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STUDIES ON CELLULAR ENGRAFTMENT AND HEPATOCYTIC DIFFERENTIATION IN LIVER INJURY AND REPAIR

BY

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TO MY WIFE

LYNNE

DECLARATION

I hereby declare that this thesis is based on the results of experiments performed by myself, except where acknowledged, and that the thesis is exclusively of my own composition. It has not been submitted previously for a higher degree. This work was carried out in the Liver Cell Biology Laboratories of the Department of Medicine in collaboration with the Department of Pathology at the University Of Edinburgh, Scotland. I also undertook some of this work at the MRC Human Genetics Unit at the Western General Hospital, Edinburgh, Scotland.

Philip N Newsome

CERTIFICATE

We certify that Philip N Newsome has completed nine terms of experimental research and that he fulfilled the conditions of ordinance 39 of the University of Edinburgh, so that he is qualified to submit the following thesis in application for the Degree of Doctor of Philosophy.

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ABBREVIATIONS

AAF; Acetylaminofluorene

ALF; acute liver failure

ANOVA; Analysis Of Variance

BCIP/NBT; 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium

BSA; Bovine Serum Albumin

cEBPβ; CCAAT/enhancer binding protein β

cDNA; complementary DNA

CFU; colony forming unit

DAB; diaminobenzidine

DAPI; 4,6-diaminidino-2-phenylindole

DMEM: Dulbecco's' Modified Eagle Medium

DPPIV; Dipeptidyl peptidase IV

dUTP; 2'-Deoxyuridine 5'-Triphosphate

ECM; extra-cellular matrix

EDTA; ethylenediaminetetraacetic acid

FACS; Fluorescence-Activated Cell Sorter

FAH; fumarylacetoacetate hydrolase

FBS; fetal bovine serum

FCS; fetal calf serum

FGF; Fibroblast growth factor

FHF; acute liver failure

FISH; fluorescent *in situ* hybridisation

FITC; fluorescein isothiocyanate

GAPDH; glyceraldehyde-3-phosphate dehydrogenase

GFP; green fluorescent protein

GM-CSF; Granulocyte-Macrophage Colony stimulating factor

GPI; Glucose Phosphate Isomerase

HBSS; Hank's Balanced Salts Solution

HEPES; 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid

HGF; Hepatocyte growth factor

HNF4α; Hepatocyte Nuclear Factor 4α

HOC; hepatic oval cell

HRP; horseradish peroxidase

HSC; Haematopoietic stem cell

ICE; Interleukin-1β-converting enzyme

ISHAGE; International Society of Hematotherapy and Graft Engineering

LDL; low density lipoprotein

LIF; leukaemia inhibitory factor

mAb; monoclonal antibody

MACS; Magnetically activated cell sorting

MDM; Modified Dulbecco's medium

MHC; Major Histocompatibility Complex

mRNA; messenger RNA

MTT; 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide

NAPQI; *N*-acetyl-*para*-benzoquinone imine

NGF; Neurotrophin growth factor

NOD-SCID; non-obese diabetic-severe combined immunodeficient

OLT; orthotopic liver transplantation

PBS; Phosphate Buffered Saline

PCR; Polymerase chain reaction

PFOA: PerFluoroOctanoic Acid

PI; Propidium Iodide

PMS; phenazine methosulfate

SCF; Stem cell factor

SPF; Specific pathogen free

SSC; Sodium Saline Citrate

TBE; Tris Boric Acid EDTA

TEMED; N,N,N',N'-Tetramethylethylenediamine

TPO; Thrombopoietin

uPA; urokinase-type plasminogen activator

VEGF; vascular endothelial growth factor

WHHL; Watanabe heritable hyperlipidemic

Z-DEVD-AMC; 7-Amino-4-methylcoumarin, N-CBZ-L-aspartyl- L-glutamyl-L-valyl-l-aspartic acid amide

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ABSTRACT

Background: In recent years, considerable attention has been directed towards the use of cellular therapies in the management of acute and chronic liver failure. The majority of clinical studies have utilised hepatocyte infusions, although there is limited understanding of the factors which limit the engraftment of cells in the face of liver injury. The use of stem cells as sources of hepatocytes which can contribute to liver repair has been examined in rodent models, but the extrapolation of these findings to human stem cells is unclear.

Aim: In this thesis the factors which regulate the adhesion and survival of hepatocytes in the face of acute liver injury environment are examined. In addition, factors which regulate the differentiation of human stem cells towards hepatocytes are examined *in vitro* and *in vivo*.

Materials and Methods: Human hepatoblastoma (HepG2) cells were used as a model of human hepatocytes to study the effect of serum from patients with acute liver failure. Various laboratory assays were used to determine effects on adhesion, cell necrosis/apoptosis, integrin expression (flow cytometry) and integrin activation. Human cord blood was used as a source of human stem cells for both *in vitro* experiments and the *in vivo* work with the NOD-SCID mice.

Results: Paracetamol-induced liver injury results in the marked up regulation of collagen on hepatic sinusoids. Adhesion of HepG2 cells to Collagen after exposure to fulminant serum was reduced within only a few hours. Apoptosis occurred approximately 24-48 hours after incubation and is associated with caspase3 activation. Furthermore, fulminant serum reduces the adhesive capabilities of HepG2 cells by a rapid down-regulation of their β_1-integrin activity. Loss of cellular adhesion and subsequent apoptosis can be reversed by treatment of HepG2 cells with the stimulatory mAb TS2/16. Human cord blood could not be directed *in vitro* down the hepatocytic lineage under any of the different combinations of cytokines/matrix. *In vivo* however infused human cord blood cells are capable of engrafting into NOD-SCID mouse liver and differentiating down the hepatocytic lineage without fusion to host hepatocytes.

Conclusion: In this thesis, mechanisms regulating the engraftment and survival of HepG2 cells during exposure to fulminant serum were identified. Human stem cells were also demonstrated *in vivo* (but not *in vitro*) to differentiate into hepatocytes within the NOD-SCID mouse liver with no evidence of cellular fusion.

AIMS AND STRUCTURE OF THE THESIS

This thesis deals with acute liver injury and the role of cellular therapies in the treatment of it. At the time of my studies, hepatocyte transplantation had been studied in experimental animal models and was being muted as an adjunctive treatment for patients with acute and chronic liver injury. Furthermore, pioneering work demonstrated that stem cells, both rodent and human, had the capacity to differentiate into hepatocytes and deliver biological benefit.

The first part of this thesis (**Chapter 1; Introduction**) provides a general background of liver disease in terms of the aetiologies and clinical management. The literature pertaining to the use of hepatocyte transplantation is then reviewed, to highlight both the benefits and the drawbacks of this technique. One of the major issues of hepatocyte transplantation remains the relatively low level of cellular engraftment and survival in the face of acute liver injury, which has limited its applicability and effectiveness. This observation forms the cornerstone of the first half of my experimental work. The remainder of the introduction summarises the literature relating to another possible cellular therapy, using marrow and cord blood derived stem cells. Stem cells have recently been shown to have far greater pluripotentiality than previously thought being able to differentiate towards multiple non-haematopoietic lineages including hepatocytes. Thus, their potential role as an alternative source of cellular therapy is discussed, whilst noting the lack of data generated with human stem cells.

The second part of my thesis (**experimental work**) uses the information above to generate questions which constitute my experimental work. My experimental work breaks down into two major components; the first part examines the mechanisms which regulate the engraftment and survival of transplanted hepatocytes as would occur in liver injury (**Chapters 2-3**). This chapter details the *in vitro* model I established to test hepatocyte transplantation/survival and the subsequent work undertaken to identify the mechanisms which affected these processes in liver injury. The second half of my thesis studies the pluripotentiality of human stem cells both *in vitro* and *in vivo* (**Chapter 4 & 5**) as a prelude to their possible usage as a cellular therapy in liver injury.

The **discussion** section (**chapter 6**) includes a critical discussion of the results, the new insights introduced by this thesis, as well as future studies emerging from this work.

CHAPTER 1

INTRODUCTION

1.1 GENERAL INTRODUCTION

1.2 HEPATOCYTE TRANSPLANTATION

1.3 STEM CELL TRANSDIFFERENTIATION AND TRANSPLANTATION

1.1 GENERAL INTRODUCTION

Context of Acute Liver Failure: Aetiology and incidence

Acute liver failure (ALF) is a dramatic clinical condition that even with advances in modern management carries a high level of morbidity and mortality (Newsome et al 2001; Ritt et al 1969; Schiodt et al 1999). ALF is, however, a relatively infrequent condition and thus appropriate studies aiming to establish the incidence of ALF are difficult to conduct, often requiring multiple centres or long study times. A large retrospective study from the United States (US) of 295 patients with ALF admitted to 13 transplant centres over a two-year period confirmed that paracetamol overdosage was the commonest cause of ALF in the US (Schiodt et al 1999). It was however, unable to provide accurate data on incidence as data in some cases were obtained solely from transplant databases and thus only patients considered for transplant were included (Riordan et al 1999). Patients who did not satisfy transplant criteria were thus excluded, an important factor considering that many studies have demonstrated that a large proportion of patients with paracetamol-induced ALF either do not meet transplant criteria or have medical/psycho-social contra-indications to transplant (Mutimer et al 1994; O'Grady et al 1989). Furthermore, the population from which ALF patients are drawn is not always well defined thus preventing appropriate calculations of prevalence and incidence (Makin et al 1995). The Scottish Liver Transplantation Unit (SLTU) is the national liver transplantation unit for Scotland, and is the referral unit for management of all patients with acute liver failure, irrespective of suitability for transplantation. 379 patients with paracetamol-induced acute liver injury were admitted to SLTU between December, 1992, and March,

2001. Of these patients, 190 developed encephalopathy, thus fulfilling the definition of acute liver failure (Newsome et al 2001). In 2001, 21 such patients were admitted from a population of 4·99 million (1991 UK Census figure), but it is known that around 50% of paracetamol-overdose patients die at home or are not referred to SLTU (Blair et al 1998). Therefore, the incidence of paracetamol-induced acute liver failure for Scotland in 2001 is 0·84 per 100 000. The incidence for all cause ALF was 1.24 per 100,000 for that year. Legislative changes were introduced in the UK in August, 1998, to try to restrict the purchase of paracetamol, and hence reduce the incidence of ALF. Preliminary data from two liver transplantation units in England suggest that this measure has reduced the number of referrals of patients with acute liver failure (Hawton et al 2001; Prince et al 2000) however, both these units lack a geographically well-defined referral population. Our study compared data from patients admitted to SLTU in 1992-98 with those admitted in 1998-2001 to see whether incidence and outcome had changed in Scotland since the introduction of legislation (Newsome et al 2001). The incidence of paracetamol-induced liver failure, severity of patients' illness, and outcome did not differ between the groups. Therefore, it appears that the legislative changes have had no impact on the incidence of ALF in Scotland.

Paracetamol

Paracetamol is often taken in overdose, and although N-acetyl-cysteine is a highly effective antidote it must be given within 15 h to be most effective (Prescott et al 1979). Unfortunately, late presentation (>15 h) of overdose patients frequently leads to the development of acute liver failure, which has a high mortality rate. Activation of a relatively small percentage of paracetamol to *N*-acetyl-*para*-benzoquinone imine (NAPQI) by cytochrome P450, predominantly CYP 2E1, has been found to be involved in the mechanism of hepatic and, perhaps, renal toxicity (Bessems et al 2001). Most paracetamol is metabolized by glucuronidation, sulphation and conjugation with glutathione, which protects the liver at therapeutic doses. Doses of 300 mg/kg per day paracetamol and higher saturate conjugation reactions, deplete glutathione and result in binding of the benzoquinone imine to cellular proteins, including enzymes and transport proteins therefore impairing their function and leading to cell death (Blazka et al 1996; Laskin et al 1986; Moore et al 1985; Vermeulen et al 1992). The predominantly centrilobular localisation of paracetamol-induced liver injury is caused by the perivenular localisation of the cytochrome P-450 (CYP) 2E1, the most important enzyme for the formation of NAPQI. Oxidative processes are also considered important in the pathogenesis of paracetamol-induced liver cell damage. NAPQI is an oxidant, leading to the oxidation of thiol groups of hepatocellular proteins. The formation of free radical metabolites or reactive oxygen species may initiate the chain reaction of lipid peroxidation, which is an important phenomenon in many types of liver injury. Severe paracetamol toxicity can be prevented by the use of *N*-acetylcysteine (NAC), which prevents the hepatic glutathione stores from being depleted (Prescott et al 1979; Prescott 1979; Prescott et

al 1980) and prevents progression to severe liver failure and death (if administered early).

Severity and prognosis of ALF

In the event that severe liver injury results from the paracetamol overdose then prognostic criteria are utilised to predict clinical outcome. The most widely used criteria are the King's College Poor Prognosis Criteria (O'Grady et al 1989). If a patient meets these criteria, they have only a 5% chance of spontaneous survival and hence they are considered for Orthotopic Liver Transplantation (OLT). The criteria can be summarised as follows:

Paracetamol overdosage- Concurrent finding of a serum creatinine >300μmol/l, prothrombin time of >100 seconds, and grade III or IV encephalopathy in patients with normal pH, or the single finding of a pH <7.3 at 24 hours or more following the overdose after adequate fluid replacement.

Non-paracetamol cause- prothrombin time >100 seconds or presence of any three of the following: serum bilirubin >300μmol/l, jaundice to encephalopathy >1 week, age <10 or >40 years, aetiology non-A, non-B hepatitis or drug-induced hepatitis, prothrombin time >50 seconds.

Therapeutic options

If patients with paracetamol overdose are identified early they can receive NAC in the first instance, but as described earlier those patients who present late are unlikely

to respond to this and can go on to develop ALF. For those patients who meet the King's College Poor Prognosis Criteria there is only one clinically effective treatment, OLT.

OLT is generally a safe procedure, although in the context of ALF the morbidity and mortality do rise and one year survivals of 70% at 1 year (Bismuth et al 1995; O'Grady et al 1991) are typical in contrast to 90+% survival for chronic liver disease patients. Whilst OLT is the only effective treatment at present, it would be inappropriate in many patients and is limited by a shortage of donor organs. Furthermore, such patients have to take life-long immunosuppression with consequent toxicities and side effects. For this reason, alternative therapies have been explored to help both patients who are unsuitable for OLT and to help patients in the setting of OLT when critically ill.

The major two modalities investigated have been Bio-Artificial Livers and hepatocyte transplantation. Several biological and non-biological liver support systems have being developed over the years and are briefly reviewed below.

Non-biological systems include haemodialysis, haemofiltration, haemodiafiltration, use of adsorbents such as charcoal and resin haemoperfusion, haemodiabsorption plasmapheresis and plasma exchange. Despite prolonged and persistent testing, these systems have not been proven to alter the course of illness (Bihari et al 1983; Gazzard et al 1974; O'Grady et al 1988).

A bio-artificial liver (BAL) or extra-corporeal liver assist device (ELAD) consists of a mass of liver cells placed in a hollow fibre bioreactor perfused by the patient's own blood or plasma. There are three critical components of a bio-artificial liver support system (BAL): a) *the biological component* (functional hepatocytes), b) the *bioreactor* which can accommodate these cells, c) the development of an *efficient circuit* between the patient and the BAL in order to provide optimal exchange of nutrients, oxygen and permit detoxification of the fulminant plasma.

Several systems have been or are in the process of being tested in human clinical trials, although as yet there are no convincing studies demonstrating clinical efficacy. There remain many questions as to the ideal cellular substrate (Tsiaoussis et al 2001) and design of the BAL system. Consequently, more attention has been directed towards the role of cellular transplantation in liver injury. As will be described in the subsequent sections such cellular infusions can consist of hepatocytes (primary or immortalised) or primitive stem cells.

1.2 HEPATOCYTE TRANSPLANTATION

Orthotopic liver transplantation (OLT) has reduced the mortality of patients with fulminant hepatic failure, end-stage liver disease, and metabolic liver diseases, but is limited by donor organ availability and the requirement for lifelong immunosuppression. It is reasonable to hypothesize that hepatocyte transplantation may assist the management of these cases in several ways (Gupta et al 1999b; Holzman et al 1993). In cases of acute liver failure, hepatocyte transplantation may function as an adjunct treatment to provide temporary liver function to sustain patients awaiting OLT. As the liver has a high regenerative ability, in some patients, hepatocyte transplantation may allow enough time for the failing liver to regenerate. However, at this time it is difficult to predict, even with best available prognostic criteria (O'Grady et al 1989), which patients with acute liver failure will not need OLT. On the other hand, for the treatment of metabolic diseases, the goal of hepatocyte transplantation is to replace the metabolic function on a long-term basis to an extent that should be sufficient for amelioration or, preferably, cure of the disease. Furthermore, hepatocyte transplantation has several potential advantages over OLT. Cell transplantation is a nonsurgical procedure with much less morbidity. Where enzyme or protein replacement is the goal, several patients could receive cells from a single donor, alleviating the donor organ shortage. Although the cost of long-term immunosuppression may be similar to that of hepatocyte transplantation and OLT, the initial expenses of hepatocyte transplantation are estimated to be about 10% that of OLT. Indeed, for the treatment of metabolic diseases, cell transplantation can be performed on an outpatient basis, avoiding the cost of hospitalization. If

hepatocyte transplantation were to achieve its full potential, many cases of whole-organ transplantation could be prevented, providing more livers for cases where cell transplantation would not suffice. However, discarded livers are the usual source of hepatocytes for cell transplants, and steatosis greater than 30% is the commonest reason why a liver is discarded (Strom et al 2003). Thus, the challenge is to salvage sufficient hepatocytes from such livers to provide an adequate supply of transplantable hepatocytes. There is an increasing acceptance that hepatocyte transplantation may not be sufficient alone to result in the survival of patients with ALF, but that it may bridge them to OLT (Strom et al 1997b).

Hepatocytes can be genetically manipulated ex vivo with gene therapy protocols if needed. In certain cases, hepatocyte transplantation may have several advantages over current gene therapy methods. If the evidence obtained in experimental animals can be reproduced in humans, hepatocytes engrafted in the host liver parenchyma should survive for the lifetime of the recipient and express normal liver functions in a stable manner. This would avoid immunologic and other complications associated with the *in vivo* use of recombinant viruses or other gene transfer vehicles and would obviate the problems of promoter "silencing." Hepatocyte transplants have been tolerated well immunologically, requiring relatively small doses of immunosuppressive agents. During liver graft rejection, the immune response is directed predominantly toward bile duct epithelium or endothelial cells. This is predominantly due to the minimal expression of class I HLA antigens on hepatocytes (Daar et al 1984), which result in a decrease in their antigenicity. Notably there is increased expression of class I HLA antigens on the hepatocytes of patients who had developed acute cellular rejection (So et al 1987). Approximately 20 years of

preclinical experience with laboratory animals has established the feasibility of hepatocyte transplantation in treating liver-based diseases, leading to a movement of hepatocyte transplantation from the bench to the bedside.

Preliminary demonstration of function after hepatocyte transplantation

Transplantation of isolated hepatocytes has been studied in the laboratory since the early 1970s. Work in experimental animals demonstrated that following transplantation into the liver, spleen, or other ectopic sites, hepatocytes displayed normal architecture at the light and electron microscopic level (Kusano et al 1982; Mito et al 1979). In addition, hepatocytes that integrated and survived at the transplantation site display differentiated hepatocyte functions, including albumin secretion (Demetriou et al 1986; Mito et al 1979; Moscioni et al 1989), glycogen storage and glycolysis (Kusano et al 1982; Mito et al 1979), bilirubin conjugation (Demetriou et al 1986; Hamaguchi et al 1994; Moscioni et al 1989; Vroemen et al 1986), ammonia metabolism (Habibullah et al 1994; Schumacher et al 1996; Takeshita et al 1993), and cytochrome P450 gene expression (Maganto et al 1990).

Animal Models of Inherited Metabolic Diseases of the Liver

Hepatocyte transplantation has been used to correct defects of metabolic function of the liver and to provide immediate liver function during acute liver insufficiency. In Gunn rats with genetic deficiency of the enzyme that mediates bilirubin glucuronidation (bilirubin-UGT), hyperbilirubinemia was ameliorated by hepatocyte transplantation into the spleen, liver, or peritoneal cavity (Demetriou et al 1986; Matas et al 1976), and bilirubin conjugates were identified in the bile of the recipient

animals, indicating function of the transplanted cells (Vroemen et al 1986). Significant increases in plasma albumin levels occurred following transplantation of hepatocytes into analbuminemic rats (Holzman et al 1993; Moscioni et al 1996). Transplantation of normal hepatocytes resulted in reduction of cholesterol levels up to 50% in hyperlipidemic rabbits with inherited low-density lipoprotein (LDL) receptor deficiency (Eguchi et al 1996; Gunsalus et al 1997) and, more importantly, prevented the formation of atherosclerotic plaques in the aorta (Gunsalus et al 1997).

Animal Models of Liver Failure

Hepatocyte transplantation also provides support in experimental models of fulminant hepatic failure. Enhanced survival or complete recovery of animals with hepatic failure induced by a variety of mechanisms has been reported (Newsome et al 2000). Transplantation of hepatocytes significantly improves the survival of animals in liver failure due to D-galactosamine-induced hepatic failure (Makowka et al 1981; Sommer et al 1979), hepatic insufficiency following 90% hepatectomy (Demetriou et al 1988), or ischemic liver injury. Hepatocyte transplantation can reverse hepatic encephalopathy (Ribeiro et al 1992; Schumacher et al 1996) and prevent intracranial hypertension in animals with ischemic liver failure (Arkadopoulos et al 1998). A major limitation of hepatocyte transplantation is the inability to isolate an adequate number of transplantable hepatocytes. A highly differentiated cell line, NKNT-3, was generated by retroviral transfer in normal primary adult human hepatocytes of an immortalizing gene that could be subsequently completely excised by Cre/Lox site-specific recombination. When transplanted into the spleen of rats under transient immunosuppression, reversibly immortalized NKNT-3 cells provided life-saving

metabolic support during acute liver failure induced by 90% hepatectomy (Kobayashi et al 2000).

Animal Models of Liver Repopulation

Studies with transgenic mice overexpressing urokinase (uPA) (Rhim et al 1994; Rhim et al 1995) and in mice deficient in the tyrosine metabolizing enzyme fumarylacetoacetate hydrolase (Overturf et al 1996; Overturf et al 1997) show that in situations where the host liver cells have reduced life-span, a small number of transplanted differentiated adult hepatocytes can correct hepatic failure and almost completely repopulate the donor liver. Small animal models will be important in the refinement and identification of relevant mechanisms in relation to hepatocyte transplantation, whereas large animal models (pig) will be more relevant for successful translation into clinical practice.

Hepatocyte Transplantation for Liver Failure in humans

Hepatocyte transplantation for the treatment of human disease was first reported by Mito and Kusano, who transplanted hepatocytes by injecting into the spleen in 10 patients in Japan (Mito et al 1992). In an intra-operative procedure, a lateral semi-segmentectomy (S3) was performed on 10 patients with liver cirrhosis or chronic hepatitis. Freshly isolated hepatocytes were infused immediately into the patients. In two patients, 10 to 600 million hepatocytes were injected directly into the inferior region of the spleen. The splenic hilum was clamped for several minutes to prevent hepatocytes from migrating out of the spleen through the splenic vein into the portal vein and liver. Epidermal growth factor was injected into the splenic artery to

stimulate hepatocyte proliferation. Hepatocytes could be detected in the spleen by the radionucleotide uptake in 8 of 9 patients examined at times ranging from 1 to 11 months. Although the presence of the cells was established, the researchers did not feel that the transplantation of hepatocytes was responsible for the clinical improvement noted in the patients, but rather reported the feasibility and safety of the procedure for future studies.

Habibullah et al. reported that the transplantation of fetal human hepatocytes into the peritoneal cavity improved the survival of patients with fulminant hepatic failure (FHF) (Habibullah et al 1994). In a study conducted in India, patients with FHF of different aetiologies of less than 2 weeks' duration and having portosystemic encephalopathy with grades III and IV encephalopathy and without other complicating factors were admitted. Patients who did not give consent for the hepatocyte transplantation served as controls. Hepatocytes were isolated from aborted foetuses of either sex with gestational ages ranging from 26 to 34 weeks and were pooled. The hepatocytes, 60 million/kg body weight, were transplanted intraperitoneally. Survival in the hepatocyte-transplanted group was 48% and was statistically different from the 33% survival rate observed in the matched controls in patients with grade III and IVa encephalopathy. Survival was 100% in the transplant group in patients with grade III encephalopathy. Hepatocyte transplantation did not improve the survival of patients with grade IVb encephalopathy, suggesting that hepatocyte transplantation is most effective when used earlier in the clinical course. In survivors, recovery from portosystemic encephalopathy was observed after an average of 48 hours of cell transplantation and was accompanied by decreases in blood ammonia and bilirubin levels. No patients with prothrombin times twofold of

normal or higher survived. The authors suggested that the data demonstrated the beneficial effect of fetal hepatocyte transplantation in FHF, with the most pronounced effects observed in patients with grade III hepatic encephalopathy.

Work in the United States, set out to determine if hepatocyte transplantation could be used to support hepatic function in end-stage liver failure in patients awaiting OLT. Eleven patients, seven with fulminant hepatic failure and four additional patients with chronic liver failure with acute decompensation, received hepatocyte transplants into the splenic artery (Strom et al 1997a). Relatively few cells (not exceeding 1.2×10^9) could be transplanted owing to the impairment of blood flow caused by hepatocytes lodging in the splenic capillaries. Two patients died awaiting OLT, six of the nine patients with FHF survived long enough to receive whole-organ transplants. Four patients with chronic liver disease with acute decompensation of liver function received hepatocyte transplantation. One received a successful OLT, one patient died of complications with an intracranial monitor, life support was terminated on one patient on day 7 following hepatocyte transplantation because of intracranial haemorrhage and a fourth patient with FHF resulting from a trisegmentectomy died 48 hours following hepatocyte transplantation.

The average survival time in the patients who received successful OLT was 3.8 ± 3.3 days (mean $\pm$ standard deviation) with a range of 1 to 10 days. In the patients who died awaiting OLT, survival was 4.6 days $\pm$ 2.5 days, with a range of 2 to 7 days. Survival in control patients who elected to not receive hepatocyte transplantation was 2.8 days $\pm$ 2.5 days, with a range of 1 to 7 days. Because of the lack of statistical significance of the difference in the duration of patient survival in the transplanted control groups, it is difficult to prove that hepatocyte transplantation improved

survival. However, some changes in clinical parameters suggest that the transplantation of hepatocytes, even in small numbers, may be beneficial. In many cases, blood ammonia levels decreased, cerebral blood flow improved, intracranial pressure was reduced, and there was an improvement in encephalopathy after hepatocyte transplantation (Strom et al 1997b; Strom et al 1997a). Increased cardiopulmonary stability also was documented following hepatocyte transplantation, by the decreased need or withdrawal of vasopressor drug support. In the first few days following hepatocyte transplantation, the effects on synthetic functions, such as circulating clotting factors and metabolic functions, such as bilirubin conjugation, were variable or absent.

Hepatocytes as a Vehicle for Ex Vivo Gene Therapy

Prevention of rejection of allografted hepatocytes requires the administration of immunosuppressive agents, which can be avoided by using autologous hepatocytes harvested from the patient and transplanted back into the donor after phenotypic correction by introduction of the therapeutic gene (Patel et al 1989). The first long-term preclinical study of liver-directed ex vivo gene therapy was performed in LDL receptor-deficient Watanabe heritable hyperlipidemic (WHHL) rabbits (Chowdhury et al 1991). Primary hepatocytes harvested from WHHL rabbits were transduced with a recombinant retrovirus expressing the human LDL receptor gene. The phenotypically corrected hepatocytes were then transplanted into the donor rabbit, resulting in long-term reduction of serum LDL levels. This was followed by a clinical trial in patients with familial hypercholesterolemia, who also have abnormalities of the LDL receptor (Grossman et al 1994; Grossman et al 1995).

Results of the study in four patients showed a modest reduction of plasma cholesterol levels and persistence of the transplanted cells and transgene function. Although it is generally felt that the effect was not adequate for a therapeutic benefit, the experiments established the feasibility of hepatocyte transplantation and the longevity of the transplanted cells.

The precise number of hepatocytes needed to sustain the needs of a human recipient has not been clearly established. Results from animal studies have suggested that only 0.5 to 3% is required (Lake 1998) but this is at odds with clinical studies which have demonstrated that the minimum amount of hepatic tissue required to sustain the human body after partial hepatectomy is approximately 20% (300g) of the normal liver mass (Lo et al 1997). Human studies of hepatocyte transplantation have not answered the slightly different question of how much transplanted liver cell mass is needed to maintain the body's basic metabolic needs in a disease (ALF) characterised by the almost complete loss of functional hepatocytes. Studies in patients with ALF have shown that smaller amounts of transplanted liver cell mass (5 to 80g) prolong life without leading to survival (Bilir et al 2000) so further studies are required to identify the minimum amount of transplanted liver cell mass needed to result in survival of patients with liver failure. The issue of minimum cell number is especially pertinent given that the availability of hepatocytes is limited. Of concern are studies suggesting that hepatocyte transplantation in the context of ALF is associated with very low levels of engraftment in the recipient liver (Allen et al 2001; Gupta et al 1999b).

The optimum site of hepatocyte transplantation has also been the subject of much research, with initial studies looking at ectopic sites because transplanted cells could not otherwise be distinguished from host hepatocytes. Early studies established that syngeneic hepatocytes could survive, proliferate and express genes normally in several ectopic sites, such as the spleen (Gupta et al 1999b). Notably, hepatocytes did not survive when transplanted into the splenic arterial bed (Mito et al 1978), whereas cells trapped in vascular spaces, sinusoids and venous radicles were able to engraft (Darby et al 1986). This dependence on vascular channels is nicely demonstrated by the mouse spleen, which lacks sinusoids, and hence transplanted hepatocytes rarely survive (Gupta et al 1999a). It is likely that spleen (with the exception of mice) confers appropriate vascular channels with extracellular matrix expression, without the high velocity flow found in other organs such as the lung (Gupta et al 1999a). Whilst hepatocytes appear to function well in the spleen, other sites such as the dorsal fat pad and the peritoneal cavity were less effective in supporting hepatocytes. This most likely reflected a combination of decreased cell survival and gene expression and also greater immune responses against soluble gene products released by the transplanted cell (Gupta et al 1994; Vemuru et al 1992). To this end studies tried with only partial success to determine whether encapsulating transplanted hepatocytes in biomaterials would diminish their immunogenicity thus preventing rejection (Dixit et al 1990). These failed in part because encapsulation prevented the necessary anchorage of hepatocytes to ECM (thus leading to cell death and/or de-differentiation) and also because the biomaterials were degraded by the body in due course leading to delayed immunogenicity. Other studies demonstrate that co-transplantation of hepatocytes and biliary/endothelial cells into the peritoneal cavity

led to prolonged survival in syngeneic recipients (Selden et al 1995). However, given that endothelial cells play a central role in generating allograft rejection it is unlikely that this will have widespread applicability. In contrast to ectopic sites, the liver microenvironment offers many unique advantages for transplanted hepatocytes such as the correct ECM, growth factors, nutrients as well as allowing the interaction with other liver cells. Some of the most successful examples of hepatocyte transplantation have been in animal models where the hepatocytes were infused intra-portally into the liver. These include the amelioration of jaundice in Gunn rats, which are a model of Crigler-Najjar syndrome (Groth et al 1977; Matas et al 1976; Rugstad et al 1970), and also improved survival of animals with acute liver injury (Sommer et al 1979; Sutherland et al 1977). Concerns also arose about the efficacy of cell transplantation as studies demonstrated that intact cells were not necessary for improving outcomes in animals with acute liver injury (Miyazaki et al 1983). Experiments with subcellular fractions of hepatocytes demonstrated that an intact cell was not required and that a heat stable "factor" (or factors) present in the cytosol fraction, which is not insulin or glucagon, is responsible for the increase in survival observed. This factor appears to increase the rate of endogenous regeneration of the injured recipient liver (Makowka et al 1980).

Within the sinusoidal bed can be found the optimum microenvironment for an engrafting hepatocyte, in terms of extracellular matrix components, growth factors, nutrients and interactions with other cells. Several hours after cell transplantation the sinusoidal endothelium is disrupted thus facilitating the entry of hepatocytes into the space of Disse. This effect is in part induced by the release of vascular endothelial growth factor (VEGF) from both transplanted and host hepatocytes, which acts as a

vascular permeability factor (Gupta et al 1999c). Engraftment of transplanted cells in the liver requires their deposition in the hepatic sinusoids. Whilst most transplanted cells (70%–80%) are destroyed in the liver within 24–48 hours, the remaining transplanted cell fraction is observed to enter the liver plate after several hours, with such cells eventually integrating within liver parenchyma (Gupta et al 1999c). However, deposition of cells in hepatic sinusoids activates ischemia-reperfusion events because sinusoidal blood flow is occluded transiently because of cells serving as emboli. This leads to microcirculatory perturbations in the liver, activation of γ-glutamyl transpeptidase expression, and disruption of gap junctions in native hepatocytes (Gupta et al 2000b). Cell transplantation activates macrophages, as demonstrated by their appearance in cellular infiltrates surrounding transplanted hepatocytes (Gupta et al 1999c). Most transplanted cells that fail to engraft are entrapped in portal vein radicles and these cells appear to be primarily cleared by macrophage-mediated processes. Within normal liver architecture, hepatocytes bind to basement membrane components such as laminin and collagen through cell surface glycoproteins termed adhesion molecules. The adhesion to extracellular matrix (ECM) is essential for the growth and survival of hepatocytes, such that their displacement from ECM leads to dedifferentiation and apoptosis, a phenomenon known as anoikis (Bretland et al 2001; Frisch et al 2001).

Extracellular matrix disruption in liver disease

The extracellular matrix of both diseased and normal liver has been examined more closely in the light of suggestions that not only does ECM provide passive structural support but that it also plays a major role in the maintenance of differentiated

hepatocyte phenotype and function of liver parenchyma. The pattern of ECM expression may also be important in cellular engraftment representing a scaffold to which engrafting cells bind. Whilst there are documented variations in major ECM expression across different types of chronic liver injury (see table 1.1) compared with normal liver (Van Eyken et al 1990), there are no published data on their expression in acute liver injury.

Table 1.1 Extracellular matrix expression in normal and injured liver

	Collagen IV	Fibronectin	Laminin	Tenascin
Normal liver	Positive staining of Sinusoids & Basal membranes of blood vessels/bile ducts	Positive staining of Sinusoids & Portal tracts Weak granular cytoplasmic staining of hepatocytes	Weakly positive /negative sinusoidal staining Strong staining of basal membranes of blood vessels/ bile ducts	Faint staining of sinusoids
Alcoholic liver disease	Positive sinusoidal staining	Positive sinusoidal staining Granular cytoplasmic staining of hepatocytes	Positive sinusoidal staining, most marked near fibrous septae	Positive sinusoidal staining Portal tracts negative
Cholestatic liver disease	Positive sinusoidal and septal staining	Positive sinusoidal and septal staining	Positive sinusoidal and septal staining	Weak positive sinusoidal staining
Acute hepatitis	As per normal liver	As per normal liver	As per normal liver	Strongly positive sinusoidal staining
Chronic hepatitis	Positive sinusoidal staining	Positive sinusoidal staining	Positive sinusoidal staining	Positive sinusoidal staining

Adhesion to matrix; molecular mediators

Integrins appear to be the major receptors by which cells attach to extracellular matrix, and are also important in mediating cell-cell adhesion events (Hynes 1992). Integrin mediated adhesion is fundamental to cell transplantation as only after this has been successfully negotiated can cells respond to appropriate migratory signals and migrate across the endothelium and into the liver plate. Integrins are heterodimeric transmembrane proteins consisting of non-covalently linked alpha and beta chains each with a large extracellular, single transmembrane and short cytoplasmic domain (Hynes 2002). Integrins are activated by contact with ECM proteins (Wu et al 1995) which leads to inhibition of interleukin-1beta converting enzyme expression preventing apoptosis and thus providing them with a major role in determining cell survival (Giancotti et al 1999). Once activated, integrins lead to protein tyrosine phosphorylation which has been shown to play a protective role in the regulation of apoptosis (Evans et al 1993; McGahon et al 1995; Simizu et al 1996; Tallett et al 1996). In the setting of small cell lung cancer cells, ECM-mediated protection from etoposide-induced caspase-3 activation can be blocked by either β1-integrin function-blocking antibody or by a tyrosine kinase inhibitor (Sethi et al 1999; Tallett et al 1996) Other groups have demonstrated that ECM regulates apoptosis (mammary epithelial cells) through an integrin-dependent negative regulation of Interleukin-1β-converting enzyme (ICE) expression, whereby expression of ICE was correlated with the loss of ECM and inhibitors of ICE activity prevented apoptosis (Boudreau et al 1995). Thus, integrin activation prevents the ICE-mediated induction of caspase-3 activity. Therefore factors which modulate the

interaction and hence activity of integrins play an important role in determining cell adhesion and survival (Diamond et al 1994; Kim et al 1997). Our understanding of the factors controlling integrin affinity are incomplete but work suggests that control is exerted in an energy dependent fashion on their cytoplasmic domain (Kim et al 1997). CD98 has been suggested as a potential controller of β_1-integrin affinity; its crosslinking leading to activation of the β_1-integrin and hence maintenance of integrin survival signaling (Cho et al 2001).

A clear understanding however does not exist as to what factors in liver injury modulate the engraftment and survival of transplanted cells, with most work concentrating on the toxicity of the host environment to donor cells.

Integrin expression on hepatocytes and hepatocellular carcinoma cells

Integrin α5 β1 and α2 β1 are the major integrin receptors in human hepatocytes. However, in human hepatocellular carcinoma (HCC) cells it was found that the expression of integrin α5 β1 was decreased and another integrin α6 β1 increased (Masumoto et al 1999). Several studies have described altered expression of β 1 integrins in hepatocellular carcinoma (HCC). Reduced expression of α 2, α 3, and α 5 subunits (Jaskiewicz et al 1995; Yao et al 1997) and overexpression of α 6 subunits of β 1 integrins (Begum et al 1995) have been shown in HCC with aggressive phenotypes.

In vitro models of hepatocyte transplantation

With a view to establishing *in vitro* models of hepatocyte transplantation groups have studied the effect of incubating hepatocytes in culture with either toxins or sera from liver injured patients. Serum from patients with fulminant hepatic failure has been shown to induce a dose-dependent inhibition of proliferation (Anilkumar et al 1997; Demetriou et al 1974; Gohda et al 1986; Gove et al 1982; Tada et al 1986; Williams et al 1977; Yamada et al 1994) and protein synthesis (Shi et al 1998), and in some studies induces cell death (Fujioka et al 1997; Hughes et al 1976; Williams et al 1977) of cultured hepatocytes.

Cell death: Apoptosis and Necrosis

Apoptosis and necrosis are two major processes by which cells die. Apoptosis is the ordered disassembly of the cell from within resulting in changes in the phospholipid content of the plasma membrane outer leaflet (Thornberry et al 1998). Phosphatidylserine (PS) is exposed on the outer leaflet and phagocytic cells recognizing this change engulf the apoptotic cell (Fadok et al 1992). Necrosis however, normally results from a severe cellular insult, and is characterised by the presence of an inflammatory response. Both internal organelle and plasma membrane integrity are lost, resulting in spilling of cytosolic and organellar contents into the surrounding environment. Immune cells are attracted to the area and begin producing cytokines that generate an inflammatory response. Other techniques that have been used to distinguish apoptosis from necrosis in cultured cells and in tissue sections include detecting PS at the cell surface with annexin V binding (Ferlini et al 1997), DNA laddering (Facchinetti et al 1991), and staining cleaved DNA fragments that

contain characteristic ends. At the extremes, apoptosis and necrosis clearly involve different molecular mechanisms, but it is not clear if there is cellular death involving both the molecular mechanisms of apoptosis and those of necrosis. Many pathological features can be used to differentiate between apoptosis and necrosis, although they are not all well suited for rapid quantitation of the phenomena as they occur (see table 1.2 below).

