes a viability marker was also used in light of my previous findings regarding cell viability (Table 3.1) to ensure that non-viable cells were not included. Representative flow cytometry plots showing high surface expression of β1 integrin by primary human hepatocytes are shown in Figure 4.2. The percentage surface expression and MFI values for the integrins listed above for Huh 7.5 and Hep G2 hepatoma cell lines are shown in Figure 4.3 and for primary human hepatocytes in Figure 4.4.

Integrin expression by primary human hepatocytes was limited with only α1 and β1 subunits expressed at high levels. There were also low levels of α2 and α9 but this was somewhat variable between experiments The hepatoma cell lines expressed a slightly

wider range of integrins on their surface with moderate to high expression of α2, β4 and αVβ5 in addition to α1 and β1.

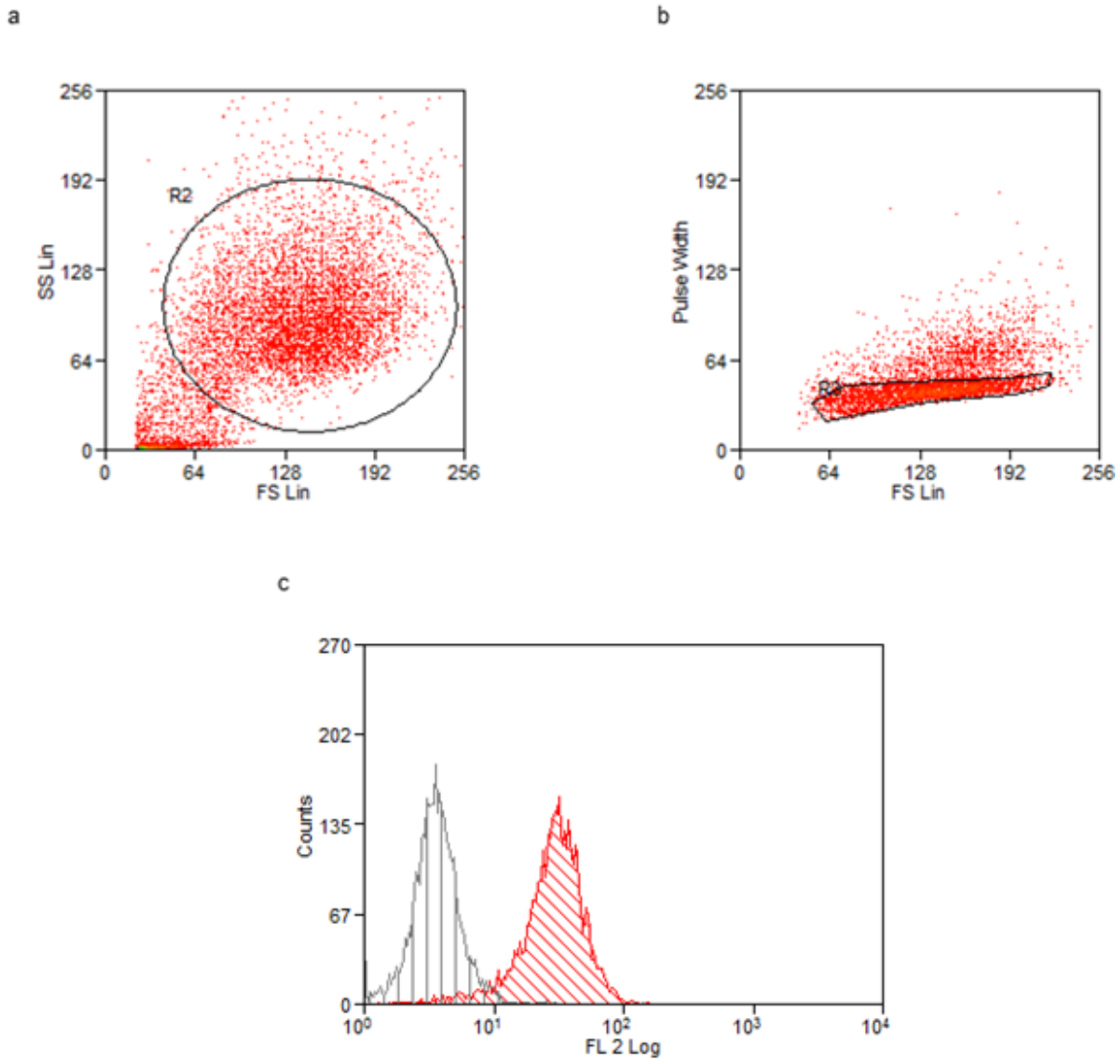


Figure 4.1 Typical flow cytometric analysis of surface expression of integrins by hepatoma cell lines showing β1 integrin expression by Huh 7.5 cells

Illustrative plots for Huh 7.5 cells are shown: (a) typical side scatter/forward scatter plot with gate around cell population; (b) gating on pulse width to exclude doublets; (c) β1 integrin (red peak) compared to IgG isotype control (grey peak).

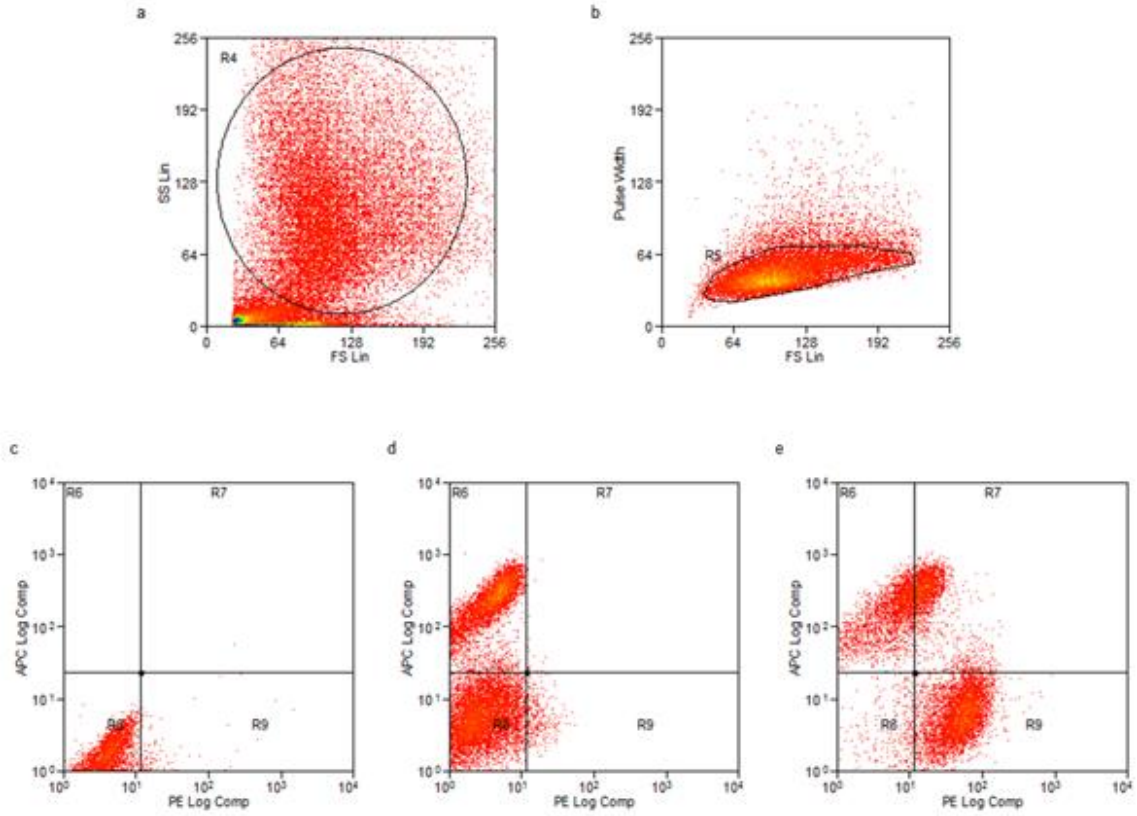
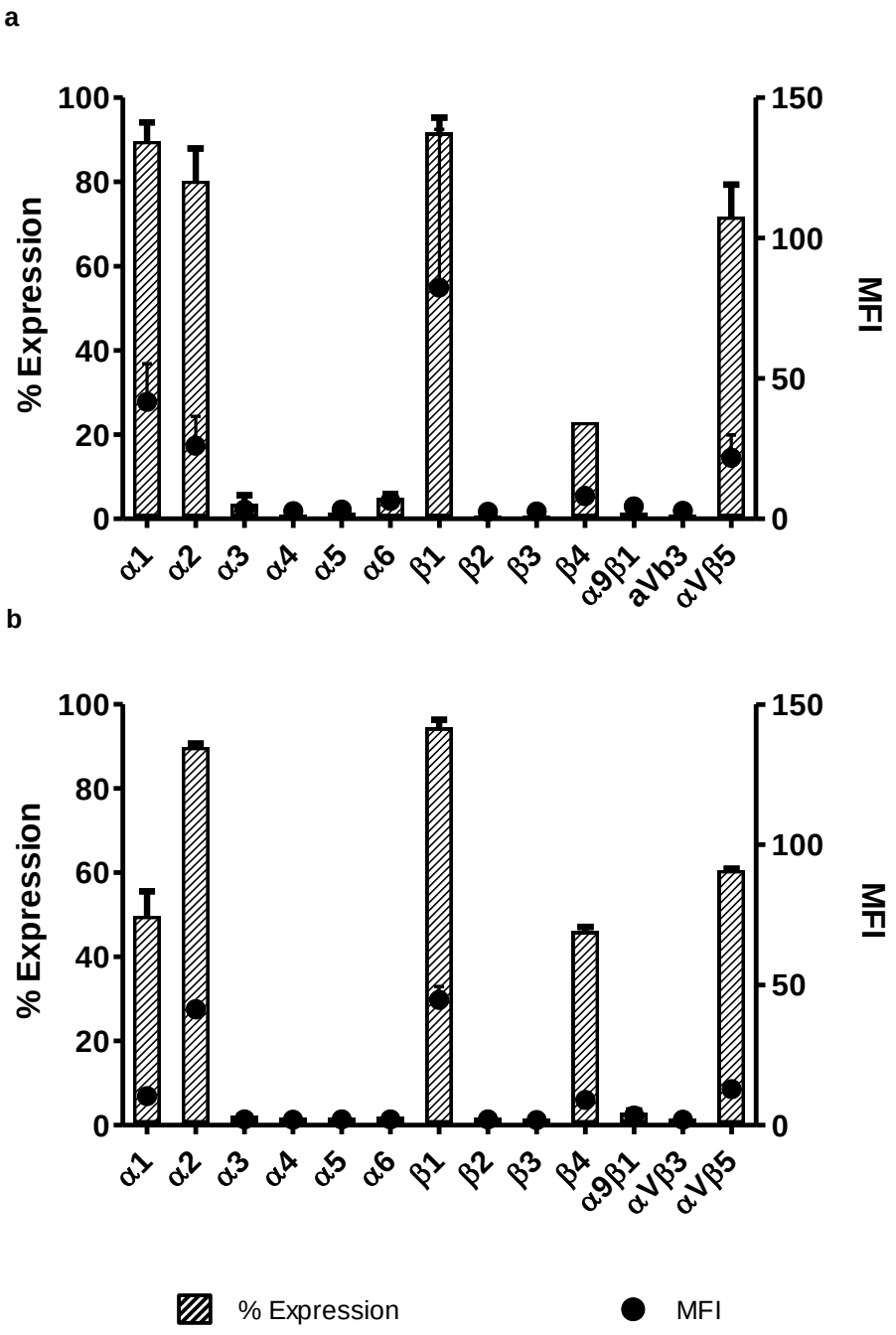


Figure 4.2 Flow cytometric analysis of integrin surface expression by freshly isolated primary human hepatocytes.

Illustrative plots are shown: (a) typical side scatter/forward scatter plot; (b) gating on pulse width to exclude doublets; (c) PE-conjugated IgG isotype control; (d) cells labelled with the viability marker only showing two distinct populations with dim (viable) and bright (dead) fluorescence in the APC channel; (e) cells labelled with the viability marker and a PE-conjugated anti-β1 integrin antibody.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β2
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β4
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αVβ3
αVβ5
% Expression
MFI

Figure 4.3 Optimisation of flow cytometric analysis of integrin expression by Huh 7.5 and Hep G2 cell lines.

This graph shows the expression of a limited panel of integrins by (a) Huh 7.5 and (b) Hep G2 cells. Data shown are % expression and median fluorescence intensity for indicated integrin subunits. Data expressed as the mean and standard error of 3 experiments.

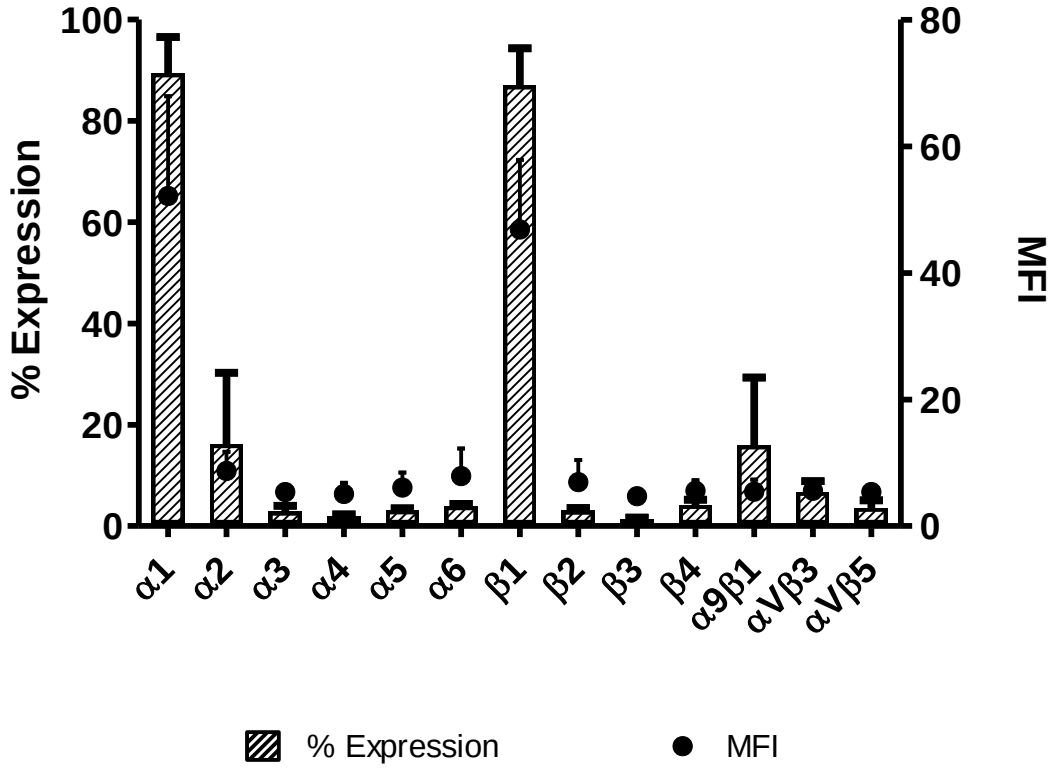


Figure 4.4 Flow cytometric analysis of integrin expression by primary human hepatocytes.

This graph shows the expression of a limited panel of integrins by primary human hepatocytes. Data shown are % expression and median fluorescence intensity for indicated integrin subunits. Data expressed as the mean and standard error of at least 3 experiments.

4.2.2 Surface Expression of Adhesion Molecules/Chemokine Receptors on Primary Hepatocytes

Following the initial optimisation experiments, freshly isolated primary human hepatocytes were analysed for surface expression of a wider range of integrin subunits including α7, α8, α10, α11, αIIb, αL, αM, αV, αVβ6, and β7. These were chosen as previous literature suggests that they are potential partners of the subunits already identified as being expressed during the optimisation experiments or because they bind ligands/ECM components known to be expressed by the liver or HSEC. In addition, a new antibody to α5 was used as it had become evident from other work in our laboratory that the previously used antibody was not working. Hepatocytes were also analysed for the surface expression of chemokine receptors CCR 1-10 and CXCR 1-7. Staining was performed with PE conjugated antibodies where available or an unconjugated antibody followed by a PE conjugated secondary antibody. Antibody-labelled samples were compared with respective IgG controls. A far red viability marker (detected on the APC channel) was also included to ensure that only viable cells were analysed.

4.2.2.1 Surface expression of integrin subunits

Primary human hepatocyte integrin surface expression data is shown in Figure 4.5. Primary hepatocytes express high levels of α1 (89%; MFI 52), αV (77%; MFI 20) and β1 (87%; MFI 47), moderate expression of α5 (30%; MFI 12), α7 (44%; MFI 20), α10 (39%; MFI 30), α11 (39%; MFI 37) and αM (54%; MFI 17) and low levels of α2 (16%; MFI 9), αIIb (13%; MFI 7), α9β1 (16%; MFI 5) and αVβ3 (7%; MFI 6) .

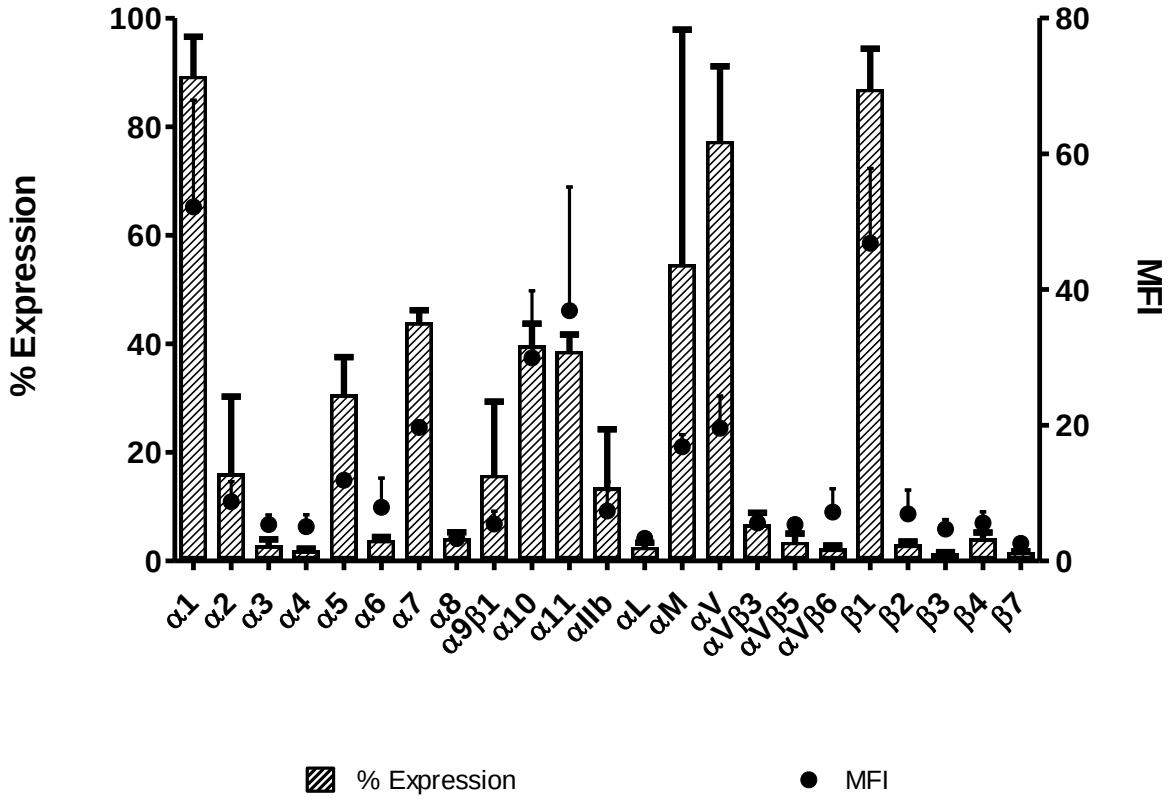


Figure 4.5 Flow cytometric analysis of integrin surface expression by primary human hepatocytes.

Following initial optimisation experiments, primary human hepatocytes were analysed for expression of a large panel of integrin subunits. Data shown are the mean ± standard error of at least 3 experiments expressed as % expression and median fluorescence intensity.

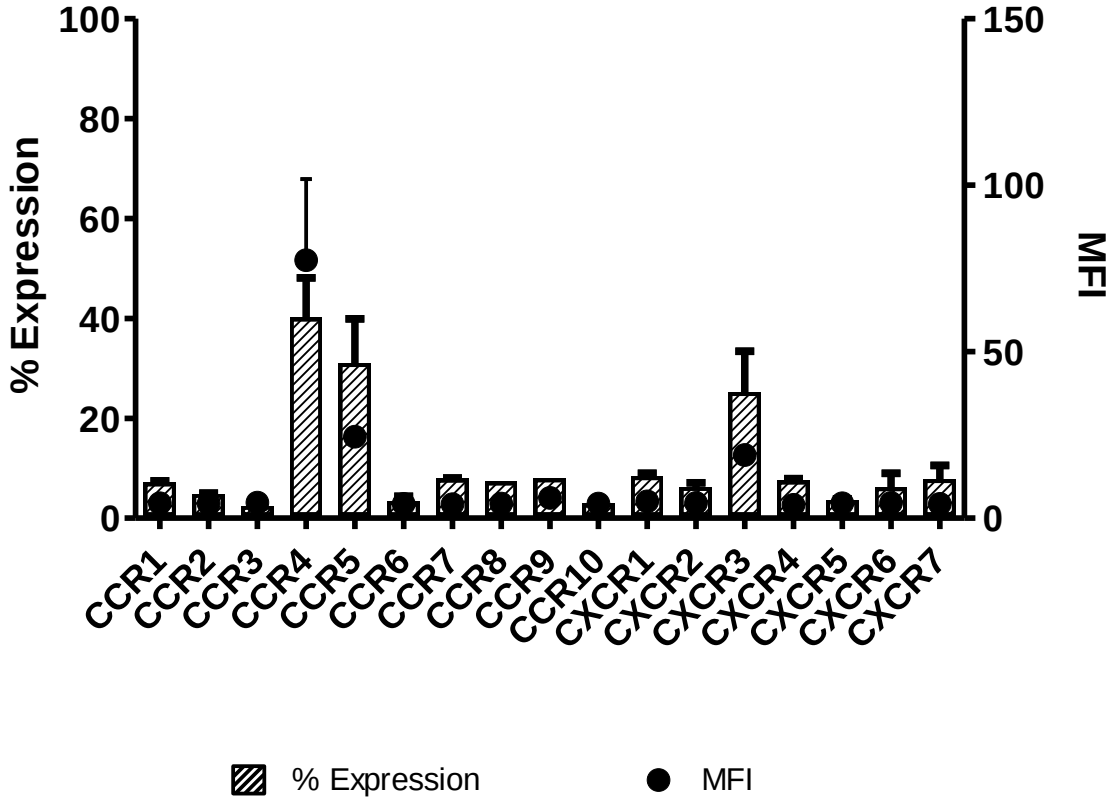


Figure 4.6 Flow cytometric analysis of chemokine receptor surface expression by primary human hepatocytes.

Chemokine receptor expression by primary human hepatocytes was analysed by flow cytometry. Data shown is the mean ± standard error of at least 3 experiments expressed as % expression and median fluorescence intensity.

4.2.2.2 Surface expression of chemokine receptors

Primary human hepatocyte chemokine receptor surface expression data is shown in Figure 4.6. Chemokine receptor expression by primary human hepatocytes was limited with only moderate expression of CCR4 (40%; MFI 77), CCR5 (31%; MFI 24) and CXCR3 (25%; MFI 19).

4.2.3 Surface Expression of Adhesion Molecules/Chemokine Receptors on Hepatoma Cell Lines

Huh 7.5 and Hep G2 cells were stained for surface expression of integrin subunits and chemokine receptors using PE conjugated antibodies where available or an unconjugated antibody followed by a PE conjugated secondary antibody. Antibody labelled samples were compared with respective IgG controls.

4.2.3.1 Surface expression of integrin subunits

Both cell lines expressed a similar repertoire of integrins to primary human hepatocytes, with α1, α2, α7, α10, α11, αIIb, αM, αV, αVβ5, β1 and β4 and integrins expressed. Whilst the same integrins were expressed by both cell lines, the levels of expression differed slightly. Huh 7.5 cells expressed high levels of α1 (89%; MFI 42), α2 (80%; MFI 26), α10 (81%; MFI 17), αIIb (97%; MFI 47), αV (100%; MFI 167), β1 (91%; MFI 82) and αVβ5 (72%; MFI 22), moderate levels of α7 (44%; MFI 44) and α11 (54%; MFI 15) and low levels of α5 (24%; MFI 9), (αM (26%; MFI 8) and β4 (23%; MFI 8) (Figure 4.7). Hep G2 cells expressed high levels of α2 (90%; MFI 41), α10 (62%; MFI 22) αIIb (92%; MFI 52), αV (85%; MFI 154) and β1 (94%; MFI 45), moderate levels of α1 (50%; MFI 10), α7 (41%; MFI 44), α11 (52%; MFI 16), αV β5 (60%; MFI 13) and β4 (46%; MFI 9) and low levels of α5 (20%; MFI 7) and αM (23%; MFI 10) (Figure 4.8).

