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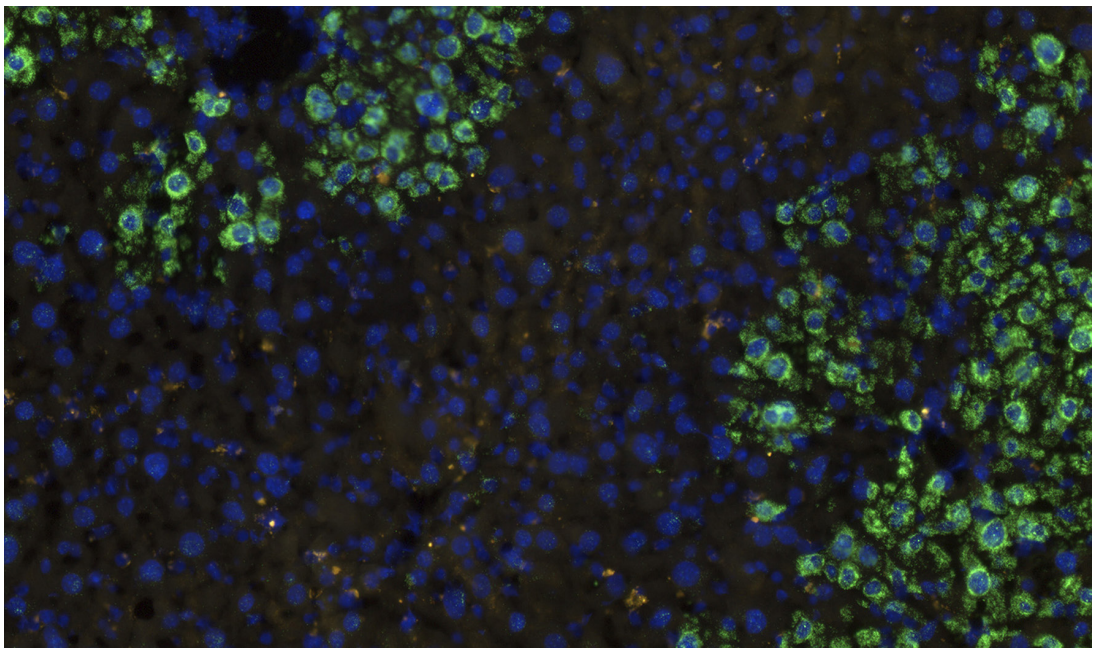
Hepatocyte Transplantation: Experimental and Clinical Studies



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HEPATOCYTE TRANSPLANTATION: EXPERIMENTAL AND CLINICAL STUDIES

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Cover: ApoE in situ hybridization of liver sections from ApoE knock out mouse transplanted with hepatocytes from wild-type mouse.

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Hepatocyte Transplantation: Experimental and Clinical Studies

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ABSTRACT

Hepatocyte transplantation is an experimental treatment for patients with end-stage liver disease and inborn metabolic liver disorders. Studies in animal models and human trials have shown that allogenic hepatocytes infused through the portal vein physiologically integrate into the liver parenchyma, replacing missing liver function. Current research data provide a proof of principle for clinical hepatocyte transplantation in a wide range of liver diseases. However, most patients ultimately undergo whole organ liver transplantation due to insufficient graft function. Thus, the efficacy and long-term results of clinical hepatocyte transplantation must be improved before this treatment can be introduced into routine clinical care.

The present thesis summarizes experimental and clinical studies with the general aim of identifying current limitations and improving outcomes of hepatocyte transplantation.

Paper I investigates strategies for improving short-term preservation of isolated human hepatocytes. Human hepatocytes are usually cold stored for prolonged periods between isolation and infusion. We found that isolated human hepatocytes undergo cell death and lose hepatocyte-specific function during this cold storage period. An alternative technique of liver tissue storage and repeated isolations led to improved viability and function of isolated hepatocytes before infusion.