Table 1.2 Features distinguishing apoptosis and necrosis

	Apoptosis	**Necrosis**
Cell size	Decreases with evidence of fragmentation	Swelling of cells
Plasma membrane	Blebbing of membrane. Phosphatidylserine expressed on surface	Lysis occurs early on
Mitochondria	Morphology and structure preserved	Swollen and architecture disrupted
Nuclei	Clumping and fragmentation of chromatin	Disruption of membrane
Cell degradation	No inflammation, just phagocytosis	Inflammatory response visible

Cell death induced by free radicals, however, may have characteristics of apoptosis and necrosis (Kane et al 1993).

Cytosolic Aspartate-Specific Proteases, called caspases, are responsible for the deliberate disassembly of a cell into apoptotic bodies. Caspases are present as inactive pro-enzymes, most of which are activated by proteolytic cleavage. Caspase-8, caspase-9, and caspase-3 are situated at pivotal junctions in apoptotic pathways. Caspase-8 initiates disassembly in response to extracellular apoptosis-inducing ligands and is activated in a complex associated with the receptors' cytoplasmic death domains (Ashkenazi et al 1998). Caspase-9 activates disassembly in response to agents or insults that trigger release of cytochrome c from the mitochondria (Liu et al 1996) and is activated when complexed with dATP, APAF-1, and extramitochondrial cytochrome c (Li et al 1997). Caspase-3 appears to amplify caspase-8 and caspase-9 signals into full-fledged commitment to disassembly (Thornberry et al 1998). Both caspase-8 and caspase-9 can activate caspase-3 by proteolytic cleavage and caspase-3 may then cleave vital cellular proteins or activate additional caspases by proteolytic cleavage.

There are two main pathways that lead to apoptosis: i) positive induction by ligand binding to a plasma membrane receptor and ii) negative induction by loss of a suppressor activity. Each leads to activation of cysteine proteases with homology to IL-1β converting enzyme (ICE) (*i.e.*, caspases) (Thornberry et al 1998). Positive-induction involves ligands related to TNF. Ligands are typically trimeric and bind to cell surface receptors causing aggregation (trimerization) of cell surface receptors (Ashkenazi et al 1998). The intracellular portion of these receptors contains an 80 amino acid death domain (DD) that through homophilic interactions recruits adaptor proteins to form a signaling complex on the cytosolic surface of the receptor

(Ashkenazi et al 1999). The bringing together of three receptors, thereby orienting the intracellular DDs, appears to be the critical feature for signaling by these receptors. The adaptor complex then recruits caspase-8, caspase-8 is activated, and the cascade of caspase-mediated disassembly proceeds. Negative induction of apoptosis by loss of a suppressor activity involves the mitochondria (Green et al 1998). Release of cytochrome c from the mitochondria into the cytosol serves as a trigger to activate caspases (Liu et al 1996). Permeability of the mitochondrial outer membrane is essential to initiation of apoptosis through this pathway. Proteins belonging to the Bcl-2 family appear to regulate the membrane permeability to ions and possibly to cytochrome c as well (Reed 1997). Although these proteins can themselves form channels in membranes, the actual molecular mechanisms by which they regulate mitochondrial permeability and the solutes that are released are less clear. The Bcl-2 family is composed of a large group of anti-apoptosis members that when overexpressed prevent apoptosis and a large group of pro-apoptosis members that when overexpressed induce apoptosis. The balance between the anti-apoptotic and pro-apoptotic Bcl-2 family members may be critical to determining if a cell undergoes apoptosis. Thus, the suppressor activity of the anti-apoptotic Bcl-2 family appears to be negated by the pro-apoptotic members.

1.3 STEM CELL DIFFERENTIATION AND TRANSPLANTATION

It is widely accepted that peripheral blood cells originate from a small population of pluripotent stem cells which are found in the bone marrow. These cells are characterised by their ability for extensive proliferation (without loss of pluripotency in the *in vivo* setting) and their ability to differentiate into any of the haematopoietic lineages. Haematopoietic stem cells have been characterised *in vitro* by their ability to generate different haematopoietic lineages in assays such as the Colony Forming Unit assay (CFU). *In vivo* assays have included infusion of sorted human stem cells into sub-lethally irradiated NOD-SCID mice. NOD-SCID mice are class I deficient, B and T cell deficient, C-5 deficient, and have low NK cells, and are an ideal model for xenograft transplantation studies (Shultz et al 2000). Notably, they are both insulitis- and diabetes-free throughout life. In this setting, they can be shown to reconstitute all of the human haematopoietic lineages within the mouse. Attempts to identify and purify these haemopoietic stem cells have centred on antigens expressed on their cell surface. The most commonly used marker for the isolation and manipulation of stem cells is the CD34 antigen (Sutherland et al 1992). Recently a novel antigen, AC133, expressed on human haemopoietic stem cells was reported. This antigen is a unique five-transmembrane (5-TM) molecule and the monoclonal antibody raised against the protein recognises a subset of $CD34^+$ cells in bone marrow (Miraglia et al 1997; Yin et al 1997). AC133 has been shown to select cells which engraft successfully in a fetal sheep transplantation model in both primary and secondary recipients, indicating the presence of long-term repopulating cells.

Recent studies have demonstrated that haematopoietic stem cells have greater plasticity than previously thought and can differentiate down multiple non-haematopoietic cell lineages in rodents (Graf 2002; Krause et al 2001; Petersen et al 1999; Theise et al 2000a). Petersen *et al* demonstrated that when mis-matched bone marrow was infused into liver-damaged syngeneic mice these donor cells were able to differentiate into hepatic oval cells and hepatocytes (Petersen et al 1999). The method of liver injury used included Acetylaminofluorene (AAF) followed by 2/3 partial hepatectomy, which is designed to prevent a reparative response from differentiated hepatocytes. Instead regeneration occurs due to the proliferation and subsequent differentiation of hepatic oval cells (HOCs) which are bipotent primitive hepatocytes residing in the canals of Hering (Theise et al 1999). AAF is metabolised by mature hepatocytes to a toxic metabolite which prevents cell division. As HOCs do not possess the enzymes required to metabolise AAF they are immune to its effects and thus have a proliferative advantage compared with differentiated hepatocytes. Several types of mis-matching were performed including gender (male to female), Dipeptidyl peptidase IV+ (DPPIV+) to DPPIV – mice and liver transplantation (liver transplanted from Brown Norway rats into Lewis rats). DPPIV + and - mice are transgenic mice which either express the enzyme DPPIV or not. Similar work by Theise *et al* demonstrated that such differentiation could occur in the absence of any intentional liver injury (Theise et al 2000a). There is debate as to the effect of irradiation on the liver, but at the levels used for marrow transplantation it causes only a mild effect on hepatocyte proliferation. Whilst much of this work has used bone marrow derived stem cells there are several reports suggesting that stem cells obtained from muscle (Asakura et al 2001; Wada et al 2002) and brain also

share these pluripotent properties (Shih et al 2002). Lagasse *et al* were able to demonstrate that this phenomenon had functional significance when they showed that infusion of syngeneic murine adult bone marrow cells into the fumarylacetoacetate hydrolase (-/-) FAH mouse, an animal model of tyrosinemia type I, rescued the mouse and restored the biochemical function of its liver (Lagasse et al 2000). Histological analysis of the livers of these animals demonstrated almost total reconstitution with donor-derived hepatocytes and is the most convincing evidence to date that stem cells may have a therapeutic role in liver injury. Notably, this is the only model in which infused stem cells had a survival advantage over host cells, in this case hepatocytes. That adult human stem cells also share some of these pluripotent properties has been demonstrated in studies utilising archival biopsy samples (Alison et al 2000) in which donor-derived hepatocytes and cholangiocytes have been observed in the recipient. In both of these studies, the presumed origin of stem cells was the bone marrow, and both studies used cross-sex transplants to differentiate host from recipient hepatocytes. A study by Korbling *et al* (Korbling et al 2002) demonstrated that human stem cells mobilised by Granulocyte-Macrophage Colony stimulating factor (GM-CSF) (as used in the management of haematological conditions) also retained the ability to differentiate down hepatocytic and other non-haematopoietic lineages. As yet, the biological relevance of human stem cell transdifferentiation remains to be clarified, as therapeutic efficacy has not yet been demonstrated with human stem cells.

After the initial optimism regarding stem cell plasticity there have been reports which suggest that the actual level of differentiation (into hepatocytes) may be lower

than previously thought. Wagers *et al* infused a single Green fluorescent protein$^+$ (GFP) c-kit$^+$ Thy1.1^{lo} Lin$^-$ Sca-1$^+$ cell from murine bone marrow into a lethally irradiated GFP$^-$ syngeneic recipient and only observed 7 GFP$^+$ hepatocytes in the recipient liver, in the face of 17.6-20.2% peripheral blood reconstitution (Wagers et al 2002). c-kit$^+$ Thy1.1^{lo} Lin$^-$ Sca-1$^+$ are widely regarded as the most pluripotent murine haematopoietic stem cell in transplantation models (Morrison et al 1994; Morrison et al 1997; Spangrude et al 1988). The conclusion drawn from these studies was either that haematopoietic stem cells only exceedingly rarely contributed to non-haematopoietic lineages, or that there are lineage-committed stem cells in the marrow which differentiate down the non-haematopoietic lineages (ie they are not haematopoietic stem cells). To test whether such lineage-committed cells existed they utilised parabiotic animals. These animals are surgically joined so that they rapidly develop a common anastomosed circulatory system and develop haematopoietic chimerism. This model permits analysis of haematopoietic cell function in the absence of any injury, as irradiation is not required. GFP transgenic and non-transgenic littermate were surgically joined together but they were unable to identify any evidence of hepatocyte or other non-haematopoietic GFP$^+$ cells in the GFP$^-$ partner, suggesting that if such cells exist they do not contribute to non-haematopoietic tissues in the absence of injury.

Furthermore, somatic stem cell plasticity has recently been questioned, and it has been suggested that this presumed differentiation may be the product of cell fusion between stem cells and pre-existing differentiated cells (Terada et al 2002; Vogel 2002; Ying et al 2002). These observations have important implications for stem cell

usage and if confirmed in *in vivo* studies may limit the benefits of stem cell transplantation.

However, the lack of suitable *in vivo* or *in vitro* model systems has hindered detailed study of stem cell plasticity. With respect to murine *in vitro* studies several groups have demonstrated that embryonic stem cells can be directed down non-haematopoietic lineages including hepatocytes, neuronal cells and myocytes (Ishizaka et al 2002; Jones et al 2002). Extensive work with murine multipotent adult progenitor cells (MAPCs) which are a type of mesenchymal stem cell found in bone marrow has demonstrated the ability of such cells to differentiate into haematopoietic, hepatocytic, pneumocytic and enterocytic lineages (Jiang et al 2002).

It would be important to develop systems whereby human stem cells could be directed towards non-haematopoietic lineage in the *in vitro* setting to facilitate both a greater understanding of the mechanisms controlling this process and to expand the clinical utility of human stem cells. In addition, the development of an *in vivo* model of human stem cell differentiation down non-haematopoietic lineages is an essential step in advancing our knowledge of the mechanisms of these processes as they occur in the body.

CHAPTER 2

STUDY OF ACUTE LIVER INJURY ON HEPATOCYTE ADHESION AND SURVIVAL

2.1 BACKGROUND AND AIMS

Cell transplantation of hepatocytes, has been studied extensively *in vivo* both in animal models and humans (Lee 2001) and has been shown to contribute to hepatic function, although proven benefit has been largely limited to metabolic liver diseases (Muraca et al 2002). However, when hepatocyte transplantation has been used in models of acute liver injury and patients with fulminant hepatic failure there is a significant reduction in both engraftment and subsequent efficacy (Birraux et al 1998; Gupta et al 2000a). A clear understanding however does not exist as to what factors in liver injury modulate the engraftment and survival of transplanted cells, with most work concentrating on the toxicity of the host environment to donor cells. Serum from patients with fulminant hepatic failure has been shown to induce a dose-dependent inhibition of proliferation (Anilkumar et al 1997; Demetriou et al 1974; Gohda et al 1986; Gove et al 1982; Tada et al 1986; Williams et al 1977; Yamada et al 1994) and protein synthesis (Shi et al 1998), and in some studies induces cell death (Fujioka et al 1997; Hughes et al 1976; Williams et al 1977).

The present study was undertaken to answer the following questions:

1) Is there a change in the extra-cellular matrix protein expression in acute liver injury?

2) Do hepatocytes exposed to acute liver injury serum have altered adhesive properties?

3) If so, does apoptosis contribute to the observed changes in cellular adhesion?

2.2 MATERIALS

FHF serum was pooled from patients with acute liver failure due to paracetamol overdose. All patients were in the intensive therapy unit (ITU) at time of sampling and fulfilled the poor prognosis criteria for liver transplantation (O'Grady et al 1989). Samples were taken within 48-96 h of paracetamol overdose and none of the patients had detectable paracetamol in their serum at the time of sampling. Normal serum was obtained from healthy individuals. Tissue from liver biopsy specimens was obtained from the Department of Pathology: the biopsies were from patients who had acute liver failure fulfilling the poor prognosis criteria for liver transplantation (O'Grady et al 1989). This study had local ethics committee approval.

2.3 METHODS

Cell culture

HepG2 is a human hepatoma derived line and it was routinely grown in mono/multiplayer cultures in Dulbecco's' Modified Eagle Medium (DMEM) containing 10% (vol/vol) fetal calf serum (FCS) in an atmosphere of 5% CO_2 in air. This concentration of serum is standard in the literature (Shi et al 1998). Cells were subcultured every 7 days at a split ratio of 1:3 using 0.05% (wt/vol) trypsin and EDTA to detach the cells. Cells used were within 10 and 20 passages of purchase.

Cell necrosis and apoptosis experiments

Cell necrosis (Propidium Iodide)

1.25 x 10^6 HepG2 cells were grown on 25cm^2 flasks and exposed to media containing 20% (vol/vol) FHF serum, 20% normal serum (vol/vol), or 10% FCS (vol/vol) for 1, 4, 12, 24, 48 and 72 hours. All cells (adherent and non-adherent) were collected and resuspended in phosphate-buffered saline (PBS) (pH 7.2) before determining the cell number and viability with Propidium iodide exclusion on a Coulter XL flow cytometer (Coulter Electronics, Luton, UK). Briefly, to 1ml of cell suspension 100µl of 0.025% Propidium iodide solution was added 10 minutes before analysis and samples were analysed where non-viable were read as those cells positive for propidium iodide (Hart et al 2000). The cell count was assessed with a Neubauer cytometer.

Feulgen Nuclear morphology (adherent cells)

1 x 10^5 HepG2 cells were grown on collagen coated glass slide-flasks (NUNC, Loughborough, UK) and exposed to media containing 20% (vol/vol) FHF serum, 20% normal serum (vol/vol), or 10% FCS (vol/vol) for 1, 4, 12, 24, 48 and 72 hours. Slides were incubated in Bouin's reagent (Sigma, UK) for 2 hours and then placed in 1M hydrochloric acid for 1h. Slides were then washed in water prior to 45min incubation in Schiff's reagent (see Appendix page 207). They were washed again prior to counterstaining in light green dye for 10 sec. Slides were then washed, dehydrated through alcohols, immersed in xylene and then coverslipped. The number of apoptotic nuclei was counted per 100 cells. This was performed 6 times per slide

and each experiment repeated 9 times. Values were expressed as percentage of apoptotic cells. Two blinded experienced observers counted slides.

Caspase assay

The Enzchek™ Caspase-3 Assay Kit (Molecular Probes Inc, Eugene, Oregon, USA) was used. This kit allows for the detection of apoptosis by assaying for increases in caspase-3 and other DEVD-specific (amino acid sequence Asp-Glu-Val-Asp) protease activities such as caspase-7. The basis for the assay is the 7-amino-4-methylcoumarin-derived substrate 7-Amino-4-methylcoumarin, N-CBZ-L-aspartyl-L-glutamyl-L-valyl-l-aspartic acid amide (Z-DEVD-AMC, where Z represents a benzyloxycarbonyl group), which is weakly fluorescent in the UV range (excitation/emission ~330/390nm), but which yields a bright blue-fluorescent product (excitation/emission ~ 342/441nm) upon proteolytic cleavage.

The method was as follows:

1) 5 x 10^6 HepG2 cells were grown on 75cm^2 flasks and exposed to media containing 20% (vol/vol) FHF serum or 20% normal serum (vol/vol) for 24 hours. Cells were then removed with Trypsin/EDTA, PBS washed and then pelleted.

2) 1x Cell lysis buffer was then prepared by adding 950µl of dH_2O to 50µl of the 20x Cell lysis buffer. 20x Cell lysis buffer consists of 1.5ml of 200nM TRIS, ph 7.5, 2M NaCl, 20mM EDTA and 0.2% Triton X-100. Samples were incubated on ice with 1x lysis buffer for 30 minutes.

3) The lysed cells were then centrifuged at 5000rpm for 5 minutes in a microcentrifuge. 50µl of the supernatant from each sample was then transferred to individual microplate wells. 50µl of the 1x lysis buffer was used as a no enzyme control to determine the background fluorescence of the substrate. As an additional control, 1µl of the 1mM Ac-DEVD-CHO inhibitor stock solution was added to selected samples. The plate was then covered and incubated at room temperature for 10 minutes.

4) 50µl of the 2x substrate working solution was added to each well. This consisted of 20µl of 10mM Z-DEVD-AMC substrate and 980µl of the Reaction buffer (20ml of 50mM PIPES, pH 7.4, 10mM EDTA, 0.5% CHAPS).

5) Plate was then covered and incubated at room temperature for 30 minutes. Fluorescence was measured in a fluorescent microplate reader using excitation at 485±12.5nm after 60 minutes. Values are expressed as the difference between samples incubated with and without the Ac-DEVD-CHO inhibitor to ensure that the values reflect activity of caspase-3-like proteases.

Annexin V staining

Annexin V is expressed on the cell surface as an early marker of apoptosis. Expression of Annexin V is measured flow cytometrically and expressed as increasing fluorescence. PFOA, a known apoptotic agent to HepG2 cells, is used as a

positive control. Cells were grown on 25cm^2 flasks and exposed to media as described previously. Cover the cells with Dispase solution for 5 minutes at 37°C. Decant the solution and incubate for an additional 10 minutes at 37°C. Monitor detachment using a microscope. If necessary, incubate for an additional 15 minutes or until detachment is complete. Suspend the cells in culture medium and pellet cells by centrifugation, before washing the cells with culture medium.

Dispase was used as trypsin detachment can result in internalisation of Annexin V. Cells were then washed twice with cold PBS and resuspended cells 1X Binding Buffer (see Appendix page 236) at a concentration of 1 x 10^6 cells/ml. A 100 µl of the solution (~1 x 10^5 cells) was then transferred to a 5 ml culture tube and incubated with 2 µl FITC-labelled Annexin V (Sigma, Dorset, UK) for 10 minutes on ice before washing (with 1 X Binding Buffer) and centrifugation. Pellets were re-suspended in 400µl Binding Buffer prior to analysis on a FACS machine (Hart et al 2000).

Confocal actin microscopy

Cells were cultured on collagen-coated coverslips, fixed with 4% formaldehyde for 20 minutes, rinsed with PBS, permeabilized with 0.5% Triton X, before addition of fluorescent phalloidin for a further 20 minutes. Coverslips were then washed again with PBS prior to mounting and analysis on a confocal microscope.

Scanning Electron Microscopy (SEM)

HepG2 cells were grown on Collagen coated Perspex coverslips (NUNC, UK) according to the same treatment schedule described in the cell culture section. After 24 h culture samples were fixed using 2.5% glutaraldehyde, and then dehydrated and coated with gold for scanning electron microscopy (SEM).

Transmission Electron Microscopy (TEM)

HepG2 cells were grown under standard conditions as described in the cell culture section. Cells were removed from flasks by trypsinisation/FCS and then pelleted into appropriate tubes. Processing for TEM was then performed as described below.

1. Fixation

3%Glutaraldehyde in 0.1M Sodium Cacodylate Buffer	**2-24hours**

2. Wash

0.1M Sodium Cacodylate Buffer	**3×20min**

3. Post-Fixation

1% Osmium Tetroxide in 0.1M Sodium Cacodylate Buffer	**45-60min**

4. Wash

0.1M Sodium Cacodylate Buffer	**3×20min**

5. Dehydration

50% Acetone	**1×10min**
70% Acetone	**1×10min**
90% Acetone	**1×10min**
100% Acetone (Analar)	**1×10min**

6. Mixing

Araldite Mix: Acetone (Analar)	50:50	**1×30min**

7. Infiltration

Place samples in 50:50 mix above without lids in 60°C oven overnight

8. Infiltration

Araldite Mix	**3×60min**

9. Infiltration

Araldite Mix with Accelerator	**2×20min**

10. Embedding

Araldite Mix with Accelerator	**48hours @ 60°C**

Immunohistochemistry

Archival sections from explanted livers from patients with FHF and from healthy livers were obtained. Slides were dewaxed in xylene and progressively dehydrated through graded alcohols and water. Antigen retrieval was performed using 0.1% trypsin (15 min at 37°C) or Vector Target Retrieval solution (microwaved for 15 min). Slides were then processed with the Envision Kit (DAKO Laboratories, Ely, UK) for Collagen IV, Laminin, Fibronectin and Tenascin (DAKO Laboratories, Ely, UK). ENVISION staining steps are summarised as follows on the next page.

This is a two-step IHC staining technique. This system is based on an HRP labelled polymer which is conjugated with secondary antibodies. The labelled polymer does not contain avidin or biotin. Consequently, nonspecific staining resulting from endogenous avidin-biotin activity in liver, kidney, lymphoid tissues and cryostat sections is eliminated or significantly reduced. This protocol offers an enhanced signal generating system for the detection of antigens. Primary antibodies produced in mouse react well with the labelled polymer.

Principles of procedure

Any endogenous peroxidase activity is quenched by incubating the specimen for five minutes with Peroxidase Block. The specimen is then incubated with an appropriately characterized and diluted mouse primary antibody, followed by incubation with the labelled polymer, using two sequential 30-minute incubations. Staining is completed by a 5-10 minute incubation with 3,3'-diaminobenzidine (DAB)+ substrate-chromagen which results in a brown-colored precipitate at the antigen site.

ENVISION staining steps:

1. Dewax in xylene for 5minutes

2. Rehydrate through alcohol into water

3. Antigen retrieval as above

4. Wash in water

5. Apply peroxidase block (10ml H_2O_2 in 290ml tap water) for 10 minutes

6. Load in sequenzas

7. Wash with PBS

8. Apply primary antibody in DAKO diluent for 30 minutes

9. Wash with PBS

10. Apply labelled polymer for 30 minutes.

11. Wash with PBS

12. Apply DAB Chromagen for 5 minutes

13. Wash with double-distilled water

14. Wash in water

15. Counterstain in Haematoxylin for 30 seconds

16. Wash in tap water

17. Wash in Acid-Alcohol

18. Wash in tap water

19. Wash in Scott's water for 15 seconds. Scott's tap water is used to "blue" nuclear structures subsequent to staining with haematoxylin. This mild, buffered bluing agent will not loosen or float away your tissue or smear preparation. It blues quickly and any excess is easily removed with a tap or distilled water rinse.

20. Wash in tap water

21. Dehydrate through alcohols

22. Place in clearing xylene, then 2nd Xylene and then mounting Xylene

23. Mount

Adhesion assay

HepG2 cells were grown on 25cm^2 flasks and exposed to media containing 20% (vol/vol) FHF serum, 20% normal serum (vol/vol), or 10% FCS (vol/vol) for 1, 4, 12, 24, 48 and 72 hours. All cells were then removed from the flasks (Trypsin/EDTA), washed with PBS and placed onto Collagen IV (1 μg/cm^2) coated wells (BD Biosciences) of a 96 well plate at a concentration of 2x 10^5 cells /100μl for a period of 1h. Collagen IV coating involved placing 100μl of a 1 μg/cm^2 Collagen IV solution into wells, leaving for 2 hours before aspirating any remaining solution. Wells were then PBS-washed, fixed with methanol, stained with methylene blue and then lysed with 1M hydrochloric acid. Plates were then read on a spectrophotometer to quantify cellular adhesion. Results were expressed as a percentage of the adhesion results obtained with cells cultured with DMEM and 5% FCS (vol/vol) (Sethi et al 1999).

Cell count assay

After PBS washing HepG2 cells were re-suspended in PBS. 100μl of cell suspension were then mixed with 100μl of Trypan Blue solution. 30μl of the cell suspension was then placed under the coverslip on the haemocytometer (Neubauer) and counted in the standard fashion. Stained (non-viable) and unstained (viable) cells were counted.

2.4 EXPERIMENTS

For the immunohistochemical analysis, 6 separate acute liver failure liver explants were examined for their expression of ECM markers. An initial dose-ranging study of fulminant and normal serum was performed to identify the optimal concentration to use in subsequent experiments. In the cell adhesion work experiments were done in multiples of eight on at least three separate occasions. Apoptosis work was done on in triplicate on at least three separate occasions.

2.5 STATISTICAL ANALYSIS OF DATA

T test assuming unequal variances was used to compare groups. Where multiple analyses were required ANOVA with post-hoc analysis was performed. A p value of <0.05 was used to define significance.

2.6 RESULTS

Liver injury leads to up-regulation of Collagen IV in the hepatic sinusoids and portal tracts.

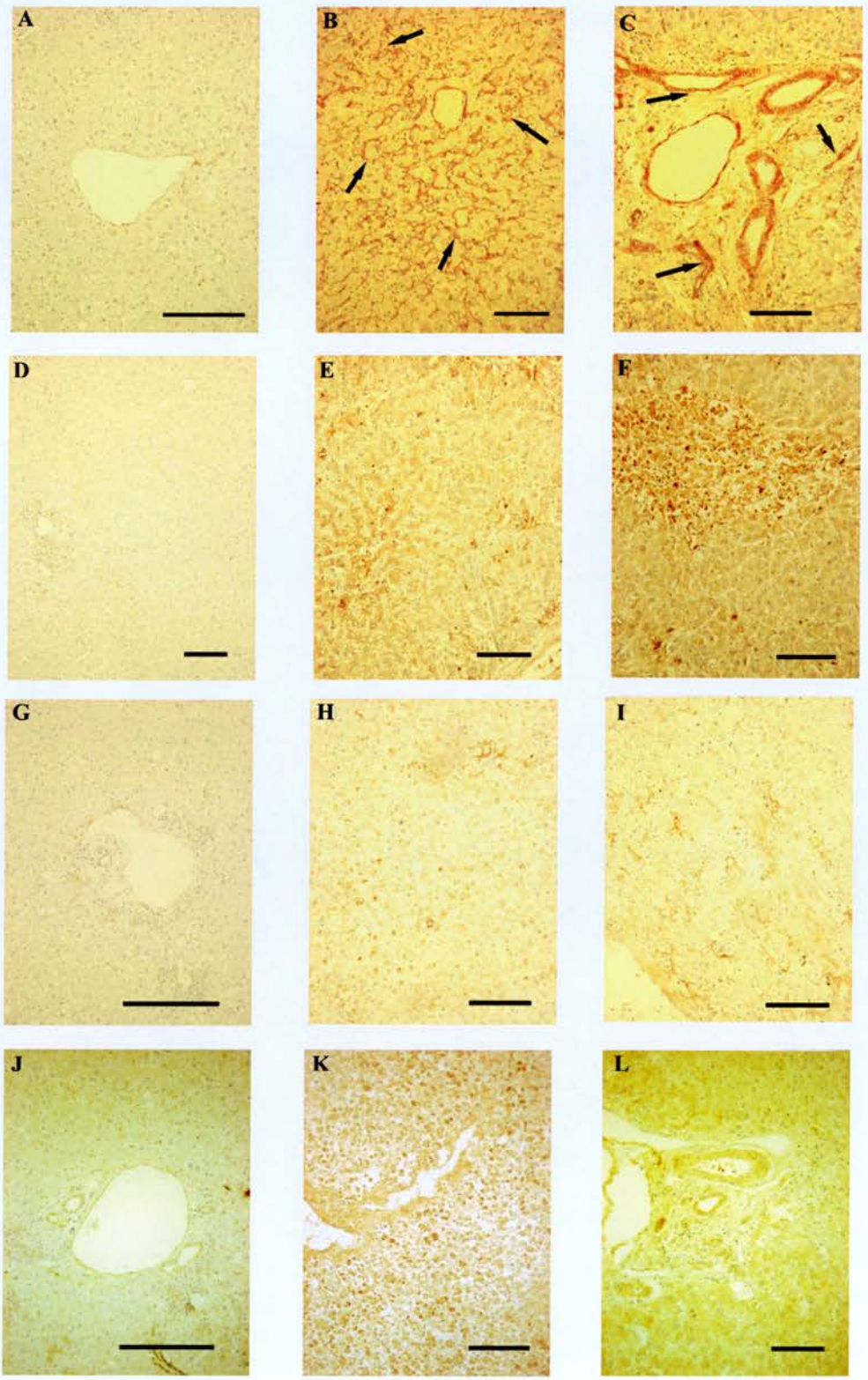
We determined the ECM composition of unselected liver specimens from patients with paracetamol-induced fulminant hepatic failure by immunohistochemical staining for collagen IV, fibronectin, laminin and tenascin. There is a marked increase of collagen IV in the hepatic sinusoids of patients with paracetamol-induced FHF (Figure 2.1, B/C) compared with healthy controls (A). This contrasts with the immunostaining of laminin (J-L) and fibronectin (D-F), which is only seen to occur in areas of hepatocyte necrosis, and probably reflects non-specific uptake of antibody/chromagen by dead cells. Tenascin is expressed in the same distribution as Collagen IV although at much lower levels (G-I).

20% is the optimal concentration of Fulminant Serum

My initial work aimed to identify the optimal concentration of serum to use in my subsequent experiments. Using Normal serum at concentrations of 10-30% there was evidence of increased cell death (Figure 2.2) at the highest concentration (30%), whilst the lower concentrations (10 & 20%) were not associated with any toxicity. The levels of cell death were compared with cultures grown with 10% Fetal Calf Serum (FCS). Study of Fulminant serum at the lowest concentrations (10%) demonstrated no significant toxicity, and for this reason all subsequent experiments were done with sera at a concentration of 20%. The difference in cell death between cultures with 20% Normal and Fulminant serum was only significant at 48 hours ($p<0.0001$); 4.32 ± 0.67 vs 18.2 ± 1.84% respectively.

Figure 2.1

Immunohistochemical analysis of matrix proteins

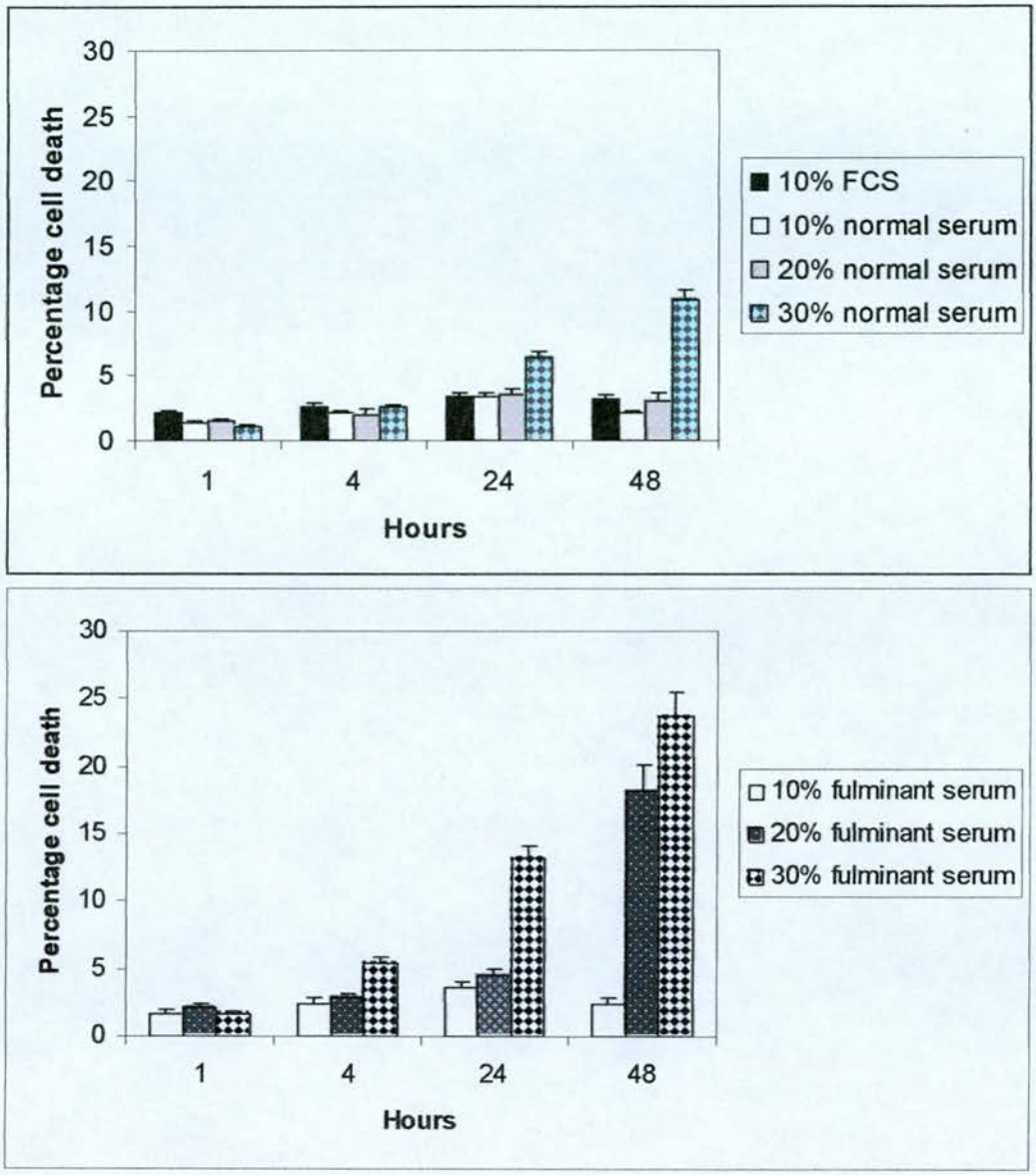


Legend for figure 2.1

The left hand column details the expression of Collagen IV (A), Fibronectin (D), Tenascin (G) and Laminin (J) in normal healthy liver. Expression of Collagen IV can be seen to be strikingly up regulated in B/C, with most of its expression localised to sinusoidal endothelium and portal tracts (see arrows). In contrast, Fibronectin (E/F) and Laminin (K/L) display only evidence of non-specific uptake by necrotic hepatocytes. Tenascin is expressed in the same distribution as Collagen IV although at much lower levels (G-I). Scale bar 20μm.

Figure 2.2

Effect of increasing serum concentration on cell death



Legend for figure 2.2

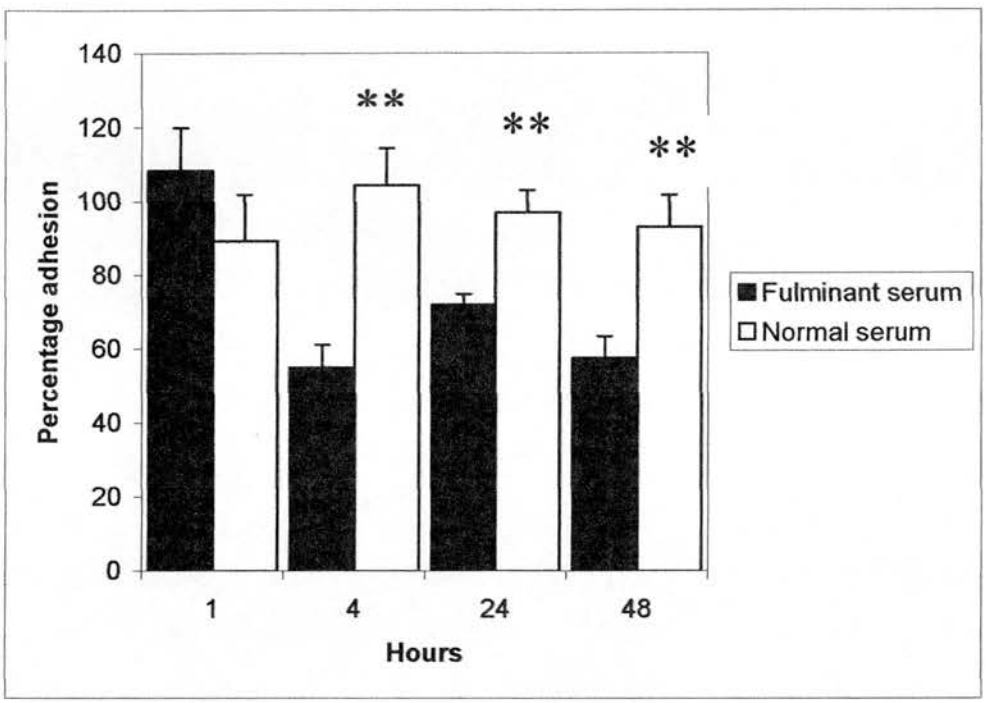
These figures demonstrate cell death as measured by the inability of dead cells to exclude propidium iodide. Error bars indicate the Standard Errors. In the upper figure, the amount of cell death is similar for cells cultured with 10% fetal calf serum (FCS), or 10 & 20% normal human serum. However, 30% normal human serum does lead to increased cell death. In the lower figure, differing concentrations of fulminant serum are used. Only 20 and 30% can be seen to cause increased cell death. The difference in cell death between cultures with 20% Normal and Fulminant serum was only significant at 48 hours ($p<0.0001$); 4.32 ± 0.67 vs $18.2 \pm 1.84\%$ respectively.

Adhesion of HepG2 cells is impaired by exposure to fulminant serum

In view of the marked up-regulation of Collagen IV expression in liver, I studied the binding of HepG2 cells to collagen-coated plates after previous culture with fulminant serum. Exposure to fulminant serum for only 4 hours caused a marked loss of adhesion on to Collagen coated plates (figure 2.3), which contrasted with normal serum, which did not alter adhesive properties (54.9 +/- 6.3 % vs. 92.7 +/- 10.1 %; $p<0.01$). This effect of fulminant serum was assessed morphologically by scanning electron microscopy (SEM) images which revealed striking differences in morphology after 4 hours culture as demonstrated by rounding up of cells cultured with fulminant serum (figure 2.4), in marked contrast to the well-spread cells cultured with normal serum. Panels A & B from Figure 4 depict adhesion of HepG2 cells after 4 hours exposure to Normal serum, whilst Panels C & D depict HepG2 cells 4 hours after exposure to Fulminant serum. Further analysis revealed changes to cytoskeletal structure as demonstrated by disruption of actin cytoskeleton (figure 2.5). Panels A & B in Figure 2.5 depict intact actin cytoskeleton of HepG2 cells after 24 hours exposure to Normal serum. The fine microfilaments can be seen clearly stretching across the cell maintain the cell's structure. In Panels C & D the effect of Fulminant serum can be seen on cells. Specifically there is no longer evidence of the delicate microfilament structure and cells appear more rounded in morphology. This key observation that serum from patients with FHF markedly down-regulates the adhesion of cells to collagen maybe of significant importance in cellular transplantation.