4.2.3.2 Surface expression of chemokine receptors

As with integrin expression, hepatocyte cell lines expressed a wider range of chemokine receptors than primary hepatocytes but expression levels varied between the two different cell lines. Huh 7.5 cells expressed high levels of CCR4 (95%; MFI 47), moderate levels of CCR 5 (35%; MFI 44), CCR10 (30%; MFI 21) and CXCR3 (27%; MFI 18) and low levels of CCR3 (19%; MFI 22) and CXCR1 (10%; MFI 21) (Figure 4.9). Hep G2 cells expressed moderate levels of CCR4 (49%; MFI 90), CCR5 (32%; MFI 28) and CXCR3 (24%; MFI 15) and low levels of CCR3 (6%; MFI 3), CCR9 (6%; MFI 3) and CCR10 (18%; MFI 15) (Figure 4.10).

4.2.4 Adhesion Molecule Gene Expression by Primary Human Hepatocytes and Huh 7.5 Cells

RNA was obtained from primary human hepatocytes isolated from normal liver tissue and Huh7.5 cells. cDNA was synthesised and subjected to RT-PCR using a TaqMan Custom Array. A list of genes investigated and the adhesion molecules which they encode is shown in

Table 4.1. Primary human hepatocytes expressed all the genes tested (Figure 4.11) while Huh7.5 gene expression was more limited with no expression of ITGA3, ITGA4, ITB2, ITGAD, ITGAX, HEPACAM, CLEC4M, CD209 or SELP (Figure 4.12).

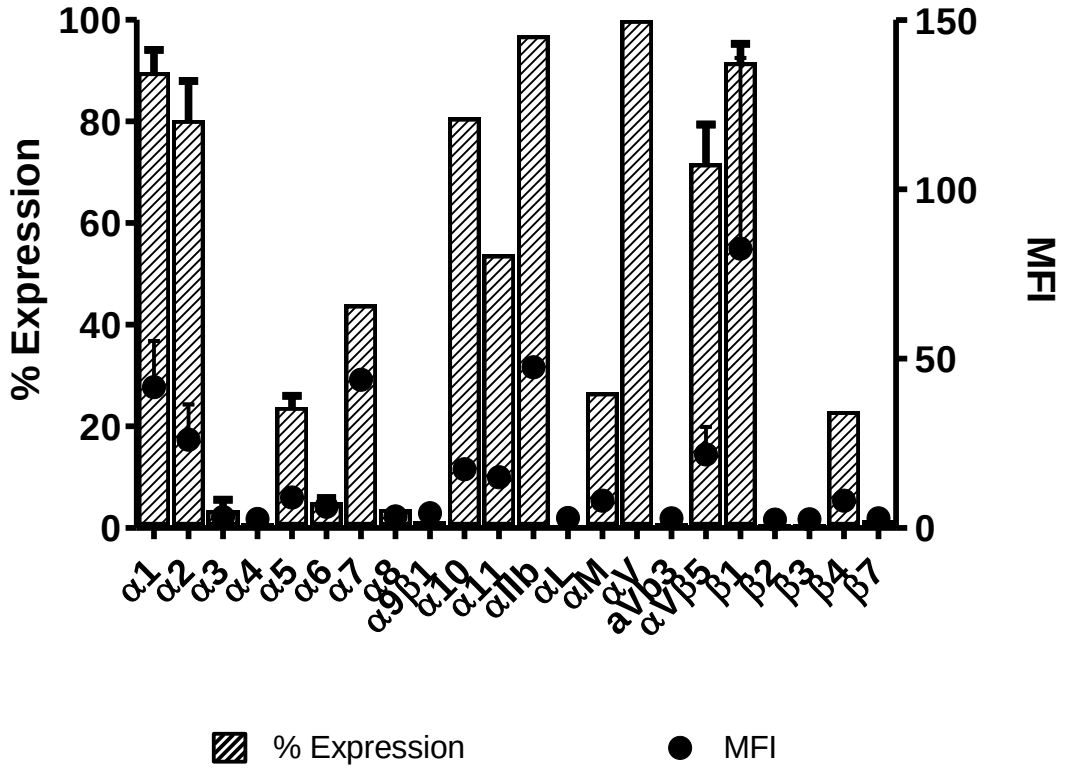


Figure 4.7 Flow cytometric analysis of integrin surface expression by Huh 7.5 cells.

Following initial optimisation experiments, Huh 7.5 cells were analysed for expression of a large panel of integrin subunits. Data shown is the mean ± standard error of at least 3 experiments expressed as % expression and mean fluorescence intensity.

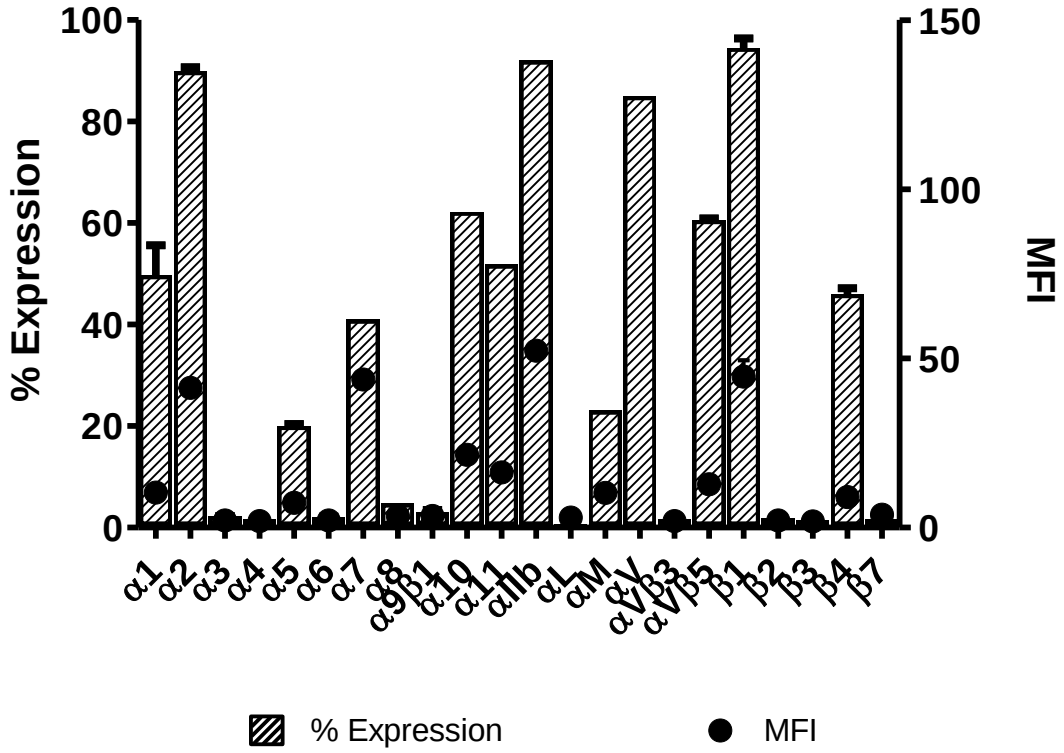


Figure 4.8 Flow cytometric analysis of integrin surface expression by Hep G2 cells.

Following initial optimisation experiments, Hep G2 cells were analysed for expression of a large panel of integrin subunits. Data shown is the mean ± standard error of at least 3 experiments expressed as % expression and mean fluorescence intensity.

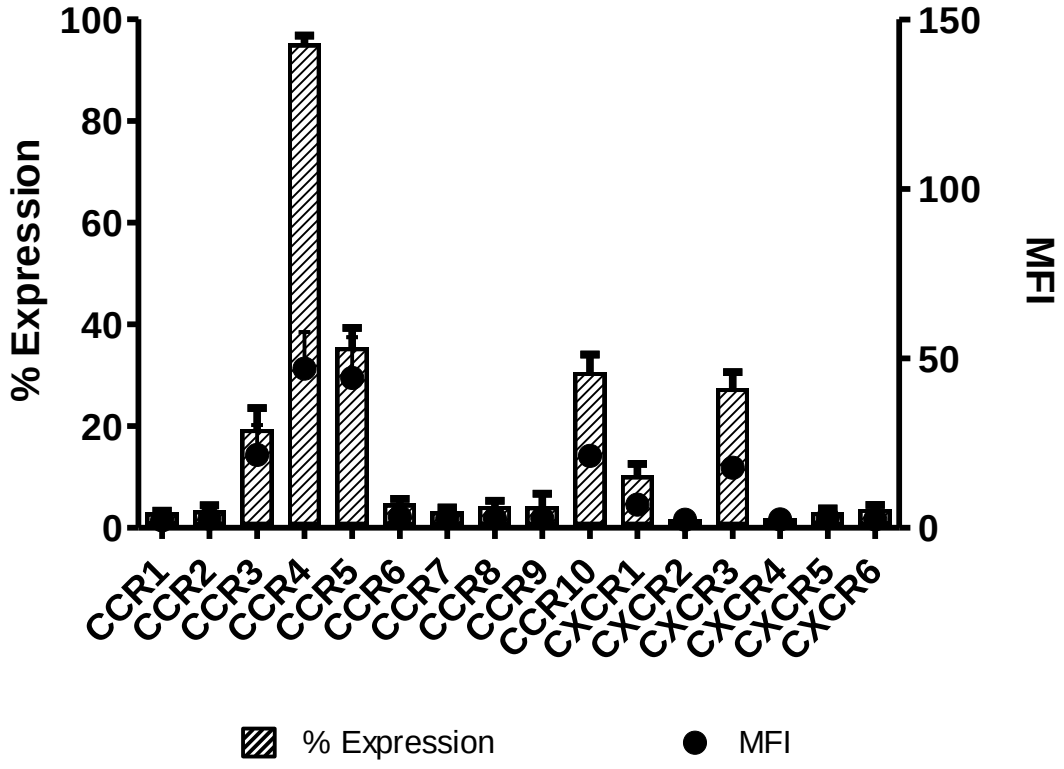


Figure 4.9 Flow cytometric analysis of chemokine receptor surface expression by Huh 7.5 cells.

Chemokine receptor expression by Huh 7.5 cells was analysed by flow cytometry. Data shown is the mean ± standard error of at least 3 experiments expressed as % expression and mean fluorescence intensity.

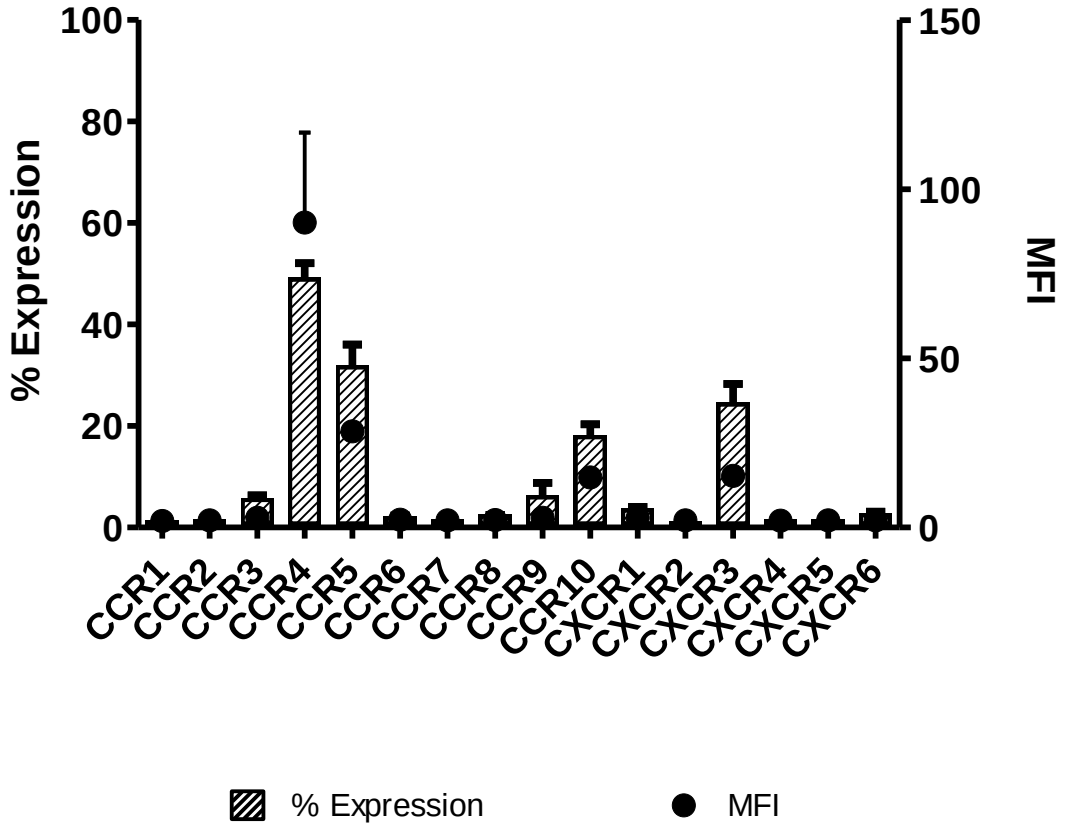


Figure 4.10 Flow cytometric analysis of chemokine receptor surface expression by Hep G2 cells.

Chemokine receptor expression by Hep G2 cells was analysed by flow cytometry. Data shown is the mean ± standard error of at least 3 experiments expressed as % expression and mean fluorescence intensity.

Gene	Molecule
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase (housekeeping gene)
ITGA1	α1 integrin
ITGA2	α2 integrin
ITGA3	α3 integrin
ITGA4	α4 integrin
ITGA5	α5 integrin
ITGA6	α6 integrin
ITGA7	α7 integrin
ITGA8	α8 integrin
ITGA9	α9 integrin
ITGA10	α10 integrin
ITGA11	α11 integrin
ITGB1	β1 integrin
ITGB2	β2 integrin
ITGB3	β3 integrin
ITGB4	β4 integrin
ITGB5	β5 integrin
ITGB6	β6 integrin
ITGB7	β7 integrin
ITGB8	β8 integrin
ITGAL	αL integrin
ITGAM	αM integrin
ITGAD	αD integrin
ITGAX	αX integrin
ITGAV	αV integrin
ITGAE	αE integrin
ITGA2B	αIIb integrin
EPCAM	epithelial cell adhesion molecule
HEPACAM	hepatocyte cell adhesion molecule 1
HEPACAM2	hepatocyte cell adhesion molecule 2
BCAM	basal cell adhesion molecule

Gene	Molecule
VCAM1	vascular cell adhesion molecule 1
PECAM1	platelet/endothelial cell adhesion molecule 1
ICAM1	intercellular adhesion molecule 1
ICAM2	intercellular adhesion molecule 2
ICAM3	intercellular adhesion molecule 3
ICAM4	intercellular adhesion molecule 4
AOC3	vascular adhesion protein 1 (VAP-1)
CD44	CD44
CDH1	epithelial cadherin (E-cadherin)
CDH2	neural cadherin (N-cadherin)
CDH17	liver-intestine cadherin (LI-cadherin)
CLEC4M	liver/lymph node-specific intercellular adhesion molecule-3-grabbing non-integrin (L-SIGN)
CD209	dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN)
SELP	P-selectin

Table 4.1 List of adhesion molecule genes investigated by PCR of human hepatocyte and Huh 7.5 cDNA.

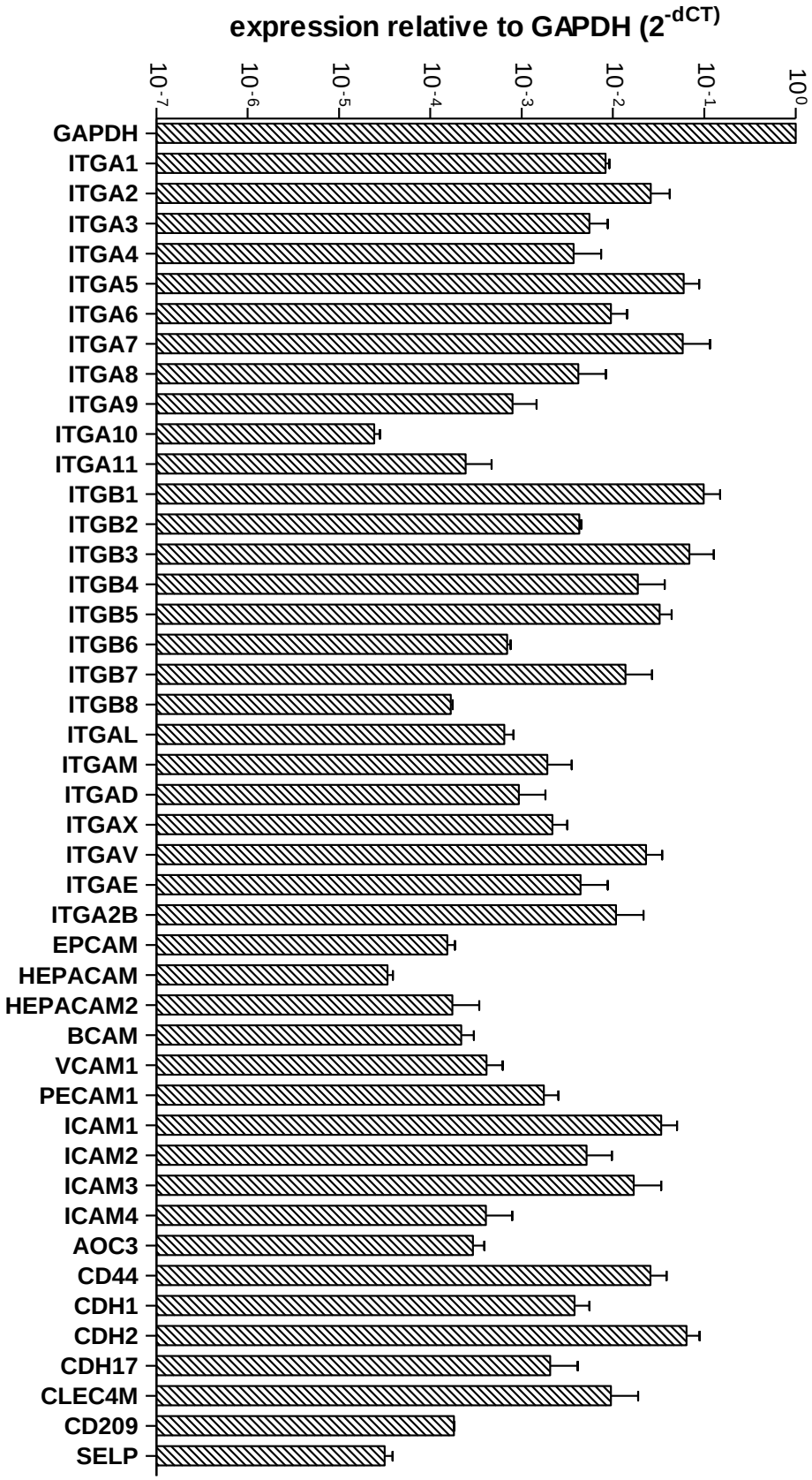

expression relative to GAPDH (2-dCT)
10-7
10-6
10-5
10-4
10-3
10-2
10-1
100
GAPDH
ITGA1
ITGA2
ITGA3
ITGA4
ITGA5
ITGA6
ITGA7
ITGA8
ITGA9
ITGA10
ITGA11
ITGB1
ITGB2
ITGB3
ITGB4
ITGB5
ITGB6
ITGB7
ITGB8
ITGAL
ITGAM
ITGAD
ITGAX
ITGAV
ITGAE
ITGA2B
EPCAM
HEPACAM
HEPACAM2
BCAM
VCAM1
PECAM1
ICAM1
ICAM2
ICAM3
ICAM4
AOC3
CD44
CDH1
CDH2
CDH17
CLEC4M
CD209
SELP

Figure 4.11 PCR analysis of adhesion molecule gene expression by primary hepatocytes.

Gene expression by primary human hepatocytes isolated from normal liver tissue for a variety of adhesion molecules was analysed by PCR and compared to GAPDH. Data are expressed as the mean and SE of 3 experiments.

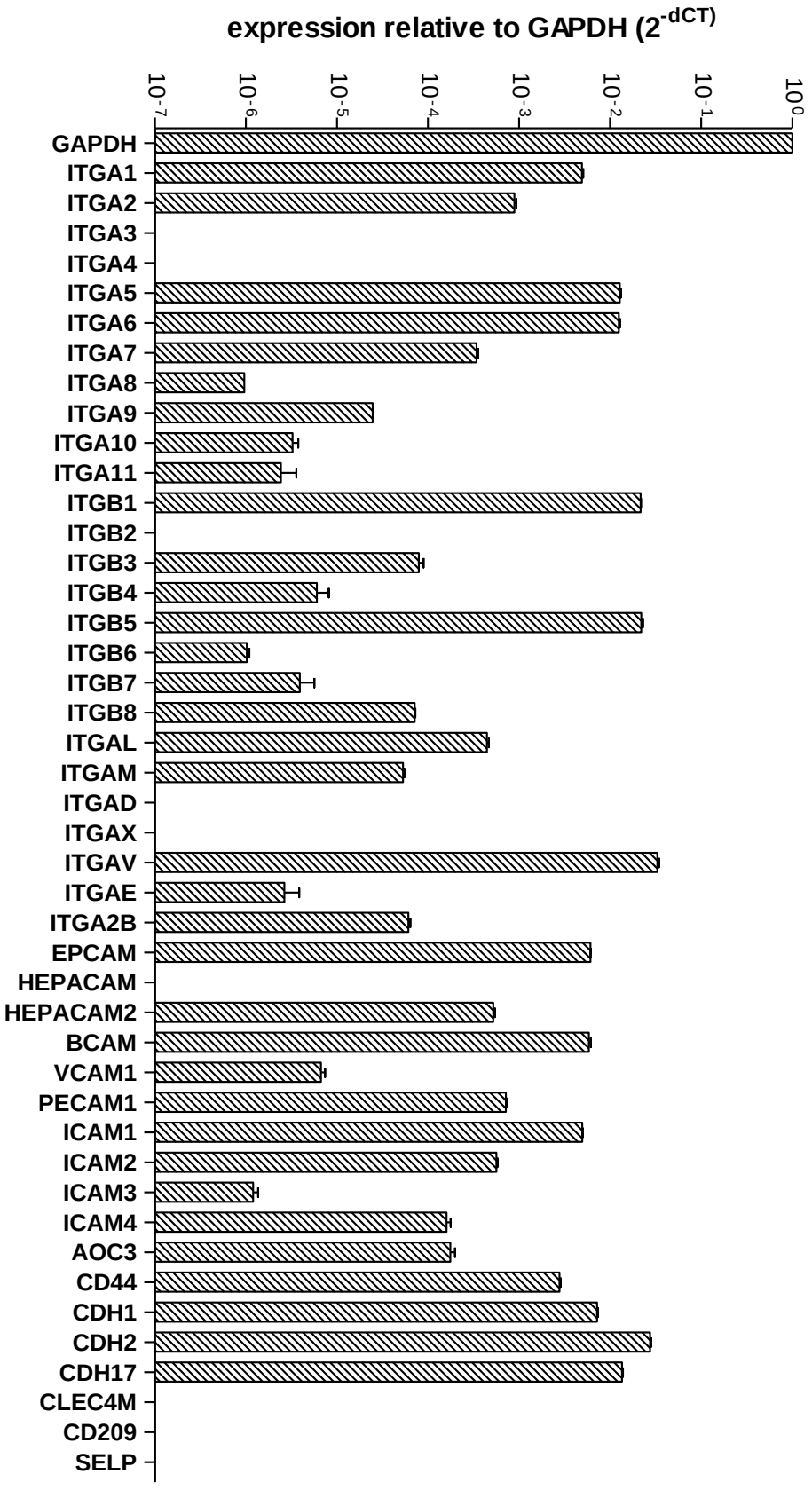

expression relative to GAPDH (2-dCT)
10-7
10-6
10-5
10-4
10-3
10-2
10-1
100
GAPDH
ITGA1
ITGA2
ITGA3
ITGA4
ITGA5
ITGA6
ITGA7
ITGA8
ITGA9
ITGA10
ITGA11
ITGB1
ITGB2
ITGB3
ITGB4
ITGB5
ITGB6
ITGB7
ITGB8
ITGAL
ITGAM
ITGAD
ITGAX
ITGAV
ITGAE
ITGA2B
EPCAM
HEPACAM
HEPACAM2
BCAM
VCAM1
PECAM1
ICAM1
ICAM2
ICAM3
ICAM4
AOC3
CD44
CDH1
CDH2
CDH17
CLEC4M
CD209
SELP

Figure 4.12 PCR analysis of adhesion molecule gene expression by Huh 7.5 cells

Gene expression by Huh 7.5 for a variety of adhesion molecules was analysed by PCR and compared to GAPDH. Data are expressed as the mean and SE of 3 experiments.