In Papers II and III, a hepatocyte transplantation model was established in the ApoE knockout mouse. Clinically relevant animal models are necessary for developing new treatment strategies. The ApoE knockout mouse is an ideal model of an inherited metabolic liver disease. ApoE is mainly produced by hepatocytes and its deficiency results in extrahepatic disease. ApoE (−/−) mice display severe hypercholesterolemia leading to premature atherosclerosis. We observed that transplanted wild-type hepatocytes integrated into the liver and excreted ApoE, and that this serum ApoE correlated with hepatic donor cell engraftment. Transplantation without preconditioning treatment resulted in serum ApoE levels of 1–2% of wild-type levels, which did not affect hypercholesterolemia. However, pretreatment with retrorsine gave donor hepatocytes a growth advantage, resulting in progressive repopulation of up to 55% of the recipient liver. This increased repopulation by donor hepatocytes led to normalization of hypercholesterolemia and prevention of atherosclerosis.

Paper IV evaluated the safety and efficacy of partial hepatectomy preconditioning with hepatocyte transplantation in two patients with Crigler-Najjar syndrome type I. Partial hepatectomy in combination with hepatocyte transplantation was safe and induced a regenerative response. Serum bilirubin decreased to approximately 50% of pretransplant concentrations, and allograft function was further confirmed by detection of bilirubin diglucuronides in bile after transplantation. However, both patients lost graft function in association with the emergence of donor-specific HLA antibodies.

LIST OF SCIENTIFIC PAPERS

I. **Jorns C**, Gramignoli R, Saliem M, Zemack H, Mörk LM, Isaksson B, Nowak G, Ericzon BG, Strom S, Ellis E. Strategies for short-term storage of hepatocytes for repeated clinical infusions.
Cell Transplant. 2014;23(8):1009–18.

II. **Jorns C**, Takahashi T, Callaghan E, Zemack H, Larsson L, Nowak G, Parini P, Ericzon BG, Ellis E. Serum apolipoprotein E as a marker to monitor graft function after hepatocyte transplantation in a clinically relevant mouse model.
Transplant Proc. 2013 Jun;45(5):1780–6

III. **Jorns C**, Watanabe M, Larsson L, Zemack H, Strom S, Ericzon BG, Parini P, Ellis E. Hepatocyte transplantation normalizes hypercholesterolemia in apolipoprotein E knockout mice.
Manuscript

IV. **Jorns C**, Nowak G, Nemeth A, Zemack H, Mörk LM, Johansson H, Gramignoli R, Watanabe M, Alheim M, Hauzenberger D, van Dijk R, Bosma PJ, Ebbesen F, Szakos A, Fischler B, Strom S, Ellis E, Ericzon BG. De novo donor-specific HLA antibody formation in two patients with Crigler-Najjar type I following human hepatocyte transplantation with partial hepatectomy preconditioning.
Revised version submitted

SUPPLEMENTAL PUBLICATION

I. **Jorns C,** Ellis EC, Nowak G, Fischler B, Nemeth A, Strom SC, Ericzon BG Hepatocyte transplantation for inherited metabolic diseases of the liver.
J Intern Med. 2012 Sep;272(3):201–23

CONTENTS

LIST OF ABBREVIATIONS

ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
ApoE	Apolipoprotein E
AST	Aspartate aminotransferas
AUC	Area under the curve
BNF	Beta-naphthoflavone
CDC	Complement-dependent cytotoxicity
CE	Cholesteryl ester
CMV	Cytomegalovirus
CN-I	Crigler-Najjar syndrome type I
CUSA	Cavitron ultrasonic surgical aspirator
CYP	Cytochrome P450
DAB	3,3'-Diaminobenzidine
DMSO	Dimethylsulfoxide
DSA	Donor-specific antibodies
dsDNA	Double-stranded DNA
EGF	Epidermal growth factor
EGTA	Ethylene glycol tetraacetic acid
EMEM	Eagle's Minimum Essential Medium
EROD	7-ethoxyresorufin-O-deethylase
FACS	Fluorescence-activating cell sorting
GMP	Good manufacturing practice
HBSS	Hank's Buffered Salt Solution
HDL	High-density lipoprotein
HGF	Hepatocyte growth factor
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HPLC	High-performance liquid chromatography