Figure 2.3

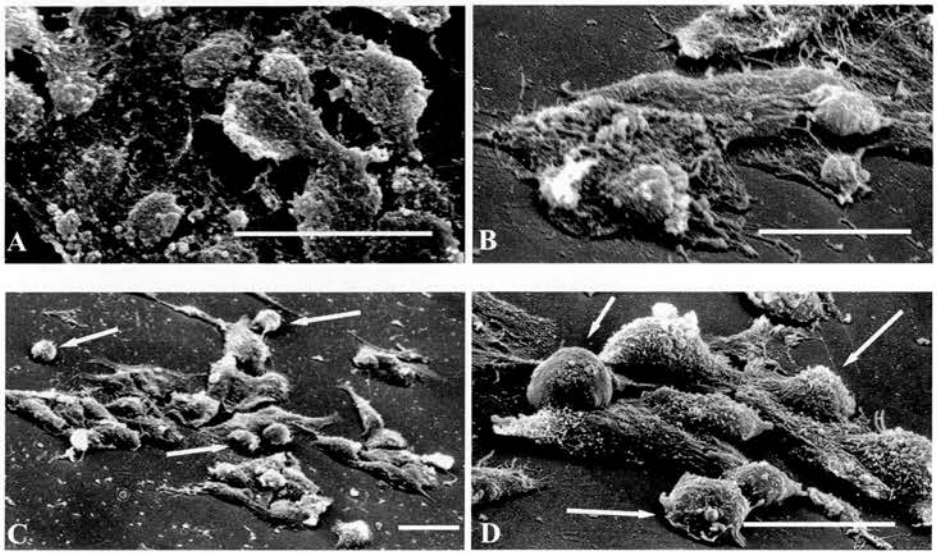
Effect of 20% serum on cellular adhesion to collagen coated plates



Legend for figure 2.3

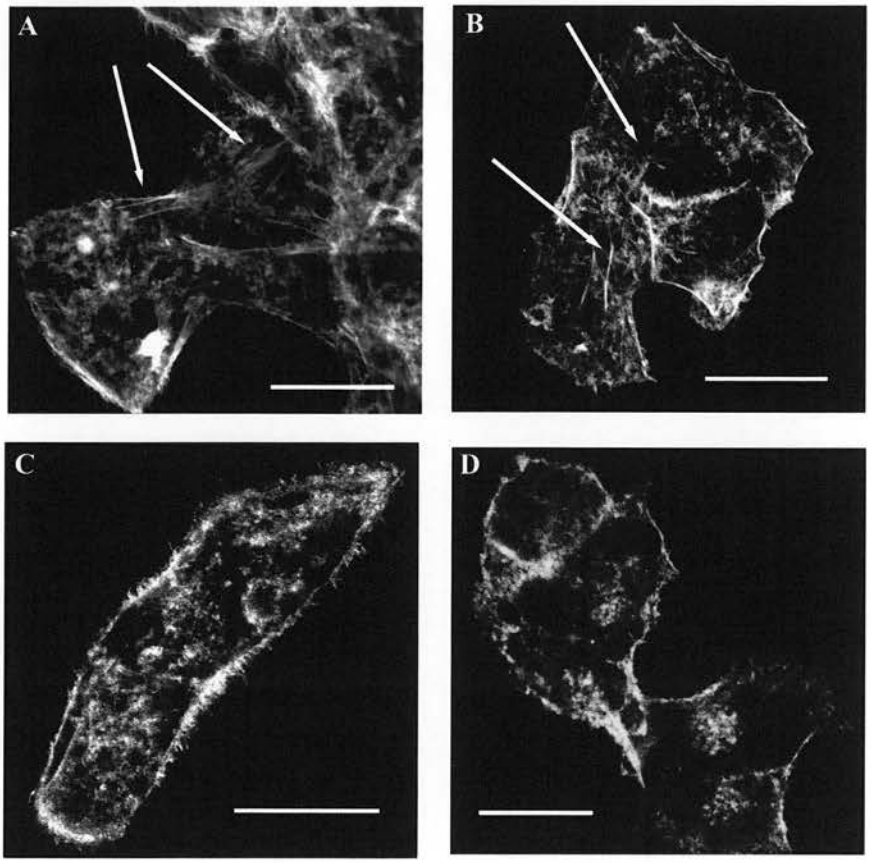
This chart demonstrates the early loss of cellular adhesion in cells cultured with fulminant serum (black bars) compared with those cultured in normal serum (white bars). This difference becomes significant after 4 hours culture and persists at 24 and 48 hours culture. Error bars are +/- SE. ** $p < 0.01$

Figure 2.4
Scanning electron microscopy of HepG2 cells exposed to 20% serum



Charts A and B demonstrate the characteristic flattening and attachment of HepG2 cells to collagen coated plates. These cells have been cultured with standard media containing 20% normal human serum. In contrast charts C and D demonstrate cells grown with 20% fulminant serum. There is evidence of rounding-up and a suggestion of imminent detaching of the hepatocytes (see arrows). Scale bar 20μm.

Figure 2.5
Actin Confocal Microscopy of HepG2 cells exposed to 20%serum



Charts A and B demonstrate the intact actin cytoskeleton of HepG2 cells grown on collagen coated plates (fluorescent phalloidin viewed with confocal microscope). These cells have been cultured with standard media containing 20% normal human serum. The actin microfilaments can be clearly seen to extend throughout the cell (see arrows). Charts C and D demonstrate cells grown with 20% fulminant serum. In contrast the microfilaments can no longer be clearly seen and there is a granular texture to the cytoplasm. Scale bar 10μm.

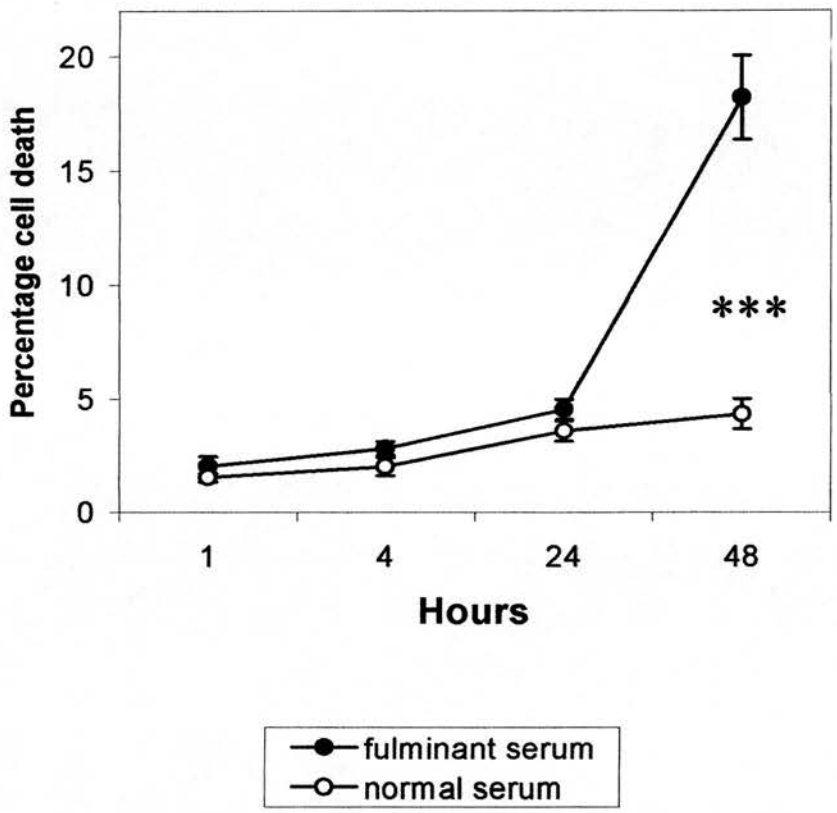
The loss of cellular adhesion precedes cell death and is due to caspase-3 mediated apoptosis.

The next step was to determine whether loss of adhesion was the result of impaired cell viability, or whether it led to cell death. Cell death studies on collagen coated plates (Propidium iodide exclusion) demonstrated that whilst fulminant serum causes cell death this occurs over 44 hours after the loss of cellular adhesion becomes apparent (Figure 2.6). At 24 hours, no significant difference exists between cells exposed to fulminant and normal serum. However after 48 hours culture the percentage of PI-permeable cells was 18.2 ± 1.84 and 4.32 ± 0.67 for fulminant and normal serum-exposed cells respectively ($p<0.001$). Studies of early markers of apoptosis, Annexin V staining, showed that significant apoptosis does not occur in cells incubated in fulminant serum until 24 to 48 hours exposure (Figure 2.7). Values are expressed as mean Annexin V fluorescence, with increasing apoptosis leading to a shift to the right of the curve and an increase in the mean fluorescent intensity. PerFluoroOctanoic Acid (PFOA) at a concentration of 450μmolar is a known pro-apoptotic agent, which can be shown in the figure to clearly produce a peak of cells positive for Annexin V after only 24 hours exposure. At 48 hours cells exposed to fulminant and normal serum have respective values of 42.3 ± 2.17 and 18.1 ± 1.35 (geometric means of fluorescence, $p< 0.001$). The curves for fulminant serum at 24 and 48 hours can be seen to be shifted to the right when compared to the curves for normal serum. That apoptosis is delayed (after loss of adhesion) after exposure to fulminant serum was confirmed by Feulgen staining of adherent cells (figure 2.8). The amount of apoptosis rises such that it is 6.36 +/- 1.34 % by 48 hours (vs. 0.87 +/- 1.3% for normal serum; $p<0.001$). HepG2 cells activate caspase 3, congruent with

this being the process by which apoptosis occurs. (Figure 2.9). Values are expressed in fluorescent units, as the assay is read on a plate fluorometer. Whilst the difference is non-significant at 24 hours, after 48 hours exposure the results from fulminant and normal serum exposed cells is 28700 ± 1410 and 15800 ± 1100 respectively ($p<0.001$). Figure 2.10 provides evidence for fulminant serum causing apoptosis, as depicted in Panels B-C, where there is morphological evidence of apoptosis (48 hours incubation) in HepG2 cells exposed to fulminant serum. In addition, there is evidence of cytoplasmic and nuclear vacuolation, confirming the ongoing injury to cells at this late stage. Panel A depicts HepG2 cells grown with 20% Normal serum. The effect of apoptosis can also be seen to result in a decrease in viable cell count in cells exposed to fulminant serum as detailed in Figure 2.11. Whilst 1.25×10^6 cells incubated with Normal human serum reach a viable count of $3.15 \pm 0.17 \times 10^6$ cells after 48 hours culture, those exposed to fulminant serum for a similar duration are unchanged at a lower comparative number, $1.25 \pm 0.16 \times 10^6$ cells ($p<0.001$). We have therefore demonstrated that the down-regulation of adhesion occurs much earlier than the first evidence of apoptosis. This suggests that loss of adhesive status is one of the causes of apoptosis rather than the result of it.

Figure 2.6

Effect of 20% serum on Cell Death (Propidium Iodide exclusion)



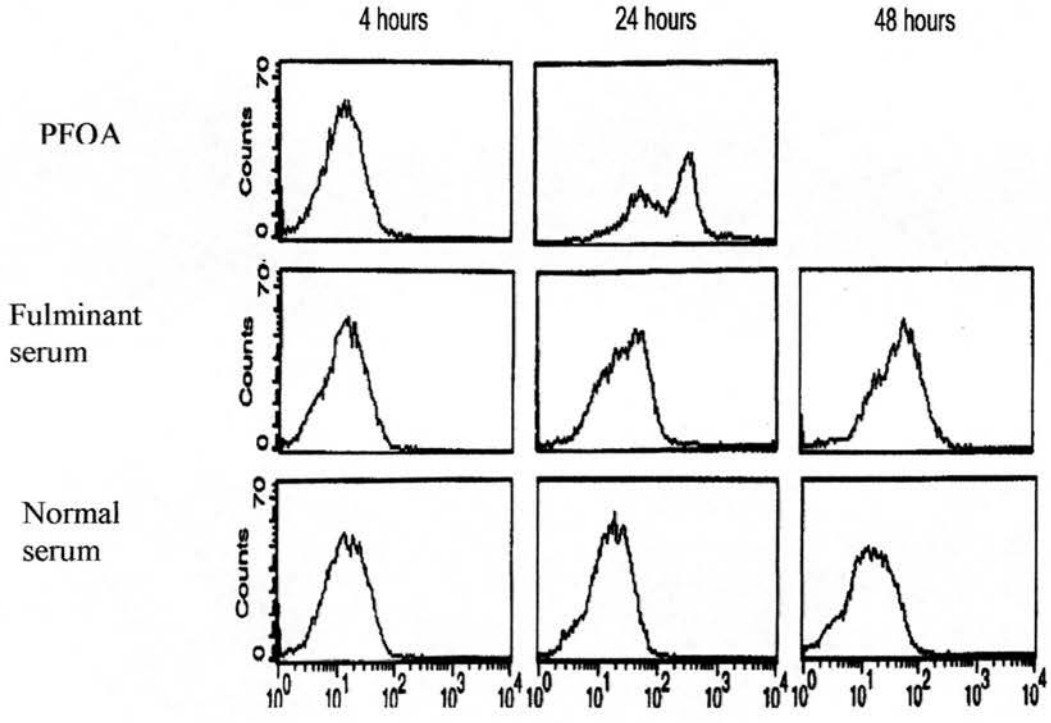
Legend for figure 2.6

This chart depicts the effect of serum on cell death, as judged by their inability to exclude Propidium Iodide. Here fulminant serum is seen to cause a significant increase in cell death, compared with normal serum, but only after 48 hours culture which is 44 hours after the loss of cellular adhesion.

Error bars are +/- SE. *** $p < 0.001$

Figure 2.7

Assessment of effect of 20% serum on apoptosis (Annexin V) by flow cytometry

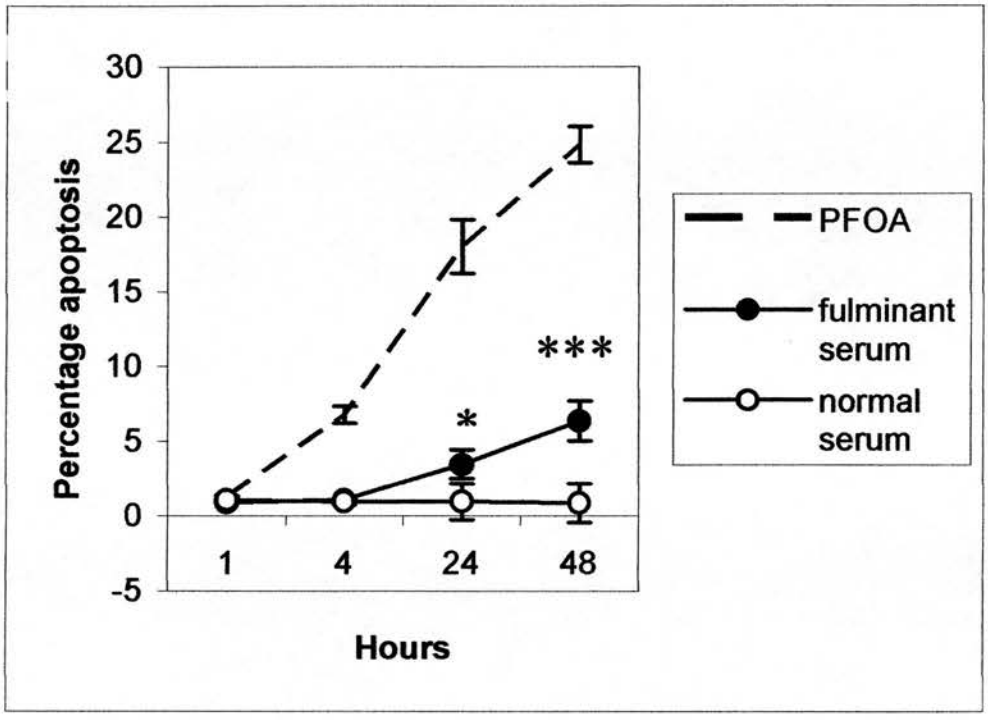


Legend for figure 2.7

This chart depicts fluorescent Annexin V staining of HepG2 cells exposed to PFOA, Fulminant serum and Normal serum after increasing incubation times. Annexin V binding is an early marker of apoptosis. PFOA, a known apoptotic agent to HepG2 cells, is used as a positive control. Expression of Annexin V is measured flow cytometrically and expressed as increasing fluorescence. Differences in Annexin V staining between cells exposed to normal and fulminant serum are minimal at 4 and 24 hours incubation with differences becoming more marked by 48 hours.

Figure 2.8

Assessment of 20% serum on apoptosis (Feulgen nuclear staining)



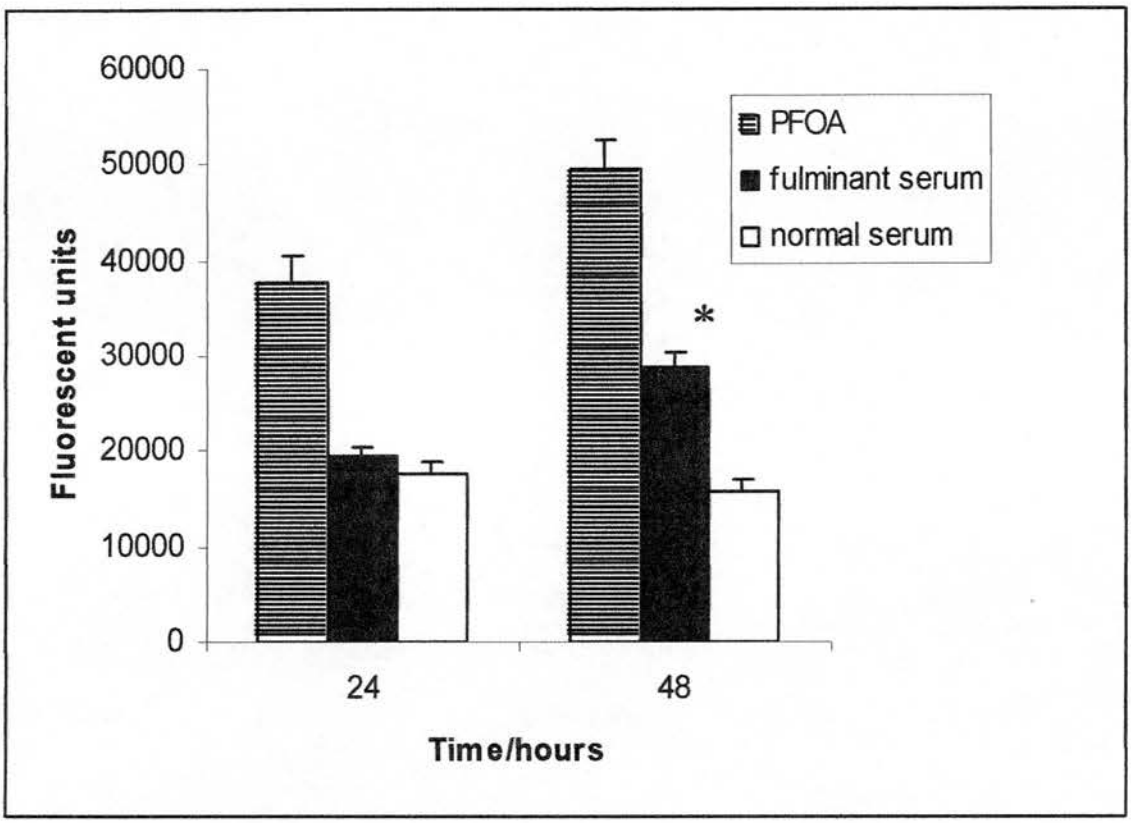
Legend for figure 2.8

This chart uses Feulgen nuclear morphology to quantify the amount of cellular apoptosis. Fulminant serum causes increased cellular apoptosis, peaking at levels of 6% after 48 hours exposure, compared to normal serum which does not lead to any increase at all. Whilst this effect becomes statistically significant after 24 hours culture, it becomes much more profound after 48 hours culture. These levels of apoptosis can be compared to the much higher levels of apoptosis seen with exposure to the known apoptotic agent perfluoroctanoic acid (PFOA). Of note, the apoptosis seen with fulminant serum does not become appreciable until after 24 hours, which is over 20 hours after the impairment of cellular adhesion was seen.

$* p < 0.05$, $*** p < 0.001$ (apoptosis in cells cultured with fulminant serum versus cells cultured with normal serum).

Figure 2.9

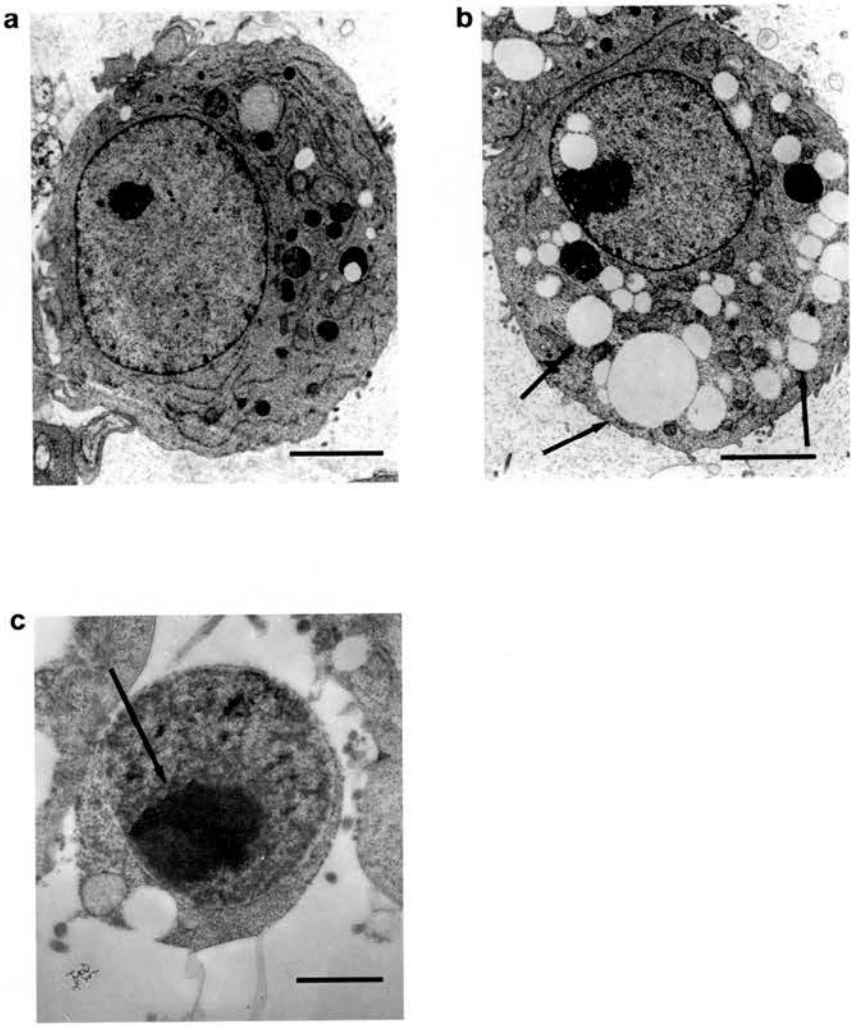
Effect of 20% serum on caspase-3 activation



Legend for figure 2.9

This chart illustrates levels of caspase 3 activity after 24 and 48 hours exposure to different sera. Only after 48 hours does fulminant serum lead to a significant increase in caspase 3 activity. * $p < 0.05$ (normal vs fulminant serum after 48 hours incubation).

Figure 2.10
Transmission Electron Microscopy of HepG2 cells

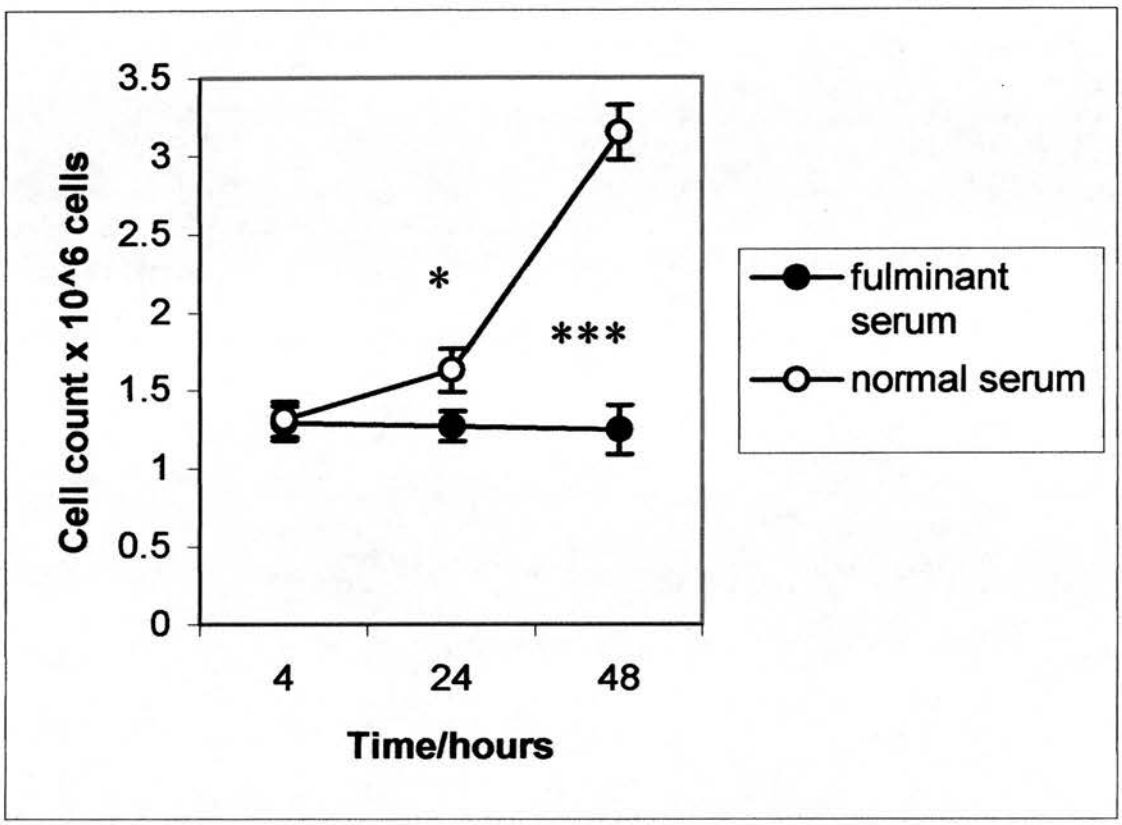


Legend for figure 2.10

This chart depicts Transmission Electron Microscopy pictures of HepG2 cells after exposure to fulminant serum. Picture A represents culture with serum from normal controls. Pictures B and C represent cytoplasmic vacuolation and production of apoptotic pyknotic nucleii in cells exposed to serum from patients with fulminant hepatic failure. Scale bar 5μm.

Figure 2.11

Effect of 20% serum on viable cell count



Legend for figure 2.11

This graph compares viable cell count in cultures exposed to fulminant and normal serum. There is a significant decrease in cell count which becomes significant at 24 hours and most marked at 48 hours. * $p < 0.05$, *** $p < 0.001$ (apoptosis in cells cultured with fulminant serum versus cells cultured with normal serum).

2.7 CONCLUSIONS

In this study I have demonstrated that paracetamol-induced liver injury results in the marked up regulation of collagen IV on hepatic sinusoids which are known to be an important portal of adhesion and subsequent entry of potentially engrafting cells into the liver plate (Gupta et al 1999a). Whilst previous studies have examined the expression of different ECM markers in chronically damaged liver injury (Van Eyken et al 1990) such data are lacking however in acute liver injury, and therefore this study provides novel information on ECM distribution in this setting. With this knowledge, I tested adhesion of hepatocytes to Collagen after their exposure to fulminant serum and was able to show that serum from patients with FHF reduces their adhesive capability within only a few hours. My studies have indicated that loss of adhesive ability precedes apoptosis and is not a consequence of it. Apoptosis does occur approximately 24-48 hours after incubation with fulminant serum. Further confirmation of this comes from the caspase 3 data, which is the final part of the caspase activation process.

Several different factors have been identified as being elevated in FHF serum, including TNF, IL-6, IL-18, Fas &TGFβ_1, but it is difficult to discern what their relative contributions are (Gove et al 1982; Harrison et al 1994; Sullivan et al 1981; Yamada et al 1994). Although some have demonstrated that some of these properties lie within the sub-10kDa ultrafiltrate there is every likelihood that these properties are the additive effects of many biological compounds (Yamada et al 1994), and thus it is rather simplistic to assume that one compound underlies all the observed effects. Furthermore, the removal of compounds found in high concentrations, or indeed the

supplementation of others that liver failure serum may be deficient of, is not a practical strategy to improve outcomes of hepatocyte transplantation. A more realistic treatment is to identify the deranged cellular mechanisms, which are the downstream consequence of liver injury and attempt to pre-emptively treat cells to improve engraftment and hepatocyte survival.

In this work I have demonstrated that 10-20% normal human serum is not harmful to HepG2 cells as there are levels of necrosis similar to HepG2 cultures grown with 10% Fetal Calf Serum. Whilst I have shown that Fulminant serum is toxic at concentrations of 20% or higher I have not shown this is specific to liver injured patients as I did not examine serum from patients with other illnesses. Having established the model and observation, I proceeded in my next chapter to identify the mechanism which underlies this.

CHAPTER 3

STUDY OF MECHANISMS IMPAIRING HEPATOCYTE ADHESION AND SURVIVAL IN ACUTE LIVER INJURY

3.1 BACKGROUND AND AIMS

In the light of my findings in the previous chapter regarding the impairment in cellular engraftment that results from exposure to liver injury serum, my aim was to identify the mechanisms which may underpin this phenomenon. To this end, it is important to review the location within the liver where engraftment occurs. In the sinusoidal bed can be found the optimum microenvironment for an engrafting hepatocyte, in terms of extra-cellular matrix (ECM) components, growth factors, nutrients and interactions with other cells. The adhesion to ECM is essential for the growth and survival of hepatocytes, such that their displacement from ECM leads to dedifferentiation and apoptosis, a phenomenon known as anoikis (Bretland et al 2001; Frisch et al 2001). Integrins appear to be the major receptors by which cells attach to ECM, and are also important in mediating cell-cell adhesion events (Hynes 1992). Integrins are heterodimeric transmembrane proteins consisting of non-covalently linked alpha and beta chains each with a large extracellular, single transmembrane and short cytoplasmic domain (Hynes 2002). Integrins are activated by contact with ECM proteins (Wu et al 1995) which leads to inhibition of interleukin-1beta converting enzyme expression preventing apoptosis and thus providing them with a major role in determining cell survival (Giancotti et al 1999). Therefore factors which modulate the interaction and hence activity of integrins play an important role in determining cell adhesion and survival (Diamond et al 1994; Kim et al 1997). Our understanding of the factors controlling integrin affinity are incomplete but work suggests that control is exerted in an energy dependent fashion on their cytoplasmic domain (Kim et al 1997). To identify the mechanisms which

control adhesive capacity of HepG2 cells I studied the expression of those integrin receptors which regulate adhesion to collagen IV, and also which are found on HepG2 cells (Torimura et al 1997). β_1, α_2, & α_6 are the major integrins that control the binding to collagen IV and these were subsequently studied. CD98 has been suggested as a potential controller of β_1-integrin affinity; its crosslinking leading to activation of the β_1-integrin and hence maintenance of integrin survival signaling (Cho et al 2001). Activity of the β_1-integrin receptor can be modulated by administration of activating antibodies such as TS2/16, which acts by directly inducing a conformational change of β_1-integrins (Weber et al 1996).

The present study was undertaken to answer the following questions:

1) Is there a change in the expression of integrin receptors in hepatocytes exposed to acute liver injury?

2) Is there a change in the function of the integrin receptors?

3) Can the effect of acute liver injury on integrin activity of hepatocytes be prevented?

3.2 MATERIALS

FHF serum was pooled from patients with acute liver failure due to paracetamol overdose. All patients were in the intensive therapy unit (ITU) at time of sampling and fulfilled the poor prognosis criteria for liver transplantation (O'Grady et al 1989). Samples were taken within 48-96 h of paracetamol overdose and none of the patients had detectable paracetamol in their serums at the time of sampling. Normal serum was obtained from healthy individuals. Liver biopsy specimens were obtained from the Department of Pathology and were from patients who had acute liver failure fulfilling the poor prognosis criteria for liver transplantation (O'Grady et al 1989). This study had local ethics committee approval.

3.3 METHODS

Cell culture

HepG2 is a human hepatoma derived line and it was routinely grown in mono/multiplayer cultures in DMEM containing 10% (vol/vol) FCS in an atmosphere of 5% CO_2 in air. Cells were subcultured every 7 days at a split ratio of 1:3 using 0.05% (wt/vol) trypsin and EDTA to detach the cells. Cells were used between passages 10 and 20.

Integrin analysis

HepG2 cells were grown on 25cm^2 flasks and exposed to media containing 20% (vol/vol) FHF serum, 20% normal serum (vol/vol), or 10% FCS (vol/vol) for 24 and 48 hours.

Cells were removed from these flasks with trypsin/EDTA, PBS washed and then incubated with Fluorescein conjugated mouse monoclonal antibodies directed against β_1, α_2 and α_6 integrin molecules (Serotec Ltd, Oxford, UK). Isotype controls were used for these antibodies (Serotec Ltd, Oxford, UK). Expression of these markers was then assessed using a Coulter FACS machine (Sethi et al 1999).

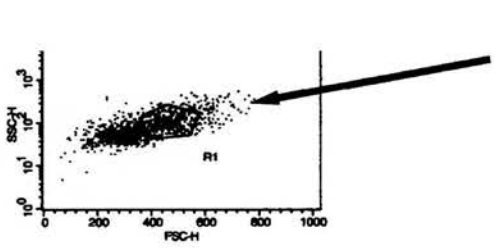
β_1-integrin activation analysis

Cells were grown on 25cm^2 flasks and exposed to media containing 20% (vol/vol) FHF serum, 20% normal serum (vol/vol), or 10% FCS (vol/vol) for 1, 4 and 24 hours. Cells were removed from these flasks, washed and incubated with HEPES buffer. The cell pellet obtained was split into 3 aliquots. All aliquots were incubated

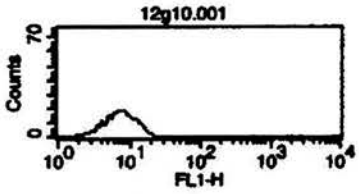
with HEPES buffer containing the 12g10 antibody (Sigma, UK) for 30 minutes (this antibody recognizes the active configuration of the β_1-integrin). Aliquot 1 (termed *native*) had no additions, aliquot 2 (termed *inhibited*) contained EDTA (to inhibit β_1-integrin activity) and aliquot 3 (termed *activated*) contained Manganese (to activate β_1-integrin activity). After this samples were washed, pelleted and incubated with a secondary FITC antibody (anti-mouse) directed against the 12g10 antibody. These samples were then processed flow cytometrically to quantify the amount of 12g10 binding (which assesses the activity of the β_1-integrins). In the forward vs side scatter box a gate (R1) was placed to catch viable whole cells, thus excluding sub-cellular debris. The amount of FITC staining in the gated population was then expressed in the histograms shown in Figure 3.1. Using the following equation an estimate of the relative activity of the β_1-integrin in a given culture condition could be calculated using the geometric means (Sethi et al 1999):

$$\% \beta_1\text{-integrin activity} = \frac{\text{Native} - \text{Inhibited}}{\text{Activated} - \text{Inhibited}} \times 100$$

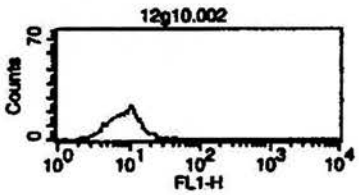
Figure 3.1
Calculation of percentage of beta-1 integrin receptors in active conformational state



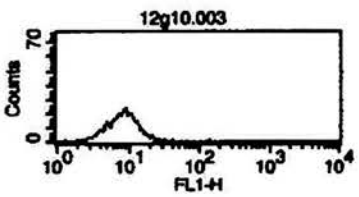
No antibody



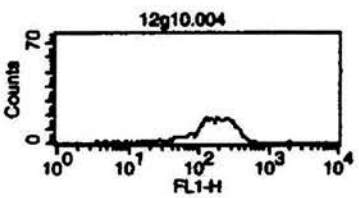
No primary antibody



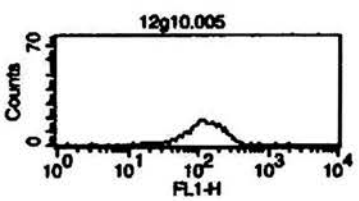
Isotype antibody



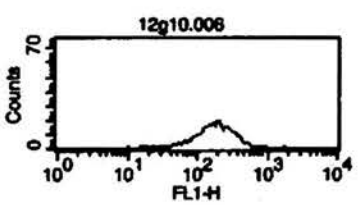
Native state



Inhibited state



Activated state



Legend for figure 3.1

This figure depicts the binding of the 12g10 antibody to HepG2 cells. 12g10 only binds to β_1-integrin receptors which are in the active conformational state. The amount of 12g10 binding is measured by the amount of fluorescence detected with the flow cytometer. Increased fluorescence, and hence 12g10 binding, is denoted by a shift to the right on the histogram. No antibody chart represents the inherent autofluorescence of HepG2 cells. No primary antibody chart represents HepG2 cells incubated only secondary antibody without the 12g10 antibody. Isotype antibody represents HepG2 cells incubated an isotype antibody (instead of 12g10) followed by the secondary antibody. Native state refers to HepG2 cells incubated with 12g10 followed by the secondary antibody. Inhibited state and Activated states are as Native state, with the exception that they received prior incubation with EDTA and Manganese respectively.

Adhesion assay

This assay is described on page 61 in chapter 2.

Apoptosis assays

These assays are described on pages 53-56 in chapter 2.

TS2/16 antibody

This antibody is derived from a hybridoma and was used at a concentration of 25μg/ml in tissue culture (on the basis of maximal flow cytometric binding data) (Weber et al 1996). It was a gift from Dr Tariq Sethi (CIR, University of Edinburgh). Differing concentrations of the TS2/16 antibody were added to 200,00 HepG2 cells for 30 minutes. Cells were then washed with PBS and pelleted. Pellets were resuspended with a 1/10 dilution of an anti-mouse secondary antibody conjugated with FITC. Again cells were incubated for 30 minutes prior to PBS-washing and further pelleting. Samples were then analysed flow cytometrically, measuring fluorescent intensity (as a proxy for tS2/16 binding). For assessment of adhesion, cells were incubated with sera for varying time-points, then washed and then incubated with the TS2/16 antibody. For assessment of apoptosis, the antibody was added at the beginning of the experiments with the different serum.

3.4 EXPERIMENTS

Flow cytometry work for integrin expression and activation was performed in triplicate on three separate occasions. In the cell adhesion work experiments were done in multiples of eight on at least three separate occasions. Apoptosis work was done on in triplicate on at least three separate occasions.

3.5 STATISTICAL ANALYSIS OF DATA

T test assuming unequal variances was used to compare groups. Where multiple analyses were required, ANOVA with post-hoc analysis was performed. A p value of <0.05 was used to define significance.

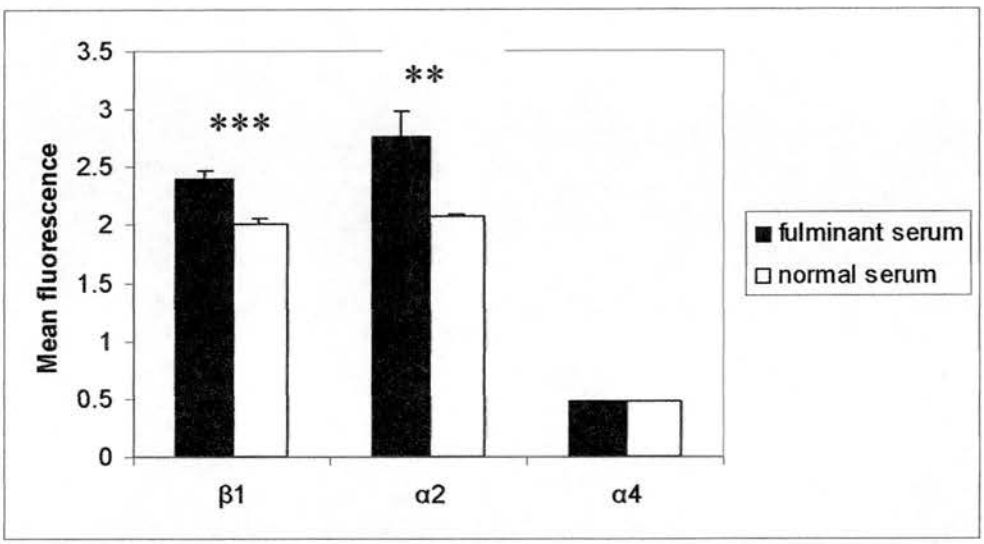
3.6 RESULTS

Down-regulation of β_1-integrin receptor activities rather than loss of total β_1-integrin receptor expression is associated with decreased cellular adhesion

After 24 hours culture with fulminant serum, when adhesion was significantly impaired, there was increased expression of the β_1- and α_6-integrins (Figure 3.2). To assess the activity of the β_1 integrin an antibody that recognises only its active conformational state, 12g10, was used. Importantly however, as shown in Figure 3.3 the proportion of β_1-integrin receptors in their active conformational state was much lower after culture for 24 hours with fulminant serum (33.2 +/- 6.2% vs. 68.6 +/- 2.4 %; $p<0.01$) compared with cells cultured with normal serum. Furthermore, Figure 3.4 demonstrates that this down-regulation occurs early, between one and four hours after exposure to fulminant serum, at the same time as loss of adhesion of these cells is demonstrated. I have therefore demonstrated that whilst the levels of the integrins that mediate binding to collagen are increased after exposure to serum from patients with FHF, the activity of the integrin, β_1, is rapidly and significantly down regulated. I conclude therefore that a possible mechanism behind the loss of cellular adhesion and hence apoptosis is down-regulation of the β_1 integrin activity on HepG2 cells.