4.3 Discussion

4.3.1 Integrin expression by primary human hepatocytes isolated from normal and diseased human liver

There is currently little data in the literature concerning integrin expression by human hepatocytes, with the majority of work investigating hepatocyte adhesion having been performed using rat or murine cells. However the few studies that have focussed on human tissue suggest that human hepatocytes express only a limited repertoire of integrin subunits. Couvelard et al (Couvelard et al. 1998) investigated the changes in integrin expression during liver development using immunohistochemistry. Foetal hepatocytes stained strongly for the β1 subunit with moderate staining for α1, α5, α6 and α9. However, mature hepatocytes only showed weak staining for the β1 and α1 subunits and moderate staining for α5 and α9. Staining for the α6 subunit was lost altogether after 16-25 weeks gestation (Couvelard et al. 1998). Several studies have investigated changes in integrin surface expression by hepatocytes in diseased liver and shown that expression of some integrin subunits may be upregulated compared to normal liver. Nejjari et al examined liver tissue from patients with chronic hepatitis C and demonstrated increased expression of α1, α5 and β1 integrins and de novo induction of α2 and α6 expression. The α9 subunit was also present but levels were similar in normal and diseased liver (Nejjari et al. 2001). Other studies have shown de novo expression of α2 and α6 in cirrhosis and hepatocellular carcinoma (HCC) (Le et al. 1997), and α3 and α6 in inflammatory liver diseases (Volpes et al. 1991).

My results confirm that human hepatocytes are able to express α1, α2, α5, α9 and β1 integrins at varying levels although I was not able to demonstrate significant expression of α6 integrin. However, I demonstrate a much wider range of integrin expression on

hepatocytes than previously described in the published literature. In particular, in addition to high levels of α1 and β1 subunits, αV was also very highly expressed. There was also expression of α7, α10, α11, αIIb and αM integrins which, to the best of my knowledge, has not been previously described. The αV subunit is able to dimerise with several β subunits including β1 forming αVβ1, a fibronectin receptor (Zhang et al. 1993). Whilst αVβ5 was also highly expressed by cell lines, it was not expressed by primary hepatocytes, nor were αV αVβ3 or αVβ6. α7 integrin is expressed mainly in skeletal and cardiac muscle and combines to form α7β1 integrin which is a laminin receptor (Mayer et al. 1997). α10 and α11, along with α1 and α2, dimerise with β1 forming collagen receptors (Springer and Dustin 2012). Integrin αIIb is only known to associate with β3 forming a fibrinogen receptor which is expressed on platelets and plays a crucial role in coagulation (Bledzka et al. 2013). The αM subunit associates with β2, known as complement receptor 3 (CR3), and is a leukocyte integrin with a wide range of ligands including several ECM components (Li 1999). The presence of these subunits on the surface of both primary hepatocytes and hepatocyte cell lines in the absence of the corresponding β subunits has a number of possible explanations. It is possible that the αIIb and αM subunits are both also able to associate with β1 but that this is yet to be reported. Alternatively, it may be that, for some reason, certain cells express integrin subunits which do not have a partner or that hepatocytes do indeed express β2 and β3 integrin but that these particular subunits are especially sensitive to the isolation process or the process of removing cells from culture. Finally, it is possible that the antibodies used are not specific and generated falsely positive results.

There are several reasons why my results may differ from those of previous studies. Firstly, the majority of studies published to date used frozen or fixed tissue sections and

immunohistochemical staining. The process of enzymatic dissociation of liver tissue, the isolation of hepatocytes and their subsequent handling may have an effect on surface expression of adhesion molecules causing either upregulation of expression or cleaving the molecule from the cell surface resulting in loss of expression. Such effects have been demonstrated in our laboratory with other cells such as mesenchymal stem cells (Garg et al. 2014) The analysis of a suspension of a single cell type by flow cytometry is also likely to produce slightly different results to those obtained by studying staining on whole liver sections.

The use of primary human hepatocytes for the purpose of hepatocyte transplantation necessitates their isolation from liver tissue. The method of delivery is likely to be as a cell suspension of freshly isolated, cultured or cryopreserved hepatocytes. Therefore the expression data obtained from freshly isolated cells/cells recently removed from culture are likely to reflect the situation in which hepatocytes would be used in clinical practice and are therefore relevant for investigating the adhesive mechanisms involved in engraftment of transplanted hepatocytes.

4.3.2 Chemokine receptor expression by primary human hepatocytes isolated from normal and diseased liver

Under normal physiological conditions hepatocytes are not required to home to the liver from the circulation, and so there is unsurprisingly little in the literature regarding hepatocyte migration to the liver and the potential role of chemokines and chemokine receptors.

Hepatocyte migration has, however, been studied in the context of liver regeneration. For example, Ma et al showed that following injury to a hepatocyte monolayer, basic fibroblast

growth factor (bFGF) promotes wound repair by stimulating proliferation and migration of hepatocytes (Ma et al. 1999). Furthermore, following hepatocyte transplant into the spleen, hepatocytes migrate to the liver where they integrate into hepatic parenchyma (Ponder et al. 1991). However, such studies have not demonstrated a definite role for chemokine receptors.

There are several studies in the literature describing the expression and role of particular chemokine receptors by hepatocytes. CXCR1 and CXCR2 are thought to be involved in the regulation of hepatocyte proliferation and regeneration following ischaemia/reperfusion injury (Clarke et al. 2011;Kuboki et al. 2008) and HIV can cause hepatocyte death by signalling through CXCR4 (Vlahakis et al. 2003). Interestingly I found none of these receptors on primary hepatocytes or cell lines, except for low levels of CXCR1 expression by Huh 7.5 cells. Similarly, the receptors that were expressed on my primary human hepatocytes (CCR4, CCR5 and CXCR3) have not been reported in the literature. These chemokine receptors are associated with lymphocytes with CCR4 expressed at higher levels on Th2 lymphocytes and some regulatory T cells while CCR5 and CXCR3 are expressed by memory effector T cells and Th1 lymphocytes (Bonecchi et al. 1998). One possible explanation for this previously unreported expression is that the freshly isolated hepatocytes were contaminated by other cell types including lymphocytes that survived the isolation process. However, this does not explain why these receptors were also expressed by the cell lines, and if the hepatocytes used for analysis were indeed contaminated then one might expect expression of other lymphocyte-associated chemokine receptors. Another explanation as suggested with integrin expression is that the isolation process and/or underlying liver disease alter chemokine receptor expression. Further, it is quite possible that nobody has actually investigated chemokine receptor expression by

human hepatocytes and so the presence of CCR4, CCR5 and CXCR3 on the surface of hepatocytes has gone unnoticed until now.

4.3.3 Integrin and chemokine receptor expression by hepatocyte cell lines

The hepatoma cell lines expressed a similar range of integrins to primary hepatocytes with the addition of αVβ5 and β4 which were not expressed at all by primary cells. Similarly, both cell lines expressed the same chemokine receptors as primary cells but with the addition of CCR3, CCR10 (both cell lines), CXCR1 (Huh 7.5) and CCR9 (HepG2).

Both cell lines differed somewhat from primary hepatocytes in terms of chemokine receptor expression. Whilst the integrin expression of both cell lines was similar, Huh 7.5 cells most closely reflected the levels of expression by primary hepatocytes. Furthermore, I found Huh 7.5 cells easier to handle and maintain in culture compared to HepG2 cells which tended to form clumps and were less suited to further planned experiments. I therefore decided to select the Huh7.5 hepatoma cell line for all further preliminary functional studies, where necessary, in place of hepatocytes.

4.3.4 Adhesion molecule gene expression

I analysed hepatocyte gene expression for a wide range of different adhesion molecules including the full repertoire of known integrin subunits as well as various CAMs, cadherins and selectins all of which are known to be expressed by other epithelial cell types, have ligands known to be expressed in the liver or by HSEC or are involved in the recruitment of other cell types into the liver. The gene expression profile of Huh 7.5 cells was different to that of primary hepatocytes in that several genes were not expressed by Huh 7.5 and those that were expressed were generally expressed at a lower level than in primary cells. These differences are likely to reflect the difference between primary cells

and a cell line but may also be a result of contamination by other cell types of the primary cells which were freshly isolated from whole liver tissue.

The integrin gene expression data backs up my surface expression data, and although I have not been able to investigate all of the other adhesion molecules further, this data provides a useful resource for future studies. Ideally to gain a complete picture of the adhesion molecule profile of primary human hepatocytes flow cytometric analysis for surface expression as well as gene expression analysis would be performed for all candidate adhesion molecules as well as chemokine receptors.

CHAPTER 5: DEFINING THE FUNCTIONAL ROLE OF ADHESION MOLECULES EXPRESSED BY HUMAN HEPATOCYTES IN THEIR ADHESION TO LIVER TISSUE AND SINUSOIDAL ENDOTHELIUM *IN VITRO* AND *IN VIVO*

5.1 Introduction

During hepatocyte transplantation, hepatocytes arriving in the liver must travel from the sinusoidal blood across the sinusoidal endothelium in order to engraft within the liver parenchyma. As discussed in the main introduction, the exact mechanisms underlying this process so far remain unclear. It is possible that hepatocytes interact with HSEC through cell-cell adhesion interactions modulated by specific adhesion molecules. Alternatively, it is also possible that hepatocytes become mechanically lodged within the sinusoid with subsequent damage to or remodelling of the endothelium and exposure of the underlying matrix to which they are able to adhere. As discussed in section 1.4.4, evidence from animal studies suggest that specific interactions with HSEC may be at least partly involved with both integrin-mediated and non-integrin mediated adhesion pathways likely to be important.

Integrins are crucial for leukocyte recruitment to liver through binding to ligands such as VCAM-1 and ICAM-1 expressed on HSEC (Adams and Eksteen 2006;Lalor et al. 2002;Shetty et al. 2008) and our group has recently demonstrated a role for β1 integrin in the recruitment of human mesenchymal stem cells (MSCs) to injured liver (Aldridge et al. 2012). Chemokine receptors are also essential for leukocyte migration and, as discussed previously, may have a role in the recruitment of epithelial cells including breast cancer metastases and melanoma to tissue.

Having demonstrated surface expression of a range of integrins and chemokine receptors by primary human hepatocytes I therefore decided to concentrate the next stage of my investigation on their possible role in hepatocyte engraftment from the sinusoid into the liver. As the β1 integrin subunit was the only β subunit demonstrated at high levels on the surface of primary hepatocytes, I decided to focus on this subunit as well as α1 and αV

integrins due to the similarly high levels of expression. Because I also detected chemokine receptors on primary human hepatocytes at low to moderate levels I decided to investigate their role in adhesion also. In order to quickly ascertain any role for chemokine receptors I decided to use pertussis toxin (PTX) to inhibit G-protein signalling resulting from activation of chemokine receptors (Goldman et al. 1985).

5.2 Results

5.2.1 Static Adhesion of Hepatocytes to Normal and Diseased Liver Tissue and Extracellular Matrix Components

5.2.1.1 Adhesion to liver tissue

The adhesion of Huh 7.5 cells to normal and various types of diseased liver tissue was compared using modified Stamper-Woodruff assays. Adhesion to liver parenchyma and portal tract areas was quantified separately. The mean number of cells adhering to parenchymal and portal tract areas of different types of liver tissue is shown in Figure 5.1. There was no significant difference in the mean number of cells adherent per field of view between the various diseases, nor between parenchymal and portal areas of the same disease.

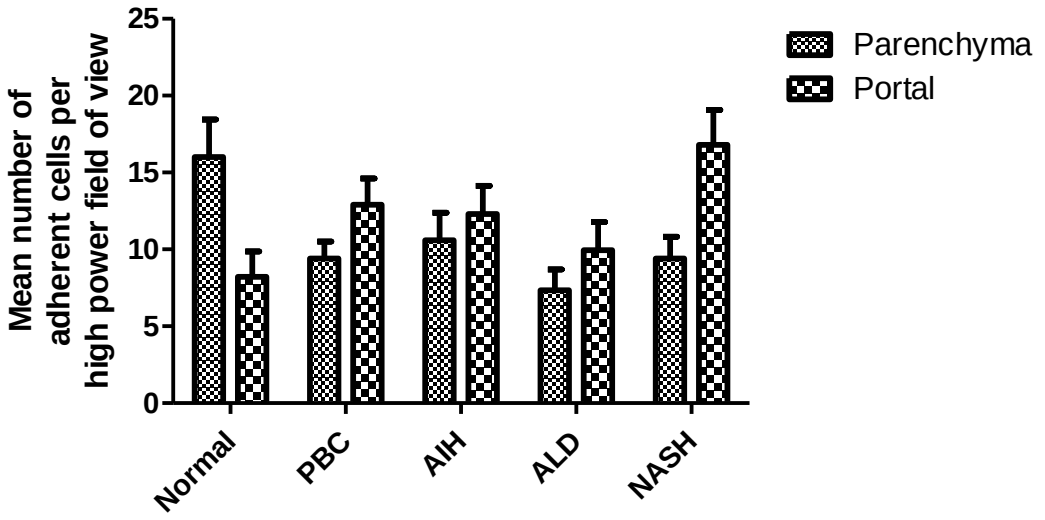


Figure 5.1 Static adhesion of Huh 7.5 cells to liver tissue in a modified Stamper Woodruff assay.

The adhesion of Huh 7.5 cells to parenchymal and portal tract areas of different types of liver tissue is shown. The data are shown as the mean number of cells adherent per high power field of view and is the mean and standard error of 4 experiments.

5.2.1.2 The effect of integrin blockade on the adhesion of hepatocytes to liver tissue

Function blocking antibodies to α1 and β1 integrins, the two major subunits expressed by primary human hepatocytes, were used to investigate their contribution to adhesion of Huh 7.5 cells to liver (Figure 5.2). Blockade of the α1 integrin subunit significantly reduced adhesion (percentage of IgG control) to normal (46.6% ± 9.1, p=0.01), PBC (47.4% ± 12.0, p=0.02), ALD (30.6% ± 3.2, p=0.0002) and NASH (40.6% ± 6.0, p=0.002) liver parenchyma. Adhesion to portal tract areas was also significantly reduced in ALD (26.8% ± 7.6, p=0.002) and AIH (26.8% ± 9.4, p=0.004) livers with α1 integrin blockade. Blockade of the αV integrin subunit significantly reduced adhesion to normal (47.1% ± 6.0, p=0.003), AIH (41.2% ± 10.2, p=0.01) and ALD (36.5% ± 10.2, p=0.01) liver parenchyma and normal (48.6% ± 10.9, p=0.02) and NASH (51.5 ± 13.2, p=0.04) portal tract areas. Blockade of the β1 integrin subunit had little effect except on AIH liver where adhesion to parenchyma was significantly reduced (45.7% ± 8.4, p=0.008).

5.2.1.3 The effect of chemokine receptor blockade using PTX on the adhesion of hepatocytes to liver tissue

Huh 7.5 cells were incubated with PTX to block chemokine receptor signalling in order to establish any role for chemokine receptors in the adhesion of hepatocytes to liver tissue (Figure 5.3).

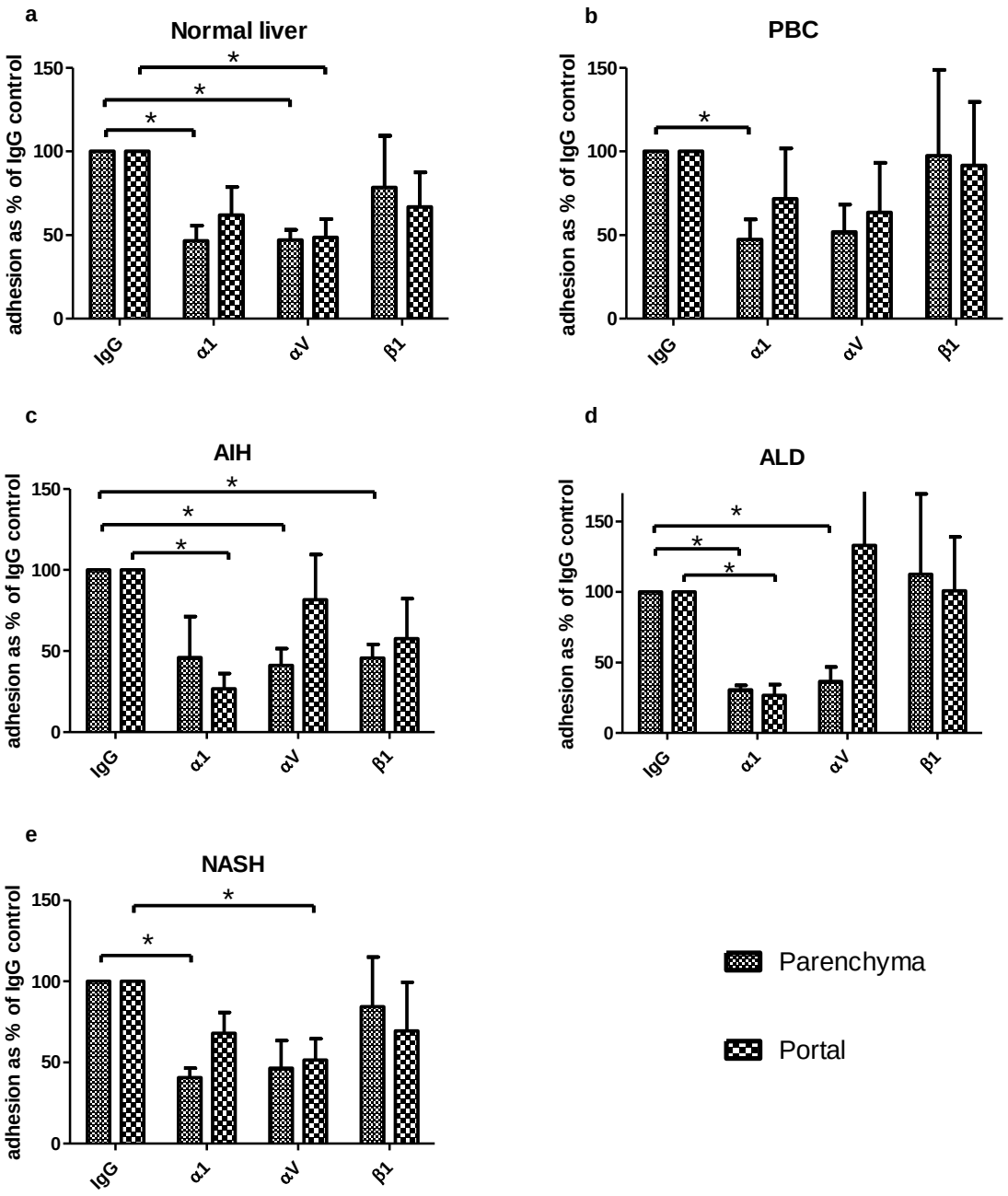

a
Normal liver
adhesion as % of IgG control
150
100
50
0
IgG
α1
αV
β1
b
PBC
c
AIH
d
ALD
e
NASH
Parenchyma
Portal

Figure 5.2 Static adhesion assay of Huh7.5 to liver tissue with integrin blockade.

This figure shows the effect of α1, αV and β1 integrin blockade on the adhesion of Huh 7.5 cells to normal and diseased liver tissue. Adhesion is expressed as % of IgG control. Data shown are the mean and standard error of 4 experiments. * $P<0.05$ paired two-tailed t test.

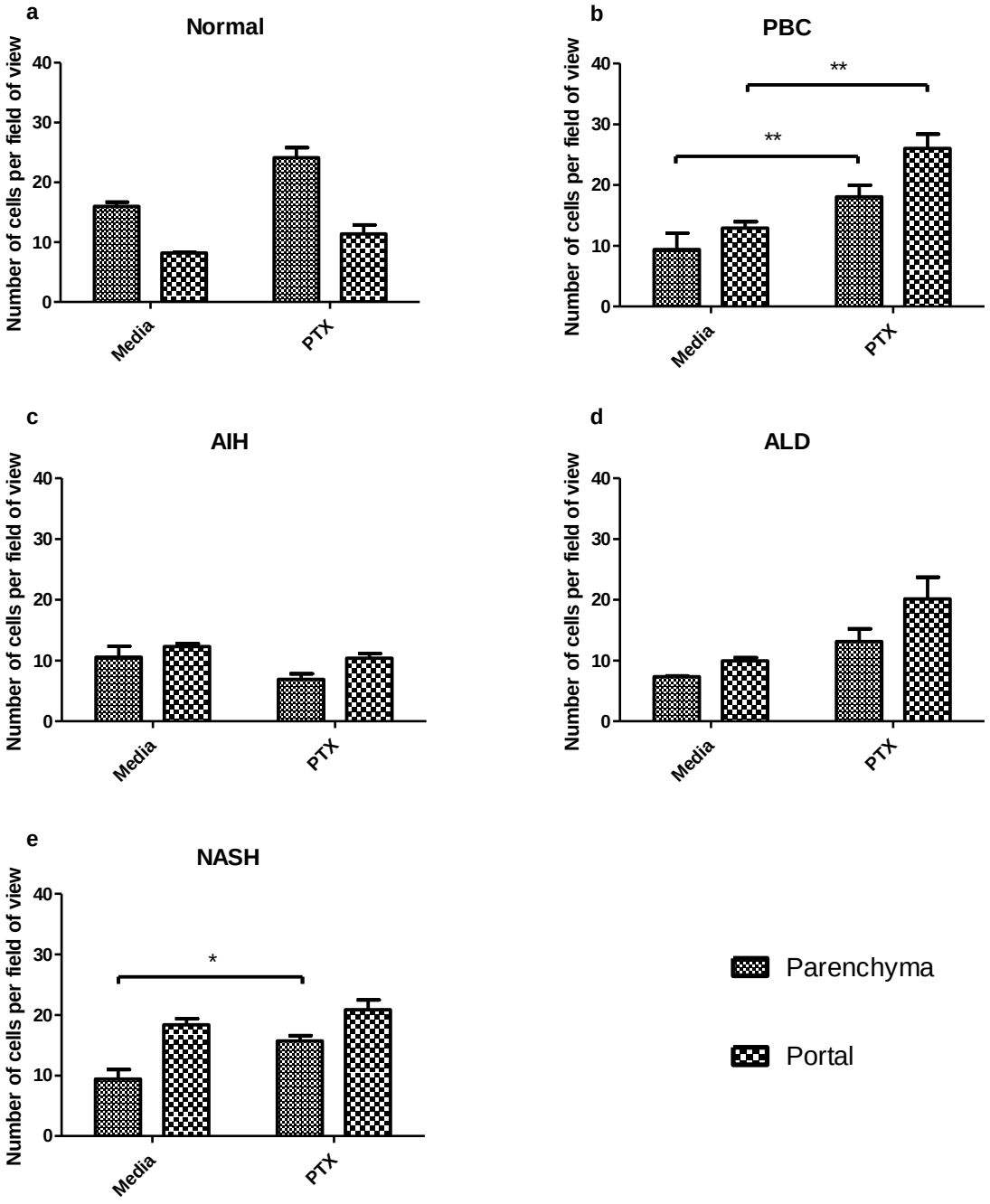

a
Normal
Number of cells per field of view
40
30
20
10
0
Media
PTX
b
PBC
**
**
c
AIH
d
ALD
e
NASH
*
Parenchyma
Portal

Figure 5.3 Static adhesion assay of Huh7.5 to liver tissue with PTX treatment

This figure shows the effect of PTX treatment on the adhesion of Huh 7.5 cells to normal and diseased liver tissue. Adhesion is expressed as the mean and standard error number of cells per field of view for Huh 7.5 treated with PTX and media only (control). * $p<0.05$, **$p<0.01$ paired two-tailed t test.

PTX treatment did not reduce the adhesion of Huh 7.5 cells to any of the normal or diseased tissue. However, for PBC tissue PTX treatment resulted in an increase in the mean number of cells bound per field of view compared to media only treated cells from 9.4 ± 2.7 to 18.1 ± 1.9 in parenchymal areas (p=0.008) and 12.9 ± 1.1 to 26.1 ± 2.3 in portal tract areas (p=0.009). Similarly, PTX treatment increased the mean number of adherent cells bound to parenchymal areas of NASH liver from 9.4 ± 1.6 to 15.7 ± 0.9 (p=0.02).

5.2.1.4 Adhesion to extracellular matrix

The adhesion of Huh 7.5 cells and primary hepatocytes to extracellular matrix components was investigated using a fluorometric assay with binding to matrix proteins compared to a BSA control. For Huh 7.5 cells there was a statistically significant increase in binding to collagen types I, II and IV, fibronectin, laminin, tenascin and vitronectin compared to control but no significant difference when comparing adhesion between the different ECM proteins (Figure 5.4). Adhesion to VCAM-1 and hyaluronic acid (HA) was also investigated but was not significantly different to the control.