IL-6	Interleukin 6
LDL	Low-density lipoprotein
LCU	Luminescent counting unit
MMF	Mycophenolate mofetil
MPA	Mycophenolic acid
PB	Phenobarbital
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PE	Plating efficiency
PRA	Panel reactive antibodies
Rif	Rifampicin
SEM	Standard error of the mean
TC	Total cholesterol
TGF	Transforming growth factor
TNF-α	Tumor necrosis factor α
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
UGT1A1	Uridine diphosphoglucuronate glucuronosyltransferase 1A1
UW	University of Wisconsin Solution
VLDL	Very low-density lipoprotein

1 INTRODUCTION

1.1 HEPATOCYTE TRANSPLANTATION

The first attempts of hepatocyte transplantation were performed in animal models in the 70´s. Rugstad et al. reported transplantation of hepatoma cell lines into the Gunn rat, a model of Crigler-Najjar syndrome type I (CN-I) (1). CN-I is a rare inborn metabolic liver disease caused by deficiency of the hepatic enzyme uridine diphosphoglucuronate glucuronosyltransferase 1A1 (UGT1A1). If untreated, UGT1A1 deficiency results in fatal brain injury due to unconjugated hyperbilirubinemia. Rugstad et al. demonstrated that UGT1A1 enzyme activity could be transferred to the Gunn rat, improving hyperbilirubinemia. This was the first reported use of cell transplantation to ameliorate a metabolic liver disease. Matas et al. refined these early findings by transplanting mechanically isolated liver fragments to the Gunn rat with similar results (2). The first true hepatocyte transplantation was performed by Groth et al. who used collagenase perfusion to isolate hepatocytes from wild-type Wistar rats and infused these isolated hepatocytes into the portal vein of the Gunn rat. They demonstrated that transplantation of only 0.2% of the liver mass led to a 40% decrease in serum bilirubin (3, 4).

In the 1990s, the first successful clinical hepatocyte transplantations were performed (5, 6). Liver transplantation was already considered the only curative treatment for many patients with end-stage liver disease, acute liver failure, and many forms of metabolic liver diseases (7, 8). Over the following decades, results of whole liver transplantation have improved dramatically. However, the procedure still carries substantial morbidity and mortality. Hepatocyte transplantation has been proposed as an alternative treatment as it has a number of potential advantages compared to whole organ transplantation. Importantly, the hepatocyte transplantation procedure is less invasive with lower morbidity and mortality (9). Hepatocytes also have enormous proliferative potential and a small proportion of the liver mass can repopulate an entire liver in cases where the donor cells have a growth advantage over the native hepatocytes (10-13). For many of its functions, the liver has an overcapacity and replacement of a small fraction of the missing function is often sufficient to improve or even correct the underlying disease (6, 14, 15). One donor liver can provide enough cells for several recipients, improving the mismatch between the available organs and recipients on the waiting list (16). Moreover, in contrast to whole organs, hepatocytes can be cryopreserved and are available on demand (5, 9, 17, 18). Finally, in cases of hepatocyte transplantation, the patient keeps their own liver as a back-up in the event of graft failure or the future availability of better therapies.

1.1.1 Hepatocyte isolation

Techniques for isolating viable hepatocytes from liver tissue were crucial for the development of hepatocyte transplantation. However, the pivotal studies were performed in the pancreas rather than the liver. In 1967, Lacy et al. reported the use of collagenase perfusion to isolate islets of Langerhans from a rat pancreas (19). Transplantation of these islets to diabetic animals resulted in hyperglycemia normalization, paving the way for clinical islet transplantation (20). This collagenase perfusion method was later adopted by Berry et al. for isolating viable hepatocytes from rat liver (21, 22) and was subsequently further refined for isolation of human hepatocytes by Strom et al. (23).