Figure 3.2

Integrin expression on HepG2 cells after 24 hours culture with 20% serum



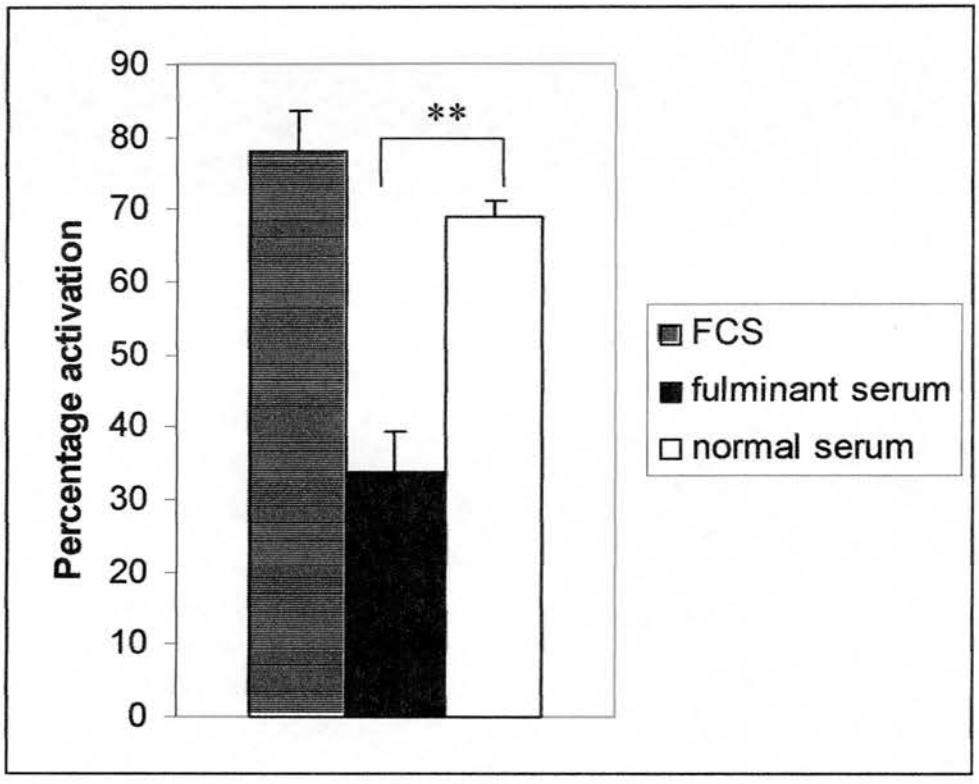
Legend for figure 3.2

This chart represents the mean fluorescence of β_1 (CD29), α_2 (CD49b) & α_4 (CD49f) integrins on HepG2 cells, after 24 hours exposure to different sera. In the cultures exposed to fulminant serum there are higher levels of β_1-integrin (CD29) and α_2-integrin (CD49b) compared to cultures with normal serum.

** $p < 0.01$, *** $p < 0.001$

Figure 3.3

Activational state of β_1–integrin after 24 hours exposure to 20% sera

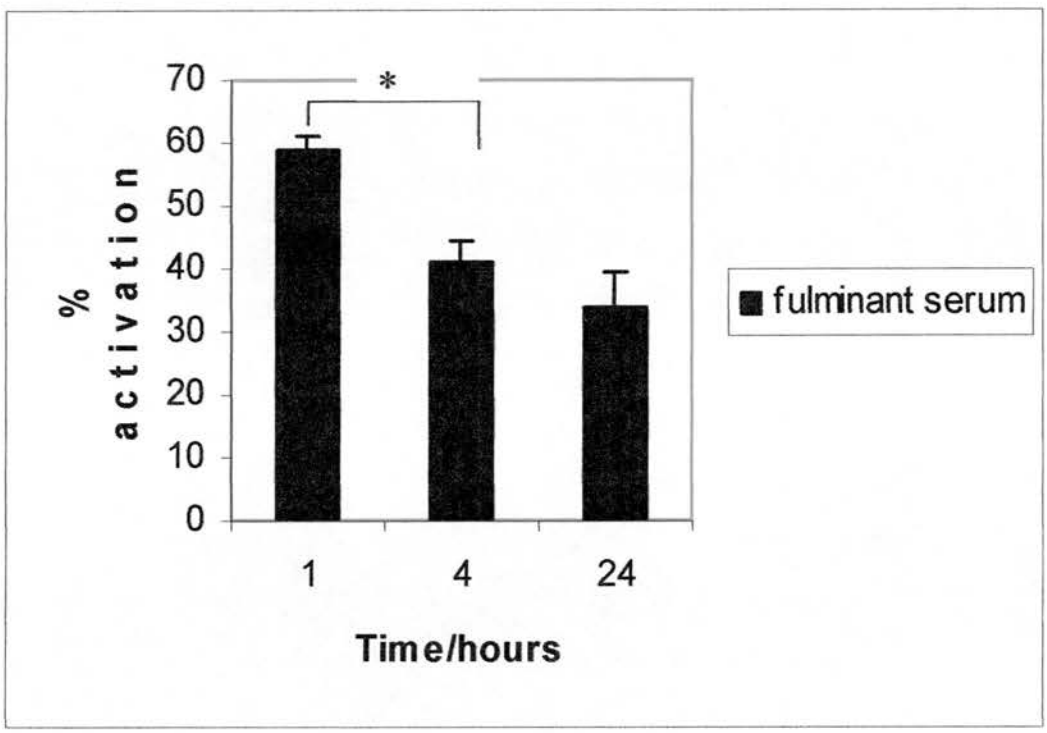


Legend for figure 3.3

This chart assesses the percentage of β_1-integrin receptors which are in their active conformational state. After culture with fulminant serum for 24 hours, the proportion in the active conformational state is much lower than those cultured with normal serum or FCS. No difference was seen between β_1-integrin receptor activity in cells cultured with FCS and normal serum. ** $p < 0.01$

Figure 3.4

Early down-regulation of β_1-integrin after exposure to 20% fulminant serum



Legend for figure 3.4

This chart demonstrates a time course analysis for β_1-integrin activity after exposure to fulminant serum. As can be seen there is a rapid down-regulation of the activity between one and four hours after exposure to fulminant serum.

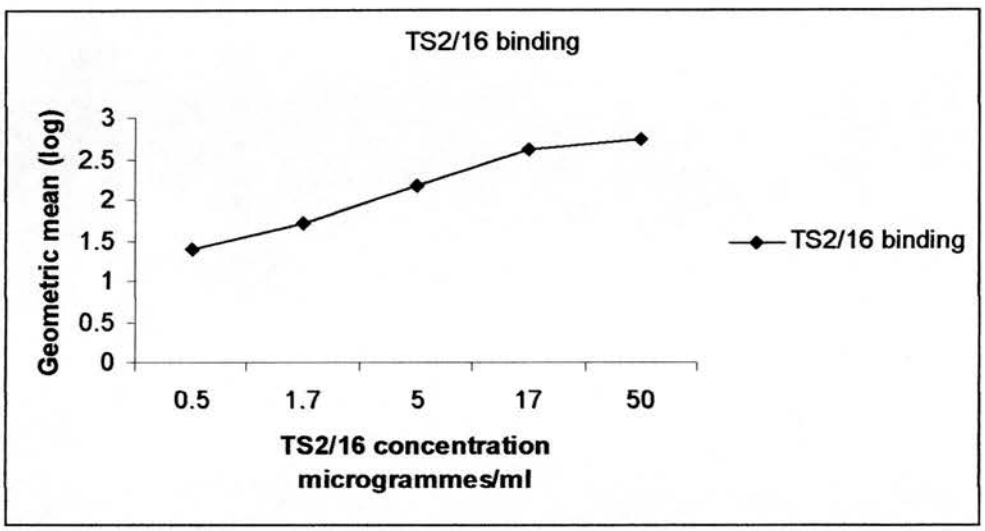
* $p < 0.05$

Activation of β_1-integrin receptor activities prevents the loss of cellular adhesion and onset of cellular adhesion seen with fulminant serum

To see whether modulation of β_1-integrin activity would influence adhesion and apoptosis I added TS2/16 antibody (β_1-integrin receptor activating antibody) to cells after incubation with fulminant serum. I decided upon the optimal concentration (25μg/ml) by a binding assay to identify the concentration at which TS2/16 saturated β_1-integrin binding (Figure 3.5). The adhesion of cells incubated with TS2/16 and fulminant serum was greater than cells incubated with fulminant serum alone at all time points. The adhesion of cells incubated with TS2/16 and fulminant serum is greater than cells incubated with normal serum at all time points until 48 hours. At the 48 hour time-point there was no significant difference between the two groups (Fulminant + TS2/16 86.4 ± 6.1 vs Normal 106.9 ± 8.9, $p= 0.19$) (Figure 3.6).

Figure 3.5

TS2/16 binding assay

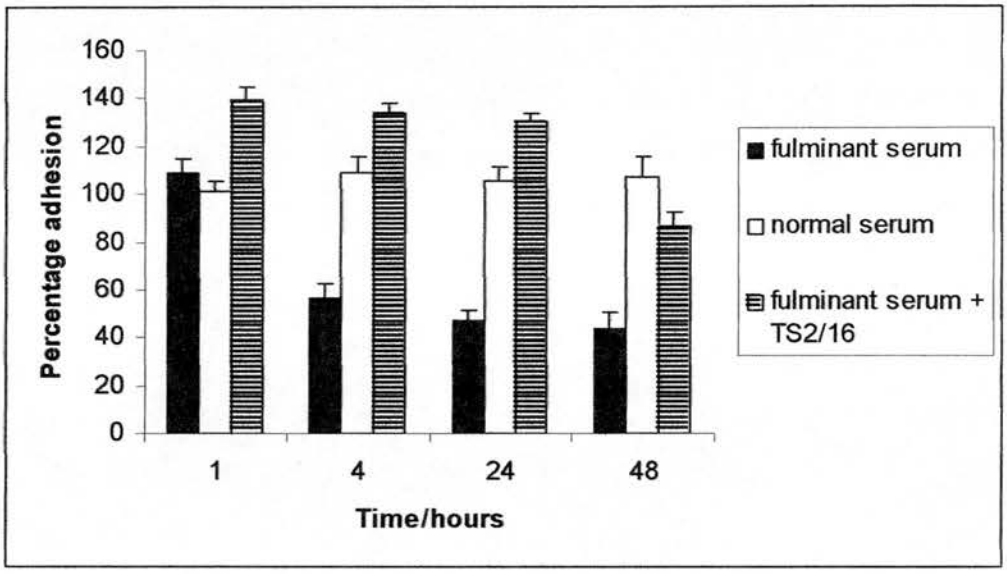


Legend for figure 3.5

This chart demonstrates the optimal binding concentration of TS2/16 antibody to HepG2 cells. Amount of TS2/16 binding was measured flow cytometrically and expressed as a geometric mean on the vertical axis.

Figure 3.6

Effect of TS2/16 incubation on adhesive properties of HepG2 cells exposed to 20% serum



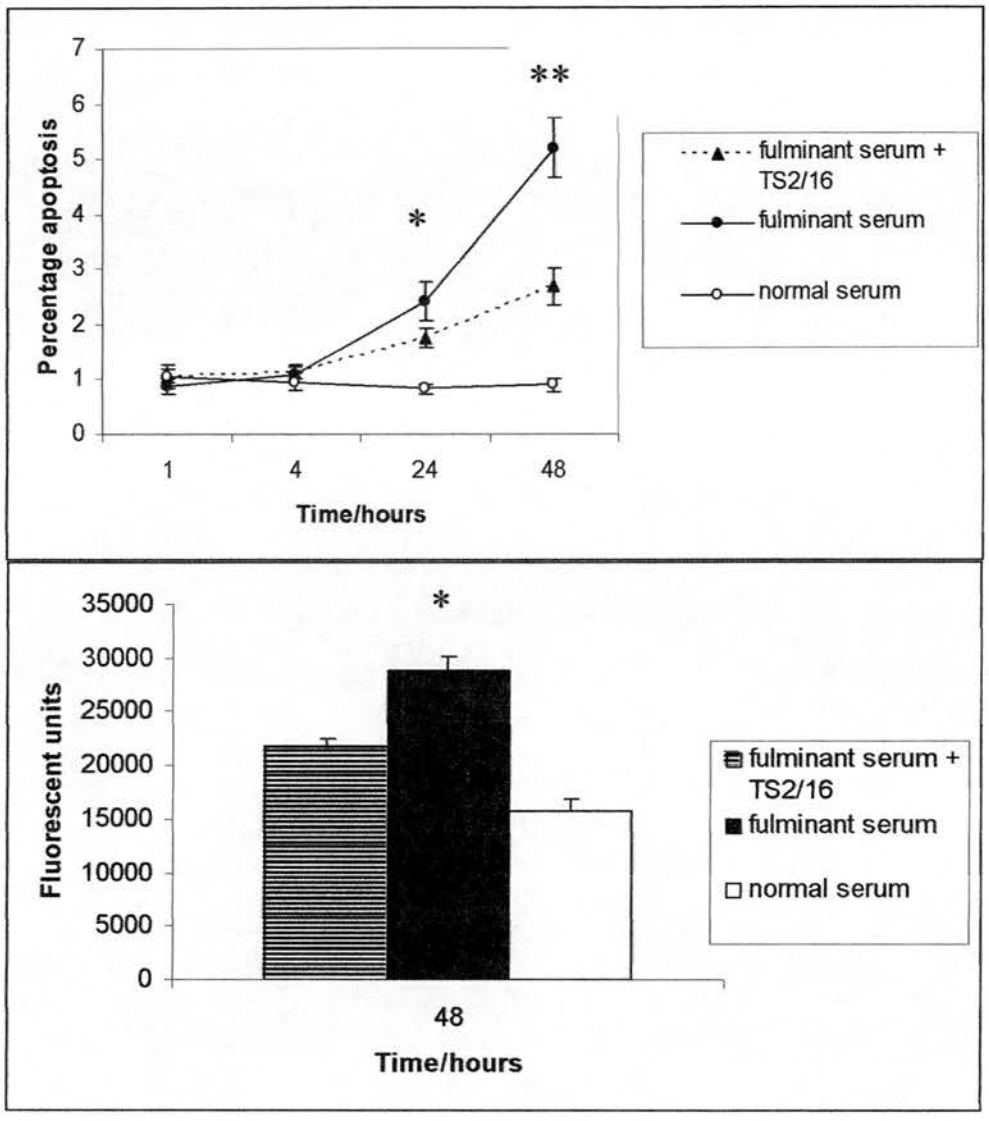
Legend for figure 3.6

This chart illustrates the effect of adding of β_1-integrin antibody (TS2-16) to HepG2 cells after defined periods of exposure to differing sera. As can be seen addition of the TS2-16 antibody can reverse the loss of cellular adhesion in cells which have been cultured in fulminant serum for up to 24 hours. At longer exposures (48 hours) the effect of the antibody on adhesion is only partial. At the 48 hour time-point there was no significant difference between the two groups (Fulminant + TS2/16 86.4 ± 6.1 vs Normal 106.9 ± 8.9, p= 0.19) (Figure 3.6).

Given that I had shown that TS2/16 antibody can reverse loss of adhesion seen with fulminant serum I then went on to look at its effect on cellular apoptosis. Using Feulgen nuclear morphology I was able to show that adding TS2/16 reduced the levels of apoptosis with fulminant serum at both 24 (1.74 ± 0.17% vs 2.43 ± .35%, p=0.049) and 48 hours (2.68 ± 0.34% vs 5.19 ± .053%, p =0.002). Although the levels of apoptosis were still higher than cells incubated with normal serum, the absolute levels seen (2.68%) with addition of the antibody constituted a 48.6% decrease on fulminant serum only cells (Figure 3.7). This anti-apoptotic effect is also supported by measurement of caspase activity after 48 hours incubation (Figure 3.7). Caspase activity with fulminant serum only was 20380 ± 1045 versus 17340 ± 940 in TS2/16 supplemented cells (p=0.02). Furthermore no significant difference in caspase activity existed between cells cultured with normal serum (14541 ± 1029) and cells supplemented with fulminant serum and TS2/16 (p=0.06). This further strengthens the causative relationship between decreased integrin activity, loss of cellular adhesion and induction of apoptosis of HepG2 cells on collagen coated plates.

Figure 3.7

Effect of TS2/16 incubation on cellular apoptosis



Legend for figure 3.7

These charts detail the effects on cellular apoptosis of administering activating β_1-integrin antibody (TS2-16) to cells cultured with fulminant serum (the antibody was added at the beginning of the incubation period). In chart A, the addition of TS2-16 antibody to fulminant serum is seen to significantly decrease cellular apoptosis after 24 and 48 hours incubation compared with cultures of fulminant serum alone. Chart B depicts caspase activity in similar groups and demonstrates a similar reduction after administration of TS2-16 antibody to cultures with fulminant serum after 48 hours incubation compared with cultures just exposed to fulminant serum.

* $p < 0.05$, ** $p < 0.01$

3.7 CONCLUSIONS

In this chapter, I have shown that serum from patients with FHF reduces the adhesive capabilities of HepG2 cells by a rapid down-regulation of their β_1-integrin activity. This loss of β_1-integrin activity and cellular adhesion results in the later activation of caspase-3 and concomitant apoptosis. Importantly, I have demonstrated that the effect of liver injury serum is a specific effect and not just a non-specific toxic effect. This is evidenced firstly by the absence of any apoptosis at any of the early time-points, when integrin affinity and adhesion are compromised. Secondly, we demonstrate that loss of cellular adhesion and subsequent apoptosis can be reversed by treatment of the hepatocytes with the stimulatory mAb TS2/16, again reinforces the point this is not a non-specific toxic phenomenon. That TS2/16 antibody can reverse loss of adhesion in cells that have already been exposed to fulminant serum for 24 hours, indicates that such cells are not committed to apoptosis at this stage.

The increase in β_1 and α_6 integrin expression seen after 24 hours culture with serum from patients with FHF is occurring at a time of impaired cellular adhesion and suggests a possible compensatory response by the cells to increase their attachment. This is confirmed by assessment of the activity of their β_1-integrin, which is seen to be lowered after 4 hours incubation. Manipulation of integrin activity has been suggested to occur by modification of its cytoplasmic component (inside-out signalling), and several mechanisms have been identified including covalent

modification, the attachment of activator proteins or the removal of repressor proteins. Examples of down-regulation of integrin activity include the oncogenic transformation by Rous sarcoma virus leads to phosphorylation of a tyrosine residue in the cytoplasmic subunit leading to reduced binding of β_1-integrins to both talin and fibronectin (Tapley et al 1989). Serine phosphorylation has been demonstrated in mitotic cells to lead to the rounding and detachment of cells as a consequence of integrin down-regulation. A further possibility is external modulation of integrin affinity (outside-in signalling), although this tends to result in up rather than down-regulation and in the context of our study may be the result of a loss of such a stimulating signal in serum from patients with FHF. Other important regulatory factors include temperature (Luque et al 1996), extracellular divalent cations (Masumoto et al 1993), and interaction with intracellular signal transduction molecules such as calreticulin (Coppolino et al 1997) and members of the small GTPase family such as R-Ras (Zhang et al 1996) and Rho A (Hotchin et al 1995).

Once activated, integrins lead to protein tyrosine phosphorylation which has been shown to inhibit apoptosis(Evans et al 1993; McGahon et al 1995; Simizu et al 1996; Tallett et al 1996). In small cell lung cancer cells, ECM-mediated protection from etoposide-induced caspase-3 activation can be blocked by either β_1-integrin function-blocking antibody or by a tyrosine kinase inhibitor(Sethi et al 1999; Tallett et al 1996). Other groups have demonstrated that ECM regulates apoptosis (mammary epithelial cells) through an integrin-dependent negative regulation of Interleukin-1β-converting enzyme (ICE) expression, whereby expression of ICE was correlated with the loss of ECM and inhibitors of ICE activity prevented apoptosis(Boudreau et al

1995). Thus, integrin activation prevents the ICE-mediated induction of caspase-3 activity. The stimulatory mAb TS2/16 acts by directly inducing a conformational change of β_1-integrins. Evidence for this comes from work which shows that TS2/16 stimulates the ligand-binding function of solubilised β_1-integrin in column binding studies(Chan et al 1993). Furthermore whilst deletion of the cytoplasmic domain of an α-subunit of β_1-integrins abolishes phorbol ester stimulation it has no effect on TS2/16 stimulation(Kassner et al 1993; Kawaguchi et al 1993). This indicates that the effect of TS2/16 does not depend on intracellular processes. Finally Fab fragments of the TS2/16 antibody have similar stimulatory properties excluding the possibility of a receptor cross-linking mechanism(Arroyo et al 1993).

The differential control and order of adhesion and anti-apoptotic signals by integrin activation is not fully understood, and in our model, it is not clear if apoptosis is resultant on decreased cellular adhesion or occurs independently. Our data suggests that cytoskeletal re-organisation (as determined by adhesion and actin cytoskeleton) precedes the onset of apoptosis and suggest it may play a role in inducing apoptosis. β_1-integrin is important not only in mediating adhesion and hence survival but also plays an important role in cellular migration. Studies with β_1-integrin knockout haematopoietic stem cells demonstrated their failure to engraft, although their subsequent haematopoietic differentiation was not impaired. Of note stimulation of CD34 cells with TS2-16 antibody (activates β_1-integrin) abrogates almost all marrow directed migration and leads to engraftment in fetal liver (Ramirez et al 2001). This observation is of particular relevance with respect to my findings and raises the possibility that β_1-integrin activity may be important in liver homing. Furthermore, I

have shown in Chapter 2 that there is up-regulation of Collagen IV expression on paracetamol-damaged liver which is a potent ligand for β_1-integrin and a possible entry point for engrafting hepatocytes during cellular transplantation. Thus the down-regulation of β_1-integrin I have observed suggests that this not only decreases hepatocyte homing but also prevents some of the collagen mediated binding on the damaged liver.

In this study the HepG2 cell line was used, which is a differentiated human hepatoma cell line. This line has many characteristics in keeping with primary hepatocytes including a moderately differentiated phenotype and similar integrin expression repertoire, which allow it to act as a suitable model for primary hepatocytes (48). Furthermore, modifications of this line are used in Bio-artificial liver support systems (49) and in hepatocyte transplantation (50). Extrapolation of this data to primary hepatocytes does however require recognition of potentially important differences. Primary human hepatocytes may for example be more susceptible to apoptosis if they are unable to bind to extracellular matrix, as immortalised/tumour cell lines have down-regulated some of their apoptotic pathways (Wiesenauer et al 2004). However, this model system is not studying anoikis; it models the early phase of hepatocyte transplantation, demonstrating that the ability of cells to adhere to matrix is reduced after only 1-4 hours exposure to fulminant serum. In this regard, differences in anchorage-independent growth between HepG2 cells and primary hepatocytes are less important in the interpretation and extrapolation of this data. Different susceptibility to serum-induced apoptosis between the two cell types may

explain the relatively modest levels of apoptosis seen with HepG2 cells, although it would suggest that this mechanism will be of greater importance with primary hepatocytes.

In conclusion, therefore I have made the important observation that serum from patients with FHF rapidly decreases their adhesive capacity as a prelude to apoptosis. I have also identified an underlying mechanism, namely a rapid down-regulation of β_1-integrin activity. Furthermore, I have shown that addition of a β_1-integrin stimulating antibody partially reverses this phenomenon and leads to prolonged hepatocyte adhesion and survival. Pre-activation of β_1-integrin could therefore offer a means of enhancing the efficacy of cell transplantation.

CHAPTER 4

STUDY OF HUMAN HAEMATOPOIETIC STEM CELL TRANS-DIFFERENTIATION *IN VITRO*

4.1 BACKGROUND AND AIMS

Recent studies have demonstrated that stem cells have greater plasticity than previously thought and can differentiate down multiple non-haematopoietic cell lineages in rodents (Graf 2002; Krause et al 2001; Petersen et al 1999; Theise et al 2000a). Human studies have also confirmed that human marrow contains cells which can differentiate into hepatocytes and biliary cells (Alison et al 2000; Korbling et al 2002; Theise et al 2000b).

In vitro studies have demonstrated that stem cells of both rodent and human origin could differentiate down epithelial lineages. At the time of this research, no studies had reported hepatocytic differentiation *in vitro*. Furthermore it is unclear if during liver injury chemicals are released which promote the attraction and differentiation of human stem cells.

Many different markers can be used to define a cell as a hepatocyte. Albumin and αFP are two commonly used markers of hepatocytic differentiation. In addition, albumin expression would demonstrate a synthetic function of the hepatocyte. Pan-cytokeratin expression is used to define epithelial differentiation, rather than hepatocytes per se. $cEBP_{\beta}$ and $HNF4_{\alpha}$ have been demonstrated to be important in the transdifferentiation of pancreatic cells into hepatocytes after administration of dexamethasone. It has been proposed that dexamethasone increases transcription of $cEBP_{\beta}$, which leads to increased fatty acid synthesis (including Acyl CoA). Acyl CoA leads to nuclear translocation of $HNF4_{\alpha}$ which is responsible for the transdifferentiation of pancreatic cells (Shen et al 2000).

This work was undertaken to answer the following questions:

1) Could human cord-blood stem cells differentiate into hepatocytes in vitro?

2) Does acute liver injury alter the differentiation profile?

4.2 MATERIAL

Human cord blood was obtained with patient consent and full approval from the Lothian Regional Ethics Committee (LREC/1993/4/76).

4.3 METHODS

Freshly collected heparinised whole cord blood samples were received from the Scottish National Blood Transfusion Service (Edinburgh, UK) following informed donor consent. Mononuclear cells were isolated using Histopaque 1077 (Sigma, UK) density centrifugation following the initial description by Boeyum (Boeyum A 1968). Briefly, heparin-treated (10^3IU/mL) whole blood was carefully layered over an equal volume of Ficoll-Hypaque^{TM} and spun at 540g for 20 minutes. The interface ('buffy coat') containing the mononuclear cells was collected and washed twice in Hank's Balanced Salts Solution (HBSS; Gibco, UK). A trypan blue exclusion assay was used to assess the viability of the harvested cells prior to their usage.

FACS Analysis

Cord blood mononuclear cell surface phenotype was characterised using standard FACS analysis. Briefly, 100,000 cells per test were pelleted by centrifugation for 5 minutes at 1000rpm and cell pellets resuspended in individual monoclonal antibody (mAb) solutions using the manufacturer's (Dako, UK; BD Pharmingen, UK; Sigma, UK) recommended test concentrations (confirmed in our laboratory to give optimum results in our hands). Test samples were immunostained for 20 minutes on ice with a

panel of directly FITC- and/or PE-conjugated mAb against human cell surface markers. The panel included mAb against the following markers: MHC1 (pan-nucleated cell) (200 μg/mL; Sigma, UK), gly-A (erythrocyte) (100 μg /mL; Dako, UK), CD3 (T Cell) (12.5 μg g/mL; BD), CD11b (granulocyte) (100 μg /mL; Dako), CD14 (monocyte) (60 μg /mL; Dako), CD19 (B cell) (25 μg /mL; BD), CD33 (myeloid progenitor) (6 μg /mL; BD), CD34 (stem cell) (25 μg /mL; BD), AC133 (stem cell) (25μg /mL; Dako), CD45 (pan-leukocyte) (50 μg /mL; BD) and CD56 (NK cell) (50 μg /mL; Dako). Cell subsets were double-immunostained using a subset-specific mAb as well as an antibody against CD45. Following immunostaining, cells were pelleted, washed in FACS handling buffer (1xPBS/1% BSA/0.1% Sodium Azide/0.5M EDTA) and resuspended in FACS fix (1% formaldehyde) prior to analysis using CellQuest software on a Becton Dickinson FACSCalibur machine.

Cell isolation and purification by positive selection for AC133

Cord blood was processed as described above and then re-suspended in Magnetic Cell Sorting (MACS) Buffer in a total volume of 300μl per 10^8 total cells. Cell suspension was then filtered through a 30μm mesh to remove clumps. This was followed by a further wash in buffer and resuspension in 300μl buffer. 100μl of FcR Blocking re-agent was added per 10^8 total cells and mixed well. Then 100μl of AC133-coated microbeads were added per 10^8 cells, mixed well and incubated for 30 minutes at 6-12°C. The final labelling volume was 500μl. After this cells were washed with 5-10 ml of MACS buffer, then centrifuged before removal of the

supernatant and re-suspension in MACS buffer to a volume of 500µl. The suspension was then applied to the column in the VarioMACS magnet. The column was washed four times with MACS buffer after this. At this time the column was removed from the magnet, 1ml of buffer was placed onto the column and the contents eluted into a fresh tube. This tube was washed and centrifuged again with MACS buffer. It was resuspended with 500µl of MACS buffer and applied to a fresh column within the VarioMACS magnet. The procedure was repeated as per the first run through the magnet.

Confirming AC133 purity

Using the FACS VANTAGE ® flow cytometry sorting machine, AC133 positive cells were quantified as a percentage of low side scatter mononuclear cells using the ISHAGE flow cytometry guidelines which uses forward/side scatter gating followed by gating on the marker CD45 and AC133.Initially events are plotted on a forward scatter vs. side scatter dotplot and a gate is placed around the low side scatter population of mononuclear cells which predominantely comprise of lymphocytes and which excludes debris, platelets, macrophages, monocytes and unlysed erythrocytes. In the next stage, the selected mononuclear cell population is then displayed on a CD45 vs. AC133 dotplot and the cluster of $AC133^{+}$/CD45 intermediate events detected are gated. Finally the $AC133^{+}$/CD45 intermediate population selected is then quantified as a percentage of the total events gated in the first stage of analysis i.e. low side scatter mononuclear cells.(Keeney et al 1998)

Cell culture

$AC133^+$ cells were divided up into aliquots of 30,000 cells/well and cultured with Stem Cell Medium (Gibco Life Technologies) in the presence or absence or different cytokines. These cytokines were Flt-3 ligand (50ng/ml), Thrombopoietin (10ng/ml), Hepatocyte Growth Factor (HGF; 20ng/ml), Neurotrophin Growth Factor (NGF; 100ng/ml) and Fibroblast Growth Factor α/β (FGF; both 20ng/ml). Cytokines were supplemented on days 3, 7, 10, whilst half-media change, occurred on day 7. At the end of 14 days, cells were either labelled for flow cytometry or stored in RNA later for subsequent rtPCR analysis. Cultures were performed on uncoated, gelatin coated and Collagen coated plates. Gelatin was purchased from Sigma (Type A porcine skin, approx 300 Bloom), and made into a 1% solution in sterile distilled water, and then diluted further to 0.1% in PBS. 100µl were added to each well and allowed to dry, prior to addition of stem cells.

For experiments studying the role of acute liver injury on differentiation Stem cell Medium with TPO/Flt-3 and varying concentrations of acute liver failure serum were used.

mRNA isolation

Cells were remove from the 24-well-plates with cell scrapers and washed twice with PBS. An aliquot of 40,000 cells was centrifuged at 3000g for 5minutes. The pellet was re-constituted with 20µl PBS, to which 180 µl RNAlater was added. Cells were then stored at minus 80°C until further analysis.

Samples were thawed and washed twice in PBS prior to pelleting and reconstitution with 100 µl Cell Lysis Buffer. It was incubated with this at 75°C for 5 minutes. 2 µl DNAase was added and the sample incubated at 37°C for 30 minutes and then 75°C for 5 minutes. Two 10 µl aliquots (4000 cells worth) were removed for usage in the reverse transcription reaction. To this was added either 2 µl of Random decamers, OligoDt or Random Decamers. OligoDts would theoretically be better for mRNA which consisted of long poly-A tails. In addition, 4 µl of dNTP mix and 16 µl of nuclease free water were added. This mixture was vortexed and then centrifuged at 500g for 30 seconds. The sample was then heated at 75°C for 3 minutes, placed on ice for 1 minute afterwards, re-centrifuged at 500g for 30 seconds and then returned to ice for a further minute. At this stage 2 µl of 10x RT buffer, 1 µl of MMLV-RT and 1 µl of RNAase inhibitor were added. Importantly the MMLV-RT enzyme was missing from the control, termed the RT Negative sample. This mixture was vortexed, centrifuged at 500g for 30 seconds and then placed at 42°C for 1 hour. It was then stored at minus 20°C prior to PCR analysis.

PCR analysis

PCR is an *in vitro* method of nucleic acid synthesis by which a particular segment of DNA can be specifically replicated. It involves two oligonucleotide primers that flank the DNA fragment to be amplified and repeated cycles of heat denaturation of the DNA, annealing of the primers to their complementary sequences, and extension of the annealed primers with DNA polymerase. These primers hybridise to opposite strands of the target sequence and are oriented so that DNA synthesis by the

polymerase proceeds across the region between the primers. Since the extension products themselves are also complementary to and capable of binding primers, successive cycles of amplification essentially double the amount of the target DNA synthesised in the previous cycle. The result is an exponential accumulation of the specific target fragment.

The polymerase chain reaction was performed by incubating 1-2 μl cDNA samples with 0.5 units of AGS GOLD DNA polymerase in 5 μl of 10 x Complete Reaction Buffer, 5 μl of AGS GOLD enhancer solution, 2 μl Primer mix, 1.6 μl dNTP mix making up to 50 μl with double-distilled water. All reagents were kept on ice and the DNA polymerase was added last. PCR cycling was carried out on a MJ Research PTC-100 thermal cycler for 35 cycles. The annealing temperature varied depending on the product and primers used. Two control tubes were run in parallel, one in which water replaced the RNA and a second omitting reverse transcriptase to ensure there was no genomic DNA contamination. Specific primers were used and these and the resulting product size are shown in Table 4.1. Primers were designed to span an intron to ensure genomic DNA was not amplified. Primers for the constitutively expressed gene GAPDH were used to confirm the integrity of the RNA and efficacy of the PCR reaction. After the PCR, the size of product was ascertained using electrophoresis. A 5 μl sample of PCR product was mixed with 3 μl of loading buffer to visualise loading and dye front on running gel. Products were loaded on a 2% agarose gel which contained ethidium bromide and placed in a tank containing TBE and run at 120 V over 45 minutes and products were visualised using ultraviolet light. A 100bp ladder (Promega, USA) was included to ensure that products seen

were of the expected size. The 500bp band is of triple intensity to the other bands. Products were purified and the identity of all PCR products was confirmed by direct sequencing as described below.

Table 4.1
PCR Primers

Gene	Primer	Sequence (5'-3')	Product size (bp)
Albumin	Forward	TGCTTGAATGTGCTGATGACAGGG	165
Albumin	Reverse	AAGGCAAGTCAGCAGGCATCTCAT	165
αFP	Forward	GTGGTTAAAGGAAACGCTCG	212
αFP	Reverse	CATACATTTCAGCAGGTGCG	212
GAPDH	Forward	GAACGGGAAGCTCACTGGCAT	310
GAPDH	Reverse	GTCCACCACCCTGTTGCTGTAG	310

PCR cycling sequence

AFP

1) 2 minutes at 94°C

2) 30 seconds at 94°C

3) 30 seconds at 48°C

4) 30 seconds at 72°C

5) 10 minutes at 72°C

Steps 2-4 were repeated 34 times

Albumin

1) 2 minutes at 94°C

2) 30 seconds at 94°C

3) 30 seconds at 58°C

4) 30 seconds at 72°C

5) 10 minutes at 72°C

Steps 2-4 were repeated 34 times

Sequencing of DNA

The first step in sequencing is to denature the double stranded DNA into single strands. The overall approach of sequencing DNA involves creating sub-fragments of all possible lengths from the DNA we want to sequence, grouping them according to which base they end in. To create these fragments a chain termination method is employed called the dideoxy method. Dideoxy analogues of normal DNA precursors cause premature termination of a growing chain of nucleotides being made by DNA polymerase. This allows generation of fragments of the stretch of DNA we want to

sequence and by using four different dideoxy analogs, one for each of the four bases, we can generate four sets of fragments. To identify these, the same DNA primer is used for each reaction but four different fluorescent dyes are used, one for each of the bases. When a sequencing gel is run, a separate colour is obtained for each base. A laser beam scans the bands and the four different dyes fluoresce in different colours. A computer which then prints the sequence records the colour of each band.

Purification of products for sequencing

This was done for all products prior to sequencing using a Hi–Pure purification kit in order to remove any primer dimers which may affect the sequencing signal. First, the amount of product present was measured and made up to 50µl with H_2O. Binding buffer (250µl) was then added to each sample and the sample then added to a column sitting in a collection tube. Next, the samples were centrifuged at 13000g for 1 minute. The elute was discarded and 500µl of washing buffer was added to the column and this was centrifuged for 1 minute at 13000g. These two steps were repeated using 200µl of washing buffer and then 40µl of elution buffer was added to a column sitting in a clean tube and centrifuged for 1 minute elute was put through the column again and products run on an agarose gel and tested in a spectrophotometer to ascertain purity and quantification.

Method for sequencing DNA

For the sequencing reaction, 30-90ng of purified PCR product was added to 4µl of sequencing mix, 4µl of ½ term and 1µl of primer at a concentration of 5pmol/µl. This was then made up to 20µl with H_2O and the mixture placed in the PCR machine at 96°C for 15 seconds, 50 °C for 25 seconds and 60 °C for 4 minutes for 25 cycles. Once finished the tubes were spun briefly and then 50µl of 96% ethanol and 2µl of 3M sodium acetate were added to each tube. The samples were vortexed thoroughly and left at room temperature for 30 minutes before spinning for 30 minutes. The supernatant was removed and pellet washed with 100µl of 70% ethanol. This was spun again for 10 minutes and supernatant removed and pellet allowed to dry. This was then resuspended in 4µl formamide: EDTA buffer (5:1 ratio of deionised formamide: 50mM EDTA) and was ready for loading on the sequencing gel.

The sequencing gel was made by mixing 50g urea and 15 ml 40% acrylamide and making up to 80 ml with water. One teaspoon of amberlite was added to this and the mixture was heated gently until the urea dissolved. This was filtered to remove the amberlite and then 10 ml of TBE added. The mixture was made up to 100 ml with water and just prior to pouring 500µl 10% APS and 45µl TEMED were added. The gel was then poured between the glass plates with spacers in situ and allowed to set prior to placing in the sequencer along with running buffer and loading with samples. Sequencing was carried out using an Applied Biosystems 373A automated sequencer.

Immunofluorescence

Cells were removed from the 24-well plates with cell scrapers and washed twice with PBS. They were reconstituted with PBS and placed on cytospins at 150g for 5 minutes. Cells were permeabilized with 0.1% Triton X for 30 minutes at 4°C. Slides were then blocked with 20% animal serum in PBS with 0.05% Tween (species is determined by the species of the secondary fluorescent antibody) for 10 minutes at room temperature. Then a 1/150 dilution of the primary antibody (in blocking solution) was added to each slide for incubation at room temperature for 1 hour. Slides were placed on a moist tissue in a sandwhich box. After the incubation, slides were washed three times with PBS containing 0.05% Tween. The secondary antibody was then added (dilution 1/300) and again incubated for 1 hour at room temperature. Finally, slides were washed three times with PBS prior to fixation and coverslipping with antifade.

Primary antibodies were HNF-4 (goat), c-EBPβ (mouse) and pan-cytokeratin, with respective secondaries were rabbit anti-goat conjugated with 488-Alexafluor and goat anti-mouse conjugated with 568-Alexafluor. These antibodies were chosen to check for evidence of early hepatocyte commitment.

4.4 EXPERIMENTS

The haematopoietic profile of human cord blood samples was characterised by FACS and CFU analysis to demonstrate that they were rich sources of human stem cells (n=10). This data is presented in chapter 5.

The next set of experiments was designed to see what the quantity and purity of $AC133^+$ cells by VarioMACS isolation was. This was performed by ISHAGE flow cytometric analysis (Keeney et al 1998), and was done on 9 separate occasions.