The adhesion of primary hepatocytes to ECM components was similarly investigated. There was generally much less binding of primary human hepatocytes to all ECM proteins compared to Huh 7.5 cells and no significant adhesion demonstrated to any of the ECM components compared to BSA control in this assay (Figure 5.5).

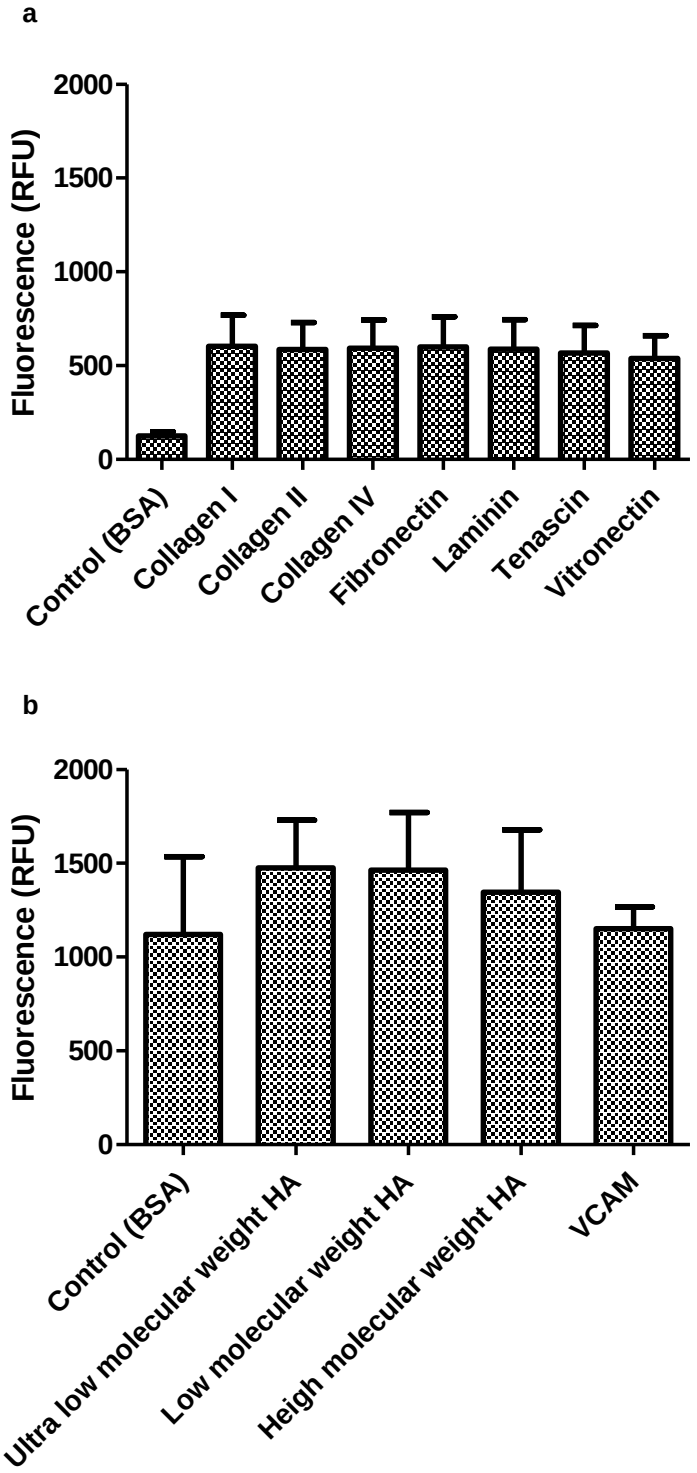

a
2000
1500
1000
500
0
Fluorescence (RFU)
Control (BSA)
Collagen I
Collagen II
Collagen IV
Fibronectin
Laminin
Tenascin
Vitronectin
b
2000
1500
1000
500
0
Fluorescence (RFU)
Control (BSA)
Ultra low molecular weight HA
Low molecular weight HA
Heigh molecular weight HA
VCAM

Figure 5.4 Static adhesion of huh 7.5 cells to ECM proteins/HA and VCAM-1.

Adhesion to ECM was assessed using a fluorometric assay kit (ECM proteins) and immobilised HA/VCAM-1. (a) Adhesion to all ECM proteins was significantly greater than to the control ($p<0.05$) but there was no significant difference in adhesion to different ECM proteins. (b) There was no significant adhesion to HA or VCAM-1. Data shown are the mean and standard error of 4 experiments.

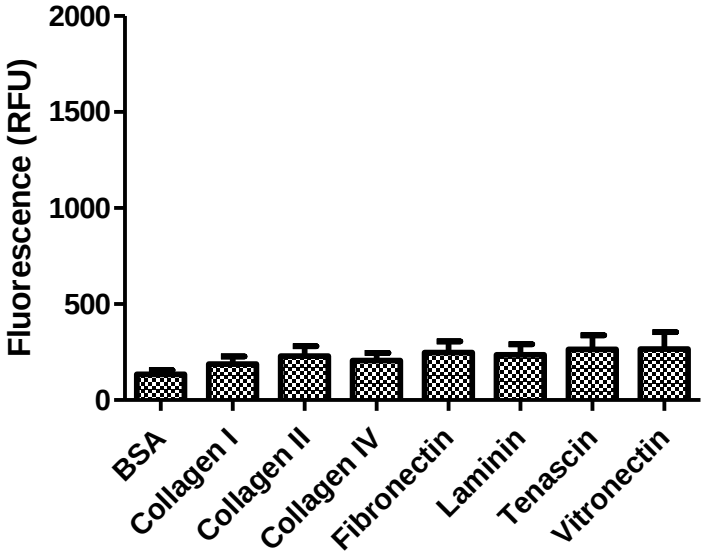


Figure 5.5 Static adhesion assay of primary hepatocytes to ECM proteins.

Adhesion to ECM was assessed using a fluorometric assay kit. There was no significant adhesion to any of the ECM components compared to BSA. Data shown are the mean and standard error of 3 experiments.

5.2.2 Adhesion of hepatocytes to hepatic sinusoidal endothelium under flow

5.2.2.1 Optimisation of the flow adhesion assay for primary human hepatocytes and Huh 7.5 cells

Huh 7.5 and primary hepatocytes were flowed over hepatic sinusoidal endothelial cells which had been treated for 24 hours with TNF-α and IFN-γ. Occasional brief tethering interactions were seen but the rolling adhesion characteristic of leukocytes and some other cell types was not observed. Under standard flow conditions, no primary hepatocytes and only very small numbers of Huh 7.5 cells were captured from flow and remained firmly adherent to the endothelium (not shown).

The assay was modified by pausing the flow for a period of time once a stable flow of cells across the endothelium had been established. Flow was then recommenced using wash buffer and the number of cells remaining adherent after 5 minutes of washing was counted. As observed in the static assay to ECM components, primary human hepatocytes were less adherent than Huh 7.5 cells. A 2 minute pause allowed sufficient Huh 7.5 cells to adhere for further blocking studies; primary hepatocytes required a longer 5 minute pause in order to obtain a similar number of adherent cells (Figure 5.6). I therefore used this modified flow adhesion assay for further experiments, with a 2 minute pause for Huh 7.5 and a 5 minute pause for primary human hepatocytes.

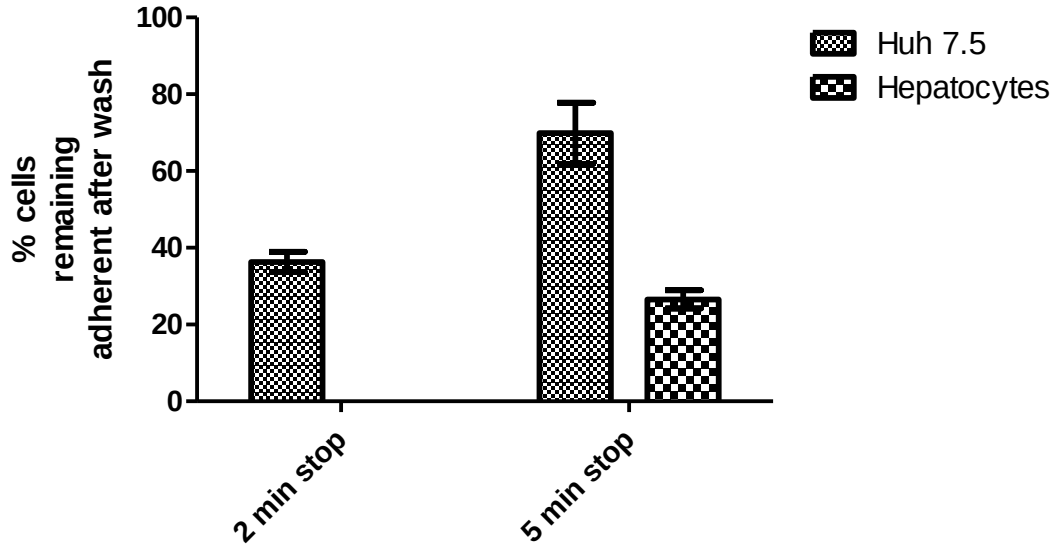


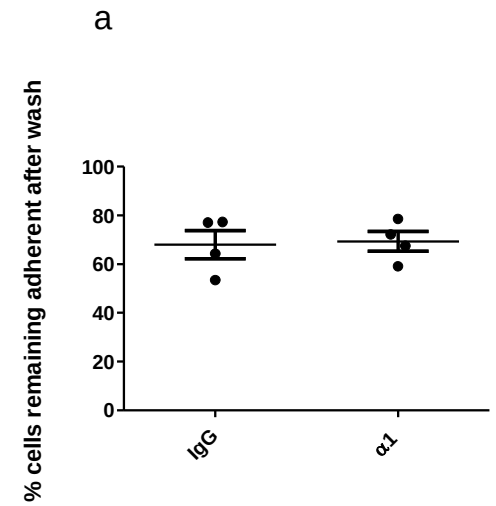
Figure 5.6 Modification of flow assay to allow hepatocytes to adhere to hepatic sinusoidal endothelium.

Flow was paused in order to allow cells to adhere to the endothelium. A 2 minute pause was sufficient for Huh 7.5 cells while a pause of 5 minutes was required to achieve a similar level of adhesion of primary hepatocytes. Data shown are the mean and standard error of 3 experiments.

5.2.2.2 The role of integrins in the maintenance of hepatocyte adhesion under flow to hepatic sinusoidal endothelium

Huh 7.5 cells were incubated with function blocking anti-α1, anti-αV and anti-β1 integrin antibodies in order to assess the role of these subunits in the adhesion of hepatocytes to HSEC (Figure 5.7). Blocking of α1-integrin had no effect on Huh 7.5 adhesion to HSEC (mean percentage of cells remaining adherent after wash 68% ± 6 with IgG control vs. 69% ± 4. with α1 block, p=0.86). Blocking of αV integrin increased adhesion to HSEC with the mean percentage of cells remaining adherent after wash increasing to 69% ± 5 compared to 30% ± 10 with IgG control (p=0.04). Similarly, β1 integrin blockade also increased adhesion from a mean of 54% ± 8 to 77% ± 6 (p=0.0006).

I was interested to find that blocking the αV and β1 integrin subunits consistently increased the adhesion of Huh 7.5 cells to HSEC under flow. I therefore repeated these experiments with primary hepatocytes (Figure 5.8). Blocking of αV integrin had no effect on the adhesion of primary hepatocytes to HSEC (mean percentage of cells remaining adherent after wash 22% ± 1 with IgG control vs. 19% ± 3 with αV block; p=0.34). However, as with Huh 7.5 cells, β1 integrin blockade increased the percentage of cells remaining adherent after wash from a mean of 13% ± 4 to 31% ± 6 (p=0.031).


a
% cells remaining adherent after wash
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60
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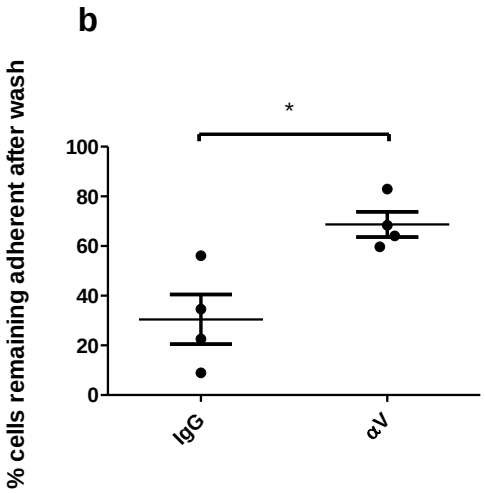
b
% cells remaining adherent after wash
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100
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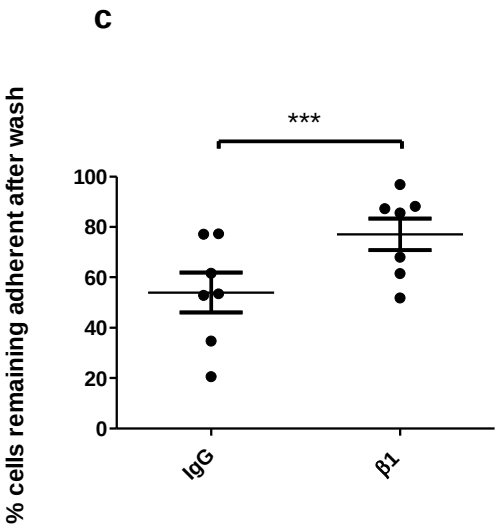

c
% cells remaining adherent after wash
***
100
80
60
40
20
0
IgG
β1

Figure 5.7 The effect of integrin blockade on the maintenance of Huh 7.5 cell adhesion to HSEC under flow.

Huh 7.5 were incubated with blocking antibodies to integrin subunits prior to flowing over HSEC. α1-integrin blockade had no effect on adhesion (a); blockade of the αV-integrin (b) and β1-integrin (c) subunit increased adhesion to HSEC. Data shown are the mean and standard error of at least 3 experiments (dots represent individual experiments). * $p<0.05$; *** $p<0.001$.

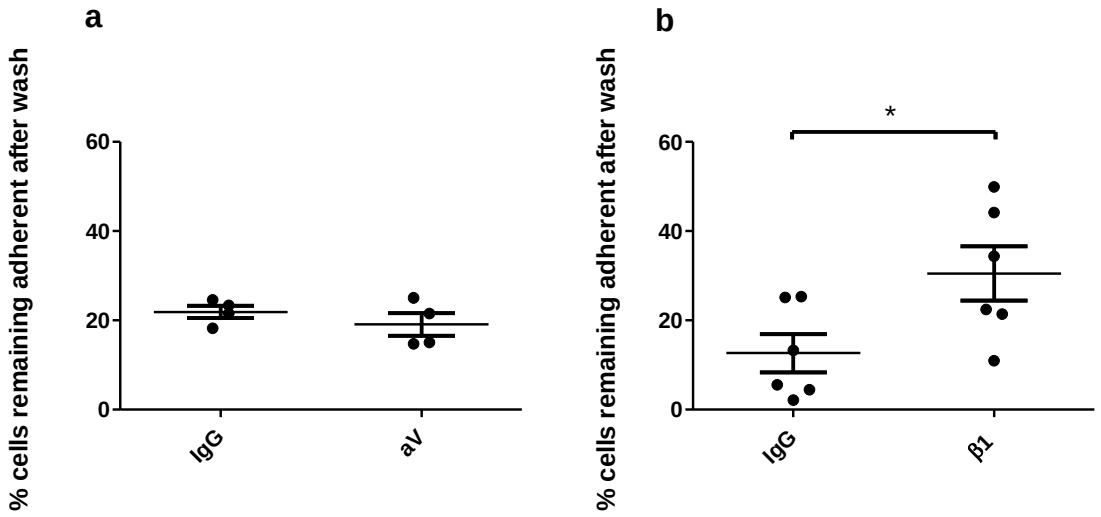


Figure 5.8 The effect of integrin blockade on the maintenance of primary human hepatocyte adhesion to HSEC under flow.

Primary human hepatocytes were incubated with blocking antibodies to integrin subunits prior to flowing over HSEC. αV-integrin blockade had no effect on adhesion (a); blockade of the β1-integrin subunit (b) increased adhesion to HSEC. Data shown are the mean and standard error of at least 3 experiments (dots represent individual experiments). * $p<0.05$.

5.2.2.3 Blocking of RGD-binding domains and activation of integrins does not affect the adhesion of hepatocytes to HSEC under flow

To further assess the role of integrins in the adhesion of hepatocytes to HSEC under flow I performed a series of further flow adhesion experiments using Huh 7.5 cells. Firstly I investigated the effect of RGD peptide to block integrin binding. During incubation with synthetic RGD peptides, RGD-binding integrins present on the cell surface bind the synthetic peptide thereby interfering with subsequent integrin-mediated adhesion (Villard et al. 2006). There was no significant difference in the number of cells remaining adherent when comparing those treated with RGD peptide (mean 44% ± 5.6) to those treated with a scrambled RGD (sRGD, control) peptide (34% ± 4) (p=0.12, Figure 5.9).

I next used various activating agents to attempt to increase integrin binding. Manganese chloride ($MnCl_2$) induces integrin activation most likely by stabilising an active conformational state (Bazzoni and Hemler 1998). Phorbol 12-myristate 13-acetate (PMA) is a protein kinase C (PKC) activator that promotes adhesion by targeting downstream events following ligand binding without significantly affecting the affinity of the receptor for the ligand (Bazzoni and Hemler 1998;Liu et al. 2002). Incubation of Huh 7.5 cells with $MnCl_2$ had no effect on adhesion under flow (control cells mean 73% ±16 vs treated cells mean 71% ± 12, p=0.79), nor did treatment with PMA (control cells mean 73% ±16 vs treated cells mean 75% ±18, p=0.46) (Figure 5.10).

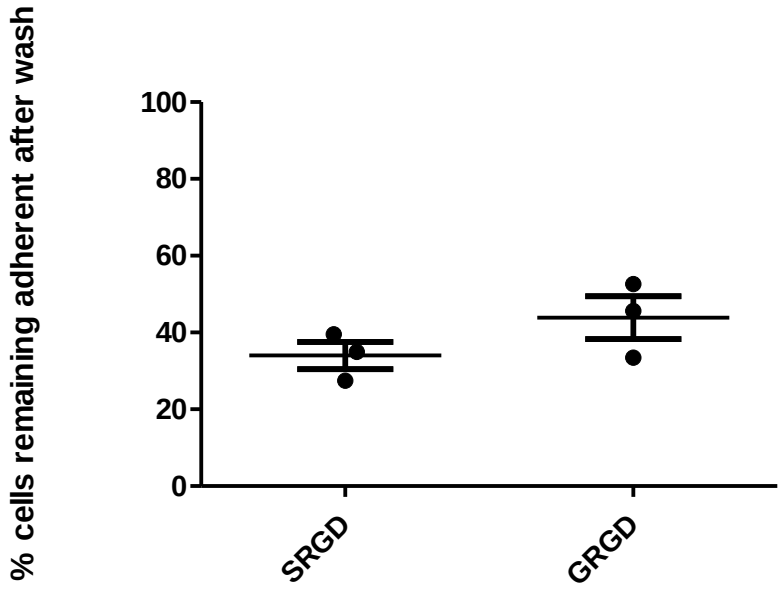


Figure 5.9 The effect of RGD peptides on the adhesion of Huh 7.5 cells to HSEC under flow

Huh 7.5 cells were incubated with RGD peptide to block potential RGD-binding integrins prior to flowing over HSEC. Incubation with RGD peptide had no effect on adhesion compared to cells incubated with a scrambled RGD peptide (control). Data shown are the mean and standard error of 3 experiments (dots represent individual experiments).

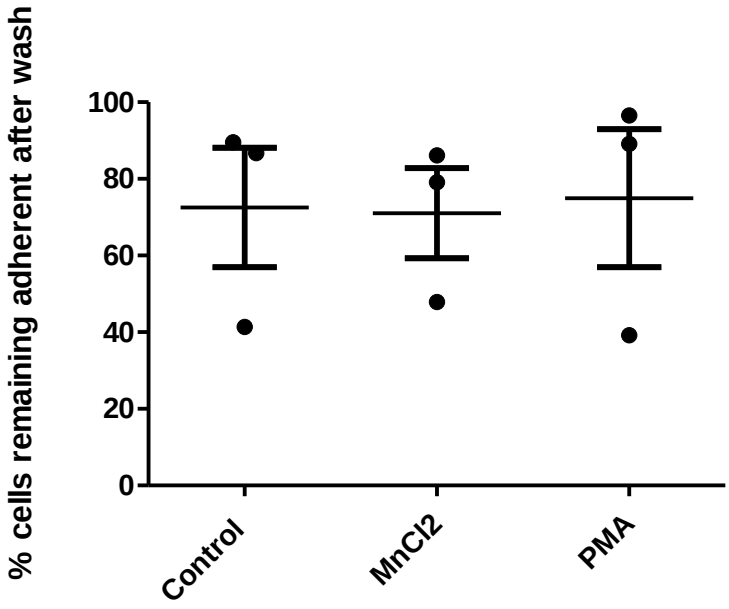


Figure 5.10 The effect of $MnCl_2$/PMA treatment on the adhesion of Huh 7.5 cells to HSEC under flow

Huh 7.5 cells were incubated with the integrin activating agents $MnCl_2$ or PMA in an attempt to increase adhesion to HSEC under flow. Neither agent increased adhesion compared to control (media only) cells. Data shown are the mean and standard error of 3 experiments (dots represent individual experiments).

Lastly, I incubated Huh 7.5 cells with the anti-β1-integrin antibody TS2/16. This is a β1-integrin activating antibody which is thought to induce an active conformation by forcing shape change (Byron et al. 2009). There was no difference in the adhesion of cells treated with the TS2/16 antibody (mean 51% ± 11) compared to untreated cells (mean 42% ± 9) (p=0.41, Figure 5.11).

5.2.2.4 The increased adhesion of hepatocytes treated with β1-integrin blocking antibody to HSEC under flow is maintained at a variety of antibody concentrations and incubation times.

I was surprised by the finding that β1-integrin blockade resulted in increased hepatocyte adhesion to HSEC under flow and therefore decided to investigate whether altering the concentration of the antibody or the length of incubation time might alter the effect and block adhesion. I firstly performed a titration experiment with Huh 7.5 cells across a range of antibody concentrations from 1ng/ml to 100ng/ml (Figure 5.12). All antibody concentrations within the range tested resulted in a significant increase in Huh 7.5 adhesion compared to isotype matched control. The increase in adhesion compared to isotype matched control was greater with increasing antibody concentration, with the highest concentration (100ng/ml) resulting in a mean percentage of cells remaining adherent of 102% ± 5 compared to 56% ± 5 with isotype matched control.

I then went on to perform a time course experiment using the standard concentration of antibody (10ng/ml) with incubation times ranging from 10 minutes to 4 hours (Figure 5.13). A 1 hour incubation time appeared to result in the greatest adhesion in the flow assay (mean 83% ± 2).

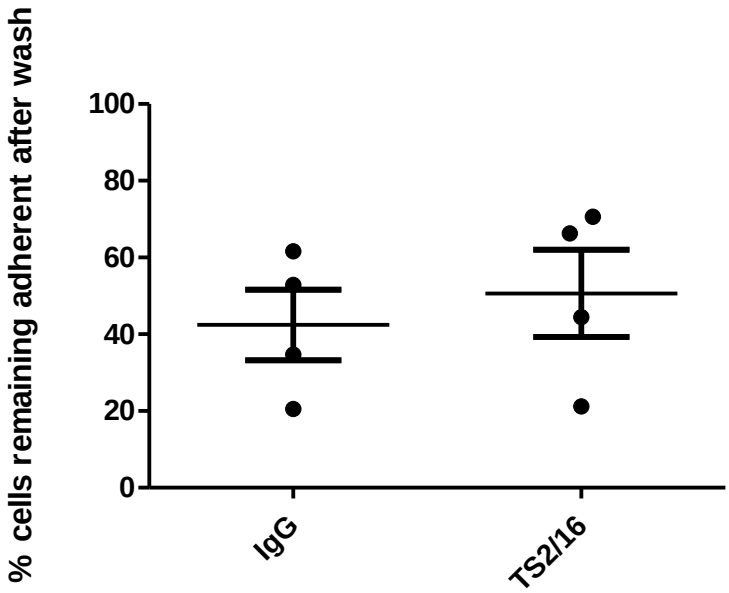


Figure 5.11 The effect of the TS2/16 antibody on the adhesion of Huh 7.5 cells to HSEC under flow

Huh 7.5 cells were incubated with the β1-integrin activating antibody TS2/16 prior to flowing over HSEC. Treatment with TS2/16 had no effect compared to cells treated with an isotype matched control. Data shown are the mean and standard error of 4 experiments (dots represent individual experiments).