Today, the isolation process is still based on the same principles with minor modifications. Liver tissue is perfused through catheters sutured into the main hepatic vessels—either portal or hepatic veins. Perfusion is performed with three solutions. The first solution contains EGTA, an iron chelator that removes calcium and thus disrupts intercellular connections. The second is a buffered electrolyte solution that removes EGTA as calcium is needed for adequate collagenase function. The third solution contains a collagenase-digesting extracellular matrix. Perfusion solutions are preheated to 37°C and often the liver is maintained at 37°C to optimize enzymatic function. In contrast to in islet isolation, the liver tissue is continuously perfused. Complete digestion usually requires 15–35 minutes of collagenase perfusion. The liver capsule is cut and the cell suspension is released into ice-cold medium. Finally, the hepatocytes are washed and purified by low-speed centrifugation.

The quality of the utilized collagenase is a main factor determining isolation success. Most commercially available collagenases are derived from *Clostridium histolyticum*. These crude enzymes have the advantage of high potency, but at the cost of great variability of enzyme activity from batch to batch. Furthermore, the bacterial origin with large amounts of endotoxins and culture conditions can be controversial if the collagenase is to be applied for clinical isolations under good manufacturing practice (GMP) conditions. A highly purified, defined, and GMP-approved enzyme (CIzyme™,Vitacyte) has recently become available for clinical hepatocyte isolations (24).

1.1.2 Characterization of hepatocyte function and viability

Hepatocyte transplantation outcome depends on the quality of the isolated hepatocytes (25). Hepatocytes participate in a wide variety of functions, including detoxification, glycogen storage, bile production and excretion, production of coagulation factors, ammonia metabolism, serum protein production, lipoprotein metabolism, etc. Most of these functions can be tested in vitro. However, there remains a need to evaluate the significance and cut-off values of each individual assay as well as the ability to predict post-transplantation outcome.

The trypan blue exclusion test is the most commonly used assay and is the only assay demonstrated to predict outcome after hepatocyte transplantation (25). This assay evaluates

cell membrane integrity as dead cells are stained blue. It has the advantages of being fast and easy to perform and of providing additional morphological information if performed under a microscope. The limitation of this assay is that it cannot detect early apoptotic cells. Apoptosis can be evaluated using TUNEL staining or evaluation of caspase activity (see methodology).

Another assay commonly used to evaluate hepatocyte function is plating efficiency (PE), which tests the ability of single hepatocytes to attach to an extracellular matrix, e.g., collagen, laminin, fibronectin, or Matrigel. Hepatocytes rely on cell anchorage, an active process requiring cellular receptors, including integrins and cadherins (26, 27). The assay purportedly measures the ability of hepatocytes to engraft after transplantation, although this has not been confirmed in animal studies (24).

Another important hepatic function is the urea cycle, in which neurotoxic ammonia is converted into urea. Urea cycle deficiencies exist in patients with urea cycle defects or acute liver failure, which are both indications for hepatocyte transplantation. Ammonia metabolic ability can be measured in hepatocyte cultures by adding ammonia to the cell culture media and measuring its disappearance (see methodology).

Several other main hepatic functions—including production of steroids, bile, vitamin D, and cholesterol, as well as the metabolism of many drugs—are catalyzed by enzymes belonging to the cytochrome P450 (Cyp450) family. The Cyp450 family comprises heme-containing enzymes that give the liver its color as they absorb light with a peak at 450 nm (28-30). This enzyme family shows inter-individual and inter-species variations in expression and function, and has thus been a main target in pharmacological research (31). Consequently, a wide range of assays have been developed to study their activity in vitro (32) (see methodology).

1.1.3 Route of administration

Clinical hepatocyte transplantations have been performed using three different transplantation sites: liver, spleen, and peritoneal cavity. The most common option is to transplant cells into the liver by infusing the cells into the portal vein. Portal vein access can be accomplished via an ultrasound-guided percutaneous trans-hepatic route or with open surgical access through the umbilical vein or a mesenteric vein (9). An advantage of transplanting cells into the liver is that donor hepatocytes physiologically integrate with the liver parenchyma. After integration, donor cells are histologically indistinguishable from recipient hepatocytes and can proliferate and repopulate the host liver driven by local growth factors and cytokines (33).