The remainder of the work set out to study the role of different culture conditions on differentiation of $AC133^+$ cells. Cells were cultured in triplicate wells on three separate occasions in the following culture conditions:

1) Thrombopoietin (TPO; 10ng/ml) & Flt-3 ligand (50ng/ml)

2) TPO/Flt-3 & HGF (20ng/ml)

3) TPO/Flt-3 & NGF (100ng/ml)

4) TPO/Flt-3 & FGFα (20 ng/ml)

5) TPO/Flt-3 & FGFβ (20ng/ml)

6) TPO/Flt-3 & NGF & HGF

7) TPO/Flt-3 & NGF & HGF & FGFα/β

Analysis then took place as described above in the methods section: flow cytometry, immunofluorescence, rtPCR, morphology.

4.5 STATISTICAL ANALYSIS OF DATA

Data is expressed as Mean +/- standard error of the mean (SEM).

4.6 RESULTS

Yield and purity of sorted AC133 stem cells

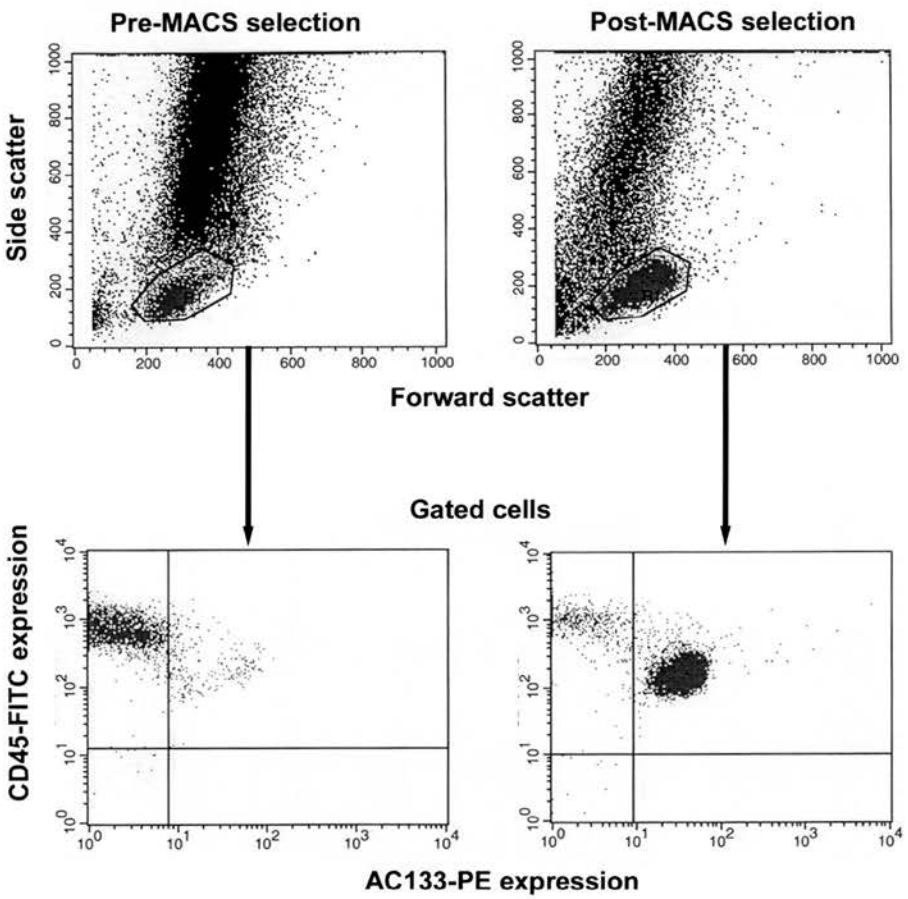
Table 4.2 displays the mean number of mononuclear cells obtained after mononuclear cell prep and the number of $AC133^+$ cells obtained after one and two runs through the VarioMACS column. The mean purity of $AC133^+$ cells obtained after column isolation is also shown in the table. After one run through the column, only 12.4% of the cells were $AC133^+$, and for this reason, cells were eluted through two columns to ensure satisfactory purity (91.3%).

Table 4.2

Cell count and purity pre- and post- MACS isolation

		Mean cell count ± SEM	$AC133^+$ cells as a % of original cell count	Purity of final $AC133^+$ population
One run through column	Pre	$86.0 \times 10^6 \pm 38.4 \times 10^6$		
	Post	$5.11 \times 10^5 \pm 2.49 \times 10^5$	0.55	12.4 ± 3.45
Two runs through column	Pre	$91.2 \times 10^6 \pm 2.93 \times 10^6$		
	Post	$1.34 \times 10^5 \pm 3.82 \times 10^4$	0.39	91.3 ± 17.9

Figure 4.1
AC133 purification with VarioMACS system



The top two panels depict the forward versus side scatter for the mononuclear cell preparation before and after VarioMACS column processing. The post-column panel can be seen to have excluded large numbers of cells with a similar forward scatter to the gated population, the remaining cells representing lymphocytes and sub-cellular debris. The lower panels look at the double staining pattern of the gated population for CD45 and AC133. After column isolation there is a clearly defined AC133+ population which is CD45dim.

Legend for figure 4.1

This figure gives a graphical illustration of the flow cytometric confirmation of the isolation and purity of $AC133^{+}$ cells after use of VarioMACS. The true $AC133^{+}$ stem cell population can be seen to be $AC133^{bright}$ and $CD45^{dim}$. There are some $CD45^{bright}$ contaminating lymphocytes seen in the post-column isolate but this is much fewer than in the starting preparation.

Cell culture

Total cell number was measured by Neubauer cytometer after 2 weeks culture, as shown in table 3. From this table it can be seen that the use of matrix and multiple cytokines leads to maximal cellular expansion.

Table 4.3

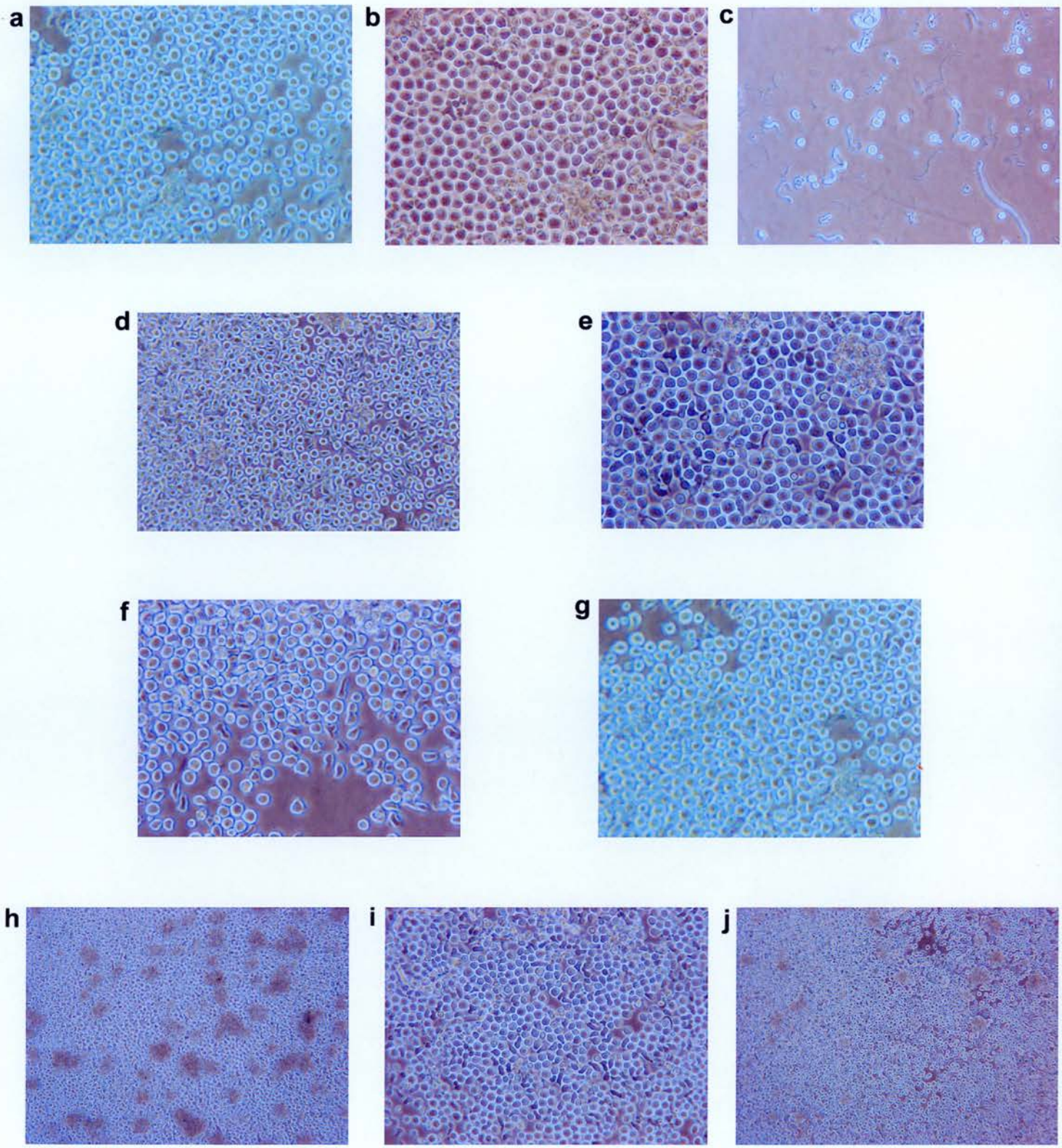
Cell quantification after culture

	Final cell number after 2 weeks culture (x 10^5)		
	Uncoated	**Collagen-coated**	**Gelatin-coated**
TPO/Flt-3	1.93 ± 0.62	4.96 ± 1.23	4.56 ± 1.22
TPO/Flt3 + HGF	1.35 ± 0.64	4.32 ± 0.97	5.12 ± 1.56
TPO/Flt3 + NGF	1.70 ± 0.37	4.42 ± 1.72	3.90 ± 1.21
TPO/Flt3 + FGFα	4.00 ± 1.59	6.67 ± 2.11	6.80 ± 2.76
TPO/Flt3 + FGFβ	4.23 ± 1.87	7.55 ± 2.65	7.20 ± 2.31
TPO/Flt3 + NGF + HGF	3.90 ± 0.75	6.78 ± 2.33	5.99 ± 3.02
TPO/Flt3 + NGF + HGF + FGFα/β	2.30 ± 0.84	7.77 ± 2.52	8.09 ± 3.62

Morphology

Pictures of $AC133^+$ cells were taken under different culture conditions, and depicted in figure 4.2. Panels A and B represent cells cultured with TPO/flt-3 whilst panel C demonstrates that without these 2 anti-apoptotic cytokines there are few cells remaining, most of which look spindle-shaped and necrotic. Panels D-J represent the morphology of cultures as grown under different cytokine combinations (see methods; D-J correspond to 1-7 with respect to cytokine combinations). There was no marked difference in morphology seen in any of the different combinations.

Figure 4.2
Stem cell morphology



Legend for figure 4.2

Light microscopy pictures taken of cultured $AC133^{+}$ cells. Panels A and B represent culture with TPO/Flt-3. Panel C is medium alone without the aforementioned growth factors. Panels D- J are correspond to 1-7 with respect to cytokine combinations (see methods).

Flow cytometry analysis

Analysis of freshly isolated samples obtained after AC133 isolation revealed them to be 91% $AC133^{+}$, with 2.5% CD3, 2% CD14 and 1% Glycophorin positive. There was no evidence of positivity for CD19. After 2 weeks culture cells were removed from plated and analysed flow cytometrically. The results are summarised in Tables 4.4 and 4.5, and depict the percentage of cells in each culture condition which had differentiated down haematopoietic lineages. For collagen-coated plates, there was no marked variation in cellular differentiation across the different cytokine combinations. The majority of cells appear to differentiate down T cell or mononuclear cell lineages, with only minimal red cell (Glycophorin) or B cell differentiation. On gelatin-cultured plates there appears to be a shift towards mononuclear lineages as compared with collagen-coated plates which have greater numbers of T cells (CD3).

Table 4.4

Flow cytometric analysis after culture on Collagen coated plates

Cytokine combination	Cell surface marker	Range (%)	Mean (± SE)	Median
TPO/Flt-3	GlyA	1-10	5 (1)	5
	CD3	41-57	46 (8)	42
	CD14	30-41	35 (6)	32
	CD19	0-8	4 (1)	3
TPO/Flt3 + HGF	GlyA	0-12	5 (1)	4
	CD3	46-73	61 (10)	58
	CD14	28-41	36 (5)	40
	CD19	0-7	3 (1)	2
TPO/Flt3 + NGF	GlyA	1-8	6 (1)	7
	CD3	36-61	45 (8)	
	CD14	42-58	43 (7)	41
	CD19	0-4	2 (1)	2
TPO/Flt3 + FGFα	GlyA	1-8	4 (1)	4
	CD3	43-60	53 (7)	52
	CD14	25-47	35 (8)	35
	CD19	0-5	2 (1)	2
TPO/Flt3 + FGFβ	GlyA	1-12	5 (2)	5
	CD3	38-56	48 (12)	49
	CD14	22-47	30 (9)	31
	CD19	0-5	3 (2)	3
TPO/Flt3 + NGF + HGF	GlyA	0-6	3 (2)	3
	CD3	27-52	40 (8)	38
	CD14	22-46	30 (13)	30
	CD19	1-6	3 (1)	3
TPO/Flt3 + NGF + HGF + FGFα/β	GlyA	0-7	4 (2)	5
	CD3	37-63	50 (9)	49
	CD14	18-34	25 (5)	26
	CD19	1-8	4 (1)	5

Table 4.5

Flow cytometric analysis after culture on Gelatin coated plates

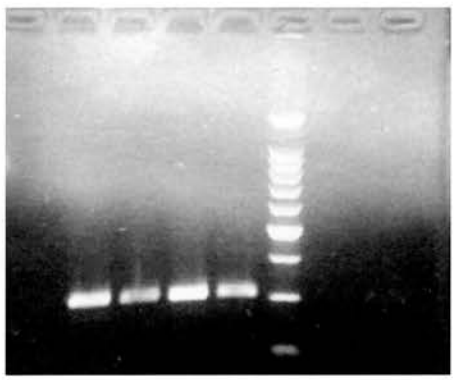
Cytokine combination	Cell surface marker	Range (%)	Mean (± SE)	Median
TPO/Flt-3	GlyA	1-12	6 (1)	5
	CD3	27-52	36 (5)	36
	CD14	38-73	60 (9)	58
	CD19	1-5	3 (1)	4
TPO/Flt3 + HGF	GlyA	0-10	4 (1)	5
	CD3	21-53	30 (7)	32
	CD14	32-66	51 (9)	50
	CD19	1-15	9 (1)	10
TPO/Flt3 + NGF	GlyA	1-9	5 (1)	5
	CD3	32-65	38 (7)	39
	CD14	42-75	63 (12)	66
	CD19	2-12	8 (1)	7
TPO/Flt3 + FGFα	GlyA	1-9	5 (1)	4
	CD3	23-49	33 (6)	33
	CD14	45-64	57 (7)	58
	CD19	1-9	6 (1)	5
TPO/Flt3 + FGFβ	GlyA	1-9	4 (2)	4
	CD3	28-52	34 (6)	35
	CD14	30-66	52 (5)	51
	CD19	1-9	5 (2)	4
TPO/Flt3 + NGF + HGF	GlyA	0-7	4 (1)	4
	CD3	21-62	37 (11)	36
	CD14	31-49	38 (6)	37
	CD19	1-11	7 (1)	8
TPO/Flt3 + NGF + HGF + FGFα/β	GlyA	0-9	5 (1)	4
	CD3	32-59	47 (7)	46
	CD14	12-43	27 (6)	28
	CD19	1-6	3 (1)	4

Across all the culture groups, there are cells which did not stain with the above markers. Further analysis of non-haematopoietic differentiation was undertaken further on in this chapter using immunofluorescent analysis.

Figure 4.3
Optimisation of rtPCR on HepG2 cells

GAPDH

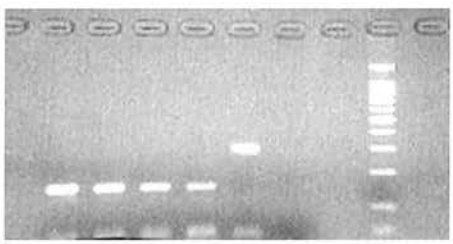
a



1 2 3 4 5 6 7

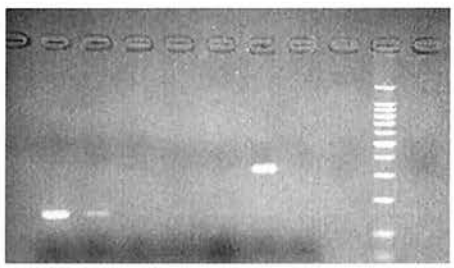
ALBUMIN

b



1 2 3 4 5 6 7 8

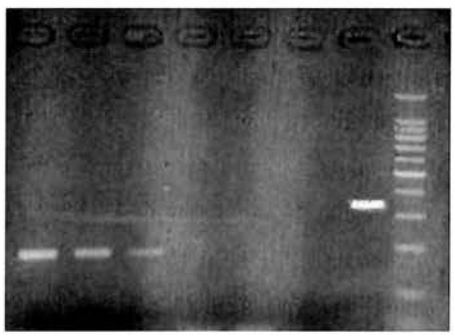
c



1 2 3 4 5 6 7 8 9

AFP

d



1 2 3 4 5 6 7 8

Legend for figure 4.3

This figure demonstrates the sensitivity of the rtPCR system for detecting GAPDH, albumin and alpha-feto-protein transcripts in HepG2 cells. Picture **a** depicts presence of GAPDH transcripts as produced with either Random Decamers (lanes 1-2) or OligodT primers (lanes 3-4). Lane 6 is the RT negative control and lane 7 is the water blank PCR control. Lane 5 is the ladder, with the thick band representing 500 base pairs. Lanes 1 and 3 represent 100 cells worth of cDNA whilst lanes 2 and 4 represent 10 cells worth. Pictures **b** and **c** depict sensitivity for albumin transcripts. Lanes 6 and 7 are the controls as per picture **a**, and lane 5 represents the GAPDH control band. Lanes 1-4 in picture **b** represent 200, 100, 50 and 25 cells worth of cDNA, whereas Lanes 1-4 in picture **c** represent 25, 10, 5 and 1 cells worth of cDNA. Picture **d** depicts sensitivity for alpha-feto-protein transcripts. Lanes 5 and 6 are the controls as per picture **a**, with lane 7 representing the GAPDH control. Lanes 1-4 in picture **d** represent 100, 50, 25 and 10 cells worth of cDNA.

rtPCR data

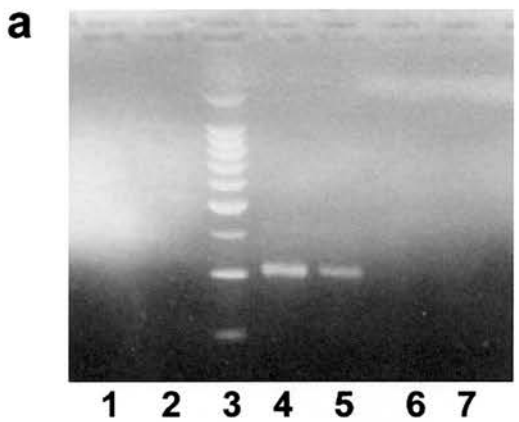
HepG2 cells were cultured in the standard manner (described in chapters 2 &3). Samples of HepG2 cells were processed for cDNA synthesis and rtPCR as described in the methods section. In the first stage, the presence of the ubiquitous housekeeping gene GAPDH was tested for. Two different primers were used for cDNA synthesis; oligodT and Random Decamers. 10 and 100 cells worth of cDNA produced with these respective primers were placed in the PCR reaction, and the products produced were as shown in figure 4.3 picture1A. These suggested that the GAPDH product was of higher quality with the OligodT primers, and consequently this was used subsequently in cDNA synthesis.

To ascertain the sensitivity of the system for detecting albumin and alpha-feto-protein transcripts diminishing amounts of cDNA were placed in the PCR reaction. As shown in figure 4.3 picture 3B and 3C as little as 10 cells worth of albumin cDNA produced by HepG2 cells could be identified. This was slightly higher than alpha-feto-protein transcripts, which required at least 25 cells worth of cDNA to be present (figure 4.3 picture 1D).

Having established the limits of the detection system samples of cultured $AC133^+$ cord blood cells were processed in similar fashion to determine whether there was any evidence of differentiation down the hepatocytic lineage. Figure 4.4A demonstrates that freshly isolated human $AC133^+$ cells do not produce albumin or alpha-feto-protein transcripts, and that GAPDH transcripts can be easily detected. Furthermore, only 25 cells worth of GAPDH is required for the system to detect it.

Figure 4.4B confirms that $AC133^{+}$ cells cultured in a variety of different cytokine combinations produce relatively similar amounts of GAPDH. However, as figures 4C and 4.4D demonstrate there was no evidence of albumin or alpha-feto-protein transcript production in any of these. In these experiments 100 cells worth of cDNA was used in the PCR reaction. This work was performed on uncoated, collagen coated and gelatin coated wells, with similar (ie negative) results in each case.

Figure 4.4
rtPCR on cultured AC133+ stem cells



Legend for Figure 4.4

This figure demonstrates the detection of GAPDH, albumin and alpha-feto-protein transcripts in $AC133^+$ cells. Picture **a** looks at the presence of GAPDH in 100 and 10 cells worth of cDNA (lanes 4 and 5 respectively). Lanes 1 and 2 are the RT negative control and the PCR water blank control. Lanes 6 and 7 represent the absence of albumin and alpha-feto-protein transcripts in freshly isolated $AC133^+$ cells. Picture **b** represents the controls in lanes 1 and 2. Lanes 3-9 are the GAPDH controls from different cytokine combinations, and match up with the lanes 1-7 in pictures **c** and **d**. Lanes 1-7 correspond to the cytokine combinations detailed in the methods section. Lane 8 in pictures **c** and **d** represent the result obtained from HepG2 cells and acts as a positive control. Pictures **c** and **d** demonstrate that there is no evidence of albumin and alpha-feto-protein transcript production in any of the culture combination tried.

Immunofluorescence

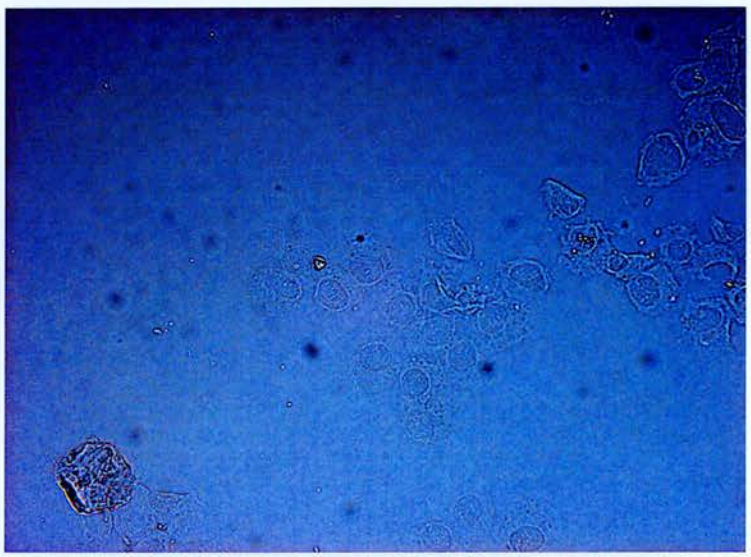
Figure 4.5 depicts the staining of HepG2 cells for a pan-cytokeratin antibody detected with a FITC secondary antibody. Panel A represents contrast light microscopy, panel B is DAPI nuclear staining and panel C the cytokeratin staining. There was no evidence of positive staining of $AC133^+$ cells at either isolation or after any combination of cytokine cultures (Olympus microscope x100).

Figure 4.6 depicts the staining of cEBPβ on HepG2 cells with a Texas Red secondary. Panel A represents contrast light microscopy, panel B is DAPI nuclear staining and panel C the cEBPβ staining. There was no evidence of positive staining of $AC133^+$ cells at either isolation or after any combination of cytokine cultures (Olympus microscope x100).

Figure 4.7 depicts the staining of HNF4α on HepG2 cells with a FITC secondary. Panel A represents contrast light microscopy, panel B is DAPI nuclear staining and panel C the HNF4α staining. There was no evidence of positive staining of $AC133^+$ cells at either isolation or after any combination of cytokine cultures (Olympus microscope x100).

Figure 4.5
Pan-cytokeratin staining on HepG2 cells

a

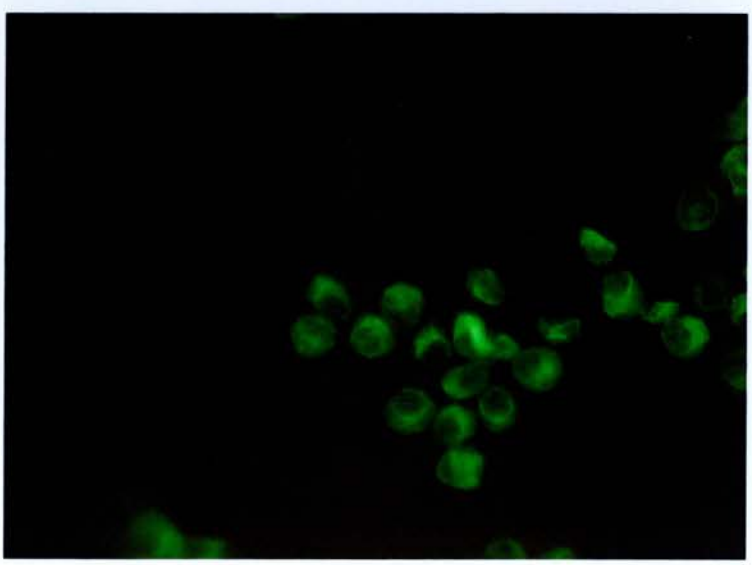


Light

b

DAPI

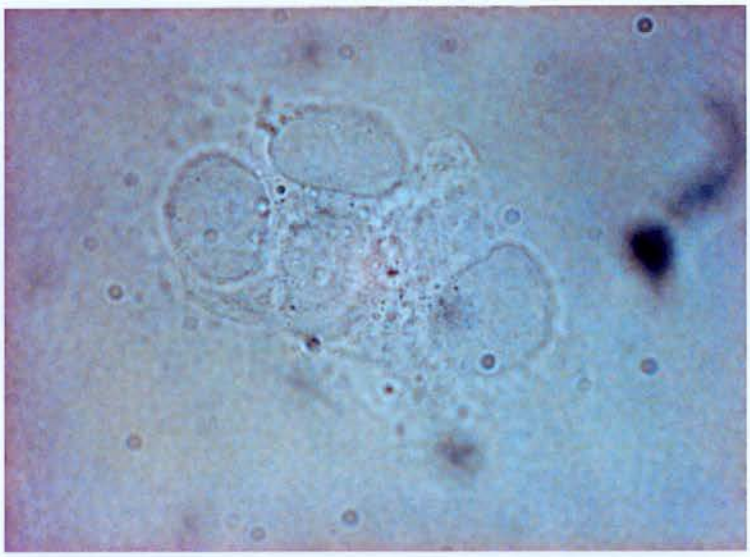
c



Pan-cytokeratin

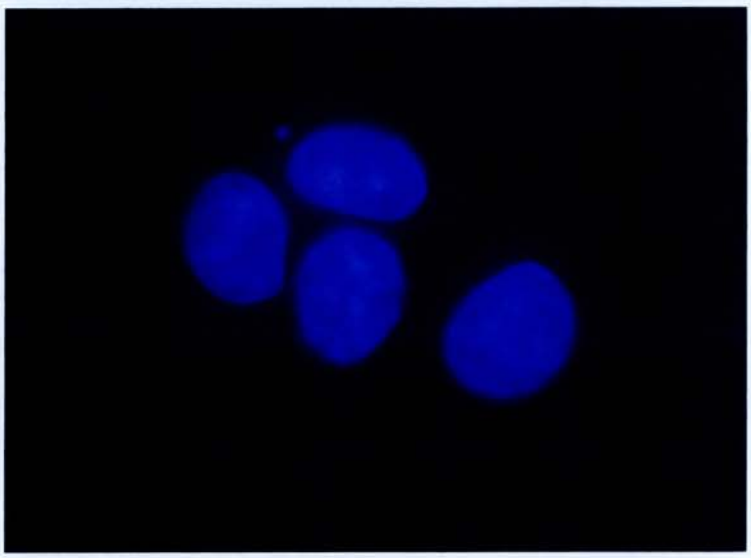
Figure 4.6
cEBP-beta staining in HepG2 cells

a



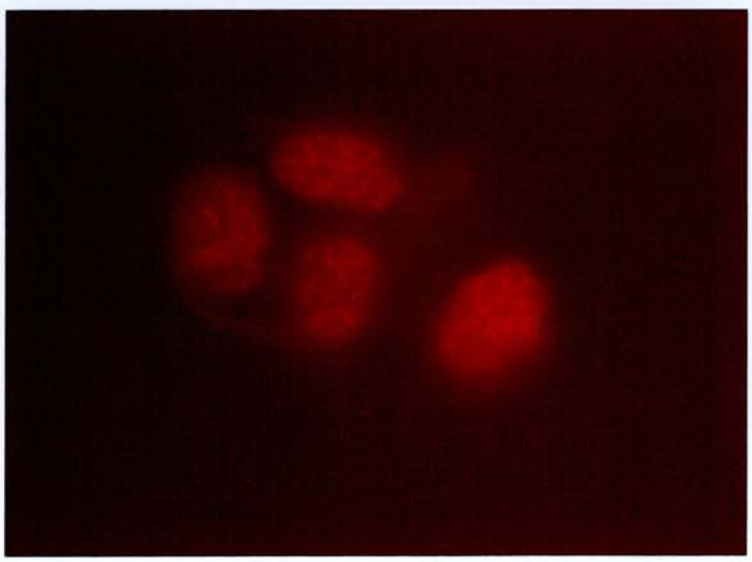
Light

b



DAPI

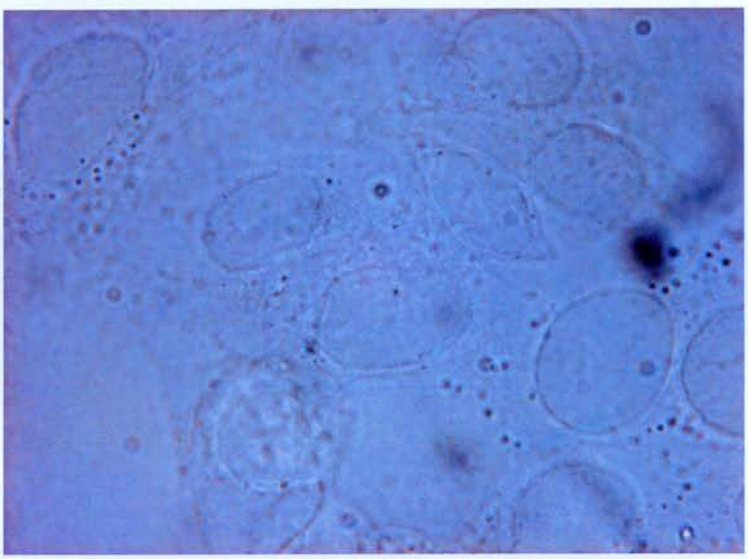
c



cEBPbeta

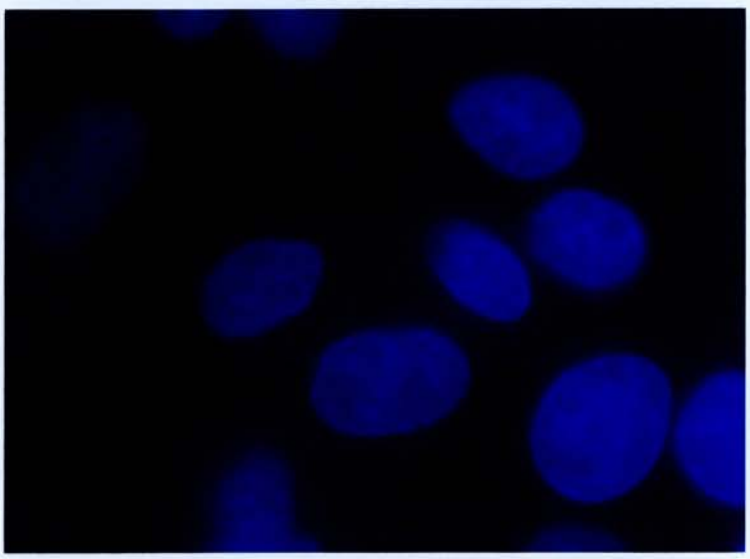
Figure 4.7
HNF4alpha staining in HepG2 cells

a



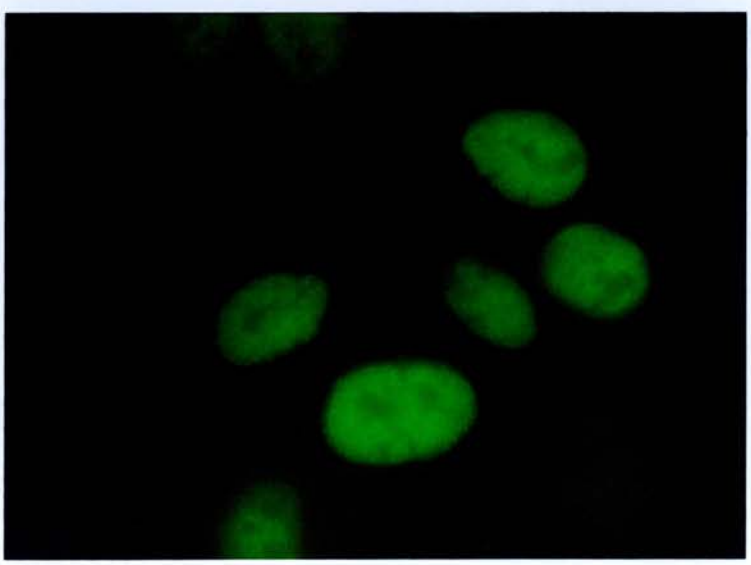
Light

b



DAPI

c



HNF4alpha

Acute liver injury serum- effect on stem cell survival and differentiation

Cells were cultured in standard media as described in the methods section, with varying concentrations of normal human or fulminant human serum. After 2 weeks culture the number of cells was counted by Neubauer cytometer as shown in table 4.6 (n=7).

Table 4.6

Cell quantification after culture with sera

Culture condition	Number of cells (x 10^5) ± SEM	Percentage cell death ± SEM
TPO/FLT 3 ligand alone	2.09 ± 0.91	9.54 ± 1.46
1% Normal serum	4.60 ± 0.82	10.2 ± 2.14
10% Normal Serum	4.24 ± 0.61	12.5 ± 2.62
1% Fulminant serum	4.61 ± 0.53	11.2 ± 2.14
2% Fulminant serum	4.22 ± 0.74	10.3 ± 2.33
5% Fulminant serum	5.25 ± 0.54	14.9 ± 3.36
10% Fulminant serum	4.90 ± 0.85	19.6 ± 2.21

Analysis of cell death was carried out using Propidium Iodide exclusion (method described in chapter 2). The results of this are shown in table 4.6. They indicate that fulminant serum at a concentration of 10% does induce increased cell death as compared with equal percentage normal serum ($p < 0.05$).

Table 4.7

Flow cytometric analysis after culture with sera

Cytokine combination	Cell surface marker	Range (%)	Mean (± SE)	Median
TPO/Flt-3	GlyA	3-8	6 (1)	7
	CD3	29-48	38 (5)	36
	CD14	27-49	39 (4)	41
	CD19	3-7	4 (1)	4
TPO/Flt3 + 1% Normal serum	GlyA	3-6	5 (2)	6
	CD3	15-36	22 (4)	21
	CD14	15-37	26 (7)	26
	CD19	2-5	3 (1)	4
TPO/Flt3 + 10% Normal Serum	GlyA	1-6	4 (2)	5
	CD3	12-29	18 (2)	17
	CD14	23-38	32 (5)	31
	CD19	3-6	5 (1)	4
TPO/Flt3 + 1% Fulminant serum	GlyA	2-7	5 (2)	4
	CD3	9-28	19 (4)	18
	CD14	28-53	41 (11)	40
	CD19	1-6	4 (2)	5
TPO/Flt3 + 2% Fulminant serum	GlyA	2-7	5 (2)	6
	CD3	9-24	17 (3)	16
	CD14	29-49	38 (7)	37
	CD19	1-5	3 (1)	3
TPO/Flt3 + 5% Fulminant serum	GlyA	0-6	3 (1)	4
	CD3	7-14	10 (1)	11
	CD14	28-43	35 (7)	34
	CD19	0-8	4 (1)	4
TPO/Flt3 + 10% Fulminant serum	GlyA	1-8	5 (2)	6
	CD3	5-11	8 (2)	7
	CD14	22-38	29 (5)	30
	CD19	3-7	5 (1)	6

Table 4.7 shows the respective haematopoietic lineages down which the stem cells differentiated. When compared to control cultures there were lower levels of T-cell differentiation (CD3) seen in all cultures supplemented with serum ($p < 0.05$, ANOVA). There was a significant trend towards lower levels of CD3 differentiation as percentage concentration of fulminant serum increased.

Further rtPCR analysis and immunofluorescent analysis was carried out as described previously in this chapter. There was no evidence of albumin or alpha-feto-protein transcripts in any of the culture combinations, nor was there any evidence of staining for cEBPβ, HNF4α and pan-cytokeratin.

4.7 CONCLUSIONS

Building on recent studies, this chapter set out to establish whether human cord blood stem cells could be differentiated down the hepatocytic lineage *in vitro*.

In this work, mononuclear cells were further sorted for the expression of a stem cell marker, AC133, to ensure that a more pluripotent population of cells was used. By using this more pluripotent population it was hypothesised that it would increase the chances of culturing cells which had the capacity to differentiate towards hepatocytes.

As described in the results although good purity for AC133 was achieved there was no evidence of hepatocytic differentiation in any cytokine culture combination. There are several possible reasons for this:

1) This may reflect the choice of cell type. Potentially more primitive, $AC133^{negative}$, cells have been discarded, and it may be these cells which have the capacity for such lineage differentiation.

2) The choice of cytokines may have been inadequate.

3) The choice of extra-cellular matrix may have been inadequate. Although uncoated, collagen and gelatin were used, perhaps these were not sufficient to sustain and stimulate hepatocytic differentiation. Other possible matrices which may have been important to examine include Matrigel, and also co-culture with hepatocytes/fibroblasts. In view of the negative outcome of these studies, I proceeded to examine whether such cells would have a greater chance of differentiating in an *in vivo* model system (chapter 5).

Since this work was undertaken other groups have reported successful differentiation of cord blood derived stem cells into hepatocytes in *in vitro* studies (Kakinuma et al 2003). Their most successful cytokine combinations utilised FGFα (20ng/ml), FGFβ (10ng/ml), LIF (10ng/ml), SCF (10ng/ml) and HGF (10ng/ml) in DMEM supplemented with 15% FBS, 2mM L-Glutamine, 25mM HEPES, 100U/ml penicillin, 100μg/ml streptomycin, 0.25μg/ml amphotericin B and 300μM monothioglycerol. Other groups have demonstrated that human and murine bone marrow also contains stem cells which have the ability to differentiate into hepatocytes. Notably these groups used Matrigel cultures with FGF-4 and HGF (Jiang et al 2002; Schwartz et al 2002).

In keeping with cytokine combinations acute liver injury serum did not lead to hepatocytic differentiation of cultured stem cells. Again, this may reflect the lack of adequate matrix to facilitate hepatocytic differentiation. As with HepG2 cells fulminant serum does appear to cause cellular necrosis, although in the case of stem cells a lower concentration (10%) is required to produce this.

Serum supplementation leads to decreased T cell differentiation, a finding which becomes manifest with increasing concentration of fulminant serum. It is not clear if this is a representation of increased cell death or a specific reduction in T cell differentiation. One possible explanation would be that T cells produced from stem cells are in turn susceptible to the injurious effects of acute liver injury serum. There may also be lower levels of factors necessary to facilitate differentiation of stem cells into T cells present in acute liver injury serum. These include factors such as flt-3 ligand, interleukin-3 (IL-3), stem cell factor (SCF), and IL-2 (Pawelec et al 1998),

which are know to be important in T cell differentiation. Given that there is increased sepsis in patients with acute liver failure, it is possible to speculate that diminished T cell differentiation of host stem cells plays a role in the diminished response to infection. Further studies are required to explore this hypothesis.