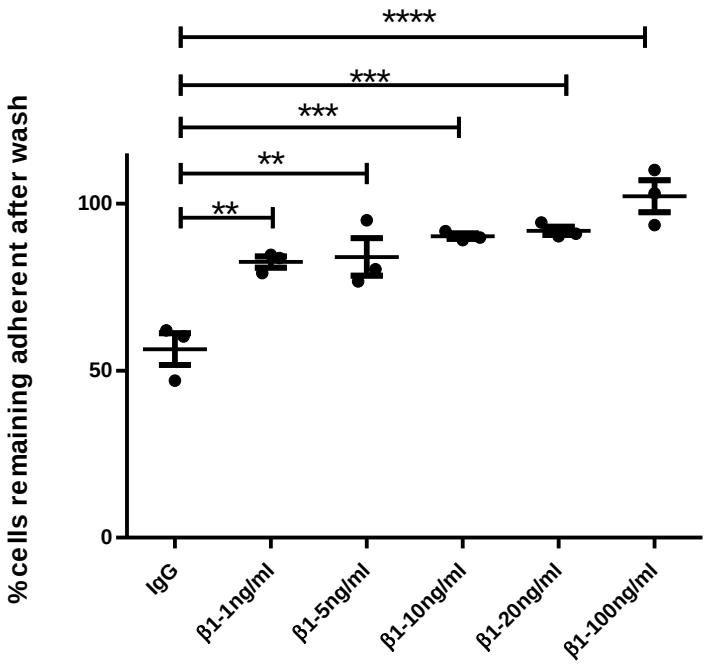


Figure 5.12 Investigating the effect of anti-β1-integrin antibody concentration on maintenance of Huh 7.5 cell adhesion under flow

Huh 7.5 cells were incubated with a β1-integrin blocking antibody at a range of concentrations. All concentrations in antibody resulted in a significant increase in adhesion to HSEC under flow compared to IgG control. Data shown are the mean and standard error of 3 experiments (dots represent individual experiments). Pairs of groups were compared using a paired t test with Bonferroni Correction for multiple comparisons. ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

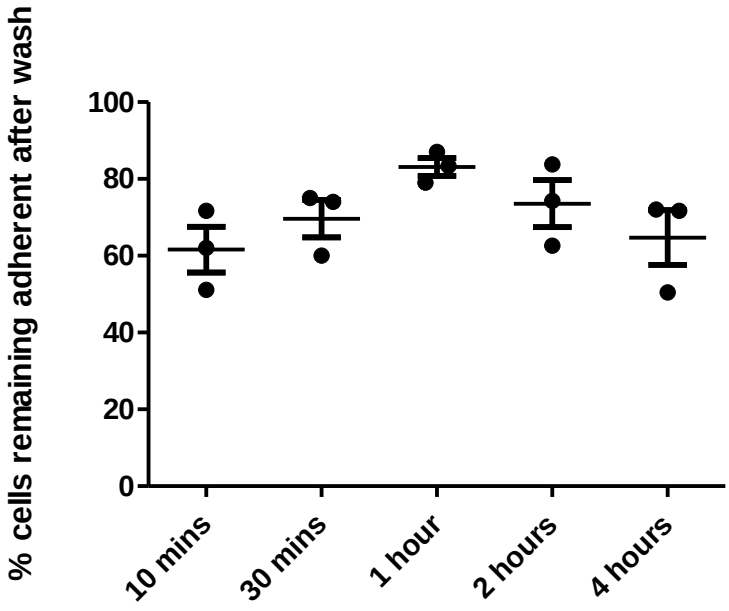


Figure 5.13 Time course analysis of Huh 7.5 cells incubated with an anti-β1-integrin blocking antibody

Huh 7.5 cells were incubated with a β1-integrin blocking antibody with incubation times ranging from 10 minutes to 4 hours. A 1 hour incubation time resulted in the greatest percentage of cells remaining adherent under flow compared to shorter or longer incubation times. However the differences between incubation times were not significant after Bonferroni Correction. Data shown are the mean and standard error of 3 experiments (dots represent individual experiments).

5.2.2.5 The increased adhesion of anti-β1-integrin treated hepatocytes to HSEC under flow is unrelated to TNFα/IFNγ HSEC stimulation and is not due to FC receptor interactions

I next examined two further factors that might have an effect on my unexpected finding of increased hepatocyte adhesion following β1-integrin blockade. Firstly, I repeated the flow adhesion assays with Huh 7.5 but without the prior stimulation of HSEC using TNF α and INF γ. β1-integrin blockade still improved adhesion of Huh 7.5 cells to HSEC under flow with a mean percentage remaining adherent of 72% ± 9 compared to 46% ± 8 for IgG treated cells (p=0.03, Figure 5.14).

I next wanted to exclude the possibility that the increased adhesion seen with anti-β1-integrin treated cells was due to FC receptor-antibody interactions. In order to do this I first treated Huh 7.5 cells and the HSEC with a FC receptor blocking reagent prior to incubation of the Huh 7.5 cells with the anti-β1-integrin antibody or isotype matched control. Again, β1-integrin blockade resulted in increased adhesion (mean 73% ± 7) compared to IgG (mean 56% ±7) (p=0.03, Figure 5.15).

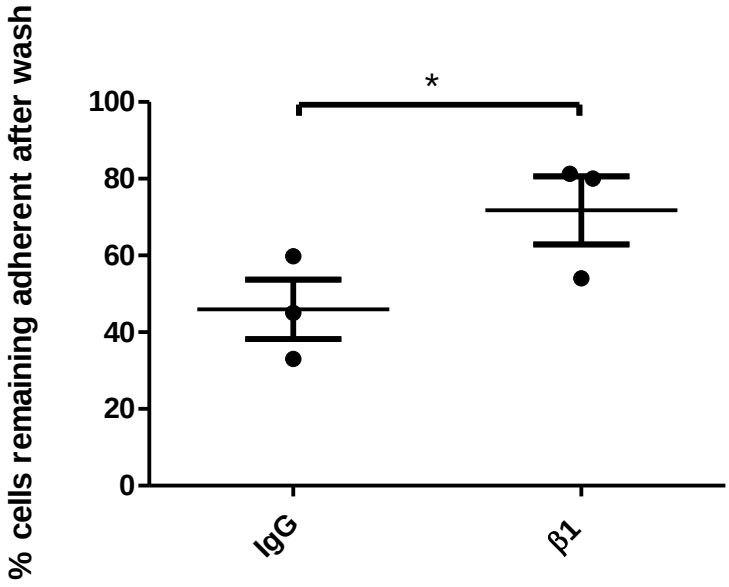


Figure 5.14 The effect of β1-integrin blockade on the adhesion of Huh 7.5 cells to unstimulated HSEC

Huh 7.5 cells pre-treated with either a β1-integrin blocking antibody or isotype matched control were flowed over unstimulated HSEC. The percentage of anti-β1-integrin treated Huh 7.5 cells remaining adherent under flow was higher compared to those treated with an isotype matched control. Data shown are the mean and standard error of 3 experiments (dots represent individual experiments). * $p<0.05$.

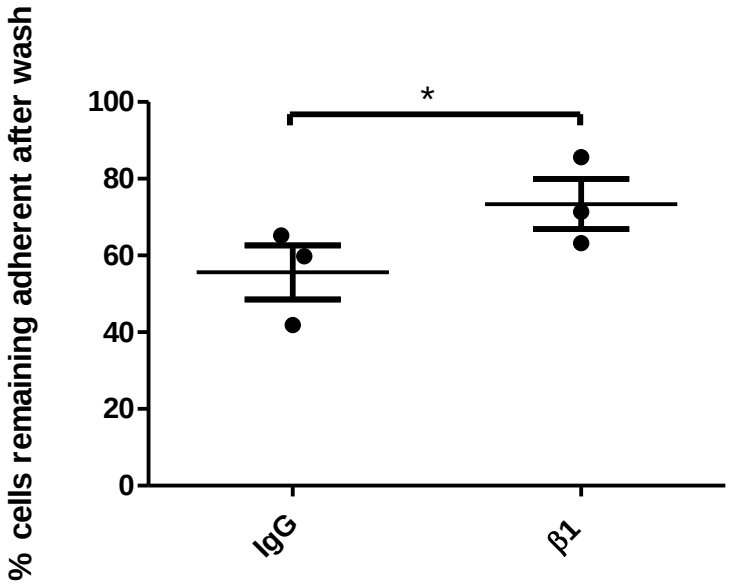


Figure 5.15 The effect of a FC receptor blocking reagent on the increased adhesion of Huh 7.5 with β1-integrin blockade

Flow adhesion assays were repeated using anti-β1-integrin or IgG treated Huh 7.5 cells following incubation of the Huh 7.5 and HSEC with a FC receptor blocking reagent. This had no effect on the previously demonstrated findings of increased adhesion with anti-β1-integrin treated cells. Data shown are the mean and standard error of 3 experiments (dots represent individual experiments). * $p<0.05$.

5.2.2.6 HSEC stimulation with TNF/IFN has no effect on binding of IgG or anti-β1-integrin treated hepatocytes to endothelium under flow

Having shown that prior stimulation of HSEC using TNF α and INF γ had no effect on the improved adhesion under flow seen with β1-integrin blockade, I investigated whether HSEC stimulation had any effect on the number of cells remaining adherent for both IgG and anti-β1-integrin treated cells. For IgG treated cells, a mean of 46% ± 8 remained adherent to unstimulated HSEC compared to a mean of 43/5 ±9 to stimulated HSEC (p=0.97). For anti-β1-integrin cells, a mean of 72% ± 9 remained adherent to unstimulated HSEC compared to 72%±9 to stimulated HSEC (p=0.84) (Figure 5.16).

5.2.2.7 The role of chemokine receptors in the adhesion of hepatocytes to hepatic sinusoidal endothelium under flow

Huh 7.5 cells were incubated with PTX in order to inhibit G-protein signalling and therefore block any possible chemokine receptor interactions that may be involved in their adhesion to HSEC under flow. PTX block had no effect on the adhesion of Huh 7.5 cells to HSEC with a mean of 63% ± 5 PTX-treated cells remaining adherent after wash compared to 63% ± 3 untreated cells (p=0.96, Figure 5.17).

5.2.3 The effect of β1-integrin blockade on the transmigration of hepatocytes through HSEC

Having established that β1-integrin blockade of hepatocytes leads to an increase in the numbers remaining adherent under flow after washing in the flow-based adhesion assays, I decided to investigate whether hepatocytes were able to migrate through a HSEC monolayer and, if so, whether this increased adhesion led to an increased number of cells migrating. Huh 7.5 cells which had been incubated with an anti-β1-integrin blocking antibody or isotype matched control were perfused across HSEC and then the flow was

stopped allowing them to settle onto the HSEC layer. A single field of view was then recorded in order to observe transmigration. After 1 hour cells were observed to start migrating into or through the HSEC monolayer (Figure 5.18). A greater number of anti-β1-integrin treated cells were seen to migrate compared to IgG treated cells (mean 35.7 ± 5.2 vs. mean 17.0 ± 1.5, p=0.03).

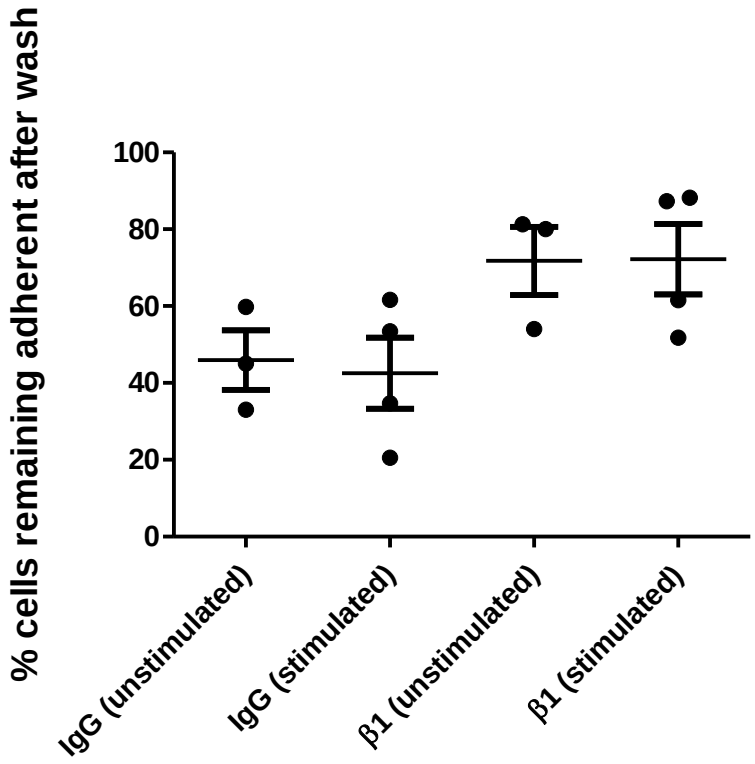


Figure 5.16 The effect of HSEC stimulation with TNF/IFN on the adhesion of IgG and anti-β1-integrin treated hepatocytes to HSEC under flow

Flow adhesion assays were repeated using anti-β1-integrin or IgG treated Huh 7.5 cells over both unstimulated HSEC and HSEC stimulated with TNF α and INF γ to investigate whether HSEC stimulation had any overall effect on the proportion of cells remaining adherent under flow. Data shown are the mean and standard error of 3 experiments (dots represent individual experiments).

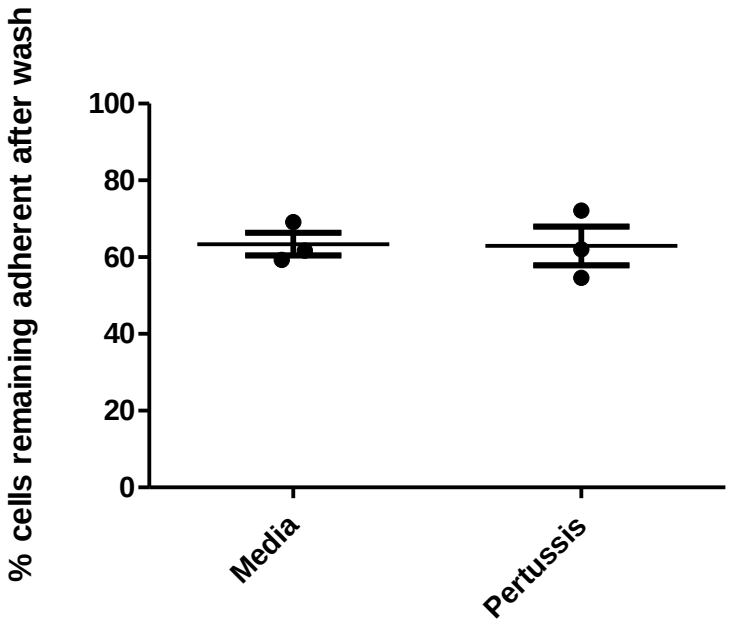
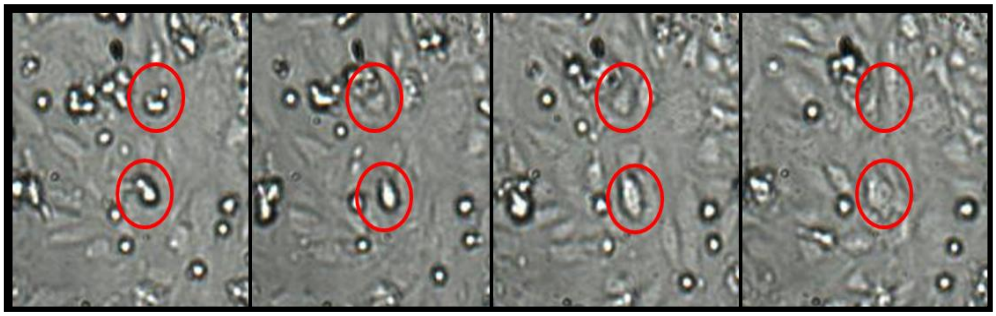


Figure 5.17 The effect of PTX to block chemokine receptor interactions on the adhesion of Huh 7.5 cells to HSEC under flow.

Huh 7.5 cells were incubated with PTX to block chemokine receptor interactions. This had no effect on their ability to adhere to HSEC under flow. Data shown are the mean and standard error of 3 experiments (dots represent individual experiments).

a



1 hour

3 hours

b

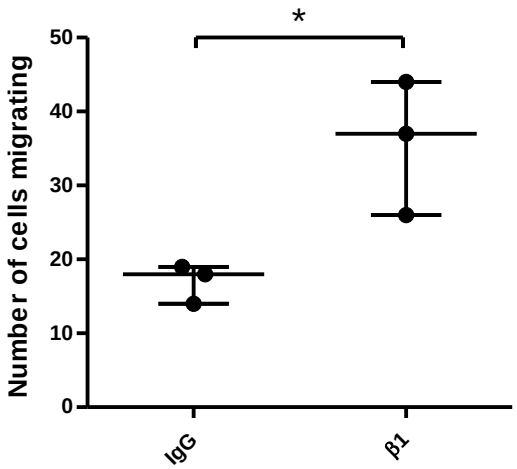


Figure 5.18 Transmigration of hepatocytes across hepatic sinusoidal endothelium

Huh7.5 cells treated with anti-β1-integrin or IgG control were flowed over a HSEC monolayer. (a) After 1 hour cells were observed to start migrating into or through the HSEC monolayer (red circle). (b) Higher numbers of anti-β1-integrin-treated cells were seen to migrate through the HSEC layer than isotype matched control-treated cells. Data expressed as the mean and standard error of 3 independent experiments (dots represent individual experiments). *$p<0.05$

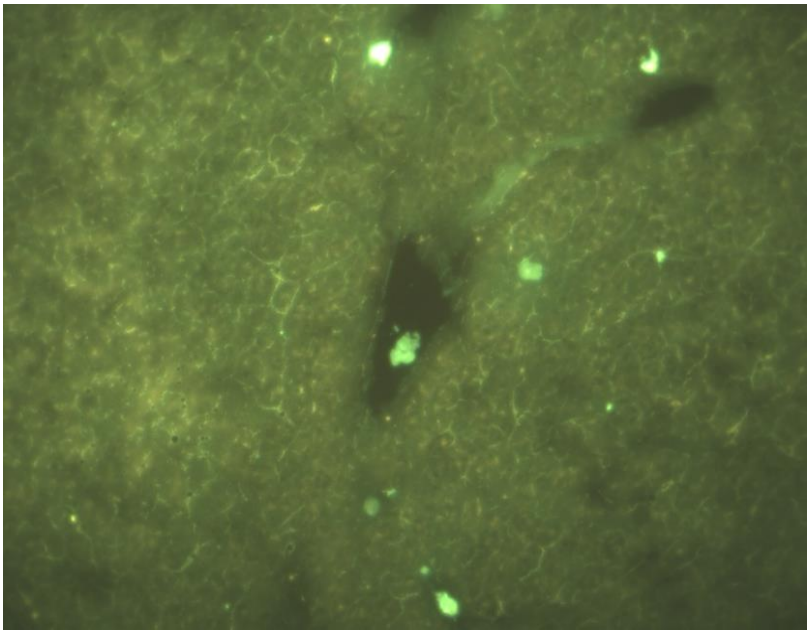
5.2.4 The effect of β1-integrin blockade on the engraftment of human hepatocytes into a murine liver

In order to study the effects of β1-integrin blockade on hepatocyte engraftment *in vivo* I transplanted human hepatocytes into wild type or CCl_4 treated mice. Hepatocytes were initially treated with anti-β1-integrin blocking antibody or isotype matched control and fluorescently labelled prior to infusion into the portal vein. After 15 minutes the mice were culled and the livers frozen then sectioned.

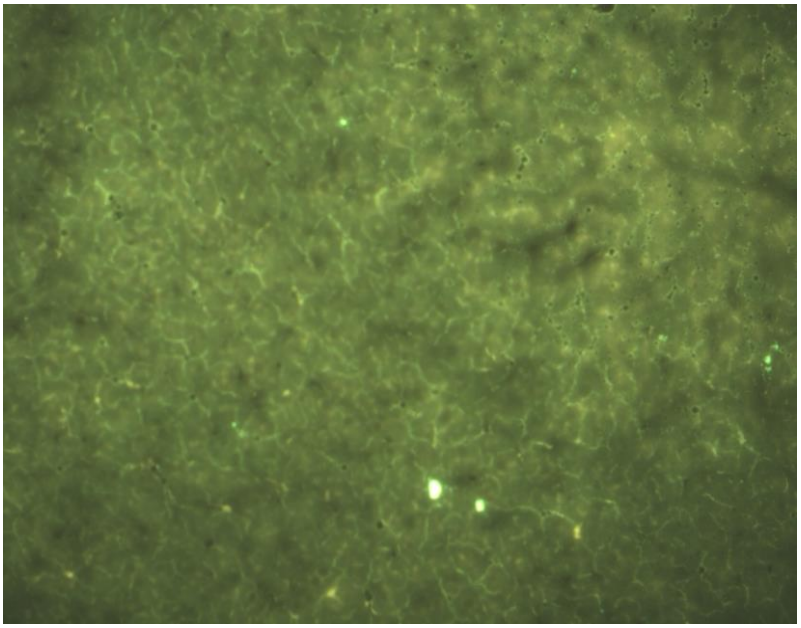
On examination of the liver sections, brightly fluorescent cells were clearly seen against the dim green autofluoresence of the murine liver. Some cells were found to be within or closely associated with a vessel whereas others appeared to be more distant from vessels.

β1-integrin blockade of primary human hepatocytes significantly increased their engraftment into murine liver. In wild type mice, the mean number of transplanted cells per field of view increased from 0.66 ± 0.14 (IgG treated cells) to 1.56 ± 0.18 (anti-β1-integrin treated cells, $p=0.017$). In CCl_4 injured mice, the mean number of transplanted cells per field of view increased from 0.71 ± 0.20 (IgG treated cells) to 1.86 ± 0.44 (anti-β1-integrin treated cells, $p=0.038$).

a



b



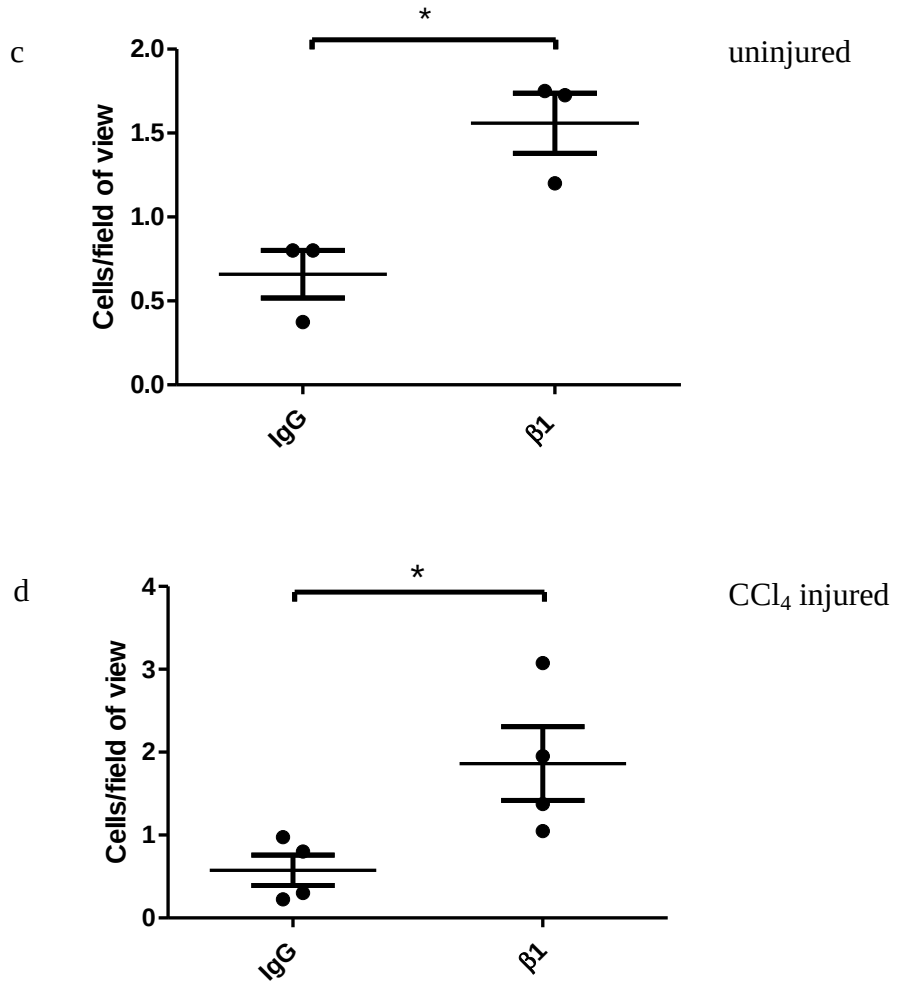


Figure 5.19 Engraftment of human hepatocytes in murine liver

Primary human hepatocytes were incubated with anti-β1-integrin antibody or IgG control. The cells were then fluorescently labelled and injected into the portal vein of uninjured or CCl_4 injured wild type mice which were culled after 15 minutes. Livers were snap frozen and cryosectioned for examination. (a) Anti-β1 integrin and (b) IgG control treated CFSE-labelled hepatocytes are clearly seen against background autofluoresence following intraportal injection into mouse liver. Significantly more fluorescent cells were seen in the livers of (c) uninjured and (d) CCl_4 injured mice. Date expressed as mean and standard error of at least 3 experiments (dots represent individual experiments), *p<0.05

5.3 Discussion

5.3.1 Adhesion of hepatocytes to liver tissue/ECM under static conditions

Having demonstrated a range of integrin and chemokine receptor expression by primary human hepatocytes I set out to investigate their role in the adhesion of hepatocytes under static conditions as well as under flow. Due to the number of experiments I needed to perform and the unpredictable nature of hepatocyte isolation I decided to use the Huh 7.5 cell line for all initial experiments. The more important findings were then confirmed using primary cells.