CHAPTER 5

STUDY OF HUMAN HAEMATOPOIETIC STEM CELL TRANS-DIFFERENTIATION IN THE NOD-SCID MOUSE

5.1 BACKGROUND AND AIMS

As detailed in the Background and Aims in Chapter 4 stem cells have been demonstrated to have greater plasticity than previously thought. That adult human stem cells also share some of these pluripotent properties has been demonstrated in studies utilising archival biopsy samples (Alison et al 2000) in which donor-derived hepatocytes and cholangiocytes have been observed in the recipient. In both of these studies, the presumed origin of stem cells was the bone marrow, and both studies used cross-sex transplants to differentiate host from recipient hepatocytes. A study by Korbling *et al* (Korbling et al 2002) demonstrated that human stem cells mobilised by gCSF (as used in the management of haematological conditions) also retained the ability to differentiate down hepatocytic and other non-haematopoietic lineages. As yet, the biological relevance of human stem cell transdifferentiation remains to be clarified; with concerns existing that human stem cells may not share the same pluripotentiality as rodent stem cells. However, no *in vivo* model systems exist to test human stem cell pluripotency prospectively thereby limiting our ability to fully appraise the role human stem cells have to play. The present study was undertaken to answer the following questions:

1) Can human cord blood cells engraft and differentiate into human hepatic type cells in the liver of immunocompromised mice?

2) Do human stem cells fuse with differentiated recipient cells to form non-haematopoietic cells?

5.2 MATERIALS

Studies were undertaken using NOD-SCID mice (obtained from Jackson Laboratories) and human cord blood obtained with patient consent and full approval from the Lothian Regional Ethics Committee (LREC). In addition samples of healthy human liver were obtained from donated liver organs, again with full LREC/MREC consent.

5.3 METHODS

Freshly collected heparinised whole cord blood samples were received from the Scottish National Blood Transfusion Service (Edinburgh, UK) following informed donor consent. Mononuclear cells were isolated using Histopaque 1077 (Sigma, UK) density centrifugation following the initial description by Boeyum (Boeyum A 1968). Briefly, heparin-treated (10^3IU/mL) whole blood was carefully layered over an equal volume of Ficoll-Hypaque^{TM} and spun at 540g for 20 minutes. The interface ('buffy coat') containing the mononuclear cells was collected and washed twice in Hank's Balanced Salts Solution (HBSS; Gibco, UK). A trypan blue exclusion assay was used to assess the viability of the harvested cells prior to their usage.

Animals

Four to 6 weeks old NOD-SCID mice were sub-lethally irradiated (250 rads) and received a single tail-vein infusion of 50 x 10^6 human cord blood-derived mononuclear cells resuspended in 100µl of sterile Hank's Balanced Salt Solution

(HBSS; Gibco, UK). Mice were, on occasions infused with samples from the same cord donor, or from pooled cord donors. Mice were kept under specific pathogen free (SPF) conditions in microinsulators and sacrificed at 1, 4, 6 and 16 weeks post cord blood cell infusion. Experimental protocols involving animals were carried out in accordance with permits and guidelines issued by the University of Edinburgh Ethical Review Committee and the UK Home Office.

In Vitro Culture Assays

In order to detect hematopoietic progenitor cells in human cord blood samples, colony-forming units (CFUs) were evaluated by placing cord blood-derived mononuclear cells in pre-tested complete semi-solid methylcellulose mixtures (Methocult GF H4534; StemCell Technologies, Inc., Canada) comprising 1% methylcellulose in Iscove's MDM, 30% fetal bovine serum, 1% bovine serum albumin, 10^{-4}M 2-mercaptoethanol, 2mM L-glutamine, 50ng/mL rh stem cell factor, 10ng/mL rh GM-CSF and 10ng/mL rh IL-3. 1,500 cells per mL of methylcellulose were plated out in triplicate wells in a multidish plate (Nunc, UK) and cultured at 37°C in 5% CO_2 for 12 days. At that time, the number of total CFUs was determined under the microscope as an average count of all 3 wells. Control cultures were included in each assay and these were grown in identical medium apart from the absence of growth factors.

FACS Analysis

Cord blood mononuclear cell surface phenotype was characterised using standard FACS analysis. Briefly, 100,000 cells per test were pelleted by centrifugation for 5

minutes at 1000rpm and cell pellets resuspended in individual monoclonal antibody (mAb) solutions using the manufacturer's (Dako, UK; BD Pharmingen, UK; Sigma, UK) recommended test concentrations (confirmed in our laboratory to give optimum results in our hands). Test samples were immunostained for 20 minutes on ice with a panel of directly FITC- and/or PE-conjugated mAb against human cell surface markers. The panel included mAb against the following markers: MHC1 (pan-nucleated cell) (200 μg/mL; Sigma, UK), gly-A (erythrocyte) (100 μg/mL; Dako, UK), CD3 (T Cell) (12.5μg/mL; BD), CD11b (granulocyte) (100μg/mL; Dako), CD14 (monocyte) (60μg/mL; Dako), CD19 (B cell) (25μg/mL; BD), CD33 (myeloid progenitor) (6μg/mL; BD), CD34 (stem cell) (25μg/mL; BD), CD45 (pan-leukocyte) (50μug/mL; BD) and CD56 (NK cell) (50μg/mL; Dako). Cell subsets were double-immunostained using a subset-specific mAb as well as an antibody against CD45. Following immunostaining, cells were pelleted, washed in FACS handling buffer (1xPBS/1% BSA/0.1% Sodium Azide/0.5M EDTA) and resuspended in FACS fix (1% formaldehyde) prior to analysis using CellQuest software on a Becton Dickinson FACSCalibur machine.

Glucose Phosphate Isomerase Assay

Electrophoresis for variants of glucose phosphate isomerase (GPI) was carried out as described by Ansell & Micklem (Ansell et al 1986). This method can distinguish between the human and murine isoforms of the enzyme on the basis of its electrophoretic mobility. Thus, it permits a biochemical assessment for the presence of human cells in murine tissue. Briefly, samples of single cell suspensions were

pelleted by centrifugation, lysed using 'sample' buffer and stored at -80°C. Electrophoresis was carried using the 'Helena' system (Helena Laboratories). Samples were applied to Titan III cellulose acetate membranes at the anode and run for 80 minutes at 350V using Supraheme buffer. Bands were developed using a staining mixture containing substrate and co-factors for GPI and visualised using tetrazolium dyes 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) and phenazine methosulfate (PMS).

Histology, Immunohistochemistry and Immunofluorescence

Histology Livers from NOD-SCID mice were removed, fixed in 4% formaldehyde overnight and embedded in paraffin blocks. 4μm sections were cut and placed onto Vector-bonded slides. Slides were dewaxed through xylene and rehydrated through graded alcohols and water. Positive controls included normal human liver obtained with ethical approval from the Scottish Liver Transplant Unit (Royal Infirmary of Edinburgh). Negative controls included mouse liver obtained from sublethally irradiated NOD-SCID mice infused iv with medium only.

Immunohistochemistry Sections were microwaved for 15 min in 0.1M citrate buffer and blocked in 1%H_2O_2 for 15 min. Slides were then incubated with a 1:4 dilution of a mouse mAb against cytokeratin 19 (as a marker of biliary epithelium) (Dako, Cambridge, UK), a 1:25 dilution ^{mouse} mAb HepPar1 that was raised against human hepatocytes (Dako, Cambridge, UK), a 1:25 dilution ^{CD31} mouse mAb against human endothelial cells (Dako, Cambridge, UK) and a 1:25 dilution ^{CD34} mouse mAb against human stem cells (Dako, Cambridge, UK). Slides were then washed and

incubated with a horseradish peroxidase (HRP) polymer-conjugated detection antibody followed by diaminobenzidine (DAB) chromagen (Envision™; Dako, UK).

Immunofluorescence Sections were microwaved for 20 min in 0.1 M citrate buffer, cooled and washed in 2 x sodium saline citrate (SSC)(Dundas et al 2001). Immunofluorescence was carried out using a 1:4 dilution of a mouse mAb against cytokeratin 19 (Dako, Cambridge, UK), or a 1:25 dilution ^{mouse} mAb HepPar1 (Dako, Cambridge, UK). Secondary antibody was a 1:100 dilution of donkey anti-mouse FITC (Vector Laboratories, US).

Fluorescent *in situ* hybridisation (FISH) Analysis

1 μg total human genomic DNA was labelled with digoxigenin 11-dUTP (Roche, Lewes, UK) and mouse genomic DNA was labelled with Alexa Fluor 594-5 dUTP (Molecular Probes, Leiden, Netherlands), by nick translation. Unincorporated nucleotides were removed by centrifugation through Quick Spin G50 Sephadex columns (Roche, UK). Specific activity of the probes was performed by dotting onto nitrocellulose filters using anti-digoxigenin-Alkaline phosphatase Fab fragments (Roche, UK). We then used a BCIP/NBT kit (Vector) which produces the purple colour change on the filter, which we then compared to known standards (DNA of known concentrations).

Slides were heated to 60°C for 20min to melt wax, and then washed in xylene 4 x for 10 min each before dehydration through an ethanol series (100%, 95%, 70%). Slides were then microwaved for 20 min in 0.1M citrate buffer pH 6·0. Slides were then denatured for 3 min at 75°C. After microwaving, liver sections were denatured for 3

min at 75°C in 70%formamide/2 x SSC, plunged into ice-cold 70% ethanol for 3 min, dehydrated through an alcohol series, and air-dried.

150 ng labelled human genomic DNA, 150 ng labelled mouse genomic DNA and 5 μg salmon sperm DNA were denatured together for 5 min at 75°C in 50% formamide, 10% dextran sulphate, 2 x SSC, 1% Tween20. Hybridisation was overnight at 37°C. Slides were washed 4x for 3min in 2 x SSC at 45°C, then incubated for 30 minutes with a 1:30 dilution of a sheep FITC anti-digoxigenin antibody (Roche, UK), followed by a further 30 minutes with a 1:100 dilution of FITC anti-sheep antibody (Vector Laboratories, US) (Fantes et al 1995). Slides were mounted in Vectashield (Vector Laboratories, US) containing 1 μg/ml 4,6-diaminidino-2-phenylindole (DAPI) counterstain.

Image analysis

Slides were visualised using a Zeiss Axioplan 2 fluorescence microscope (Thornwood, US) equipped with a triple bandpass filter (Chroma #83000). Grayscale images were collected with a cooled charge-coupled device (CCD) camera (Quantix Corp. US) and analysed using custom IPLab scripts. Images were pseudocoloured using image processing software (Adobe Photoshop, US).

Quantitation Of Cord Blood-Derived Hepatocytes

Human hepatocytes were identified in two ways: (1) With positive staining of the human specific HepPar1 antibody (figure 2). We have demonstrated that HepPar1 is human specific and does not cross-react with NOD-SCID mouse liver (or human cord blood). This observation is particular to NOD-SCID mice and is in keeping with

that of other laboratories. We also performed Immunofluorescent HepPar1 staining which permitted DAPI nuclear analysis. The nuclear DNA staining pattern of human and mouse cells is quite distinct (figures 5.3/5.4) thus permitting further differentiation between human and murine hepatocytes. The abundance of pericentric heterochromatin on mouse chromosomes is manifest as bright foci of DAPI staining (Tate et al 1998), a feature not seen in human cells, which have less pericentric heterochromatin. The combination of binding of a human specific liver antibody and human nuclear morphology were taken as indicative that the cell was a human hepatocyte. (2) With positive FISH signal for human DNA (figure 5.3) and morphology typical of human hepatocytes. Hepatocytes were characterised as large polyclonal cells, arranged in plates, which had large round nuclei. In addition their cytoplasm was characteristically bright reflecting the FITC-type cytoplasmic autofluorescence caused by bilirubin metabolic products (Theise et al 2000a). The number of positive cells obtained by these two methods was used to provide a mean value of cord blood-derived hepatocytes in each liver. Total human cells were quantified by counting the number of cells with human FISH signal in the murine liver section. Co-localisation studies of HepPar1 staining and positive FISH signal were attempted but FISH processing degraded the HepPar1 signal, and thus we were unable to detect co-localisation of these markers even in human liver tissue.

5.4 EXPERIMENTS

Human cord blood samples were characterised by FACS analysis and CFU analysis to demonstrate that they were rich sources of human stem cells (n=10).

In a subsequent set of experiments pooled sets of human cord blood were infused into sub-lethally irradiated NOD-SCID mice as described in the methods section (n=12) and these mice were kept in SPF conditions for 1, 4, 6 and 16 weeks before termination. Bone marrow was analysed at these points for evidence of human-mouse chimerism. Livers were also analysed for evidence of human hepatic cell differentiation by immunohistochemical and Immunofluorescent analysis. Evidence of fusion between human and murine cells was also tested for using simultaneous FISH.

5.5 STATISTICAL ANALYSIS OF DATA

Data are expressed as Mean +/- standard error of the mean (SEM).

5.6 RESULTS

Infused Human Cord Blood Samples Are Rich in Hematoprogenitor Cells

Initially, 10 individual human cord blood samples were characterised by FACS analysis and *in vitro* culture assays prior to injection into NOD-SCID mice (see Table 5.1). The percentage of cells expressing a variety of haematopoietic markers is detailed as well as mean values (along with their standard error and medians). The results were that an average of 1% of cord blood mononuclear cells were CD34+ve

stem cells. In line with this were the results from our in *vitro* culture assays which demonstrated an average of 48 colony forming units (CFU) per cord blood sample (see Figure 5.1). Taken together, the results thus suggested that our infused cord blood samples were rich in haematoprogenitor cells.

Table 5.1 Characterisation of infused human cord blood prior to intravenous inoculation into sub-lethally irradiated NOD/SCID mice.

Cell surface marker	Range (%)	Mean (SE)	Median
MHC1	78-96	88 (2)	88
CD45	59-93	81 (3)	83
GlyA	2-18	11 (2)	11
CD3	0-70	54 (6)	58
CD11b	5-24	14 (2)	14
CD14	1-21	6 (2)	4
CD19	0-24	8 (3)	1
CD33	0-21	4 (2)	2
CD34	0-2	1 (0)	1
CD56	0-2	1 (0)	0
Number of CFU	0-100	48 (12)	51

Legend for Table 5.1

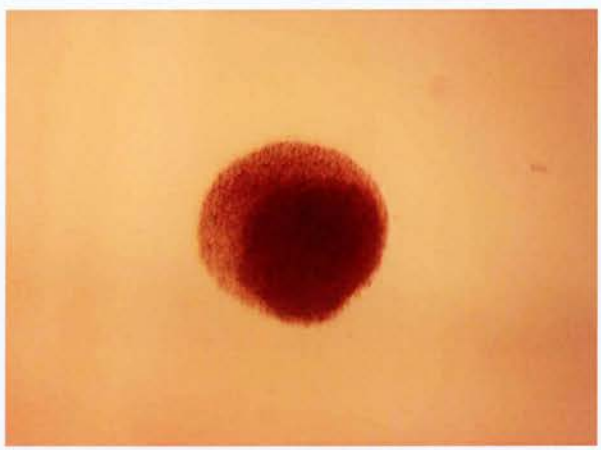
Characterisation of infused human cord blood prior to intravenous inoculation into sub-lethally irradiated NOD/SCID mice.

This table describes the analysis of 10 human cord blood preparations prior to their infusion into sub-lethally irradiated NOD/SCID mice. The percentage of cells expressing a variety of haematopoietic markers is detailed as well as mean values (along with their standard error, SE) and medians. In particular the percentage of cells expressing CD34 is seen to be 1%. Colony-forming unit (CFU) measurement confirms the presence of stem cells in the infused cord blood.

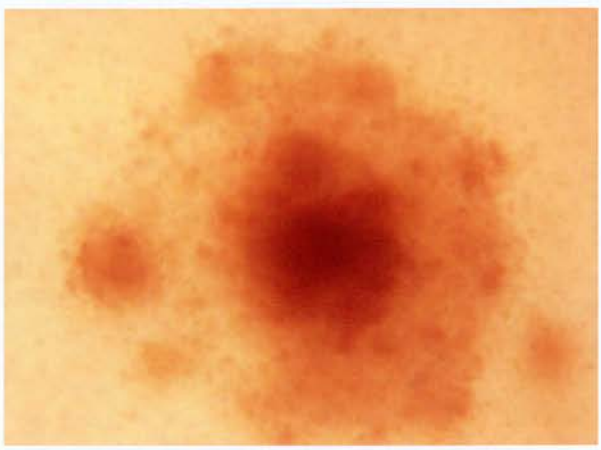
Figure 5.1

Colony forming units demonstrated with cord blood derived cells

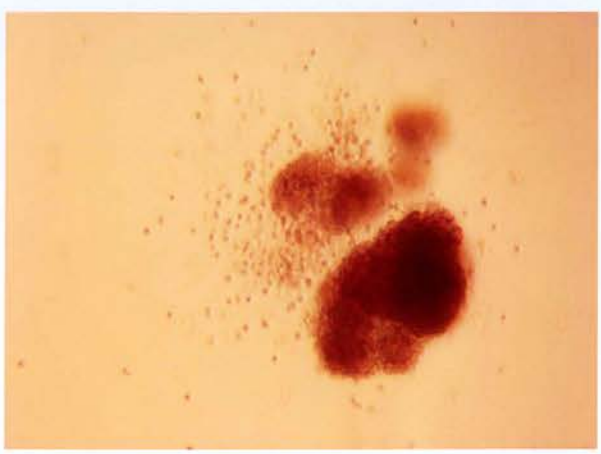
a



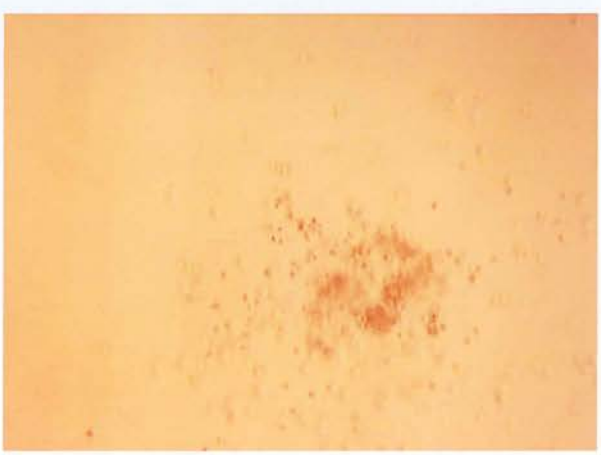
b



c



d



Human Cord Blood Samples Engraft in NOD/SCID Mice

In order to assess *in vivo* engraftment of our human samples up to 16 weeks post-inoculation, I used FACS and GPI analysis as well as *in vitro* culture assays to study bone marrow samples from our infused NOD-SCID mice. Table 5.2 represents the contribution of human cells to the bone marrow of irradiated NOD-SCID mice (n=6) 8 weeks after infusion of human cord blood cells. The column on the left denotes the panel of markers (human and murine) screened for in the murine marrow. The middle column reflects the relative contribution of human cells to different haematopoietic lineages in the NOD-SCID murine marrow (these were identified using flow cytometric analysis with human specific antibodies). The column on the right reflects murine haematopoietic cells identified by flow cytometric analysis with mouse-specific antibodies. Using FACS, the results were that mice tested had detectable levels (10-45% of counted cells; average 17%) of human CD45+ve leukocytes in their bone marrow. In addition, there was evidence of multiple haematopoietic lineages represented by human cells. The data correlated with results of our GPI assay, which confirmed the presence of the human GPI isoenzyme in the assayed samples (see Figure 5.2). Panel **a,** demonstrates the different electrophoretic mobility of human and NOD-SCID mouse iso-enzymes. Panel **b,** shows the results obtained in a representative NOD-SCID mouse bone marrow after (labelled 'recipient 1') inoculation with a human cord blood sample.

Table 5.2 Human reconstitution of NOD-SCID murine bone marrow

Haematopoietic Markers	Human		Murine	
	Mean (%)	Range (%)	Mean (%)	Range (%)
MHC 1 +ve	26	14-47	32	23-46
CD45 +ve	24	10-45	34	28-38
CD3 +ve/ CD45 +ve	4	0-13		
CD11b +ve/ CD45 +ve	7	2-12		
CD14 +ve/ CD45 +ve	2	0-6		
CD19 +ve/ CD45+ve	77	64-85		
CD33 +ve/ CD45 +ve	6	3-12		
CD34 +ve/ CD45 +ve	6	3-10		

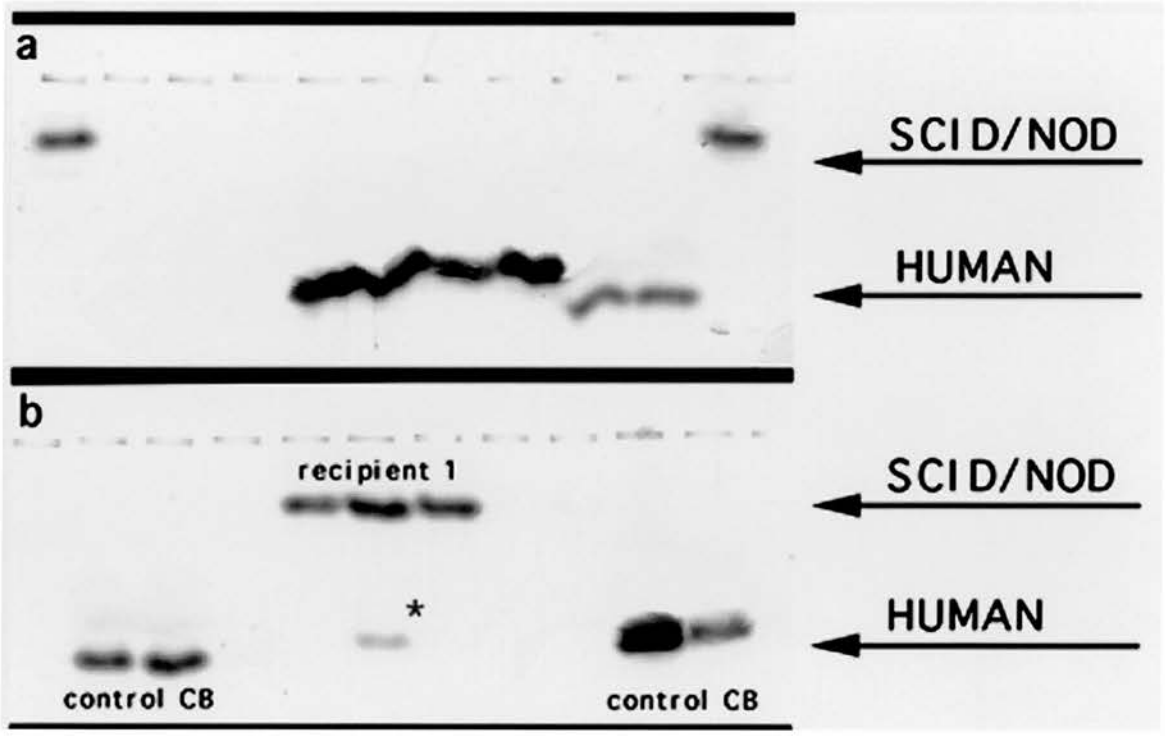
Legend for Table 5.2

Human reconstitution of NOD-SCID murine bone marrow

This table represents the contribution of human cells to the bone marrow of irradiated NOD-SCID mice (n=6) 8 weeks after infusion of human cord blood cells. The column on the left denotes the panel of markers (human and murine) screened for in the murine marrow. The middle column reflects the relative contribution of human cells to different haematopoietic lineages in the NOD-SCID murine marrow (these were identified using flow cytometric analysis with human specific antibodies). The column on the right reflects murine haematopoietic cells identified by flow cytometric analysis with mouse-specific antibodies. These data confirm that there is marrow engraftment with human cord blood cells.

Figure 5.2

Glucose phosphate isomerase assay for in vivo detection of human cells



Legend for figure 5.2. Glucose phosphate isomerase assay for *in vivo* detection of human cells

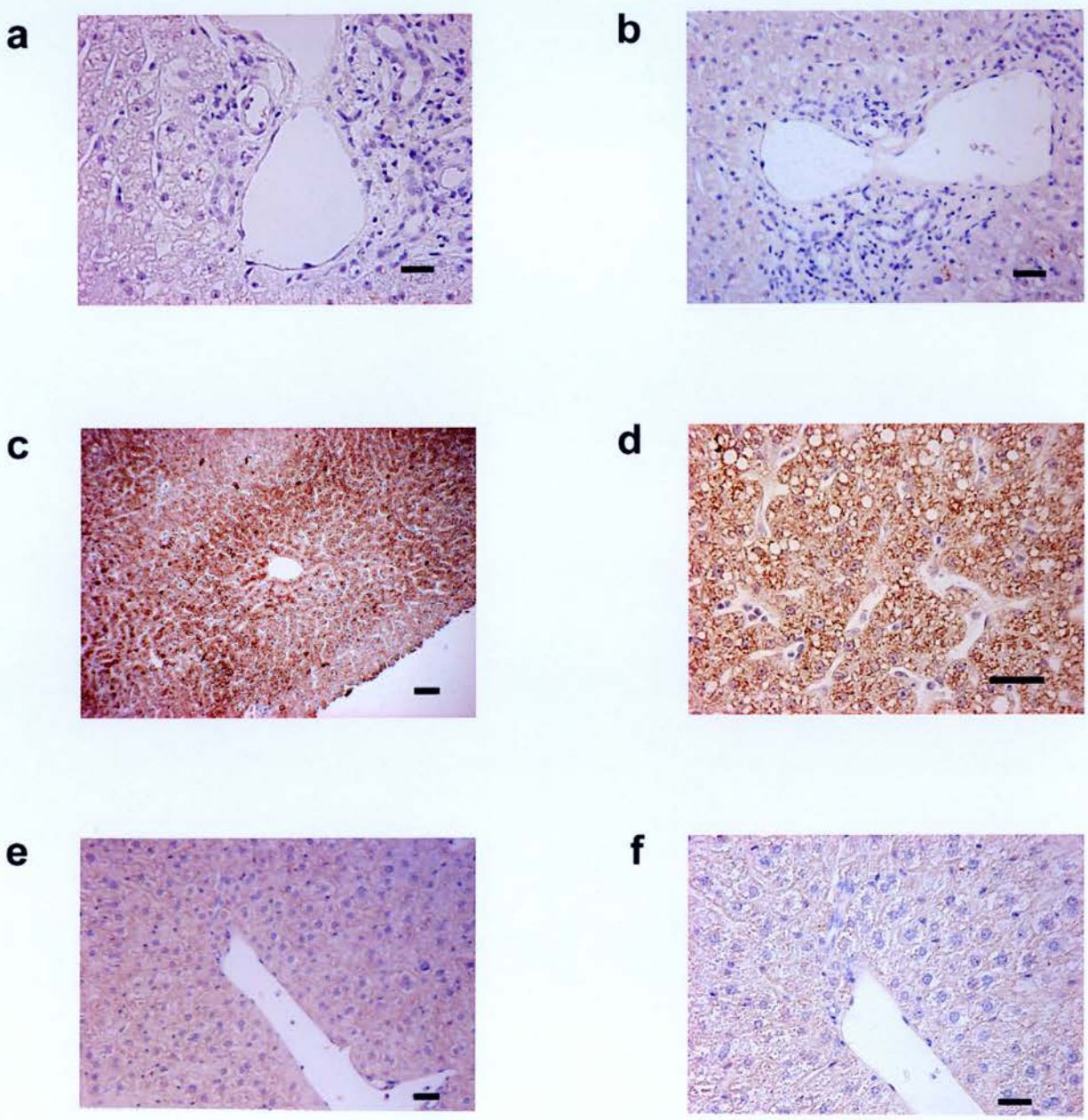
GPI analysis of bone marrow-derived samples from NOD-SCID mice infused with human cord blood cells for demonstration of the human isoenzyme. Panel **a,** demonstrates the different electrophoretic mobility of human and NOD-SCID mouse iso-enzymes. Panel **b,** shows the results obtained in a representative NOD-SCID mouse bone marrow after (labelled 'recipient 1') inoculation with a human cord blood sample. The results were in line with data obtained by FACS analysis and *in vitro* cultures.

HepPar1 Antibody Is Specific For Human Hepatocytes

In the next set of experiments, I ascertained the specificity of the HepPar1 mAb using serial tissue sections. The results, which are shown in Figure 3, demonstrated that when incubated for 1 hour at a concentration of 1:25, the HepPar1 antibody is specific for human hepatocytes and does not cross-react with human cord blood or NOD-SCID mouse liver elements. Panels a and b in Figure 5.3 illustrate negative controls on human liver in the absence of antibody, but in the presence of DAB chromagen. Panels c and d in Figure 5.3 illustrate the characteristic cytoplasmic staining of human hepatocytes with the HepPar1 antibody. Finally, panels e and f illustrate that NOD-SCID murine liver sections did not demonstrate any binding to the HepPar1 antibody, confirming that this is a human specific hepatocyte antibody. Furthermore, I showed that there was no staining of the HepPar1 antibody in human cord blood using a pelleted cord blood sample that had been mounted onto a slide.

Figure 5.3

HepPar1 is specific for human hepatocytes



Legend for figure 5.3

Immunohistochemistry on liver sections with antibody HepPar1 (brown) that detects human, but not mouse, hepatocytes. Cells were counterstained with Haematoxylin (pink). **a-d,** Human liver. **e,f**, mouse liver. **a** and **b** represent human liver with no primary antibody. **c** to **d**, positive cells in the human liver. **e** and **f** represent NOD-SCID mouse liver which has no binding of the antibody.. Scale bar, 20μm.

Human Cord Blood Cells Can Engraft And Differentiate Into Hepatocytes In The NOD-SCID Mouse Liver

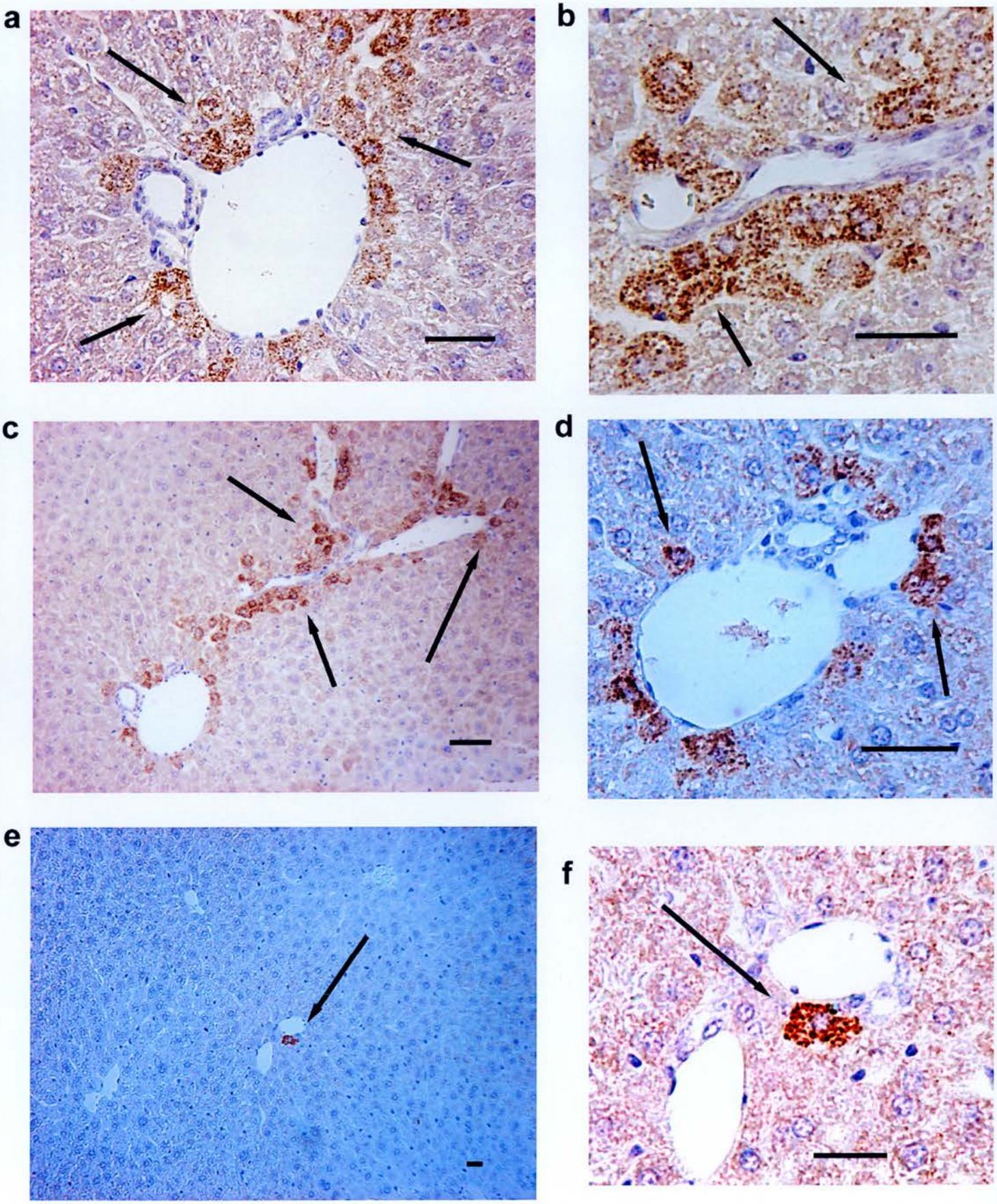
Despite no evidence of hepatocyte damage after irradiation, human-derived cells were detected in the liver of cord blood-injected NOD-SCID mice 1 week post-infusion and at all subsequent timepoints. Human hepatocytes were demonstrated in mouse liver 4 weeks post-infusion and at all subsequent timepoints.

Human hepatocyte differentiation *in vivo* was detected firstly by immunohistochemical analysis using the human specific hepatocyte marker (HepPar1) demonstrating the presence of isolated positive cells within the NOD-SCID mouse liver (see Figure 5.4). Positive cells were occasionally seen as clumps of cells (Panels a-d) or as individual cells (Panels e-f) within the liver. Scale bar on these panels is 20μm. Furthermore immunofluorescence analysis of these sections using the same HepPar1 antibody confirmed the presence of isolated positive cells integrated in the liver plate (see Figure 5.5), with characteristic human nuclear morphology. These cells tended to be located within the hepatic plate in the vicinity of portal tracts, suggesting that circulating cells derived from human cord blood had invaded the liver and differentiated into mature hepatocytes. In Figure 5.5 Panels a and b represent HepPar and DAPI nuclear staining on human and murine liver sections respectively. Panel a demonstrates the characteristic cytoplasmic staining of HepPar 1 on human hepatocytes. The panel on the right only looks at the blue DAPI signal and converts it into greyscale to demonstrate the characteristic human nuclear morphology. In contrast, panel b demonstrates the previously noted absence of staining on murine liver of the HepPar 1 antibody. To the right the characteristic

DAPI murine nuclear morphology is depicted in greyscale. Panels c and d are indicative of isolated positive human hepatocytes within the NOD-SCID mouse liver, which stain positive with the HepPar 1 antibody, but which also have characteristic human DAPI nuclear morphology. Scale bars represent 20 μm.

Further evidence for the presence of human hepatocytes derived from the presence of positive FISH signals in cells with typical hepatocyte morphology. Figure 5.6 illustrates the differences in human and murine staining with FISH probe. Panel a is human liver after FISH staining for human DNA, demonstrating that human nucleii have positive signal (FITC-labelled). In contrast, Panel b demonstrates that murine liver does not have any positive staining. On processing of the murine livers after cord-blood infusion many positive cells (ie human) were identified which had the morphology (large nuclei and abundant cytoplasm) typical of mature hepatocytes (Panels c-d, g-h). In addition, many positive cells did not display hepatocyte morphology and were presumed to be haematopoietic cells (panels e-f). Panel h represents the light microscopic appearance of Panel g, depicting the typical complex hepatocytic cytoplasm of a cell which has stained positive with the human DNA probe. Staining with the human biliary marker CK19 did not identify any human biliary cells in the murine liver. Example positive staining on human liver is demonstrated in Figure 5.7. Panels a-d depict fluorescent staining of human liver with Cytokeratin 19, a biliary marker. Panels e is a negative control in human liver (No primary antibody), whereas f represents negative staining in murine liver.

Figure 5.4
Cord blood-derived hepatocytes in NOD-SCID mouse liver

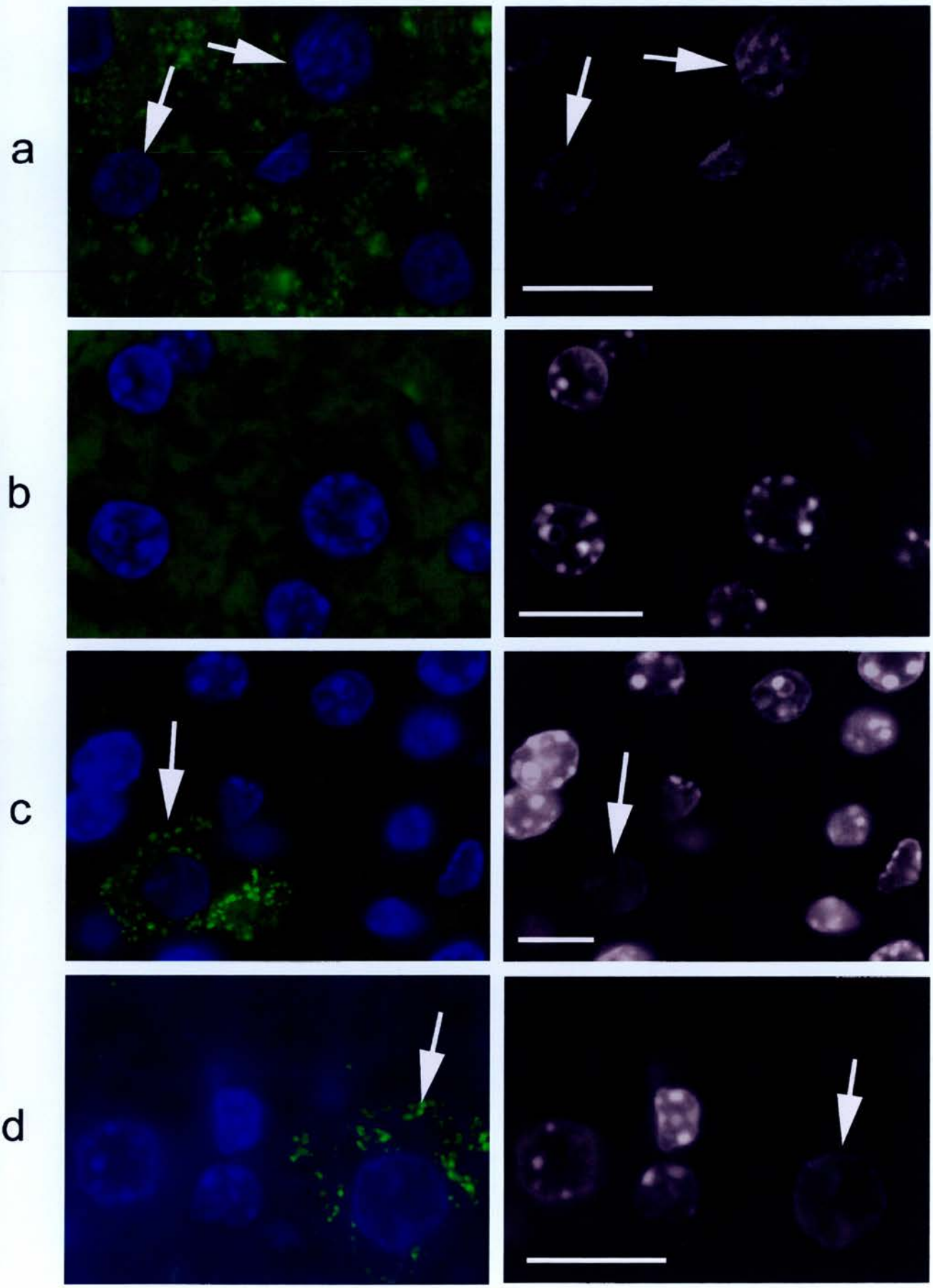


Legend for figure 5.4

Immunohistochemistry on NOD-SCID mouse liver sections with antibody HepPar1 (brown) after infusion of human cord blood cells. The panels represent positive human hepatocytes in either clumps (**a-d**), or as isolated cells (**e,f**). Scale bar, 20μm.

Figure 5.5 Immunofluorescent staining of HepPar1

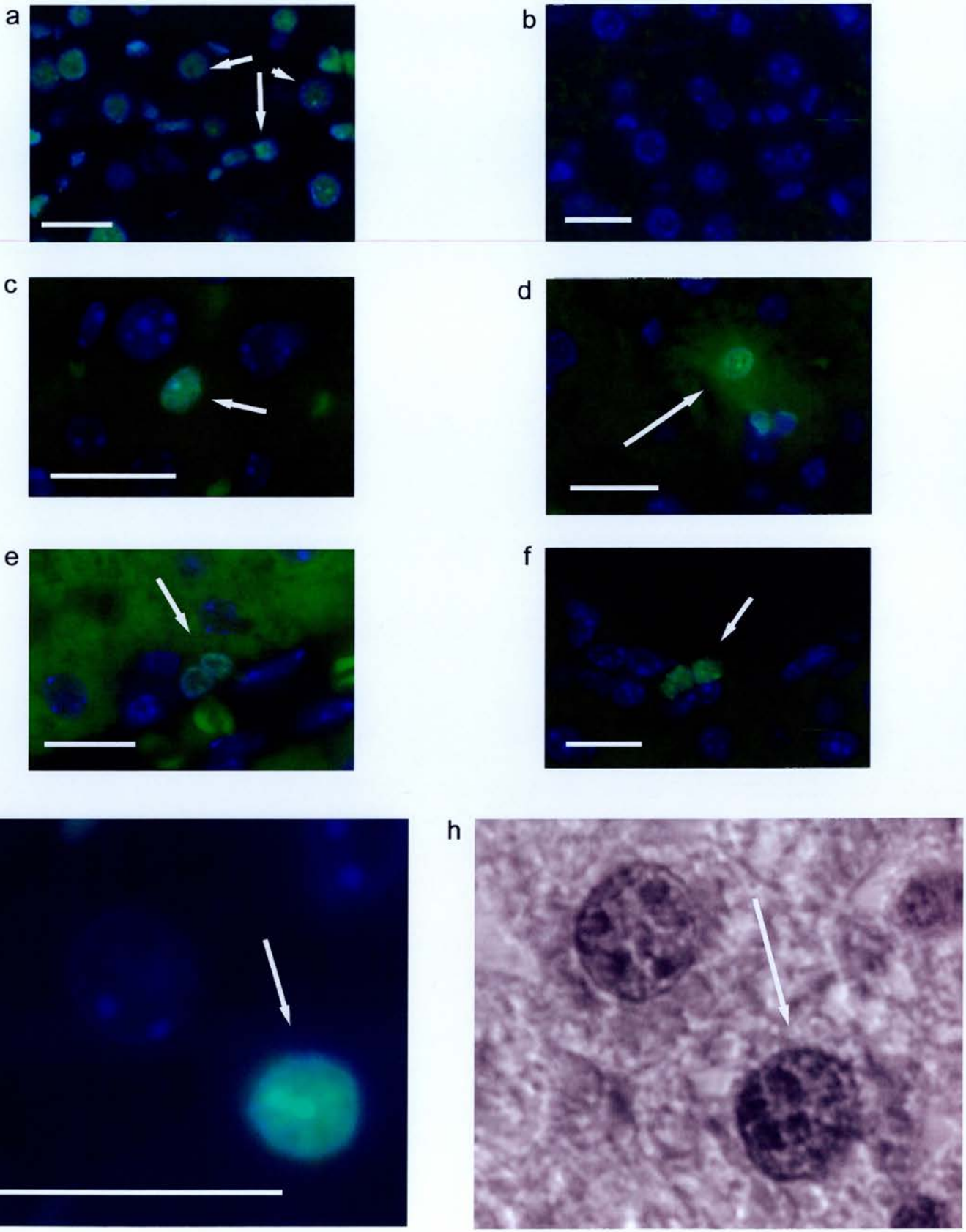
2 colour **DAPI Greyscale**



Legend for figure 5.5

Immunofluorescence on liver sections with antibody HepPar1 (green) that detects human, but not mouse, hepatocytes. Cells were counterstained with DAPI (blue). Greyscale images of DAPI staining are shown on the right . **a,** Human liver. **b**, mouse liver. **c** to **d**, positive cells integrated into the hepatic plate of livers of NOD-SCID mice. Note the DAPI nuclear staining typical of human derived-cells in the positively staining cells. Scale bar, 20μm.

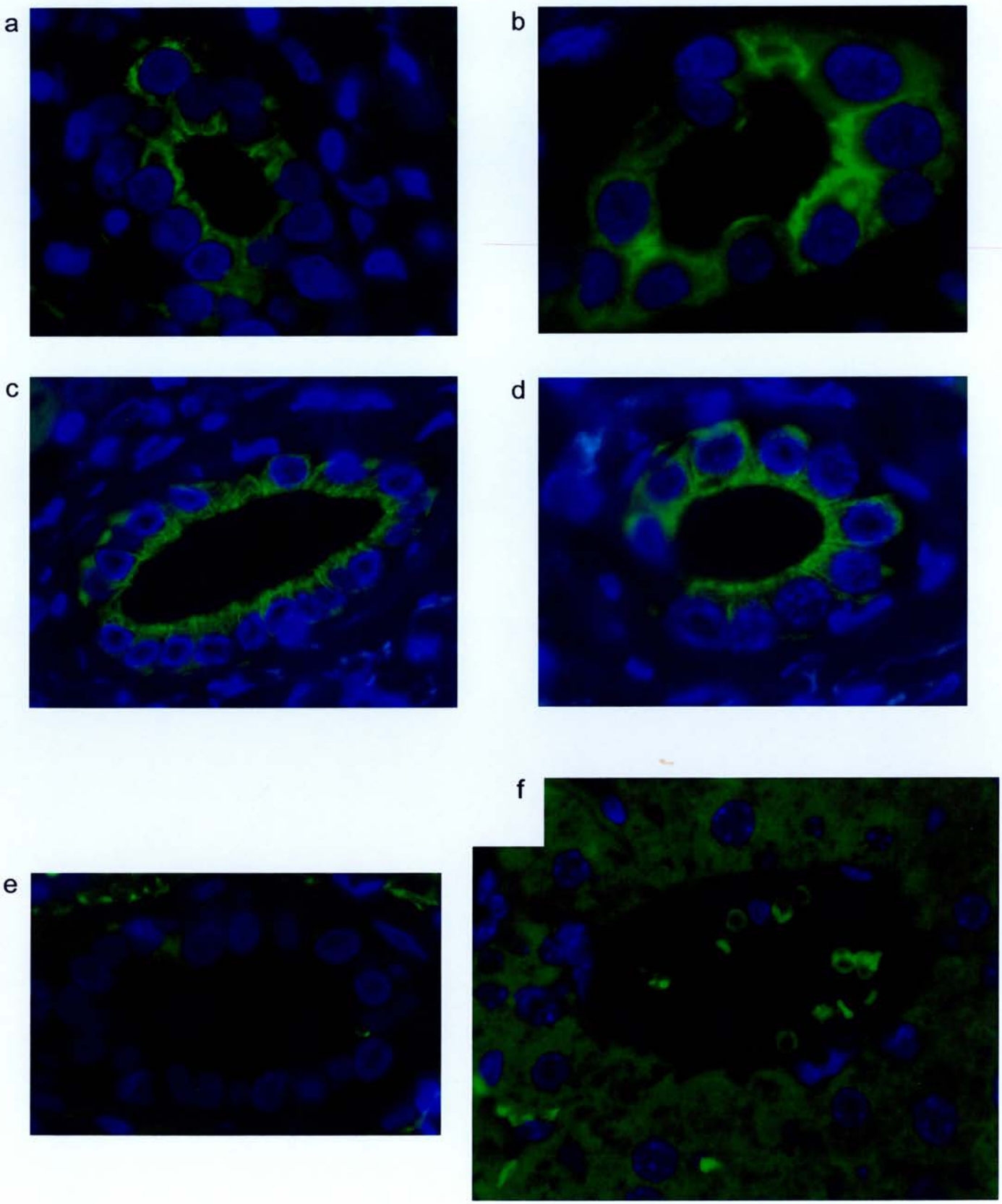
Figure 5.6
FISH staining in NOD-SCID mouse liver sections



Legend for figure 5.6

Detection of digoxigenin-labelled human DNA by FITC (green) in liver sections counterstained with DAPI (blue). Greyscale images of DAPI staining are shown on the right. **a,** Human liver. **b**, mouse liver. **c** to **d**, human hepatocytic cells integrated into the hepatic plate of livers of NOD-SCID mice. **e** to **f**, human haematopoietic cells entering the murine liver. **g** to **h**, represent greyscale H&E pictures of cells with positive FISH signal and characteristic hepatocytic morphology. These cells had morphology typical of hepatocytes with large polyclonal size with large round nuclei and complex intracellular cytoplasm. Scale bar, 20μm.

Figure 5.7
Biliary staining in human and NOD-SCID liver sections

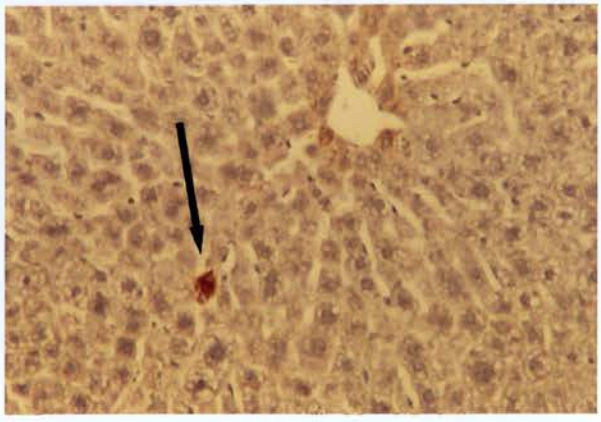


Legend for figure 5.7

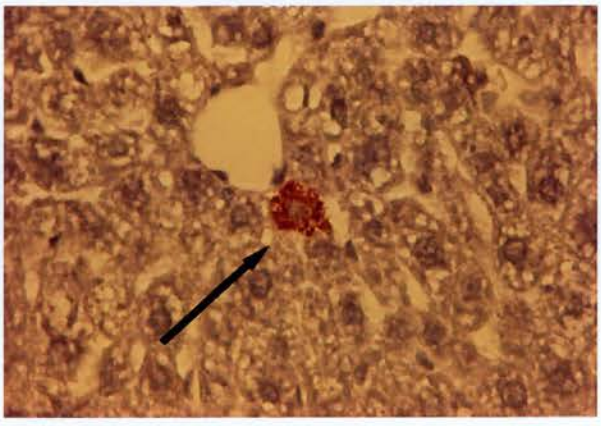
Immunofluorescence on liver sections with antibody CK19 (green) that detects human, but not mouse, biliary duct cells. Cells were counterstained with DAPI (blue). **a-d,** Human liver. **e**, mouse liver. **f**, Mouse liver after infusion of human cord blood cells, demonstrating no human cells. Scale bar, 20μm.

Figure 5.8
Staining of liver sections with CD34

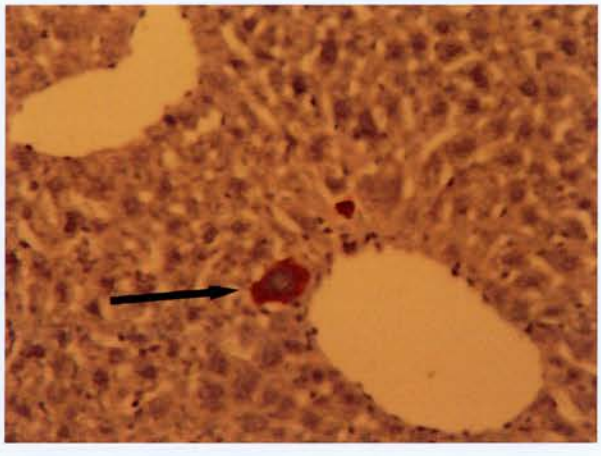
a



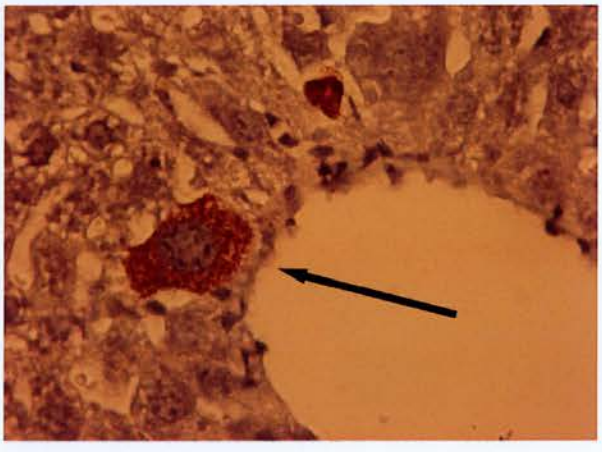
b



c



d



Legend for figure 5.8

Immunohistochemistry on NOD-SCID mouse liver sections (after infusion with human cord blood cells) with antibodies against CD34 (brown). The panels represent positive human hepatocytes in either clumps (**a-d**), or as isolated cells (**e,f**). Scale bar, 20μm

Table 5.3 quantifies both the total number of human cells and the number of human hepatocytes engrafted within the NOD-SCID mouse liver. Transdifferentiation efficiency is expressed by dividing the total number of donor-derived hepatocytes by the number of infused CD34+ cells. The mean number of human hepatocytes per section was 47.8 (range 19-73). The observed frequency of human hepatocytic transformation seen was modest, with such cells constituting on average 0.011% of all cells on each section (maximum seen was 0.017%). When the number of trans-differentiated hepatocytes is expressed as a percentage of the number of infused CD34+ cells the mean value is 2.11%. No evidence of any human hepatocytes was seen 1 week after cord blood infusion, although infiltrating CD34+ cells could be seen entering through the portal tracts (see Figure 5.6, panels e-f, and Figure 5.8). There did not appear to be an increase in the number of human hepatocytes seen in the mouse liver after 4 weeks post cord blood infusion, with similar percentages being found after 6 and 16 weeks. Furthermore, similar numbers of human cells were also observed in vascular channels and/or had morphology that was more consistent with haematopoietic lineages (see Figure 5.6, panels e-f). There was no evidence of human biliary (cytokeratin 19+ve) or endothelial (CD31+) differentiation in any of the sections (see Figure 5.7).

Table 5.3 Number of human cells/hepatocytes in NOD-SCID mice transplanted with human cord blood-derived mononuclear cells

Mouse	Time Post CBI	Mean number of human cells (all types) per mouse liver section (and value as a percentage of host cells)	Mean number of human hepatocytes per mouse liver section (and value as a percentage of host cells)	Trans-differentiation efficiency of CD34+ cells becoming hepatocytes (%)
1	7d	45 (0.0072)	0 (0)	0
2	7d	67 (0.018)	0 (0)	0
3	7d	75 (0.012)	0 (0)	0
4	4wk	91 (0.013)	62 (0.0089)	1.77
5	4wk	132 (0.04)	46 (0.014)	2.79
6	4wk	83 (0.027)	52 (0.017)	3.40
7	6wk	108 (0.015)	71 (0.0099)	1.97
8	6wk	88 (0.021)	46 (0.011)	2.19
9	6wk	80 (0.013)	40 (0.0066)	1.31
10	6wk	54 (0.0082)	19 (0.0028)	0.58
11	16wk	63 (0.017)	52 (0.014)	2.81
12	16wk	51 (0.013)	43 (0.011)	2.21
13	16wk	70 (0.014)	22 (0.0043)	0.88
14	16wk	117 (0.027)	73 (0.017)	3.38

Legend for table 5.3

This table quantifies both the total number of human cells and the number of human hepatocytes engrafted within the NOD-SCID mouse liver. Transdifferentiation efficiency is expressed by dividing the total number of donor-derived hepatocytes by the number of infused CD34+ cells. Peak trans-differentiation is seen to occur at 4-6 weeks with no significant increment after this time. Total human cells were quantified by counting the number of cells with human FISH signal in the murine liver section.

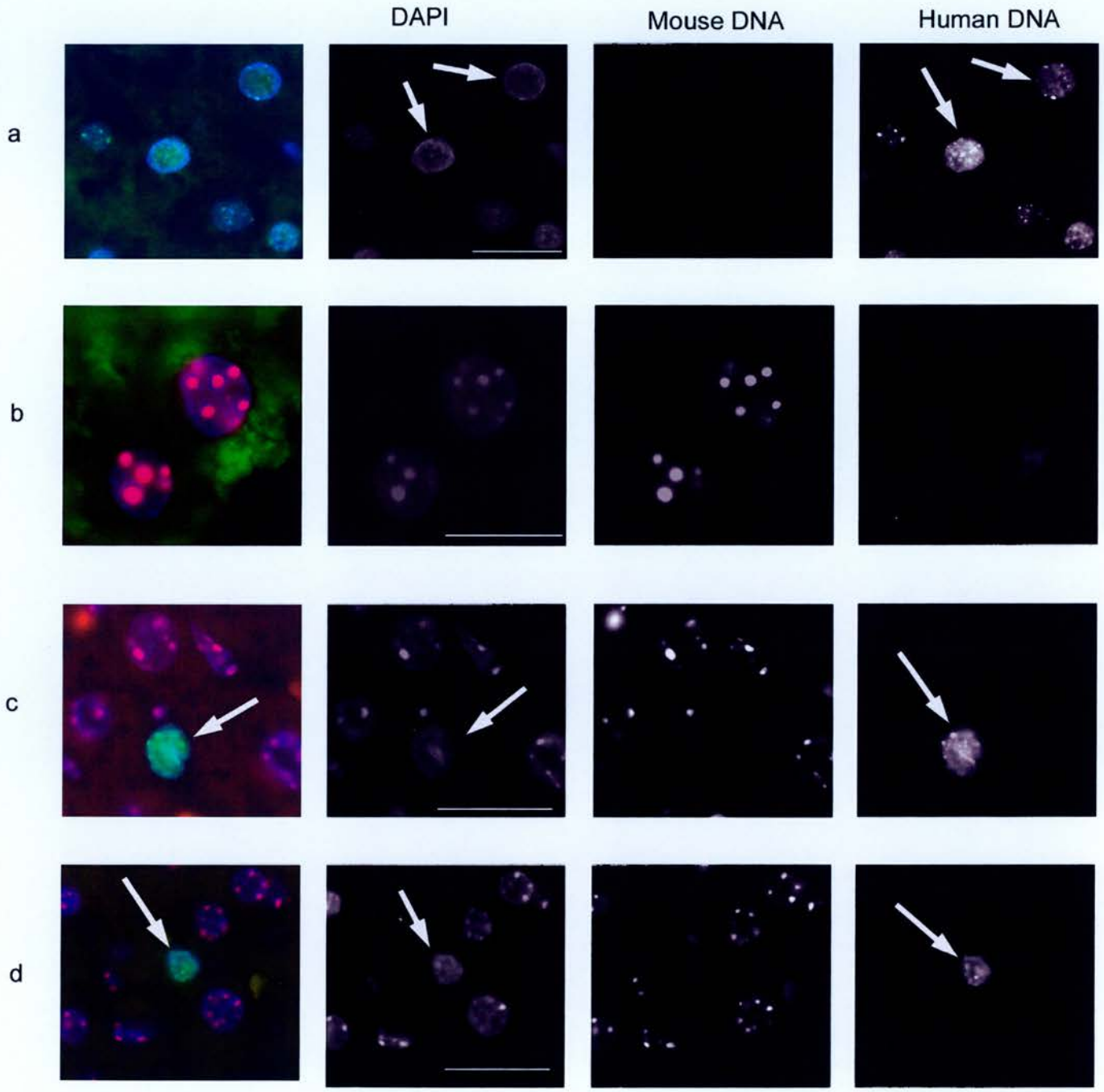
Fusion Of Human And Murine Cells Does Not Occur *In Vivo*

The nuclear DNA staining pattern of human and mouse cells is quite distinct as described previously (Tate et al 1998). Bright foci of DAPI staining were evident in sections of mouse liver, whereas nuclei in human liver sections had a more uniform staining pattern (see Figure 5.5). Cells hybridising to human DNA and engrafting into mouse livers had characteristic human nuclear staining morphology, and there was no evidence in these cells of bright DAPI foci characteristic of mouse chromosomes (see Figure 5.6). Similarly, cells staining with a human specific hepatocyte antibody (see Figure 5.4) also had nuclear morphology typical of human rather than mouse cells.

To confirm this I performed a simultaneous FISH analysis for murine and human DNAs. To optimise the method I performed it on pure human liver sections and on pure NOD-SCID mouse sections to confirm their specificity (see Figure 5.9). Figure 5.9 displays simultaneous FISH analysis of liver sections with total human (green) and mouse (red) DNAs. Cells were counterstained with DAPI (blue). Greyscale images of the DAPI staining, and mouse and human hybridisations signals are shown. **a,** Human liver. **b**, mouse liver. **c** and **d**, Human positive (green) cells integrated into the hepatic plate of livers of NOD-SCID mice. In Panels c and d it is important to note the absence of any detectable mouse DNA (red) in these nuclei and their DAPI staining typical of human derived-cells. I then examined at least 5 liver sections of each one of 14 different mouse livers (after infusion of human cord blood). Each section had a mean of 80.3 positive cells/section giving a total number of events screened of 5620. Not one of these cells was the result of fusion of human and mouse cells (see Figure 5.9). Whilst I cannot totally exclude cellular fusion it is

clearly exceedingly rare compared to human stem cell transdifferentiation. Taken together, the data suggest that it was unlikely that the donor human cord blood cells had fused with host mouse hepatocytes. The scale bar in this figure is 20μm.

Figure 5.9
Simultaneous FISH on NOD-SCID liver sections



Legend for figure 5.9

Simultaneous FISH analysis of liver sections with total human (green) and mouse (red) DNAs. Cells were counterstained with DAPI (blue). Greyscale images of the DAPI staining, and mouse and human hybridisations signals are shown. **a,** Human liver. **b**, mouse liver. **c** and **d**, Human positive (green) cells integrated into the hepatic plate of livers of NOD-SCID mice. Note the absence of any detectable mouse DNA (red) in these nuclei and their DAPI staining typical of human derived-cells. Scale bar, 20μm

5.7 CONCLUSIONS

This data demonstrate that infused human cord blood cells are capable of engrafting into NOD-SCID mouse liver and differentiating down the hepatocytic lineage without fusion to host hepatocytes. This work confirms the recently published observation by Danet et al (Danet et al 2002) who have identified a sub-population of human cord blood cells which have the ability to differentiate down the hepatocytic lineage upon infusion into the NOD-SCID mouse liver. In these experimental animals, engrafted hepatocytes were most often scattered throughout the murine liver parenchyma, usually appearing as isolated cells, although occasionally we observed some small clumps of cells. The rate of hepatocytic differentiation of cord blood cells occurred at low levels in our model (up to 0.017% of total cells), and it will be important to determine in future if liver injury increases this frequency (Vessey et al 2001). There appears to be some variation in the amount of stem cell-derived hepatocytic differentiation reported in the literature with groups reporting as few as 10 hepatocytes per murine section (Wagers et al 2002) whilst others observe levels as high as 1.1% of recipient liver tissue (Theise et al 2000a). This variation may reflect differences in species and strains of animal used, presence of liver injury (whether intentional or non-intentional such as radiation) and methods used to identify stem cell-derived hepatocytes. I have used several different methods to identify stem cell-derived hepatocytes and notably our observed frequency falls in the lower end of this range. The results further suggest that in the absence of liver injury, there is no stimulus for trans-differentiated cord blood-derived hepatocytes to proliferate since the proportion of human hepatocytes did not appear to increase with time following

the 4 weeks timepoint. This is in keeping with the literature as reported by Theise *et al* (Theise et al 2000a) who demonstrated that the proportions of donor murine bone marrow-derived hepatocytes in myeloablated mouse recipient livers were similar up to 12 months after bone marrow transplant. Whilst the observed level of engraftment in this xenogeneic model is lower than that observed *in vivo* by Theise *et al* (up to 1.1% of total cells studied), part of this may be due to human stem cells being at a relative survival/proliferative disadvantage when infused into xenogeneic environments in absence of liver injury. In contrast, infusion of human hepatocytes into mice suffering mild chronic liver injury leads to their populating up to 15% of the mouse liver, but this is seen in the context of urokinase-type plasminogen activator (uPA) transgenic mice which have a liver-toxic phenotype (Dandri et al 2001). The majority of the transdifferentiated hepatocytes in our model were seen to be located in the hepatic plate in the vicinity of portal tract areas and most of these cells were found as isolated single cells (although occasional clumps of cells were seen). In conjunction with the finding of CD34+ human cells in the vicinity the portal tracts these observations suggest that the portal tract may be a major route of entry of hepatocyte progenitors into the liver. This is supported by the finding that human cells with morphology that was more consistent with haematopoietic cells were frequently seen to be entering through the portal tracts (see Figure 5.3f-i), although tracking experiments would be required to confirm their subsequent hepatocytic commitment. In the absence of any significant liver injury, human stem cells would have no obvious reason to migrate further into the liver plate. Hence, their peri-portal trans-differentiation may reflect their earliest exposure to murine hepatocytes and other factors triggering differentiation. No evidence of human biliary (cytokeratin

19+ve) or endothelial (CD31) differentiation was seen in any of the liver sections but this may reflect the relatively low turnover of these cellular compartments (LeSage et al 2001) and the fact that they represent only ~2% of the cellular mass of the liver. In view of the modest levels of hepatocytic trans-differentiation, the chances of identifying other stem cell derived lineages would therefore be much lower.

I used unsorted human cord blood as a source of donor cells as it is known to contain higher levels of stem cells than normal peripheral blood (Glimm et al 2002). Whilst other groups have attempted to select out an enriched population of stem cells using markers such as CD34, it is possible that the CD34-ve population may in fact contain an even more pluripotent population of stem cells (Dao et al 2000; Goodell et al 1997; Nakauchi 1998). Therefore, there is ample evidence to suggest that hepatocyte progenitors belong to a stem-cell compartment.

Somatic stem cell plasticity has recently been questioned, and it has been suggested that this presumed differentiation might be the product of cell fusion between stem cells and pre-existing differentiated cells (Terada et al 2002; Vogel 2002; Ying et al 2002). In contrast to recent ex vivo reports (Terada et al 2002; Ying et al 2002), I found no evidence of cell fusion in our *in vivo* model. Whilst a type 2 error cannot be totally excluded, the fact that not one of 5620 human cells in the murine liver are the result of cellular fusion indicates that fusion is at best an exceedingly rare phenomenon. A type 2 error is made when an incorrect null hypothesis is not rejected, when a treatment effect is in fact present (eg fusion), but not observed, perhaps because of too few events (cell transdifferentiation).

This study would suggest therefore that it plays a negligible role in the phenomenon of trans-differentiation. The presence of cellular fusion in other studies may thus reflect the *ex vivo* environment that the work was carried out in and not *in vivo* conditions. If fusion is a rare event, it may become apparent in an *in vivo* model of liver injury in which more hepatocyte transdifferentiation may take place. A further demonstration that fusion was not contributing would be to show that there was loss of donor haematopoietic cell markers (in this case CD45).

This work makes the fundamental observation that human cord blood-derived cells have the ability to migrate, engraft and differentiate into hepatocytes in mouse liver when infused into NOD-SCID mice. Furthermore, I show categorically that none of our transdifferentiated hepatocytes are the result of cellular fusion of human cord blood cells with murine hepatocytes. The results thus suggest new opportunities in the study of human adult stem cell biology using pre-conditioned NOD-SCID mice and future work should aim to optimise this model system for further studies of hepatocyte progenitors.

CHAPTER 6

GENERAL CONCLUSION

6.1 ACUTE LIVER FAILURE AND HEPATOCYTE ENGRAFTMENT

6.2 STEM CELL PLURIPOTENTIALITY

6.3 FINAL DISCUSSION

6.1 ACUTE LIVER FAILURE AND HEPATOCYTE ENGRAFTMENT

The studies in chapters 2 and 3 explore the mechanisms which regulate the adhesion and survival of HepG2 cells in the environment of acute liver failure.

These studies demonstrate an up-regulation of Collagen IV expression on paracetamol-damaged liver which is a potent ligand for β_1-integrin and a possible entry point for hepatocytes post cellular transplantation. Furthermore, they demonstrate that exposure of HepG2 cells to serum from patients with acute liver failure leads to reduced ability to adhere to collagen matrix, via a β_1-integrin dependent pathway. The differential control and order of adhesion and anti-apoptotic signals by integrin activation is not fully understood, and in the model used it is not clear if apoptosis is resultant on decreased cellular adhesion or occurs independently. The data suggests that cytoskeletal disruption (as determined by adhesion and actin cytoskeleton) precedes the onset of apoptosis and suggest it may play a role in inducing apoptosis. β_1-integrin is important not only in mediating adhesion and hence survival but also plays an important role in cellular migration (Masumoto et al 1999). Thus the observed down-regulation of β_1-integrin suggests that this not only decreases hepatocyte homing but also prevents some of the collagen mediated binding on the damaged liver. It is notable therefore that prior incubation of HepG2 cells with TS2/16, a β_1-integrin activating antibody, prevented much of the loss of cellular adhesion and apoptosis.

This work has important implications for hepatocyte, and potentially for any form of cell, transplantation and identifies an avenue which may be usefully explored in the optimisation of this treatment modality.

In this study the HepG2 cell line was used, which is a differentiated human hepatoma cell line, but extrapolation of this data to primary hepatocytes does however require recognition of notable differences. Primary human hepatocytes may for example be more susceptible to apoptosis if they are unable to bind to extracellular matrix, as immortalised/tumour cell lines have down-regulated some of their apoptotic pathways (Wiesenauer et al 2004).

6.2 STEM CELL PLURIPOTENTIALITY

The studies in chapters 4 and 5 examine the pluripotency of haematopoietic stem cells *in vitro* and *in vivo*, as a first step to utilising such cells in the treatment of patients with liver injury. The two chapters look at complementary strategies; chapter 4 aims to differentiate stem cells into hepatocytes *in vitro* to produce adequate numbers of cells, which can then be infused as per hepatocyte transplantation. Chapter 5 however aims to infuse undifferentiated stem cells into immunocompromised mice, whereupon hepatocytic differentiation occurs as required *in vivo*.

Despite extensive experimentation, no hepatocytic differentiation of haematopoietic stem cells was observed *in vitro* in this thesis. This has been achieved subsequently by other groups and with corroboration by others provides a blueprint for the larger scale differentiation of such cells *in vitro* prior to therapeutic usage. The most notable difference in culture conditions was matrix; other groups utilised Matrigel as a more complex complete matrix. The usage of sarcoma-derived matrix does however pose safety issues for subsequent human usage of such cells.

The demonstration that human cells could differentiate *in vivo* in the murine liver without any observed cellular fusion was successful. This refuted the claim of other groups that cellular fusion (Terada et al 2002; Vassilopoulos et al 2003; Wang et al 2003; Ying et al 2002) rather than transdifferentiation was the major explanation behind observed stem cell plasticity.

Notably other groups, also do not find evidence of fusion as a mechanism for marrow-cell engraftment into insulin producing islet cells of the brain or pancreas after minimal injury (Ianus et al 2003). These differences are likely to be due, at least in part, to the different model systems in which the experiments have been performed. In vitro work suggests that multiple mechanisms participate (Prockop et al 2003), including fusion events and differentiation of single cells participate. In vivo, it is likely that the type and extent of target organ injury will determine not only the degree to which extra-organ stem cells engraft, but also the mechanisms by which this occurs.

Given the absence of cell fusion in other models of stem cell plasticity (Ianus et al 2003; Tran et al 2003) and the compelling evidence supporting *in vitro* plasticity of HSCs(Jiang et al 2002), it is possible that the observed fusion in this model reflects the extreme architectural disruption and hepatocyte membrane instability that occurs in the $FAH^{-/-}$ mouse. Since neither study reported that all of the marrow-derived hepatocytes were formed by fusion, and given that hepatocytes themselves are known to fuse in pathological conditions, it is important to recognise that whilst fusion may occur it does not explain the majority of new hepatocytes. The role of fusion derived hepatocytes is unclear, but in the $FAH^{-/-}$ model such cells do result in survival of the animal with no evidence of subsequent carcinogenesis (Lagasse et al 2000). Even if fusion does occur *in vivo* in human tissue it could be used as a basis for gene therapy.

Also, there has been no reported activation of the intraorgan stem cell compartment (so called "oval cells" derived from the biliary tree) as seen in other injury models (Petersen et al 1999), suggesting that normal repair mechanisms are not activated. It

should be also be noted that the current results presented by Wang et al cannot be accounted for by a single fusion event followed by subsequent proliferation. If this were the case, then the expected ratio of %FAH wildtype DNA to %Rag1 wildtype DNA would be greater than 33%, a number that assumes all fusion events occur between a diploid blood cell and a tetraploid hepatocyte. Four of the six mice have significantly lower amounts of FAH wild type DNA. These numbers can only be explained by the fusion of a transplanted hepatocyte with a recipient cell or consistent loss of one FAH allele. The latter case seems rather unlikely and while fusion could occur with a second blood cell, this offers no selective advantage, whereas hepatocyte-hepatocyte fusion might (Gupta 2000).

6.3 FINAL DISCUSSION

This thesis has studied two complementary strategies of cellular transplantation for augmenting liver function. Each of these strategies has its advantages and disadvantages. Hepatocyte transplantation has been studied much more extensively than stem cell transplantation, and has in animal studies at least been demonstrated to be efficacious. However only by developing a better understanding of the factors which control the successful migration and engraftment of these cells in clinical practice will this treatment deliver the hoped for benefit to patients.

There are many unanswered issues which require clarification. Do mice require irradiation for the stem cells to engraft and trans-differentiate? Does the source of stem cells influence the amount of trans-differentiation? Do human stem cells have to be freshly isolated or could they be cryopreserved prior to use? Furthermore which cells within the cord blood preparation are able to trans-differentiation? Is this property confined to previously identified haematopoietic stem cell groups which express markers such as CD34 or AC133? Even more important is this a stochastic phenomenon or does it occur in response to injury signals? By using models of liver injury which affect different parts of the lobule both acutely and chronically it may be possible to gain insights into the mechanisms which control this phenomenon. It can be hypothesized that the liver has 3 levels of cells in the hepatic lineage that respond to injury or carcinogenesis: 1) the mature hepatocyte, which responds to centrilobular injury, such as that induced by carbon tetrachloride (CCl_4); 2) the ductular "bipolar" progenitor cell, which responds to centrilobular injury when the

proliferation of hepatocytes is inhibited, and 3) the putative periductular stem cell, which responds to periportal injury, such as induced by allyl alcohol. Hepatocytes are numerous, respond rapidly by 1 or 2 cell cycles, but can only produce other hepatocytes. The ductular progenitor cells are less numerous, may proliferate for longer times than hepatocytes, and are generally considered "bipolar," (can give rise to biliary cells or hepatocytes). Periductular stem cells are rare in the liver, have a very long proliferation potential, and may be pluripotent. It is these cells which have been suggested to be of bone marrow origin. Studies are needed to compare the relative contributions of stem cells in such models of injury. This will facilitate the identification of mechanisms controlling stem cell recruitment and transdifferentiation, which in turn will permit greater clinical applications.
Finally does direct injection into spleen or intra-portally improve the efficiency of this phenomenon? The more local injection of stem cells may lead to more directed administration of this therapy, and is in keeping with methods used in hepatocyte transplantation (Ohashi et al 2001).

Stem cell-derived hepatocytes are most likely to benefit liver diseases characterised by abnormal gene/protein production such as Wilson's disease and the certain forms of dyslipidaemias. Conventional hepatocyte transplantation (Fox et al 1998; Muraca et al 2002) has already shown benefit in patients with certain hepatic defects. The use of stem cell-derived hepatocytes in bio-artificial liver support systems is limited by the need for very large numbers of cells for these devices, which can only be achieved by the stable expansion of such cells *in vitro* first. The potential for utilising bone marrow derived stem cells is even greater, as they could potentially offer an

autologous source of new hepatocytes. Patients with metabolic defects or viral hepatitis could have their own bone marrow samples taken followed by *ex vivo* manipulation of bone marrow derived stem cells to either produce missing enzymes or confer viral resistance. These engineered cells would have a survival advantage over host hepatocytes, leading to more extensive repopulation within the liver parenchyma (Lagasse et al 2000).

Further developments in the field will also depend on the development and usage of appropriate techniques to track both hepatocytes and stem cells. Whilst the tracking of stem cells may be difficult in rodent systems it is impossible to prospectively analyse the migration and engraftment of such cells in the humans. For this reason non-invasive strategies are required to track such cells on their respective journey from marrow to liver, or indeed vice versa. Fluorescent cell membrane labels such as PKH26 (Beerheide et al 2002; Krause et al 2001) have been important tools for cell tracking in rodents. However, safety concerns of such labels has prevented their use in clinical trials. Whilst radio-labelling techniques have been successfully and safely used in humans single-cell visualisation or subsequent cell sorting has not been achieved with radio-labelling techniques. Human haematopoietic progenitor cells can be labelled with various magnetic resonance (MR) imaging contrast agents and subsequently localised with MRI. (Daldrup-Link et al 2003; Frank et al 2003). Cells have been labelled with ferumoxides, ferumoxtran, magnetic polysaccharide nanoparticles-transferrin, P7228 liposomes, and gadopentetate dimeglumine liposomes before MR imaging with T1- and T2-weighted spin-echo and fast field-echo sequences. Detection thresholds were 5×10^5 cells for gadopentetate

dimeglumine liposomes and ferumoxtran, 2.5 x 10^5 cells for ferumoxides and P7228 liposomes, and 1 x 10^5 cells for magnetic polysaccharide nanoparticles-transferrin (Daldrup-Link et al 2003). While haematopoietic progenitor cells can be labelled with MR contrast agents and can be depicted with a standard 1.5-T MR imager, further in vivo work is required to enable rigorous testing of its potential for tracking studies.

Stem cells are at the time of writing topical, exciting and offer a myriad of potential therapeutic opportunities, but only further work will discriminate between hope and hype.

Appendix

Bouin's reagent

750 ml Picric acid, saturated aqueous solution

250 ml 37-40% formalin (formaldehyde)

50 ml Glacial acetic acid

Schiff's reagent

Basic fuchsin	1gm
Boiling water	100ml
Potassium meta-bisulfite	2gm
1M HCl	20ml
Activated charcoal (powder)	300mg

Annexin V Binding Buffer

10X Binding Buffer: 0.1 M HEPES, pH 7.4; 1.4 M Sodium Chloride; 25 mM Calcium Chloride. Dilute to 1X prior to use.

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BASIC–LIVER, PANCREAS, AND BILIARY TRACT

Human Cord Blood–Derived Cells Can Differentiate Into Hepatocytes in the Mouse Liver With No Evidence of Cellular Fusion

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Background & Aims: **Studies have indicated that stem cells have unexpected plasticity and can differentiate down a multitude of nonhematopoietic cell lineages in rodents. Our aim was to identify whether human cord blood cells, which are a rich source of stem cells, would be able to differentiate into hepatocytes when infused into nonobese diabetic-severe combined immunodeficient (NOD-SCID) mice. We also wanted to test whether such differentiated cells were the result of cellular fusion or true stem cell transdifferentiation.** ***Methods:*** **Unsorted mononuclear cell preparations of human cord blood were infused into sublethally irradiated NOD-SCID mice. After death, immunohistologic analysis of murine livers was performed using human specific hepatocyte, biliary, and endothelial markers. Fluorescent in situ hybridization (FISH) for mouse and human DNA was also performed.** ***Results:*** **We show that human cord blood cells have the ability to engraft into NOD-SCID liver and become mature hepatocytes. We were unable to identify any biliary or endothelial differentiation. Furthermore, we do not detect any evidence of cell fusion in any of the human cells found in the mouse liver, suggesting that human cord blood cells are capable of true transdifferentiation into hepatocytes in vivo.** ***Conclusions:*** **We conclude that hepatocytes can derive from human cord blood cells when infused into NOD-SCID mice in the absence of fusion. The demonstration that human stem cell differentiation can occur in this murine model permits comprehensive study of human stem cell plasticity in vivo.**

Recent studies have demonstrated that stem cells have greater plasticity than previously thought and can differentiate down multiple nonhematopoietic cell lineages in rodents.[1–4] Although much of this work has used bone marrow–derived stem cells, there are several reports suggesting that stem cells obtained from muscle[5,6] and brain also share these pluripotent properties.[7] Lagasse et al. were able to demonstrate that this phenomenon had functional significance when they showed that infusion of murine adult bone marrow cells in the FAH(−/−) mouse, an animal model of tyrosinemia type I, rescued the mouse and restored the biochemical function of its liver.[8] Notably, this is the only model in which infused stem cells have a survival advantage over host cells, in this case hepatocytes. That adult human stem cells also share some of these pluripotent properties has been demonstrated in studies utilizing archival biopsy samples[9–11] in which donor-derived hepatocytes and cholangiocytes have been observed in the recipient. As yet, the biologic relevance of human stem cell transdifferentiation remains to be clarified, with concerns existing that human stem cells may not share the same pluripotentiality as rodent stem cells. However, the lack of suitable in vivo model systems has hindered detailed study of human stem cell plasticity. Furthermore, somatic stem cell plasticity has recently been questioned, and it has been suggested that this presumed differentiation may be the product of cell fusion between stem

Abbreviations used in this paper: BCIP/NBT, 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium; DAB, diaminobenzidine; DAPI, 4,6-diaminidino-2-phenylindole; FAH, fumarylacetoacetate hydrolase; FISH, fluorescent in situ hybridization; FITC, fluorescein isothiocyanate; HBSS, Hanks' balanced salt solution; HRP, horseradish peroxidase; mAb, monoclonal antibody; NOD-SCID, nonobese diabetic-severe combined immunodeficient; SSC, sodium saline citrate; uPA, urokinase-type plasminogen activator.