I initially examined adhesion under static conditions to liver tissue sections and immobilised ECM components. Here I used a variety of liver diseases and ECM components as our group and others have previously shown differential expression of adhesion molecules, chemokines and ECM components in liver disease with consequent effects on cell recruitment. For example, α1, α5, α6 and β1 integrins and ICAM-1 are upregulated in fibrosis related to chronic hepatitis C (Nejjari et al. 2001). A distinct pattern of chemokine expression is seen in patients with ALD (Afford et al. 1998) and CXCR3 ligands expressed on HSEC mediate T cell recruitment in liver injury (Curbishley et al. 2005;Oo et al. 2010;Oo et al. 2012). In fibrotic liver disease there are changes in both the quantity and composition of ECM components with accumulation of collagens, fibronectin, laminin and hyaluronan amongst others resulting from increased synthesis and reduced degradation due to overexpression of tissue inhibitors of matrix metalloproteinases (TIMPs) (Arthur 2000;Bataller and Brenner 2005;Benyon and Iredale 2000). I therefore felt it was important to determine whether hepatocytes would preferentially adhere to tissue from a specific type of liver disease or to specific ECM

components particularly as perhaps the greatest benefit of hepatocyte transplantation would be treating patients with pre-existing liver disease.

Huh 7.5 cells were able to adhere to liver tissue sections but interestingly there was no difference in the numbers that adhered to tissue from normal and different types of liver disease. This may be due to a lack of requirement for specific adhesion pathways for these cells, which readily adhere in culture to uncoated flasks. Alternatively, it may be that the required specific mechanisms important for their adhesion to liver tissue were functional in all the sections and were not altered by the presence of any particular liver disease. This is backed up by the finding that Huh 7.5 cells adhere to individual ECM components better than BSA control, suggesting that adhesion to ECM, presumably through integrins, is important. Huh 7.5 cells express a range of integrins allowing them to bind to ECM proteins present in normal and diseased liver. My integrin expression data suggest that the reason for the lack of specificity for any particular ECM component may be that Huh 7.5 cells express all the integrin subunits required for adhesion to the ECM components tested: collagens are bound by α1β1, α2β1, α10β1 and α11β1; fibronectin is bound by αVβ1; tenascin is bound by α2β1; laminin is bound by α1β1, α2β1, α7β1 and α10β1; and vitronectin is bound by αVβ5 (Humphries et al. 2006). I also examined adhesion to immobilised VCAM-1 and hyaluronic acid because our group has previously shown that VCAM-1 and CD44 which can bind hyaluronic acid are important for the recruitment of other cells such as human mesenchymal stem cells into injured liver (Aldridge et al. 2012). The fact that there was no increased binding to VCAM-1 compared to control is not surprising given that Huh 7.5 cells lack the VCAM-1 binding integrins (αDβ2, α9β1, α4β7, α4β1). Similarly, the lack of binding to hyaluronic acid suggests these cells do not express CD44 consistent with previous studies that report that human hepatocytes in normal and

cirrhotic liver do not express CD44 although expression has been reported in hepatocellular carcinoma (Cruickshank et al. 1999;Mathew et al. 1996;Seelentag et al. 1995). Having demonstrated Huh 7.5 binding to ECM components I decided to repeat this experiment with primary human hepatocytes. Interestingly there was no significant adhesion to any of the ECM components compared to BSA control despite primary human hepatocytes expressing the necessary integrins. I suspect that this may be due to a lack of sensitivity of the experimental technique combined with the fact that, in my experience, a large proportion of hepatocytes often fail to attach in culture without optimal conditions. Most protocols for hepatocyte culture, including my own, require initial plating in media containing serum which is changed to serum free media after several hours; this process was not possible during this assay.

I next investigated the effect of blocking the 3 major integrin subunits I had identified as being expressed on the surface of primary human hepatocytes. Blockade of the α1 or αV integrins resulted in some reduction in adhesion to most types of liver tissue tested, although the exact pattern varied depending on disease. Interestingly, the results from normal and NASH liver looked almost identical with a significant reduction in adhesion to parenchymal and portal areas with αV-integrin block and parenchymal areas only with α1-integrin block. Whether these differences in the blocking effect of anti α1/αV antibodies might relate to specific patterns of ECM deposition in different diseases is unclear from the literature. Most likely the differences relate to limitations of the assay and variations in cells and tissue samples outside my control. However, it does seem likely that α1- and αV- integrins are important. Blockade of α1- integrin has been previously shown to block rat hepatocyte adhesion to collagen I and IV and also to liver sections (Kocken et al. 1997;Pinkse et al. 2004). It is possible that αVβ5, which is expressed highly by Huh 7.5

cells, is involved in their adhesion although this is unlikely to be relevant to primary cells where I have not been able to demonstrate αVβ5 integrin expression although I did detect the αV chain by flow cytometry and RT-PCR. Indeed, normal hepatocytes are thought not to express any αV-integrins, although they are expressed in hepatocellular carcinoma (Nejjari et al. 2002).

β1-integrin blockade only resulted in a significant reduction in adhesion to parenchymal areas of AIH liver with no effect on adhesion to other liver types. This was most surprising as numerous studies have reported a role for β1-integrin in hepatocyte adhesion. Rat hepatocytes treated with an anti-β1-integrin antibody show loss of attachment to isolated collagen I and IV, laminin and fibronectin as well as both vessel walls and parenchymal areas of liver sections (Kocken et al. 1997;Pinkse et al. 2004). The impairment of adhesion seen after cryopreservation of human hepatocytes appears to be partly due to a down-regulation of β1-integrin expression with a direct correlation between loss of β1-integrin and attachment (Terry et al. 2007). Serum from patients with fulminant hepatic failure leads to a down-regulation in human hepatocyte β1-integrin activity with loss of cellular adhesion; this effect is prevented when hepatocytes are treated with a β1-integrin activating antibody (Newsome et al. 2004). I had therefore expected β1-integrin blockade to significantly reduce adhesion in these experiments. However, in the absence of expression of other β subunits that are known to associate with α1- and αV- integrins, I suspect that α1β1 and αVβ1 are important for hepatocyte adhesion. It is also possible that integrins which I have demonstrated to be expressed at lower levels but have not tested in these experiments, such as α10β1 and α11β1, may also contribute. The extensive previous literature describing a role for β1-integrin in hepatocyte adhesion, albeit mainly focussing on non-human hepatocytes, as well as the fact that I did see a reduction in binding to AIH

liver, is highly suggestive that β1-integrins are involved in human hepatocyte adhesion. I therefore hypothesise that some other, as yet undetermined factor, is preventing the significant reduction in hepatocyte adhesion with β1-integrin block described elsewhere. Possible mechanisms for this include the presence of other adhesion pathways that may be upregulated in response to blocking β1-integrin. It is also possible that the particular antibody used does not function well in this particular assay, but I feel this is unlikely as our group has used the same antibody at the same concentration to successfully block adhesion of other cell types such as mesenchymal stem cells in similar experiments.

Finally I used PTX to block chemokine receptor signalling in order to demonstrate any possible role for chemokine receptors in adhesion to liver tissue. PTX block did not result in any reduction in adhesion suggesting that chemokine receptors do not contribute to hepatocyte adhesion to liver tissue; conversely adhesion was significantly increased in some experiments. Why this might be is unclear and may relate to mechanisms not involving chemokine receptors. Similar effects of PTX have been described with other cell types. For example, myelomonocytic cells show increased adhesion to vitronectin when exposed to PTX, possibly through binding to CD14 on the cell surface leading to increased activity of urokinase-type plasminogen activator receptor (uPAR) which is also a vitronectin receptor that promotes cell adhesion and migration (Li and Wong 2000;Madsen et al. 2007;Wong et al. 1996). Hepatocytes are known to express CD14 (Pan et al. 2000;Su et al. 1999) and it is possible that PTX may enhance hepatocyte adhesion through a similar pathway.

5.3.2 Adhesion of hepatocytes to HSEC under flow

I next moved on to look at hepatocyte adhesion to HSEC under flow. My initial optimisation experiments showed that neither primary human hepatocytes or Huh 7.5 cells

showed the characteristic cascade of initial capture with tethering/rolling interactions prior to firm adhesion as seen with lymphocytes (Lalor and Adams 1999). Indeed, negligible numbers of cells were able to adhere to endothelium under flow. This is not surprising as these cells would not normally migrate through the vasculature. Work in our laboratory investigating human mesenchymal stem cell recruitment to liver demonstrated similar findings which led us to develop a modified flow assay technique whereby flow is paused for a period of time to allow initial adhesion (Aldridge et al. 2012). Flow is then recommenced, thus washing away cells which are not firmly adherent. Adhesion can then be quantified as the percentage of cells present per field of view while the flow is stopped that remain adherent once flow is restarted. As discussed previously, it is likely that human hepatocytes become mechanically lodged to some extent within the liver sinusoids following intraportal infusion. I therefore feel that my modified flow adhesion assay better resembles the in vivo environment with the stopping of flow modelling the slowing/lodging of cells within the sinusoids.

Once again I concentrated on the 3 most highly expressed integrins on primary human hepatocytes and, using Huh 7.5 cells initially, established that α1-integrin did not appear to have a role in hepatocyte adhesion to HSEC under flow. Similarly, PTX toxin block had no effect on adhesion suggesting that chemokine receptors are not involved in adhesion to HSEC. This was expected given that hepatocytes did not appear to undergo the tethering/rolling under flow seen with leucocytes during which time chemokine signals lead to activation of integrins which in turn permit firm adhesion. However, I was then surprised to find that blocking αV- or β1-integrin significantly increased adhesion to HSEC. Because this finding was unexpected I repeated the experiments with primary hepatocytes. Whilst αV-integrin blockade no longer had any effect, β1-integrin blockade

again significantly increased adhesion. As discussed in Chapter 1, there is little in the literature describing the interaction between hepatocytes and HSEC other than animal data suggesting that integrin mediated pathways may be important. It is possible that some of the integrins expressed at lower levels by hepatocytes are involved, or that hepatocytes express ligands for integrins present on HSEC. Alternatively other non-integrin adhesion molecules which I have not investigated may be more important for adhesion in this context.

Why β1-integrin blockade should increase adhesion was unclear and I wanted to satisfy myself that this was a true finding. I therefore performed a series of experiments demonstrating that the effect of β1-integrin blockade persisted with lower or higher antibody concentrations and different antibody incubation times. Next, I repeated the experiment with unstimulated HSEC and again showed increased adhesion with anti-β1-integrin treated hepatocytes compared to IgG control. Lastly, as HSEC are also known to express β1-integrin and both HSEC and hepatocytes have FC receptors then I also wanted to exclude the possibility of the antibody binding β1-integrin on hepatocytes and subsequently binding HSEC via FC receptors or vice-versa. However, addition of a FC receptor blocking agent did not reverse the increased adhesion seen with β1-integrin blockade. Furthermore, if the effect seen with β1-integrin blockade was due to FC receptor binding then it would be expected that a similar effect would have been seen with anti α1- and αV-integrin antibodies. I was therefore confident that β1-integrin blockade of primary human hepatocytes does increase their adhesion to HSEC under flow.

5.3.3 Transmigration of human hepatocytes through HSEC

Increasing adhesion of hepatocytes to sinusoidal endothelium would be of little benefit if this did not translate into increased engraftment into the liver. I therefore went on to

question whether hepatocytes, having adhered to HSEC, would transmigrate through an HSEC layer and whether β1-integrin blockade might also increase the number transmigrating. I used the same technique as for the modified flow adhesion assay up until the point when the flow was paused and the cells settled onto the HSEC monolayer when the microslide was transferred to a JuLi Cell Analyser in order to capture transmigration.

Between 1 and 4 hours, cells were seen to move into the HSEC monolayer, and significantly more cells were seen to move when they were pre-treated with anti-β1-integrin blocking antibody compared to those treated with IgG control. Presumably this is due to the increase in initial adhesion to HSEC which I have demonstrated in the preceding experiments, although it is possible that signalling as a result of the β1-integrin blocking antibody also results in a more migratory phenotype through other mechanisms. Whether the movement seen represented migration into or transmigration across the HSEC monolayer is not clear from this assay. However, it is clear from animal experiments, as outlined in the introduction chapter, that hepatocytes are able to transmigrate into the liver parenchyma from the hepatic sinusoids. Taken together with the results of this experiment I hypothesised that β1-integrin blockade would increase transmigration across HSEC into the liver.

5.3.4 Engraftment of human hepatocytes in murine liver

Many groups have demonstrated that human hepatocytes are able to successfully engraft of human in murine liver. As such models are well established and the techniques involved relatively straight forward I felt that a murine model would be ideal to investigate the effect of β1-integrin blockade on engraftment of human hepatocytes *in vivo.* I initially selected the FRG mouse and successfully established a colony within our animal facility. This mouse, a model of human Tyrosinaemia Type 1, has several advantages as described

in the introduction. However, despite the successful use of this model for human hepatocyte transplantation reported by other groups, I found considerable variability in the ability of FRG mice to tolerate general anaesthesia and surgery. After discussion with our veterinary surgeon I therefore opted to use a different model.

The C57BL/6 mouse has been used extensively for murine to murine hepatocyte transplant experiments. Our group has also used this mouse model for previous work including developing a CCl_4 liver injury model and have successfully transplanted human mesenchymal stem cells into both CCl_4 injured and non-injured mice by intraportal injection (Aldridge et al. 2012). I therefore chose this model and transplanted anti-β1-integrin or IgG treated hepatocytes into both injured and non-injured mice. I chose a 15 minute end point as I wanted a long enough period to allow cells to travel throughout the liver and for cells that failed to bind successfully within the sinusoids to be washed away. However, importantly it was also short enough that mice could be easily maintained in a stable condition under general anaesthesia.

When analysing the livers following hepatocyte transplantation I wanted to ensure as far as possible that the results were representative of the situation in the whole liver. Therefore sections were taken from several positions within different lobes of the liver and fields of view were selected randomly from several different sections. I only wanted to count transplanted cells that were truly adherent or trapped within vessels/sinusoids and therefore rejected any fields of view where there were large clumps of cells or debris, or where the liver parenchyma was damaged.

Individual fluorescent cells were seen throughout the livers of all mice following transplantation of CFSE-labelled human hepatocytes. Many cells appeared to be within or

close to a vessel, although smaller numbers appeared to be within the parenchyma. The overall numbers of cells seen in any field of view were small. However, when one considers the relatively small number of cells transplanted (1×10^6 cells) and the volume of one field of view of a 5µm section compared to that of the whole mouse liver, the numbers appear appropriate. Interestingly, in keeping with my *in vitro* findings, more transplanted hepatocytes were found in the livers of mice receiving anti-β1-integrin treated cells compared to those receiving IgG-treated cells. This was true for both wild type and CCl_4 injured mice suggesting that liver fibrosis is not a barrier at least to the early stages of hepatocyte engraftment following intraportal infusion. It is possible that the presence of a degree of liver injury may be beneficial to initial engraftment due to the cytokine effects leading to upregulation or differential expression of adhesion molecules in disease states. Indeed, slightly higher numbers of IgG and anti-β1-integrin treated hepatocytes were seen in CCl_4 treated livers than wild type although this did not reach statistical significance. There is one report in the literature of similar findings following transplantation of Brown Norway rat hepatocytes into immunocompromised Lewis rats. At day 2 following intraportal infusion of 1×10^7 untreated hepatocytes, 2.39% ± 0.27% of the transplanted hepatocytes were found in the liver parenchyma. Following infusion of hepatocytes treated with an anti-β1-integrin antibody, 36.31% ± 8.15% of transplanted hepatocytes were found in the recipient liver at day 2 representing a significant ($p<0.001$) increase in survival (Kocken et al. 1997).

In conclusion, I have demonstrated in this chapter that although β1-integrins are likely to have an important role in human hepatocyte adhesion, incubation with anti-β1-integrin blocking antibodies alone has a minimal effect on adhesion under static conditions. However, pre-treatment of isolated human hepatocytes with an anti-β1-integrin antibody

increases their ability to remain adherent to sinusoidal endothelium under flow and results in an increase in early engraftment of human hepatocytes in murine liver following intraportal infusion. The underlying mechanisms as yet remain unclear.

CHAPTER 6: INVESTIGATING THE MECHANISMS UNDERLYING THE EFFECT OF B1-INTEGRIN BLOCKADE ON HUMAN HEPATOCYTE ADHESION AND ENGRAFTMENT

6.1 Introduction

The work described in the previous chapters led me to focus on β1-integrin and the effects of β1-integrin blockade on human hepatocyte adhesion and engraftment into liver. Rather than blocking adhesion of human hepatocytes, treatment with anti-β1-integrin antibodies confers an increased ability to remain adherent to sinusoidal endothelium under flow, improves migration across HSEC and leads to higher numbers of cells retained within murine livers following transplant by intraportal infusion. The mechanism by which blocking a molecule classically described as an adhesion molecule might lead to increased adhesion is not immediately clear.

The phenomenon of anoikis, or detachment-related cell death, has been demonstrated in isolated hepatocytes and is one of the stumbling blocks to successful hepatocyte transplantation (Zvibel et al. 2002). Treatment of rat hepatocytes with anti-β1-integrin antibodies increases their survival (Kocken et al. 1997;Pinkse et al. 2004). It has been suggested that this occurs through the activation of integrin-linked kinase (ILK) and subsequent phosphorylation of protein kinase B/Akt (Pinkse et al. 2005).

Activation of integrin related signalling pathways and improved survival of isolated primary human hepatocytes through the prevention of anoikis could potentially go some way to explain the improvement in adhesion and engraftment observed following anti-β1-integrin treated hepatocytes. I therefore investigated whether anti-β1-integrin antibodies result in activation of a cell survival signalling pathway in human hepatocytes.

6.1.1 Anoikis

For most cells, their position within tissue is crucial to their function and that of the organs of which they are part. Integrins on the cell surface are able to interact with ECM and

form a link with the cytoskeleton activating signalling cascades and influencing responses to other stimuli (Lee and Juliano 2004;Shattil et al. 2010). If cells lose their usual cell-ECM interactions, a specific form of caspase-dependant apoptosis, known as anoikis, occurs (Frisch and Ruoslahti 1997). Thus anoikis provides a means by which `misplaced' cells may be eliminated; overcoming anoikis is a crucial step in the development of malignancy allowing tumour cells to disseminate throughout the body (Guadamillas et al. 2011).

Anoikis is not a single specific pathway leading to cell death; rather, different cells respond to loss of adhesion to ECM via a diverse range of signalling mechanisms. Anoikis progresses via the interplay of two different apoptotic signalling pathways which ultimately converge on the activation of caspases (Gilmore 2005). These two pathways, termed the intrinsic and extrinsic pathways, are summarised in Figure 6.1. The intrinsic pathway may be triggered by intracellular signals, such as DNA damage and endoplasmic reticulum stress through the action of pro-apoptotic regulator proteins of the B cell lymphoma-2 (Bcl-2) family, including Bcl-2-associated death promoter (Bad), Bcl-2-associated X protein (Bax), Bcl-2-interacting domain (Bid) and Bcl-2-interacting mediator of cell death (Bim). These lead to mitochondrial permeabilisation and release of pro-apoptotic factors such as cytochrome c and second mitochondria-derived activator of caspases/direct inhibitor of apoptosis binding protein with low pI (Smac/DIABLO) with subsequent assembly of the so-called apoptosome and caspase activation. The extrinsic pathway is initiated by the binding of ligands to death receptors of the tumour necrosis factor receptor (TNFR) superfamily such as Fas receptor, TNFR1 and TNF-related apoptosis inducing ligand (TRAIL) receptors. This results in the formation of the death-

inducing signalling complex (DISC) which in turn leads to caspase activation (Chiarugi and Giannoni 2008;Guadamillas et al. 2011;Paoli et al. 2013).

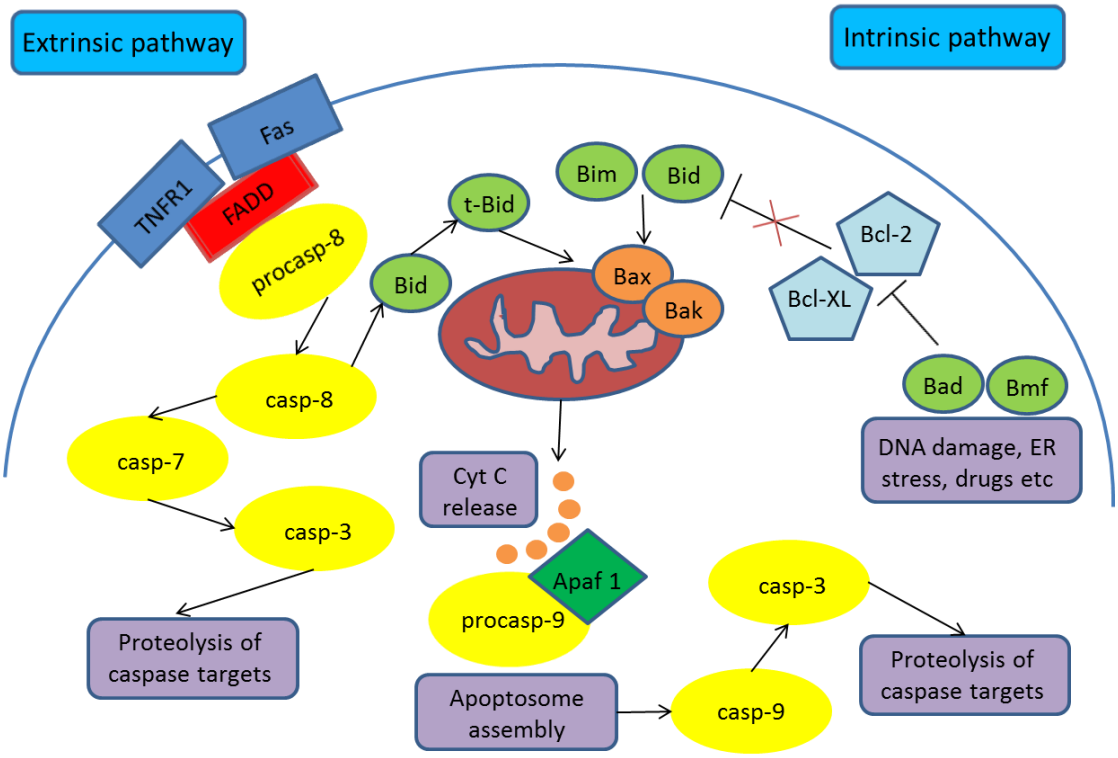


Figure 6.1 The extrinsic and intrinsic apoptotic pathways

Anoikis, or detachment-related cell death, occurs through the activation of two apoptotic pathways. In the extrinsic pathway, engagement of death receptors such as TNFR1, Fas and TRAIL receptors leads to formation of the death-inducing signalling complex (DISC). The interaction of DISC with adaptor proteins such as the Fas-associated death domain (FADD) results in the activation of caspase-8 and downstream executioner caspases. In the intrinsic pathway, activation of the pro-apoptotic BH3-only group of the Bcl-2 protein family leads to translocation of Bax/Bak to the outer mitochondrial membrane where their oligomerisation creates a channel resulting in cytochrome c release.

BH3-only activators (e.g. Bid, Bim) directly promote Bax/Bak oligomerisation whilst sensitizers (e.g. Bad, Bmf) block the anti-apoptotic effects of Bcl-2. Cytochrome c together with caspase-9 and apoptosis protease activating factor (Apaf) form the apoptosome which results in downstream caspase activation. Caspase-8 activation may also cleave Bid to a truncated form (t-Bid) which also promotes mitochondrial cytochrome c release, thereby linking the intrinsic and extrinsic pathways.

6.1.2 Caspases

Caspases are a family of proteases with a critical role in cell death and inflammation. Those involved in cell death may be divided into two groups: initiator caspases (caspase-8 and -9) exist as inactive procaspase monomers that are activated by dimerization brought about by upstream signalling events; executioner caspases (caspase-3, -6 and -7) are produced as inactive dimers which are activated through cleavage by initiator caspases and subsequently other activated executioner caspases (McIlwain et al. 2013).