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cells and preexisting differentiated cells.[12–14] These observations have important implications for stem cell usage and, if confirmed in in vivo studies, would significantly limit the benefits of stem cell transplantation. In this study, we sought to examine whether human cord blood cells could transdifferentiate into hepatocytes when infused into preconditioned, nonobese diabetic–severe combined immunodeficient (NOD-SCID) mice and whether cellular fusion of human and murine cells occurs.

Materials and Methods

Human Cord Blood Sample Material

Freshly collected, heparinized, whole cord blood samples were received from the Scottish National Blood Transfusion Service (Edinburgh, United Kingdom) following informed donor consent. Mononuclear cells were isolated using Histopaque (Sigma, Dorset, United Kingdom) density centrifugation following the initial description by Boeyum.[15] Briefly, heparin-treated (10^3 IU/mL) whole blood was carefully layered over an equal volume of Ficoll-Hypaque (Sigma) and spun at 540*g* for 20 minutes. The interface ("buffy coat") containing the mononuclear cells was collected and washed twice in Hanks' Balanced Salt Solution (HBSS; Gibco, United Kingdom). A trypan blue exclusion assay was used to assess the viability of the harvested cells prior to their usage.

Animals

Four- to 6-week-old NOD-SCID mice were sublethally irradiated (250 rads) and received a single tail-vein infusion of 50×10^6 human cord blood-derived mononuclear cells resuspended in 100 μL of sterile HBSS. Mice were kept under specific pathogen free (SPF) conditions in microinsulators and killed at 1, 4, 6, and 16 weeks after cord blood cell infusion. Experimental protocols involving animals were carried out in accordance with permits and guidelines issued by the University of Edinburgh Ethical Review Committee and the United Kingdom Home Office.

In Vitro Culture Assays

To detect hematopoietic progenitor cells in human cord blood samples, colony-forming units (CFUs) were evaluated by placing cord blood-derived mononuclear cells in pretested complete semisolid methylcellulose mixtures (Methocult GF H4534; StemCell Technologies, Inc., Canada) comprising 1% methylcellulose in Iscove's MDM, 30% fetal bovine serum, 1% bovine serum albumin, 10^{-4} mol/L 2-mercaptoethanol, 2 mmol/L L-glutamine, 50 ng/mL rh stem cell factor, 10 ng/mL rh GM-CSF, and 10 ng/mL rh IL-3. Fifteen hundred cells per milliliter of methylcellulose were plated out in triplicate wells in a multidish plate (Nunc, United Kingdom) and cultured at 37°C in 5% CO_2 for 12 days. At that time, the number of total CFUs was determined under the microscope as an average count of all 3 wells. Control cultures were included in each assay, and these were grown in identical medium apart from the absence of growth factors.

FACS Analysis

Cord blood mononuclear cell surface phenotype was characterized using standard FACS analysis. Briefly, 100,000 cells per test were pelleted by centrifugation for 5 minutes at 1000 rpm and cell pellets resuspended in individual monoclonal antibody (mAb) solutions using the manufacturer's (Dako; BD Pharmingen, United Kingdom; Sigma, United Kingdom) recommended test concentrations (confirmed in our laboratory to give optimum results in our hands). Test samples were immunostained for 20 minutes on ice with a panel of directly FITC- and/or PE-conjugated mAb against human cell surface markers. The panel included mAb against the following markers: MHC1 (pannucleated cell; 200 μg/mL; Sigma), gly-A (erythrocyte; 100 μg/mL; Dako), CD3 (T cell; 12.5 μg/mL; BD Pharmingen), CD11b (granulocyte; 100 μg/mL; Dako), CD14 (monocyte; 60 μg/mL; Dako), CD19 (B cell; 25 μg/mL; BD Pharmingen), CD33 (myeloid progenitor; 6 μg/mL; BD Pharmingen), CD34 (stem cell; 25 μg/mL; BD Pharmingen), CD45 (panleukocyte; 50 μg/mL; BD Pharmingen), and CD56 (NK cell; 50 μg/mL; Dako). Cell subsets were double immunostained using a subset-specific mAb as well as an antibody against CD45. Following immunostaining, cells were pelleted, washed in FACS-handling buffer (1× PBS/1% BSA/0.1% sodium azide/0.5 mol/L EDTA) and resuspended in FACS fix (1% formaldehyde) prior to analysis using CellQuest software on a Becton Dickinson FACSCalibur machine.

Glucose Phosphate Isomerase Assay

Electrophoresis for variants of glucose phosphate isomerase (GPI) was carried out as described by Ansell and Micklem.[16] Briefly, samples of single cell suspensions were pelleted by centrifugation, lysed using "sample" buffer, and stored at −80°C. Electrophoresis was carried using the "Helena" system (Helena Laboratories). Samples were applied to Titan III cellulose acetate membranes at the anode and run for 80 minutes at 350 V using Supraheme buffer. Bands were developed using a staining mixture containing substrate and cofactors for GPI and visualized using tetrazolium dyes MTT and PMS.

Histology, Immunohistochemistry, and Immunofluorescence

Histology. Livers from NOD-SCID mice were removed, fixed in 4% formaldehyde overnight, and embedded in paraffin blocks. Four-micrometer sections were cut and placed onto Vector-bonded slides. Slides were dewaxed through xylene and rehydrated through graded alcohols and water. Positive controls included normal human liver obtained with ethical approval from the Scottish Liver Transplant Unit (Royal Infirmary of Edinburgh). Negative controls included mouse liver obtained from sublethally irradiated NOD-SCID mice infused IV with medium only.

Immunohistochemistry. Sections were microwaved for 15 minutes in 0.1 mol/L citrate buffer and blocked in 1% H_2O_2 for 15 minutes. Slides were then incubated with a 1:4 dilution of a mouse mAb against cytokeratin 19 (as a marker of biliary epithelium; Dako), a 1:25 dilution mouse mAb HepPar1 that was raised against human hepatocytes (Dako), a 1:25 dilution CD31 mouse mAb against human endothelial cells (Dako), and a 1:25 dilution CD34 mouse mAb against human stem cells (Dako). Slides were then washed and incubated with a horseradish peroxidase (HRP) polymer-conjugated detection antibody followed by diaminobenzidine (DAB) chromagen (Envision; Dako).

Immunofluorescence. Sections were microwaved for 20 minutes in 0.1 mol/L citrate buffer, cooled, and washed in 2× sodium saline citrate (SSC).[17] Immunofluorescence was carried out using a 1:4 dilution of a mouse mAb against cytokeratin 19 (Dako) or a 1:25 dilution mouse mAb HepPar1 (Dako). Secondary antibody was a 1:100 dilution of donkey anti-mouse FITC (Vector Laboratories).

FISH Analysis

One microgram total human genomic DNA was labelled with digoxigenin 11-dUTP (Roche, Lewes, United Kingdom), and mouse genomic DNA was labelled with Alexa Fluor 594-5 dUTP (Molecular Probes, Leiden, The Netherlands) by nick translation. Unincorporated nucleotides were removed by centrifugation through Quick Spin G50 Sephadex columns (Roche). Specific activity of the probes was performed by dotting onto nitrocellulose filters using anti-digoxigenin-alkaline phosphatase Fab fragments (Roche). We then used a BCIP/NBT kit (Vector), which produces the purple change on the filter, which we then compared with known standards (DNA of known concentrations).

Slides were heated to 60°C for 20 minutes to melt wax then washed in xylene 4 times for 10 minutes each before dehydration through an ethanol series (100%, 95%, 70%). Slides were then microwaved for 20 minutes in 0.1 mol/L citrate buffer, pH 6.0. After microwaving, liver sections were denatured for 3 minutes at 75°C in 70% formamide/2× SSC, plunged into ice-cold 70% ethanol for 3 minutes, dehydrated through an alcohol series, and air-dried.

One hundred fifty nanograms labeled human genomic DNA, 150 ng labelled mouse genomic DNA, and 5 μg salmon sperm DNA were denatured together for 5 minutes at 75°C in 50% formamide, 10% dextran sulphate, 2× SSC, and 1% Tween 20. Hybridization was overnight at 37°C. Slides were washed 4 times for 3 minutes in 2× SSC at 45°C then incubated for 30 minutes with a 1:30 dilution of a sheep FITC anti-digoxigenin antibody (Roche), followed by a further 30 minutes with a 1:100 dilution of FITC anti-sheep antibody (Vector Laboratories).[18] Slides were mounted in Vectashield (Vector Laboratories) containing 1 μg/mL 4,6-diaminidino-2-phenylindole (DAPI) counterstain.

Table 1. Characterization of Infused Human Cord Blood Prior to Intravenous Inoculation Into Sublethally Irradiated NOD/SCID Mice

Cell surface marker	Range (%)	Mean (SE)	Median
MHC1	78–96	88 (2)	88
CD45	59–93	81 (3)	83
GlyA	2–18	11 (2)	11
CD3	0–70	54 (6)	58
CD11b	5–24	14 (2)	14
CD14	1–21	6 (2)	4
CD19	0–24	8 (3)	1
CD33	0–21	4 (2)	2
CD34	0–2	1 (0)	1
CD56	0–2	1 (0)	0
Number of CFU	0–100	48 (12)	51

NOTE. Table describes the analysis of 10 human cord blood preparations prior to their infusion into sublethally irradiated NOD/SCID mice. The percentage of cells expressing a variety of hematopoietic markers is detailed as well as mean values (along with their standard error, SE = SD/√n) and medians. In particular, the percentage of cells expressing CD34 is seen to be 1%. Colony-forming unit (CFU) measurement confirms the presence of stem cells in the infused cord blood.

Image Analysis

Slides were visualized using a Zeiss Axioplan 2 fluorescence microscope (Thornwood) equipped with a triple bandpass filter (Chroma No. 83000). Grayscale images were collected with a cooled charge-coupled device (CCD) camera (Quantix Corp.) and analyzed using custom IPLab scripts. Images were pseudocolored using image processing software (Adobe Photoshop).

Results

Infused Human Cord Blood Samples Are Rich in Hematoprogenitor Cells

Initially, we characterized 10 individual human cord blood samples by FACS analysis and in vitro culture assays prior to injection into NOD-SCID mice (Table 1). The results were that an average of 1% of cord blood mononuclear cells were $CD34^+$ve stem cells. In line with this were the results from our in vitro culture assays, which demonstrated an average of 48 colony-forming units (CFU) per cord blood sample. Taken together, the results thus suggested that our infused cord blood samples were rich in hematoprogenitor cells.

Human Cord Blood Samples Engraft in NOD/SCID Mice

To assess in vivo engraftment of our human samples up to 16 weeks postinoculation, we used FACS and GPI analysis as well as in vitro culture assays to study bone marrow samples from our infused NOD-SCID mice. Using FACS, the results were that mice tested had

Table 2. Human Reconstitution of NOD-SCID Murine Bone Marrow

Hematopoietic markers	Human		Murine	
	Mean (%)	Range (%)	Mean (%)	Range (%)
MHC1$^+$ve	26	14–47	32	23–46
CD45$^+$ve	24	10–45	34	28–38
CD3$^+$ve/CD45$^+$ve	4	0–13		
CD11b$^+$ve/CD45$^+$ve	7	2–12		
CD14$^+$ve/CD45$^+$ve	2	0–6		
CD19$^+$ve/CD45$^+$ve	77	64–85		
CD33$^+$ve/CD45$^+$ve	6	3–12		
CD34$^+$ve/CD45$^+$ve	6	3–10		

NOTE. Table represents the contribution of human cells to the bone marrow of irradiated NOD-SCID mice (n = 6) 8 weeks after infusion of human cord blood cells. The column on the left denotes the panel of markers (human and murine) screened for in the murine marrow. The middle column reflects the relative contribution of human cells to different hematopoietic lineages in the NOD-SCID murine marrow (these were identified using flow cytometric analysis with human specific antibodies). The column on the right reflects murine hematopoietic cells identified by flow cytometric analysis with mouse-specific antibodies. These data confirm that there is marrow engraftment with human cord blood cells.
MHC1, Major histocompatibility 1 antigen.

detectable levels (10%–45% of counted cells; average, 17%) of human CD45$^+$ve leukocytes in their bone marrow (Table 2). In addition, there was evidence of multiple hematopoietic lineages represented by human cells. The data correlated with results of our GPI assay, which confirmed the presence of the human GPI isoenzyme in the assayed samples (Figure 1). In in vitro culture assays, samples gave rise to colonies in methyl cellulose, albeit at low numbers (data not shown).

HepPar1 Antibody Is Specific for Human Hepatocytes

In the next set of experiments, we ascertained the specificity of the HepPar1 mAb using serial tissue sections. The results, which are shown in Figure 2, demonstrated that, when incubated for 1 hour at a concentration of 1:25, the HepPar1 antibody is specific for human hepatocytes and does not cross-react with human cord blood or NOD-SCID mouse liver elements.

Quantitation of Cord Blood–Derived Hepatocytes

Human hepatocytes were identified in 2 ways: The first way was with positive staining of the human specific HepPar1 antibody (Figure 2). We have demonstrated that HepPar1 is human specific and does not cross-react with NOD-SCID mouse liver (or human cord blood). This observation is particular to NOD-SCID mice and is in keeping with that of other laboratories. We also performed immunofluorescent HepPar1 staining, which permitted DAPI nuclear analysis (data not shown). The nuclear DNA staining pattern of human and mouse cells is quite distinct (Figures 3 and 4), thus permitting further differentiation between human and murine hepatocytes. The abundance of pericentric heterochromatin on mouse chromosomes is manifest as bright foci of DAPI staining,[19] a feature not seen in human cells, which have less pericentric heterochromatin. The combination of binding of a human specific liver antibody and human nuclear morphology were taken as indicative that the cell was a human hepatocyte. The second way human hepatocytes were identified was with positive FISH signal for human DNA (Figure 3) and cell morphology typical of human hepatocytes. Hepatocytes were large polyclonal cells, arranged in plates, and had large round nuclei. In addition, their cytoplasm was characteristically bright, reflecting the FITC-type cytoplasmic autofluorescence caused by bilirubin metabolic products.[2] The number of positive cells obtained by these 2 methods was used to provide a mean value of cord blood-derived hepatocytes in each liver. Total human cells were quantified by counting the number of cells with human FISH signal in the murine liver section. Colocalization studies of HepPar1 staining and positive FISH signal were attempted, but FISH processing degraded the HepPar1 signal, and, thus, we were unable to detect colocalization of these markers even in human liver tissue.

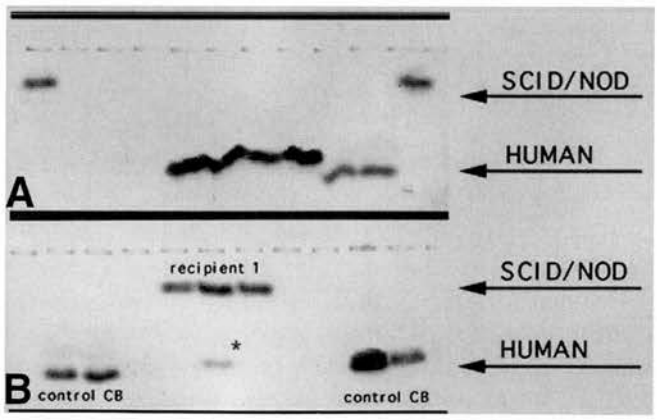

Figure 1. Glucose phosphate isomerase assay for in vivo detection of human cells. GPI analysis of bone marrow–derived samples from NOD-SCID mice infused with human cord blood cells for demonstration of the human isoenzyme. (*A*) Demonstrates the different electrophoretic mobility of human and NOD-SCID mouse iso-enzymes. (*B*) Shows the results obtained in a representative NOD-SCID mouse bone marrow after (labeled "*recipient 1*") inoculation with a human cord blood sample. The results were in line with data obtained by FACS analysis and in vitro cultures.

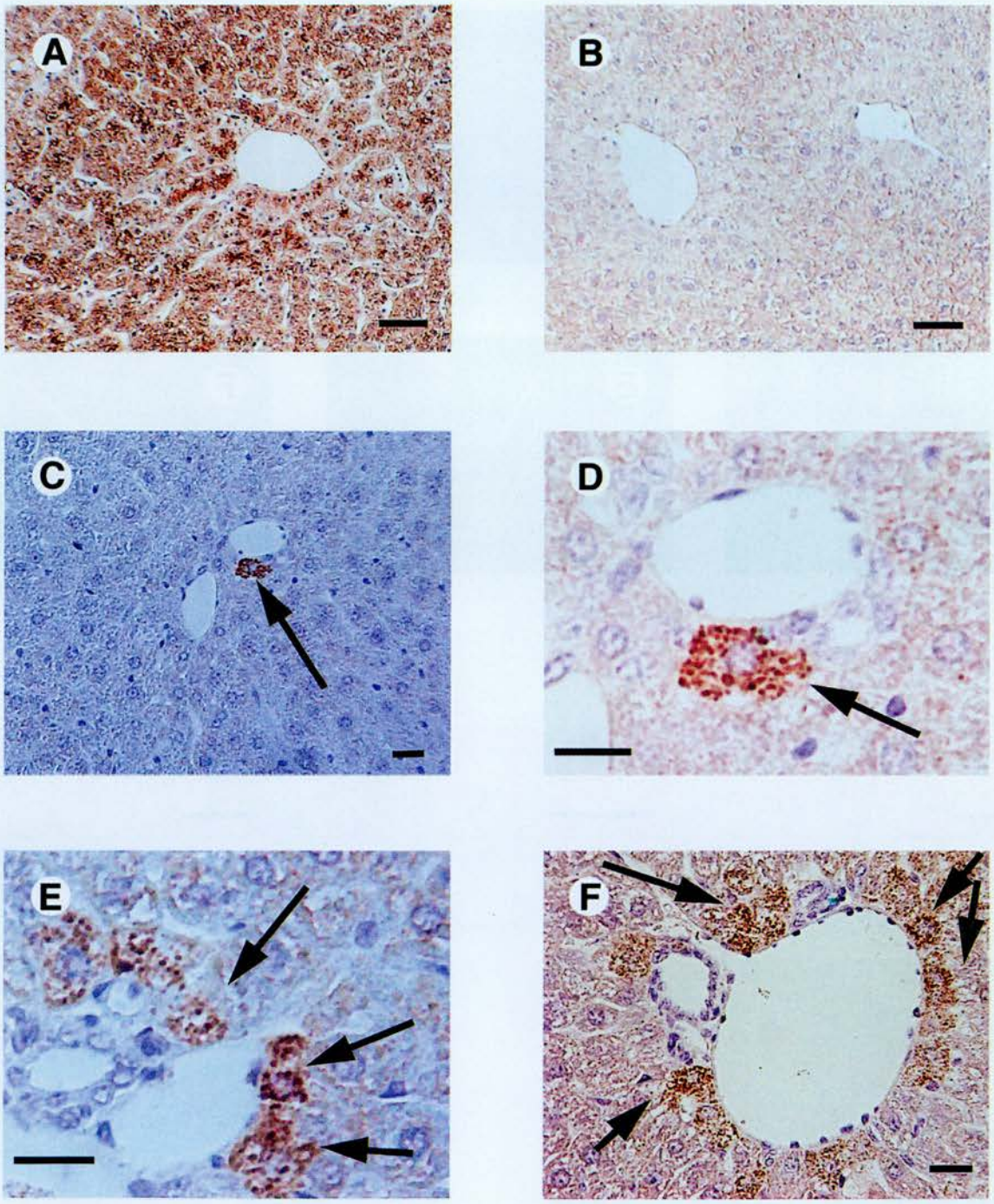

Figure 2. Detection of human hepatocytes with immunohistochemistry. Immunohistochemistry on liver sections with antibody HepPar1 (*brown*) that detects human, but not mouse, hepatocytes. Cells were counterstained with Hematoxylin (*blue*). (*A*) Human liver. (*B*) Mouse liver. (*C–F*) Positive cells integrated into the hepatic plate of livers of NOD-SCID mice. (*C* and *D*) Represent the more typical finding of isolated single positive cells (higher magnification of *C*). (*E* and *F*) Represents the occasional clump of cells seen in the parenchyma. (*Bar* = 20 μm.)

Human Cord Blood Cells Can Engraft and Differentiate Into Hepatocytes in the NOD-SCID Mouse Liver

Despite no evidence of hepatocyte damage after irradiation, human-derived cells were detected in the liver of cord blood–injected NOD-SCID mice 1 week postinfusion and at all subsequent time points. Human hepatocytes were demonstrated in mouse liver 4 weeks postinfusion and at all subsequent time points. Human hepatocyte differentiation in vivo was detected first by immunohistochemical analysis using the human-specific hepatocyte marker (HepPar1), demonstrating the pres-

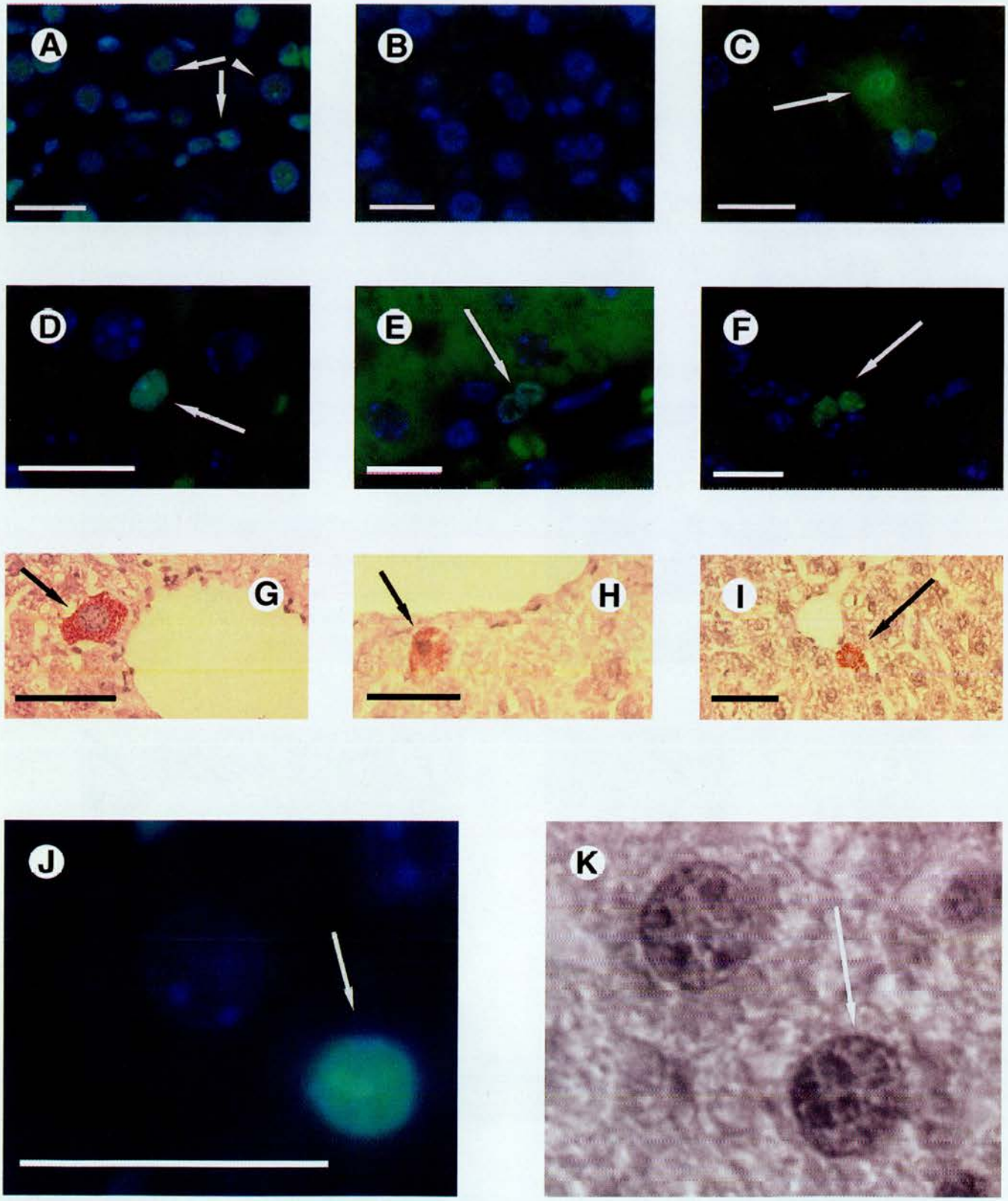

Figure 3. Detection of human cells in liver sections by FISH. Detection of digoxigenin-labeled human DNA by FITC (*green*) in liver sections counterstained with DAPI (*blue*). Greyscale images of DAPI staining are shown on the right. (*A*) Human liver. (*B*) Mouse liver. (*C* and *D*) Human hepatocytic cells integrated into the hepatic plate of livers of NOD-SCID mice. (*E* and *F*) Human hematopoietic cells entering the murine liver. (*G–I*) Human $CD34^+$ cells detected with immunohistochemistry entering the murine liver. (*J* and *K*) Represent greyscale H&E pictures of cells with positive FISH signal and characteristic hepatocytic morphology. These cells had morphology typical of hepatocytes with large polyclonal size, large round nuclei, and complex intracellular cytoplasm. (*Bar* = 20 μm.)

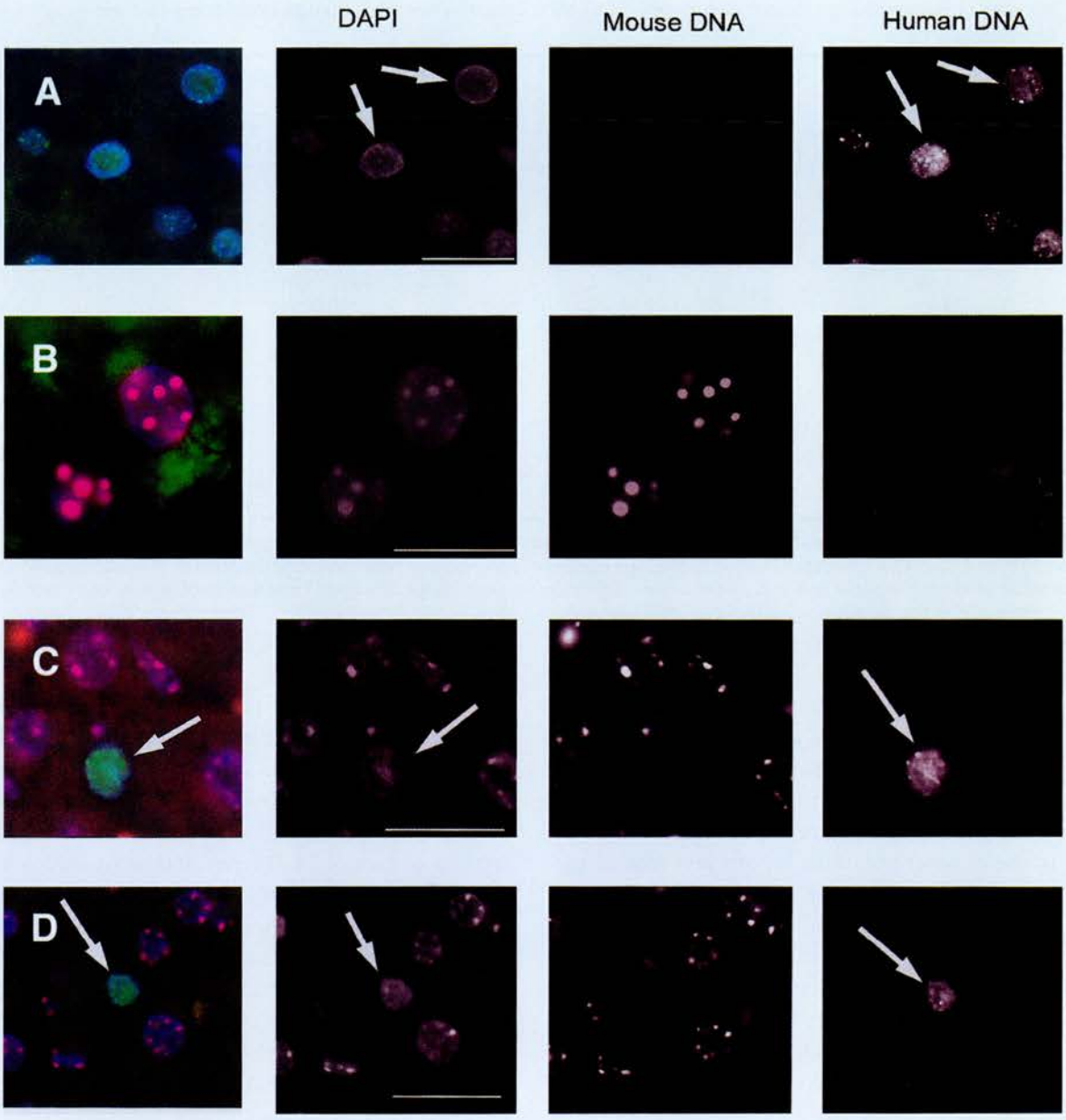

Figure 4. Assessment of donor-host cell fusion. Simultaneous FISH analysis of liver sections with total human (*green*) and mouse (*red*) DNAs. Cells were counterstained with DAPI (*blue*). Greyscale images of the DAPI staining and mouse and human hybridization signals are shown. (*A*) Human liver. (*B*) Mouse liver. (*C* and *D*) Human positive (*green*) cells integrated into the hepatic plate of livers of NOD-SCID mice. Note the absence of any detectable mouse DNA (*red*) in these nuclei and their DAPI staining typical of human derived cells. (*Bar* = 20 μm.)

ence of isolated positive cells within the NOD-SCID mouse liver (Figure 2). Occasionally, clumps of cells were seen within the liver. Furthermore, immunofluorescence analysis of these sections using the same HepPar1 antibody confirmed the presence of isolated positive cells integrated in the liver plate (data not shown), with characteristic human nuclear morphology. These cells tended to be located within the hepatic plate in the vicinity of portal tracts, suggesting that circulating cells derived from human cord blood had invaded the liver and differentiated into mature hepatocytes. Further evidence for the presence of human hepatocytes derived from the presence of positive FISH signals in cells with typical hepatocyte morphology. Many of these cells had the morphology (large nuclei and abundant cytoplasm) typical of mature hepatocytes (Figure 3). The mean number of human hepatocytes per section was 47.8 (range, 19–73). The observed frequency of human hepatocytic transformation seen was modest (Table 3), with such cells constituting on average 0.011% of all cells on each section (maximum seen was 0.017%). When the number of transdifferentiated hepatocytes is expressed as a per-

Table 3. Number of Human Cells/Hepatocytes in NOD-SCID Mice Transplanted With Human Cord Blood–Derived Mononuclear Cells

Mouse	Time Post CBI	Mean number of human cells (all types) per mouse liver section (and value as a percentage of host cells)	Mean number of human hepatocytes per mouse liver section (and value as a percentage of host cells)	Transdifferentiation efficiency of $CD34^+$ cells becoming hepatocytes (%)
1	7 d	45 (0.0072)	0 (0)	0
2	7 d	67 (0.018)	0 (0)	0
3	7 d	75 (0.012)	0 (0)	0
4	4 wk	91 (0.013)	62 (0.0089)	1.77
5	4 wk	132 (0.04)	46 (0.014)	2.79
6	4 wk	83 (0.027)	52 (0.017)	3.40
7	6 wk	108 (0.015)	71 (0.0099)	1.97
8	6 wk	88 (0.021)	46 (0.011)	2.19
9	6 wk	80 (0.013)	40 (0.0066)	1.31
10	6 wk	54 (0.0082)	19 (0.0028)	0.58
11	16 wk	63 (0.017)	52 (0.014)	2.81
12	16 wk	51 (0.013)	43 (0.011)	2.21
13	16 wk	70 (0.014)	22 (0.0043)	0.88
14	16 wk	117 (0.027)	73 (0.017)	3.38

NOTE. Table quantifies both the total number of human cells and the number of human hepatocytes engrafted within the NOD-SCID mouse liver. Transdifferentiation efficiency is expressed by dividing the total number of donor-derived hepatocytes by the number of infused $CD34^+$ cells. Peak trans differentiation is seen to occur at 4 to 6 weeks with no significant increment after this time. Total human cells were quantified by counting the number of cells with human FISH signal in the murine liver section.
CBI, Cord blood infusion.

centage of the number of infused $CD34^+$ cells, the mean value is 2.11%. No evidence of any human hepatocytes was seen 1 week after cord blood infusion, although infiltrating $CD34^+$ cells could be seen entering through the portal tracts (Figure 3). There did not appear to be an increase in the number of human hepatocytes seen in the mouse liver 4 weeks after cord blood infusion, with similar percentages being found after 6 and 16 weeks. Furthermore, similar numbers of human cells were also observed in vascular channels and/or had morphology that was more consistent with hematopoietic lineages (Figure 3). There was no evidence of human biliary (cytokeratin 19+ve) or endothelial ($CD31^+$) differentiation in any of the sections.

Fusion of Human and Murine Cells Does Not Occur In Vivo

The nuclear DNA staining pattern of human and mouse cells is quite distinct, as described previously.[19] Bright foci of DAPI staining were evident in sections of mouse liver, whereas nuclei in human liver sections had a more uniform staining pattern (Figure 4*A* and *B*). Cells hybridizing to human DNA and engrafting into mouse livers had characteristic human nuclear staining morphology, and there was no evidence in these cells of bright DAPI foci characteristic of mouse chromosomes (Figure 3*C–F*). Similarly, cells staining with a human-specific hepatocyte antibody (Figure 2*C–F*) also had nuclear morphology typical of human rather than mouse cells.

To confirm this, we performed a simultaneous FISH analysis for murine and human DNAs. The results were that we did not detect any cells, either hepatocytic or nonhepatocytic, that were positive for both human and mouse DNA (Figure 4). We examined at least 5 liver sections of each of 14 different mouse livers. Each section had a mean of 80.3 positive cells/section, giving a total number of events screened of 5620. Not one of these cells was the result of fusion of human and mouse cells. Although we cannot totally exclude cellular fusion, it is clearly exceedingly rare compared with human stem cell transdifferentiation. Taken together, the data suggest that it was unlikely that the donor human cord blood cells had fused with host mouse hepatocytes.

Discussion

Our data demonstrate that infused human cord blood cells are capable of engrafting into NOD-SCID mouse liver and differentiating down the hepatocytic lineage without fusion to host hepatocytes. This work confirms the recently published observation by Danet et al.,[20] who have identified a subpopulation of human cord blood cells that have the ability to differentiate down the hepatocytic lineage upon infusion into the NOD-SCID mouse liver. In our experimental animals, engrafted hepatocytes were most often scattered throughout the murine liver parenchyma, usually appearing as isolated cells, although occasionally, we observed some small clumps of cells. The rate of hepatocytic differentiation of

cord blood cells occurred at low levels in our model (up to 0.017% of total cells), and it will be important to determine in the future whether liver injury increases this frequency.[21] There appears to be some variation in the amount of stem cell–derived hepatocytic differentiation reported in the literature, with groups reporting as few as 10 hepatocytes per murine section,[22] whereas others observe levels as high as 1.1% of recipient liver tissue.[2] This variation may reflect differences in species and strains of animal used, presence of liver injury (whether intentional or nonintentional such as radiation), and methods used to identify stem cell–derived hepatocytes. We have used several different methods to identify stem cell–derived hepatocytes, and, notably, our observed frequency falls in the lower end of this range. The results further suggest that, in the absence of liver injury, there is no stimulus for transdifferentiated cord blood–derived hepatocytes to proliferate because the proportion of human hepatocytes did not appear to increase with time following the 4 weeks time point. This is in keeping with the literature as reported by Theise et al.,[2] who demonstrated that the proportions of donor murine bone marrow–derived hepatocytes in myeloablated mouse recipient livers were similar up to 12 months after bone marrow transplant. Although our observed level of engraftment in our xenogeneic model is lower than that observed in vivo by Theise et al. (up to 1.1% of total cells studied), part of this may be due to human stem cells being at a relative survival/proliferative disadvantage when infused into xenogeneic environments in absence of liver injury. In contrast, infusion of human hepatocytes into mice suffering mild chronic liver injury leads to their populating up to 15% of the mouse liver, but this is seen in the context of urokinase-type plasminogen activator (uPA) transgenic mice, which have a liver-toxic phenotype.[23] The majority of the transdifferentiated hepatocytes in our model were seen to be located in the hepatic plate in the vicinity of portal tract areas, and most of these cells were found as isolated single cells (although occasional clumps of cells were seen). In conjunction with the finding of $CD34^+$ human cells in the vicinity the portal tracts these observations suggest that the portal tract may be a major route of entry of hepatocyte progenitors into the liver. This is supported by our finding that human cells with morphology that was more consistent with hematopoietic cells were frequently seen to be entering through the portal tracts (Figure 3*D–I*), although tracking experiments would be required to confirm their subsequent hepatocytic commitment. In the absence of any significant liver injury, human stem cells would have no obvious reason to migrate further into the liver plate. Hence their periportal, transdifferentiation may reflect their earliest exposure to murine hepatocytes and other factors triggering differentiation. No evidence of human biliary (cytokeratin 19+ve) or endothelial (CD31) differentiation was seen in any of the liver sections, but this may reflect the relatively low turnover of these cellular compartments[24] and the fact that they represent only ~2% of the cellular mass of the liver. In view of the modest levels of hepatocytic transdifferentiation, the chances of identifying other stem cell–derived lineages would therefore be much lower.

We used unsorted human cord blood as a source of donor cells because it is known to contain higher levels of stem cells than normal peripheral blood.[25] Although other groups have attempted to select out an enriched population of stem cells using markers such as CD34, it is possible that the CD34-ve population may in fact contain an even more pluripotent population of stem cells.[26–28] Therefore, there is ample evidence to suggest that hepatocyte progenitors belong to a stem-cell compartment.

Somatic stem cell plasticity has recently been questioned, and it has been suggested that this presumed differentiation might be the product of cell fusion between stem cells and preexisting differentiated cells.[12–14] In contrast to recent ex vivo reports,[12,13] we found no evidence of cell fusion in our in vivo model. Although a type 2 error cannot be totally excluded, the fact that not one of 5620 human cells in the murine liver are the result of cellular fusion indicates that fusion is at best an exceedingly rare phenomenon. Our study would suggest, therefore, that it plays a negligible role in the phenomenon of transdifferentiation. The presence of cellular fusion in other studies may thus reflect the ex vivo environment that the work was carried out in and not in vivo conditions. If fusion is a rare event, it may become apparent in an in vivo model of liver injury in which more hepatocyte transdifferentiation may take place.

This work makes the fundamental observation that human cord blood–derived cells have the ability to migrate, engraft, and differentiate into hepatocytes in mouse liver when infused into NOD-SCID mice. Furthermore we show categorically that none of our transdifferentiated hepatocytes are the result of cellular fusion of human cord blood cells with murine hepatocytes. The results thus suggest new opportunities in the study of human adult stem cell biology using preconditioned NOD-SCID mice, and future work should aim to optimize this model system for further studies of hepatocyte progenitors.

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