Caspase-3 in particular is a key protease in mammalian cell death and is activated by a variety of agents that induce apoptosis. Many of the typical hallmarks of apoptosis are dependent on caspase-3 and it is indispensable for chromatin condensation and DNA fragmentation during cell death (Nicholson et al. 1995;Perry et al. 1997;Porter and Janicke 1999). Caspase-3 activity has been demonstrated in hepatocytes subjected to a variety of apoptotic stimuli and is therefore a useful marker of hepatocyte apoptosis (Kubota et al. 2004;Li et al. 2003;Tapalaga et al. 2002) and I therefore selected caspase-3 activity to use as a more specific marker of apoptosis in human hepatocytes along with a more generic flow cytometry viability marker.

6.1.3 The PKB/Akt signalling pathway and cell survival

Protein kinase B or Akt (PKB/Akt) is a serine/threonine kinase which has a key role in cell survival, metabolism, motility and the cell cycle. Three members of the family have been identified in mammals, termed PKBα/Akt1, PKBβ/Akt2 and PKBγ/Akt3 (Fayard et al. 2005;Manning and Cantley 2007). PKB/Akt is a downstream target for phosphoinositide 3-kinase (PI-3K) which may be activated by insulin or other growth factors, G-protein coupled receptor stimulation or integrin signalling (Foster et al. 2003;Wymann et al. 2003). PI-3K activation results in the generation of second messengers which in turn

allows the translocation of PKB/Akt to the plasma membrane from the cytoplasm. Once at the plasma membrane, PKB/Akt undergoes phosphorylation at threonine 308 (Thr308) by phosphoinositide-dependent kinase-1 (PDK-1) (Alessi et al. 1997) and serine 473 (Ser473). Ser473 phosphorylation may occur via a variety or mechanisms including mammalian target of rapamycin (mTOR) complex 2 (mTORC2), DNA-dependent protein kinase (DNA-PK), ILK or by autophosphorylation (Delcommenne et al. 1998;Feng et al. 2004;Sarbassov et al. 2005;Toker and Newton 2000). Phosphorylation of both residues is required for maximal PKB/Akt activity (Alessi et al. 1996).

Activation of the PKB/Akt signalling pathway promotes cell survival through direct action on mediators of apoptosis (such as Bad and caspase-9), regulation of transcription factors associated with pro- and anti-apoptotic genes and interactions with metabolic pathways such as the inhibition of glycogen synthase kinase 3 (GSK3) (Song et al. 2005).

6.1.4 Activation of the PKB/Akt pathway by integrin ligation

Integrins do not possess any intrinsic enzymatic activity and so integrin signalling depends on the recruitment of various associated signalling proteins (Harburger and Calderwood 2009;Schiller et al. 2011). ILK is one such protein that has been studied extensively. ILK is recruited to β1 and β3 integrins and thought to influence various processes including cytoskeletal rearrangement, cell polarisation, spreading and migration, and cell proliferation and survival (Attwell et al. 2000;Hannigan et al. 2005;Hannigan et al. 1996;Widmaier et al. 2012). It has been demonstrated that ILK may have a role in phosphorylation of PKB/Akt on Ser473 (Troussard et al. 2003) and, as mentioned above, ILK has been suggested as a possible link between β1-integrin and PKB/Akt. However, the exact nature of ILK signalling remains unclear; whilst many earlier studies reported ILK to be a serine/threonine kinase it lacks several highly conserved motifs present in

most kinases and more recent evidence suggests that ILK lacks true kinase activity (Wickstrom et al. 2010;Widmaier et al. 2012). Other authors suggest that, rather than acting as a true kinase and directly phosphorylating PKB/Akt, it may act as an adapter protein that activates another kinase or inhibits a S473 phosphatase (Lynch et al. 1999).

Other intermediate proteins that have been shown to be involved in β1-integrin signalling include focal adhesion kinase (FAK) and Src family kinases but it has been shown that β1-integrin may induce PKB/Akt phosphorylation independently of these pathways (Velling et al. 2004). β1-integrin may also modify PKB/Akt signalling through activation of phosphatases such as protein phosphatase 2A (PP2A) (Pankov et al. 2003).

Due to the uncertainty surrounding the role of ILK and the mechanism by which integrin ligation leads to PKB/Akt activation, I decided to focus on investigating PKB/Akt phosphorylation directly in order to ascertain whether this pathway is activated in human hepatocytes following ligation by anti-β1-integrin antibodies.

6.2 Results

6.2.1 Human hepatocytes treated with anti-β1-integrin antibodies show increased viability

Primary human hepatocytes were incubated in suspension for 1 hour with anti-β1-integrin antibodies or isotype matched control antibody before labelling with a viability marker for flow cytometry. Hepatocytes treated with anti-β1-integrin antibodies showed a small but significant increase in viability compared to those treated with IgG control (mean viability 24.9% ± 3.3 vs. 21.4% ± 3.4; p=0.01); similar findings were demonstrated with Huh 7.5 cells although the viability in both groups was much higher than for primary hepatocytes (mean viability 85.4% ± 1.6 vs. 79.1% ± 2.2; p=0.02) (Figure 6.2 a, b).

To confirm the findings, primary hepatocytes and Huh 7.5 cells were treated in a similar manner with anti-β1-integrin antibodies or isotype matched control and viability assessed using trypan blue dye exclusion. Again, viability was significantly higher following anti-β1-integrin antibody treatment for both primary hepatocytes (mean viability 49.7% ± 3.8 vs. 36.3% ± 5.8; p=0.03) and Huh 7.5 cells (mean viability 75.8% ± 6.2 vs. 58.4% ± 4.2; p=0.01).

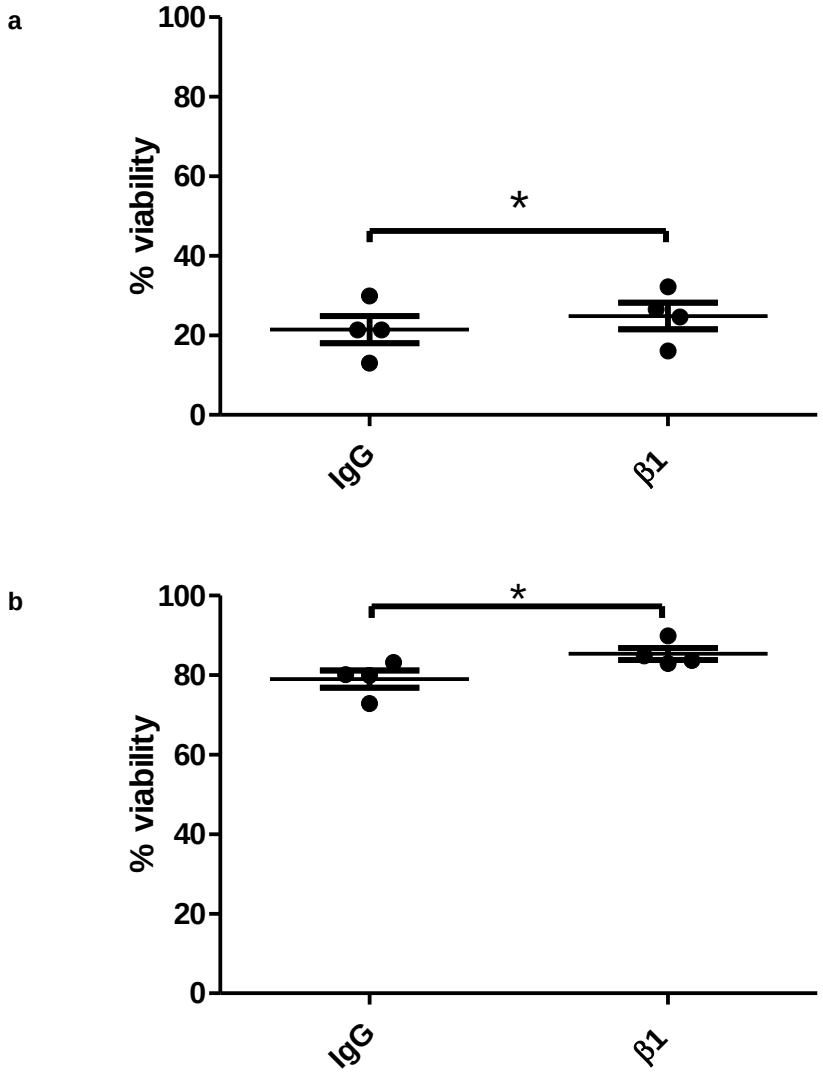


Figure 6.2 The effect of anti-β1-integrin antibodies on hepatocyte viability measured by flow cytometry

Primary hepatocyte and Huh 7.5 viability was assessed by flow cytometry using a cell viability marker. Incubation of (a) primary hepatocytes and (b) Huh 7.5 with anti-β1-integrin antibodies led to a small but significant increase in mean viability compared to those incubated with isotype matched control. Date expressed as mean and standard error of at least 3 experiments (dots represent individual experiments), *$p<0.05$.

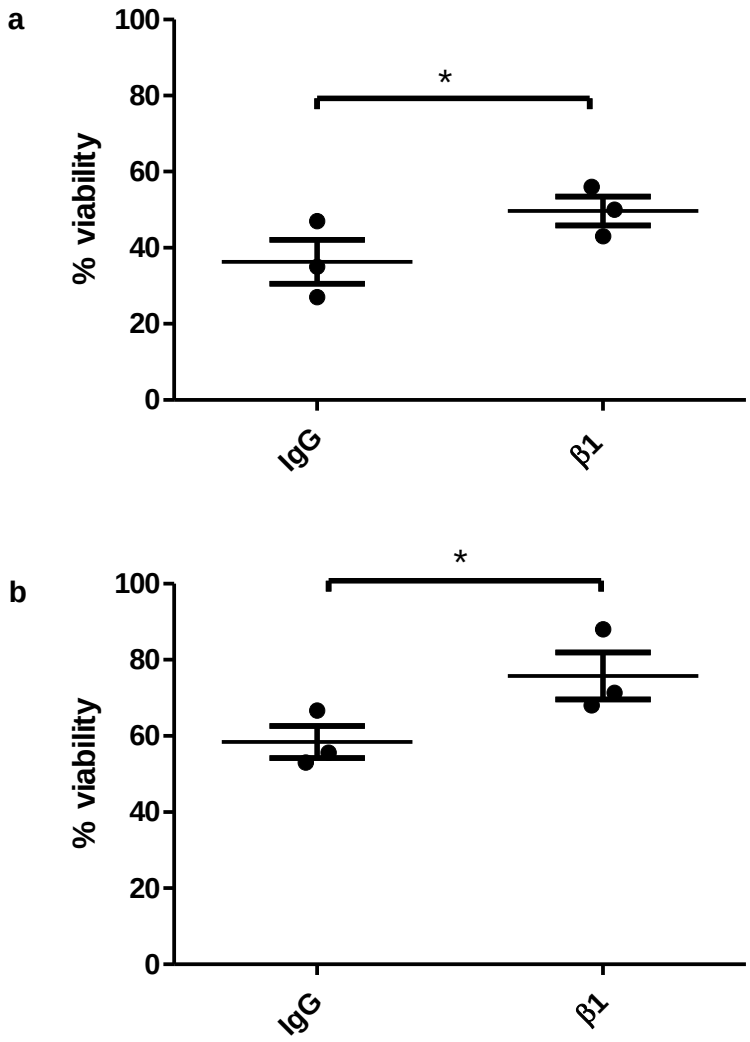


Figure 6.3 The effect of anti-β1-integrin antibodies on hepatocyte viability measured by trypan blue dye exclusion

Primary hepatocyte and Huh 7.5 viability was assessed using trypan blue dye exclusion. Incubation of (a) primary hepatocytes and (b) Huh 7.5 with anti-β1-integrin antibodies led to a small but significant increase in mean viability compared to those incubated with isotype matched control. Date expressed as mean and standard error of 3 experiments (dots represent individual experiments), *$p<0.05$.

6.2.2 Treatment of human hepatocytes with anti-β1-integrin antibodies results in a reduction in caspase-3 activity

Caspase-3 activity was then measured in lysates of hepatocytes or Huh 7.5 cells s incubated for 1 hour in suspension with anti-β1-integrin antibodies or isotype matched control. Caspase-3 activity as indicated by cleaved caspase 3 ELISA was significantly reduced in hepatocytes that had been treated with anti-β1-integrin antibodies (mean absorbance 1.37 ± 0.28 vs. 1.90 ± 0.36, p=0.02). Caspase-3 activity was similarly reduced in Huh 7.5 cells (mean absorbance 1.672 ± 0.24 vs. 1.943.± 0.26, p=0.002) (Figure 6.4 a, b).

6.2.3 Treatment of human hepatocytes with anti-β1-integrin antibodies results in activation of the protein kinase B/Akt pathway

Western blotting was used to evaluate PKB/Akt phosphorylation in hepatocytes treated with anti-β1-integrin antibodies compared to those treated with IgG control. Total Akt levels were constant across experiments; however, phosphorylated Akt levels were increased in hepatocytes treated with anti-β1-integrin antibodies (mean relative density 3.5 ± 0.73; p=0.03) suggesting that this treatment does lead to Akt activation within human hepatocytes (Figure 6.5).

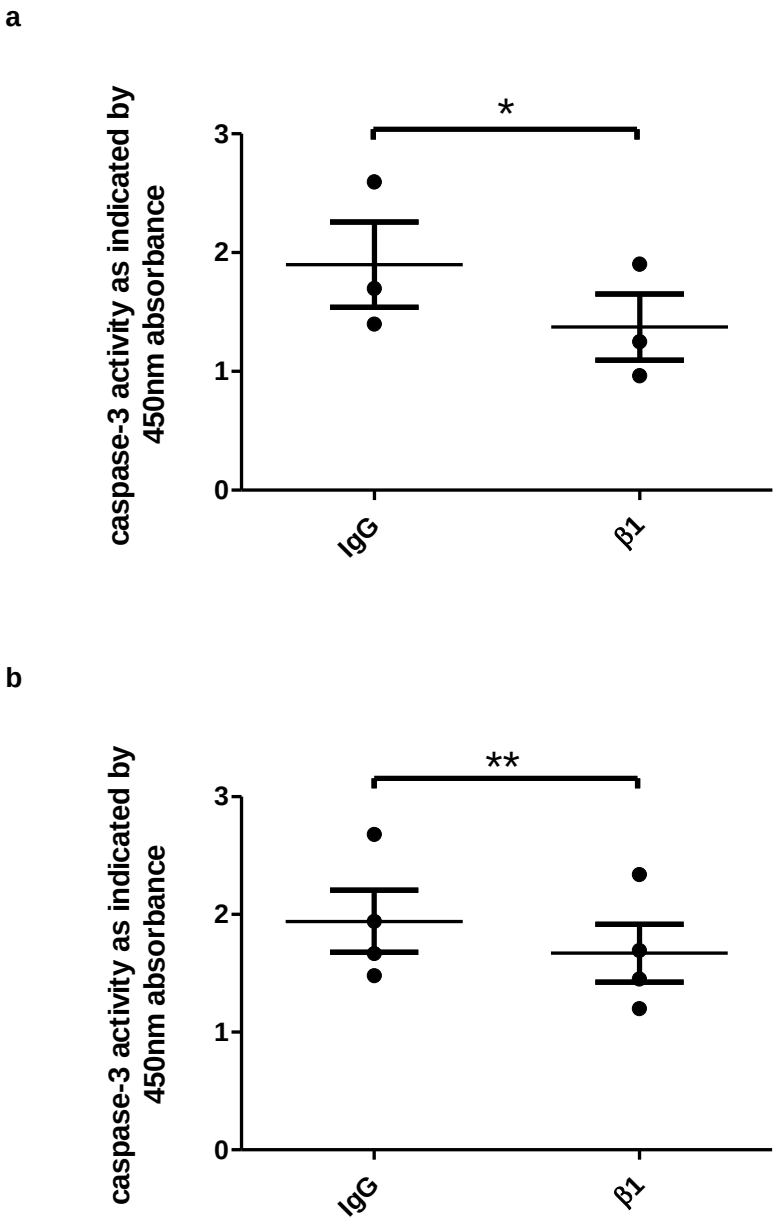


Figure 6.4 The effect of anti-β1-integrin antibodies on hepatocyte caspase-3 activity

Caspase-3 activity was measured by cleaved caspase-3 ELISA of hepatocyte lysates from (a) primary hepatocytes and (b) Huh 7.5 cells treated with anti-β1-integrin antibodies. Data expressed as mean and standard error of ≥3 independent experiments (dots represent individual experiments). *p<0.05, **p<0.01.

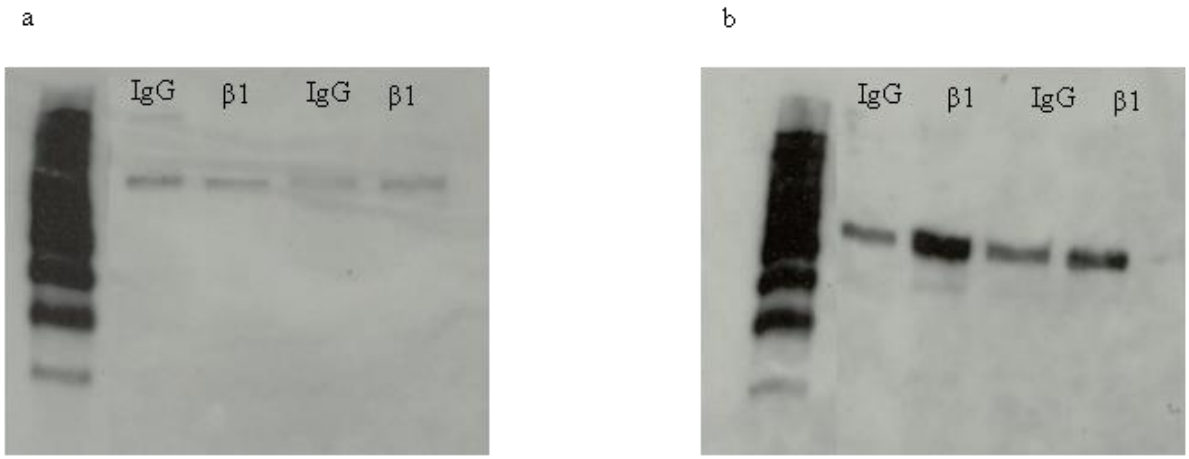


Figure 6.5 The effect of anti-β1-integrin antibodies on AKT signalling in primary human hepatocytes

Cell lysates were made form primary human hepatocytes following incubation with anti-β1-integrin antibodies or isotype matched control and the protein concentration normalised. Total and phosphorylated AKT were measured by Western Blot. Representative blots (two replicates) are shown for (a) total AKT and (b) phosphorylated AKT.

6.2.4 Human hepatocytes treated with anti-β1-integrin antibodies and allowed to adhere to HSEC demonstrate cytoskeletal reorganisation

I next investigated the interaction between hepatocytes and HSEC in vitro. Huh 7.5 cells transfected with GFP-vinculin and RFP-actin were incubated with anti-β1-integrin antibodies or isotype matched control and subsequently layered over an HSEC monolayer. Confocal microscopy was used to obtain images of individual Huh 7.5 cells once they had settled onto the HSEC. Huh 7.5 cells that had been pre-treated with anti-β1-integrin antibodies showed filamentous actin projections. Far fewer projections were seen with IgG control treated cells. Anti-β1-integrin treated cells also clearly demonstrated the formation of multiple discrete areas of actin/vinculin co-localisation at the point of contact between the Huh7.5 and HSEC suggesting the formation of focal adhesions/adherens junctions (Figure 6.6).

a

b

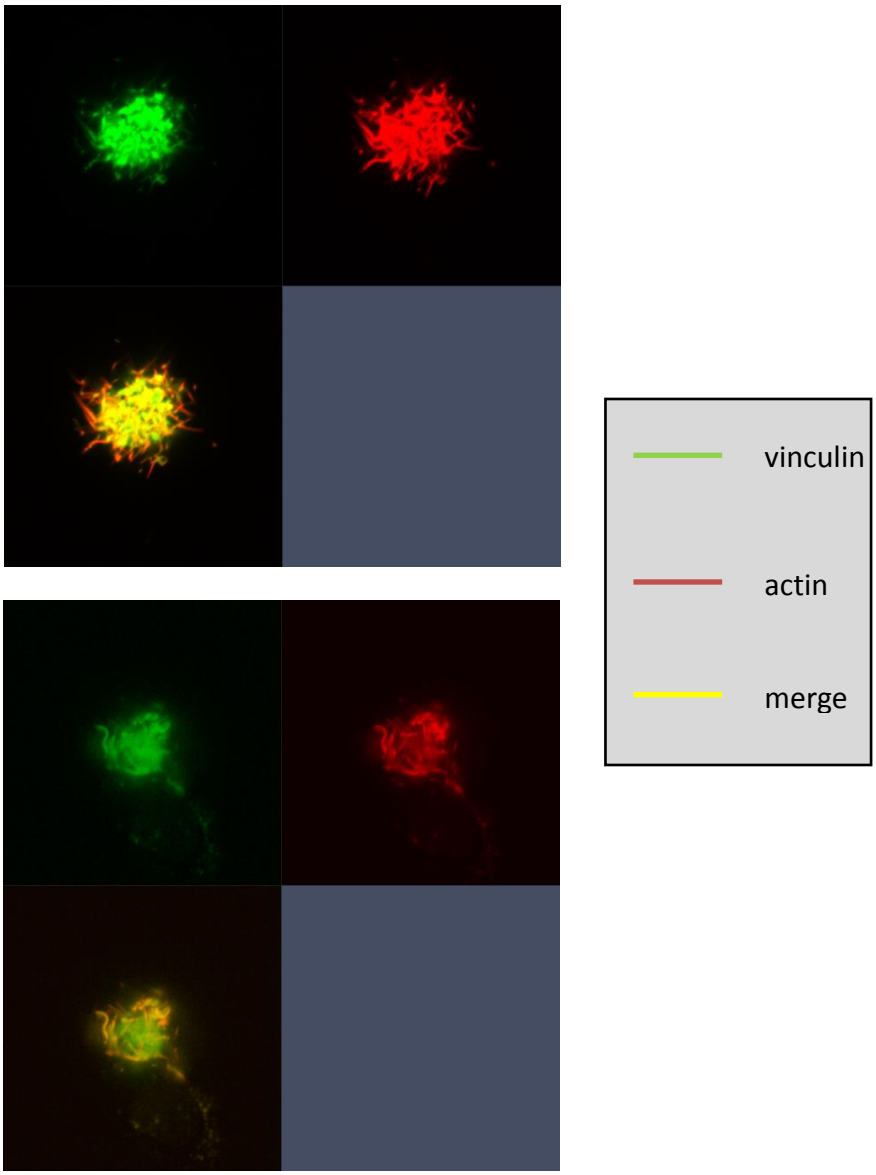


Figure 6.6 Action of anti-β1-integrin antibodies on hepatocyte cytoskeleton

Huh7.5 cells were transfected with plasmids containing GFP tagged vinculin and RFP tagged actin. The transfected cells were then allowed to adhere to a HSEC monolayer and imaged using confocal microscopy. Representative images with GFP and RFP colocalisation at the level of the interface with underlying HSEC are shown (100X magnification). (a) Huh7.5 pre-treated with anti-β1-integrin antibodies appeared to be spreading and showed filamentous projections and discrete areas of actin and vinculin colocalisation. (b) Cells treated with isotype matched control showed fewer projections and far less colocalisation of actin and vinculin.

6.3 Discussion

6.3.1 Hepatocyte survival

The importance of attachment to ECM for hepatocyte survival is well described, and β1-integrin in particular has been found to play a crucial role in hepatocytes. Rat hepatocytes cultured on polyhydroxyethylmethacrylate (polyHEMA) coated plates to prevent attachment rapidly show more extensive DNA fragmentation, a hallmark of apoptosis, than those cultured on ECM components. However, when hepatocytes that have been pre-incubated with anti-β1-integrin antibodies are cultured on polyHEMA there is a marked reduction in DNA fragmentation. Even when cultured on different ECM components to which hepatocytes are able to adhere, such as collagen, laminin and fibronectin, hepatocyte DNA fragmentation was evident within 5 hours of plating. However, no DNA fragmentation was detected up to 24 hours after plating in hepatocytes that had been pre-treated with anti-β1-integrin antibodies and cultured on ECM components (Pinkse et al. 2004). Furthermore, freshly isolated rat hepatocytes incubated with anti-β1-integrin antibodies showed a reduction in caspase-3 immunostaining compared to untreated hepatocytes (20.1±3.4% vs 39.8±3.9%, $P<0.02$), again suggesting a reduction in apoptosis (Pinkse et al. 2005).

My data confirms that the findings previously demonstrated in rat hepatocytes are replicated in human hepatocytes. I have demonstrated an increase in viability of human hepatocytes cultured in suspension with anti-β1-integrin antibodies. It is interesting that viability of primary hepatocytes was much lower in both the IgG and anti-β1-integrin groups compared to Huh 7.5 cells undergoing the same treatment. This is likely to reflect the fragility of primary hepatocytes especially when removed from culture flasks, with the necessary subjection to enzymes, and kept in suspension for a prolonged period. Huh 7.5

cells, however, are a cell line derived from hepatocellular carcinoma and are likely to have a degree of anoikis resistance. Absolute viability also varied for both primary hepatocytes and Huh7.5 cells in control and treatment groups between experiments. This was also the case in the flow adhesion assays described earlier, and likely reflects lot-to-lot variation in the quality of cells and the imprecise nature of certain steps in the experimental procedure such as removing cells from the culture flask.

The difference in viability between anti-β1-integrin treated and control cells, although significant, was small. However, repeating the experiment with both primary and Huh 7.5 cells using an alternative method of viability assessment produced similar results, and so I believe the difference to be real. Further, I have demonstrated reduced caspase-3 activity in isolated hepatocytes treated with anti-β1-integrin antibodies demonstrating a definite effect on the apoptotic pathway. Together these findings suggest that a similar β1-integrin mediated survival mechanism operates in human hepatocytes to that demonstrated previously in rat hepatocytes. One aspect that I have not studied here is the length of incubation with anti-β1-integrin antibodies, instead keeping to 1 hour which I used as a standard incubation time for all experiments. It is quite possible that longer incubation times may have revealed a greater difference in viability between anti-β1-integrin and isotype matched control treated cells. However, time course experiments described in 5.2.2.4 did not reveal an increase in hepatocyte adhesion to HSEC under flow with anti-β1-integrin antibody incubation times up to 4 hours compared to incubation for 1 hour. Therefore it is possible that only a small difference in viability may contribute to the differences in adhesion, migration and engraftment described in previous chapters. Alternatively, it may be that the effect on viability of the anti-β1-integrin antibody is unrelated to the effects seen in earlier studies.

6.3.2 PKB/Akt signalling

The PKB/Akt signalling pathway is well known to have a crucial role in cell survival and the anti-apoptotic and regenerative effects of PKB/Akt are important in hepatocyte survival and liver regeneration as well as the development of tumours such as HCC. Indeed, following partial hepatectomy, PKB/Akt is markedly activated and active PKB/Akt is essential for liver regeneration (Haga et al. 2009;Hong et al. 2000). Conversely the use of drugs such as fenofibrate, a fibric acid derivative with lipid-lowering activity, can be limited due to hepatic toxicity due to caspase dependent hepatocyte apoptosis resulting from the inhibition of PKB/Akt phosphorylation (Kubota et al. 2005). Fenofibrate may also suppress the growth of human HCC by blocking PKB/Akt activation (Yamasaki et al. 2011) whereas activation of PI-3K/PKB/Akt signalling with a drive towards cell survival mediates resistance to certain therapeutic agents for HCC (Chen et al. 2011).

My finding of an increase in PKB/Akt Ser473 phosphorylation following treatment of hepatocytes with anti-β1-integrin blocking antibodies suggests that the PKB/Akt signalling pathway is activated in human hepatocytes by β1-integrin and that anti-β1-integrin blocking antibodies are able to mimic the signal that is normally delivered through interaction with ECM. The improvement in hepatocyte viability that I have demonstrated following incubation with anti-β1-integrin blocking antibodies is likely to result from PKB/Akt activation and subsequent modulation of the apoptotic pathway and thus prevention of anoikis.

PKB/Akt signalling has previously been identified as a likely mechanism for β1-integrin mediated survival in several cell types. Treatment of rat hepatocytes cultured on polyHEMA with anti-β1-integrin antibodies results in a significant increase in the

proportion of hepatocytes expressing Ser473 phosphorylated PKB/Akt (assessed by immunostaining) compared to untreated hepatocytes (80% vs 20%; $P<0.05$) (Pinkse et al. 2005). Treatment of embryonic hippocampal neurons with an integrin-activating peptide derived from laminin (EIKLLIS) protects the cells from apoptosis and results in increased PKB/Akt activity. The effect is blocked by co-incubation with a PI-3K inhibitor or an anti-β1-integrin antibody (Gary et al. 2003). The fact that in that study a β1-integrin antibody completely abolished any increase in PKB/Akt activity following integrin activation suggests that the specific clone of blocking antibody is important. A number of different anti-β1-integrin antibodies are available; in the hippocampal neuron study, a polyclonal antibody was used. Pinkse et al treated rat hepatocytes with a hamster anti-rat monoclonal antibody (clone not specified) whilst I used a mouse anti-human β1-integrin antibody clone P5D2. A study of human lung fibroblasts found that treatment with the β1-integrin activating antibody TS2/16 in suspension culture resulted in an increase in PKB/Akt phosphorylated at Ser473. Interestingly, the authors state that this was inhibited when cells were pre-treated with the P5D2 anti-β1-integrin antibody although they do not show the data in the paper (Nho et al. 2005). It is possible that the use of two anti-β1-integrin antibodies together blocks the pro-survival effect of the P5D2 antibody; indeed, previous work by the same group suggested that the P5D2 anti-β1-integrin antibody treatment of fibroblasts, both attached and in suspension, leads to an increase in in Ser473 phosphorylation of PKB/Akt (Tian et al. 2002).

In addition to an increase in Ser473 phosphorylated PKB/Akt, Pinkse et al also reported an increase in ILK activity following treatment of rat hepatocytes with anti-β1-integrin integrin antibody and suggested that anti-β1-integrin integrin antibodies exert an effect on cell survival by activating ILK which in turn phosphorylates PKB/Akt on Ser473 (Pinkse

et al. 2005). However, the study offered no proof that ILK was directly responsible for PKB/Akt phosphorylation and it is quite possible that ILK activation is a separate phenomenon to the activation of PKB/Akt with phosphorylation of the latter occurring through a different mechanism. Nonetheless, ILK appears to have a role in survival signalling in hepatocytes; depletion of murine hepatocyte ILK results in increased apoptosis and caspase-3 activity compared to normal hepatocytes in culture and hepatitis in vivo. Reintroduction of ILK reverses this effect. However, PKB/Akt phosphorylation is not affected by the loss of ILK from hepatocytes suggesting that ILK does indeed act through a different pathway (Gkretsi et al. 2007). Conversely, Gary et al confirmed a possible role for ILK in PKB/Akt phosphorylation in their study of hippocampal neurons. Following transfection of cells with a kinase-dead ILK, integrin activation using EIKLLIS had no effect on levels of phosphorylated PKB/Akt where as a large increase was seen in untransfected neurons or neurons transfected with wild-type ILK (Gary et al. 2003). Similarly, knock down of ILK expression using small interfering RNA (siRNA) leads to a reduction of PKB/Akt phosphorylation and increases the level of apoptosis in fibroblasts (Nho et al. 2005).

In summary, whilst the exact mechanism and the possible role of ILK in promoting PKB/Akt phosphorylation either directly or indirectly is uncertain, my data lend support to the hypothesis that anti-β1-integrin antibodies improve hepatocyte survival by activating the PKB/Akt pathway.

6.3.3 Cytoskeletal reorganisation

The Rho family of GTPases are a group of small signalling proteins that have a major role in cytoskeletal reorganisation. Integrin-mediated adhesion results in the activation FAK and formation of an active FAK-Src complex and activation of subsequent signalling

pathways resulting in the regulation of several Rho-GTPases (Huveneers and Danen 2009). Key members of the Rho GTPase family involved in cell migration and cell-cell/cell-ECM adhesion include RhoA, which can modulate actin polymerisation, and Rac and Cdc42 which are involved in the formation of filopodia and lamellipodia, the actin cytoskeletal projections present on mobile cells (Bustelo et al. 2007;Etienne-Manneville and Hall 2002;Price et al. 1998). β1- and β3-integrin are particularly important in Rho-GTPase activation and may differentially activate RhoA and Rac/Cdc42, with the overexpression of β1- integrin in Chinese hamster ovary cells leading to enhanced Rac/Cdc42 activity and lamellipodia formation (Miao et al. 2002). β1-integrin ligation has been shown to result in activation of these pathways with effects on processes such as melanoma invasion (Nakahara et al. 1996;Nakahara et al. 1998) and migration of pancreatic cancer cells (Shields et al. 2012). In hepatocytes, Cdc42 may have a key role in establishment of cell polarity (Hua et al. 2012) and hepatocyte proliferation during liver regeneration (Cimica et al. 2005;Yuan et al. 2009). Hepatocytes from rats fed with an ethanol-containing diet show impaired cell spreading and attachment possibly due to a loss of Rac and Cdc42 activation (Schaffert et al. 2006;Tuma et al. 1999;Xu et al. 1992). ILK is also able to activate Rac and Cdc42 ultimately resulting in actin cytoskeleton remodelling and promotion of cell spreading and migration (Boulter et al. 2006;Filipenko et al. 2005).

My confocal microscopy data suggest that hepatocytes treated with anti-β1-integrin antibodies undergo reorganisation of the cytoskeleton when allowed to adhere to a HSEC monolayer. Discrete areas of actin/vinculin colocalisation may represent sites of adhesion between hepatocytes and endothelium or underlying matrix, with the enhanced spreading and generation of filopodia-like protrusions indicating a more migratory phenotype.

In conclusion, in this chapter I have demonstrated that treatment of human hepatocytes with anti-β1-integrin antibodies results in an increase in cell viability and most likely occurs through activation of the PKB/Akt signalling pathway leading to suppression of anoikis. Anti-β1-integrin treated hepatocytes also appear to have an enhanced spreading and migratory phenotype compared to untreated cells. A possible mechanism for this is through FAK/Src and ILK signalling with subsequent activation of Rho GTPases and cytoskeletal rearrangement.

CHAPTER 7: OVERVIEW

7.1 Overview

7.1.1 Hepatocyte isolation

The aim of the first part of this thesis was to re-establish the technique of hepatocyte isolation from human liver tissue within our laboratory. However, it soon became clear that the outcome of human hepatocyte isolation from the range of diseased liver tissue available within the laboratory was not ideal. Although we were able to isolate viable hepatocytes from a range of different types of normal and diseased liver tissue, the success rate of the technique and the viability obtained were modest (Bhogal et al. 2011). Huh 7.5 cells proved useful for optimising initial experiments and for experiments where the use of primary cells would have been extremely challenging. However, I felt it was important, where possible, to confirm results obtained with the Huh 7.5 cell line using primary hepatocytes and that this would add strength to the data. Therefore a significant part of the work presented here describes an attempt to improve the outcome of hepatocyte isolation from human liver tissue in order to obtain sufficient numbers of viable cells for experimental purposes.

Most laboratory research involving hepatocytes makes use of rat or murine hepatocytes, and there is very little literature available describing techniques for human hepatocyte isolation. However, two small studies looking separately at the use of NAC and Liberase showed that these reagents offer some improvement in the outcome of hepatocyte isolation (Donini et al. 2001;Sagias et al. 2010). Taken together with other studies concerning the mechanisms of action of NAC and the effect of Liberase on the isolation of other cell types, this suggested to me that their use in combination may improve the outcome of human hepatocyte isolation. I therefore developed an isolation protocol that included

NAC and Liberase and compared outcomes with the standard protocol that had been in use up until now in our laboratory.

The new protocol resulted in a significant improvement in the outcome of hepatocyte isolation from normal and diseased human liver tissue. I was able to greatly increase the success rate of hepatocyte isolation, meaning that the isolation process resulted in viable cells that would attach in culture and were therefore useful for experimental purposes. I also showed that two basic measures of hepatocyte function, urea and albumin synthesis, were as good as or better than that seen with hepatocytes isolated using the old protocol. Clearly there are many further aspects of hepatocyte function that warrant investigation, but these findings suggest that the new protocol does not negatively impact the functional quality of isolated hepatocytes. As well as providing the numbers of viable cells required for my subsequent experimental work, the outcome of this study in itself will be useful for others wishing to work with primary human hepatocytes.

7.1.2 Adhesion molecules expressed by primary human hepatocytes and their role in hepatocyte engraftment into host liver

Having established a reliable method for isolating primary human hepatocytes, the main aim of the rest of the thesis was identify the adhesion molecules expressed by human hepatocytes and attempt to modulate their function to improve engraftment in the host liver following transplantation. There is no comprehensive analysis of the surface expression of adhesion molecules by human hepatocytes. However, several studies describe the expression of integrins on human or other mammalian hepatocytes, with some demonstrating a role in adhesion. This, and the fact that integrins are known to play a key role in the adhesion of many cell types to ECM as well as in the transmigration of cells

across sinusoidal endothelium, suggested that examination of the integrin repertoire expressed by human hepatocytes would be a useful place to start.

I demonstrated surface expression of a wide range of integrin subunits by primary human hepatocytes, including several which, to the best of my knowledge, have not been described on hepatocytes before. For further functional I concentrated on those which are most highly expressed, namely α1, αV and β1 integrins. Using Huh 7.5 cells as a model of human hepatocytes, I showed that blockade of α1 and αV-integrin both resulted in reduction of adhesion to various types of liver tissue under static conditions suggesting that α1β1 and αVβ1-integrins have a role in hepatocyte adhesion. Interestingly, β1-integrin blockade alone did not reduce adhesion. Furthermore, in modified flow adhesion assays, β1-integrin blockade actually resulted in an increase in the proportion of hepatocytes remaining adherent to HSEC under flow. Together with the lack of effect of β1-integrin blockade in the static adhesion assay, this was a highly unexpected finding given that other groups have previously shown that β1-integrin blockade results in a loss of hepatocyte adhesion.

Despite being contrary to what I had expected, the β1-integrin blocking effect was an exciting finding as it could offer a potential means to improve hepatocyte engraftment. I therefore investigated this further, showing that by blocking β1-integrin I could increase the transmigration of hepatocytes through an HSEC monolayer. Most importantly, I showed that β1-integrin blockade also increased the initial engraftment of human hepatocytes into murine liver following intraportal injection. On review of the literature, I discovered that a similar effect had been demonstrated previously with rat hepatocytes although such a finding has not been reported previously with human hepatocytes. It is important, however, to bear in mind that the results of the animal experiments shown in

5.2.4 represent only the initial engraftment of transplanted cells during a brief 15 minute period. This, together with the very small number of mice used, clearly limits the conclusions that may be drawn. In order to confidently demonstrate the ability of β1-integrin blockade to reliably increase engraftment in mice, further longer term experiments using larger sample sizes are required. Nevertheless, with these promising initial results I therefore felt that targeting β1-integrin may provide a useful mechanism for improving the outcome of hepatocyte transplantation through increased engraftment. Furthermore, the preliminary data obtained from these studies will be useful for developing adequately powered longer-term engraftment experiments.

In order to begin to understand the effects of β1-integrin blockade in human hepatocytes, I looked at several aspects of β1-integrin signalling. Previous studies of hepatocytes and other cell types have shown a role for β1-integrin in the prevention of anoikis and that this is mediated, at least in part, through PKB/Akt signalling. Integrin signalling is also known to activate FAK/Src which in turn are able to regulate Rho-GTPases. These have an effect on cell migration and cell-cell/cell-ECM interactions. I have shown that following treatment of primary human hepatocytes with a β1-integrin blocking antibody there is activation of PKB/Akt signalling with a reduction in caspase-3 activity and an improvement in cell viability. Further, there is evidence of cytoskeletal rearrangement with perhaps a more migratory phenotype. Many of these findings are novel in human hepatocytes and offer an exciting starting point for future investigation of integrin signalling pathways in primary human hepatocytes in general, and, in particular, for the improvement of hepatocyte engraftment in hepatocyte transplantation.

7.1.3 Concluding remarks

Hepatocyte transplantation has the potential to supplement endogenous hepatocyte mass in liver failure or to correct genetic deficiencies. As such, it is one of a number of strategies that may help overcome the shortage of deceased donor organs. However, the success of this approach has been limited by inadequate hepatocyte engraftment in the native liver (Gupta et al. 1999a). Little is known about the mechanisms which regulate interactions between hepatocytes and liver sinusoidal endothelial cells despite the fact that such interactions are critical for successful engraftment. I therefore initially set out to investigate the role of adhesion molecules in this process in the hope that this would identify mechanisms that could be modulated to increase engraftment. An attempt to block β1-integrin function in order to demonstrate its role in human hepatocyte adhesion resulted in the unexpected finding that this manoeuvre actually increased adhesion to sinusoidal endothelium under flow. This led me to focus further on β1-integrin.

In conclusion, stimulation of the β1-integrin on the surface of primary human hepatocytes using anti-β1-integrin antibodies results in increased adhesion to and transmigration across HSEC, as well as increase engraftment in murine liver following intraportal injection. This is likely to be due to activation of integrin signalling pathways resulting in increased cell survival and cytoskeletal reorganisation. We and others have shown that the anti-β1-integrin antibody used in these studies is able to block β1-integrin-mediated adhesion with other cell types. Presumably, therefore, other adhesion molecules must be responsible for human hepatocyte adhesion and migration in this context. Exactly which molecules are involved cannot be answered by this study, but remains an important area for future research. However, the demonstration of an intervention to increase human hepatocyte engraftment into host liver holds promise for the future of hepatocyte transplantation.

7.1.4 Future Work

The first part of the thesis demonstrated a technique to significantly improve the outcome of hepatocyte isolation from human liver tissue. However, the 70% success rate achieved, whilst a great improvement on our previous results, still leaves room for improvement. Further research into the optimal preparation of liver tissue for hepatocyte isolation, the isolation process itself, and the subsequent culture techniques may yield further benefits.

- The preparation and storage of liver tissue for hepatocyte isolation

  We have previously shown that time to tissue processing has a significant impact on the success of hepatocyte isolation. However, further improvements in outcome may be achieved by greater attention to the handling of liver tissue prior to commencement of the isolation procedure, for example by using cold storage preservation techniques similar to those used for organs to be transplanted. Furthermore, it is not clear what constitutes the optimum `wedge' for hepatocyte procurement. Use of a whole liver, or an anatomic resection, may offer benefits through achieving a more thorough and even perfusion by using segmental vessels.

- The enzymatic digestion process

  Successful enzymatic digestion requires a balance between using an enzyme cocktail of sufficient activity to rapidly digest tissue whilst limiting damage due to an overly harsh enzyme preparation or prolonged digestion time. The data suggest that the use of Liberase and NAC goes someway to addressing this issue, but there may well be further improvements to be made.

- The techniques for culturing human hepatocytes

  Much has been published on culture techniques for hepatocytes. I used one such technique that, according to the authors, results in maintenance of various aspects of hepatocyte function for several weeks. However, the optimum culture medium and technique has yet to be identified, with most systems resulting in dedifferentiation, loss of hepatocytes phenotype and loss of viability. Cryopreservation may offer a solution to the problem of prolonged culture, but also requires further investigation and optimisation.

The remainder of the data presented herein suggests a mechanism to improve the engraftment of transplanted hepatocytes. Furthermore, it confirms for the first time in primary human hepatocytes the role of β1-integrin in cell survival signalling and cytoskeletal reorganisation that has been demonstrated by others in non-human hepatocytes and other cell types. Further work would improve understanding of β1-integrin signalling in human hepatocytes, and the mechanisms underlying human hepatocyte adhesion:

- The role of non-integrin adhesion molecules in hepatocyte adhesion

  I have demonstrated gene expression of a range of adhesion molecules by primary human hepatocytes. Further work to quantify surface expression and function of these would greatly add to our understanding of hepatocyte adhesion and may lead to other interventions to improve engraftment following hepatocyte transplantation.

- The mechanisms regulating hepatocyte migration

  I have demonstrated the expression of a number of chemokine receptors by primary human hepatocytes, and shown that they are able to transmigrate through an HSEC monolayer. Further study of hepatocyte migration and the role of individual chemokine receptors and adhesion molecules may identify alternative strategies to increase engraftment during hepatocyte transplantation. Furthermore, such studies may add to the current understanding of liver development and regeneration as well as the behaviour of hepatocellular carcinoma.

- The precise nature of integrin signalling through PKB/Akt and associated signalling molecules

  The data suggest that β1-integrin signalling results in activation of PKB/Akt with subsequent improvements in viability most likely due to the suppression of anoikis. However, a more complete understanding of the pathway by which β1-integrin ligation results in PKB/Akt phosphorylation may offer additional targets for modulation. Similarly, investigation of the mechanisms by which β1-integrin ligation results in changes in cytoskeletal arrangement would be of great interest.

- Longer term engraftment and functional studies

  The data presented demonstrate an increase in hepatocyte engraftment *in vivo* using a murine model of hepatocyte transplantation. However, I have only so far demonstrated short term engraftment. Whilst there is existing

animal data suggesting that once transplanted hepatocytes enter the liver parenchyma they are able to engraft and function in the longer term, it is important to confirm this with primary human hepatocytes using the techniques reported here. Functional outcome, using models such as the FRG mouse, would confirm that the increase in hepatocyte engraftment achieved results in a clinically meaningful benefit.

APPENDIX I

Publications resulting from this work:

1. Bartlett DC, Hodson J, Bhogal RH, Youster J, Newsome PN. ***Combined use of N-acetylcysteine and Liberase improves the viability and metabolic function of human hepatocytes isolated from human liver.*** Cytotherapy. 2014 Jun; 16(6):800-9. doi: 10.1016/j.jcyt.2014.01.006. Epub 2014 Mar 15

2. Aldridge V, Garg A, Davies N, Bartlett DC, Youster J, Beard H, Kavanagh DP, Kalia N, Frampton J, Lalor PF, Newsome PN. ***Human mesenchymal stem cells are recruited to injured liver in a β1-integrin and CD44 dependent manner.*** Hepatology. 2012 Sep; 56(3):1063-73.

3. Bhogal RH, Hodson J, Bartlett DC, Weston CJ, Curbishley SM, Haughton E, Williams KT, Reynolds GM, Newsome PN, Adams DH, Afford SC. ***Isolation of primary human hepatocytes from normal and diseased liver tissue: a one hundred liver experience.*** PLoS One. 2011 Mar 29; 6(3):e18222.

Published abstracts:

1. DC Bartlett, J Hodson, RH Bhogal, SC Afford, DH Adams, PN Newsome. ***N-acetylcysteine and Liberase improve success of hepatocyte isolation and viability of hepatocytes isolated from normal and diseased liver***. The Lancet, Volume 381, Page S21, 27 February 2013

2. DC Bartlett, VS Aldridge, A Wilhelm, N Davies, J Youster, DH Adams, PN Newsome. ***Anti β1-integrin antibodies improve survival of isolated human hepatocytes significantly increasing adhesion to hepatic sinusoidal endothelium under flow and engraftment in murine liver following transplantation.*** Journal of Hepatology Volume 56, Supplement 2, Page S163, April 2012

3. D C Bartlett; V S Aldridge; A Wilhelm; N Davies; J Youster; D H Adams; PN Newsome. ***Anti- β1-integrin antibodies improve survival of isolated human hepatocytes significantly increasing both adhesion to hepatic sinusoidal endothelium under flow and engraftment in murine liver following transplantation.*** Gut 2012 Vol: 61(Suppl 2):A119-A120

Presentations to national and international meetings:

1. Bartlett DC, Hodson J, Bhogal RH, Afford SC, Adams DH, Newsome PN. ***N-acetylcysteine and Liberase improve success of hepatocyte isolation and viability of hepatocytes isolated from normal and diseased liver.*** Poster presentation at the Academy of Medical Sciences Spring Meeting for Clinician Scientists in Training, Royal College of Physicians, London, February 2013.
2. Bartlett DC, Aldridge V, Wilhelm A, Davies N, Youster J, Adams DH, Newsome PN. ***Anti-β1-integrin antibodies improve survival of isolated human hepatocytes significantly increasing adhesion to hepatic sinusoidal endothelium under flow and engraftment in murine liver following transplantation.*** Poster presentation at DDF, Liverpool, June 2012.

3. Bartlett DC, Aldridge V, Wilhelm A, Davies N, Youster J, Adams DH, Newsome PN. ***Anti-β1-integrin antibodies improve survival of isolated human hepatocytes significantly increasing adhesion to hepatic sinusoidal endothelium under flow and engraftment in murine liver following transplantation.*** Presented (invited oral presentation and poster) at EASL, Barcelona, April 2012.
4. Bartlett DC, Aldridge V, Davies N, Youster J, Newsome PN. ***Anti β1-integrin antibodies confer increased survival to detached hepatocytes promoting their adhesion to hepatic sinusoidal endothelium under flow and improving initial engraftment in a mouse model of hepatocyte transplantation.*** Poster presentation at 24th UK Adhesion Society Meeting, Birmingham, 22nd September 2011
5. Aldridge VS, Davies N, Bartlett DC, Youster J, Kavanagh D, Kalia N, Lalor P, Frampton J, Newsome PN***. Human mesenchymal stem cells bind preferentially to injured liver in a β1 integrin and CD44 dependant manner.*** Poster presentation at BASL, London, September 2011

Awards:

1. EASL Young Investigator Bursary, January 2012
2. BASL Travel Award, October 2010

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