Much remains unknown regarding the integration process of donor hepatocytes building intercellular connections with host hepatocytes, and connecting with the biliary tree and vascular system. Animal experiments have shown that hepatocytes infused into the portal vein are embolized into the terminal portal vein radicles and sinusoids (33-35), leading to

increased portal pressure and small infarcts in the liver parenchyma. Due to their size, the infused hepatocytes are usually not dislodged to the pulmonary arteries, although lung embolization was described in one patient with acute on chronic liver failure due to portocaval shunts (36, 37). The endothelium is disrupted and donor hepatocytes can be found in the parenchyma 24 hours after infusion (34). A major proportion of infused cells are cleared by macrophages within these first 24 hours. After 3–7 days, the remaining donor hepatocytes start to integrate and the liver parenchyma is remodeled. During this time period, increased expression of vascular endothelial growth factor (VEGF) and matrix metalloproteases are observed in the vicinity of donor hepatocytes (34, 35, 38). The major disadvantage of transplanting hepatocytes through the portal vein is the limited cell number that can be infused during a single transplantation event without causing portal thrombosis.

The spleen is also sometimes used as a transplant site, especially in liver cirrhosis patients with extensive portocaval shunts and liver scar tissue limiting donor cell engraftment. Cells can be transplanted to the spleen by infusion into the splenic artery or by direct puncture of the spleen. Interestingly, donor hepatocytes have shown long-term engraftment in the spleen and can even proliferate and repopulate the spleen. However, only a limited number of cells can be infused, and hepatocyte transplantation to the spleen carries an increased risk of bleeding and splenic infarcts (5, 39).

The peritoneal cavity is another attractive site for clinical hepatocyte transplantation. This site allows easy access and transplantation of a large number of cells during a single transplantation. It is especially useful for patients with acute liver failure who are in need of temporary support until the native liver regenerates.

Experimental animal studies have also investigated alternative transplantation sites, including the kidney capsule, subcutaneous space, intramuscular, and lymph nodes (40-44).

1.1.4 Indications for human hepatocyte transplantation

Hepatocyte transplantation has been performed experimentally for three main indications: as a bridge to liver transplantation in patients with end-stage liver disease, as treatment for patients with acute liver failure without organ transplantation, and as treatment for patients with inborn metabolic liver diseases.

Eighteen centers worldwide have reported human hepatocyte transplantations, and the literature includes reports of 100 patients undergoing this procedure: 37 patients with metabolic liver diseases, 46 with acute liver failure, and 17 with end-stage liver cirrhosis (45). In all cases, hepatocyte transplantation has been investigated in a small clinical series or is described in a case report. To date, seven centers are actively recruiting patients for this procedure: Children’s Hospital University of Pittsburgh, Pennsylvania, USA; King’s College London, Great Britain; Cliniques St-Luc Brussels, Belgium; University La Fe Hospital,

Valencia, Spain; Hannover Medical School, Germany; RiMED Foundation Palermo, Italy; and Karolinska University Hospital, Sweden.

1.1.4.1 Liver cirrhosis

Liver transplantation is the only curative treatment for patients with end-stage liver disease. Few options exist for patients who are not liver transplantation candidates or for whom an organ is not available. In end-stage liver disease patients, hepatocyte transplantation has been investigated both with the aim of providing temporary liver support as a bridge to transplantation and as a means of improving liver function and quality of life (46). In chronic liver disease, the spleen is considered the optimal hepatocyte transplantation site since the liver architecture is disturbed and portocaval shunts increase the risk of lung embolization during portal infusion. Studies in animal models of liver cirrhosis show that transplantation of hepatocytes to the spleen leads to long-term engraftment and improved serum bilirubin, plasma ammonia, encephalopathy, and overall survival (47-50).

Mito et al. performed the first human hepatocyte transplantations for liver cirrhosis. In their study, 10 patients underwent liver resection of the left lateral segment and isolated hepatocytes were autotransplanted by direct injection into the spleen. Repeated Technetium-99m labeling revealed transplanted hepatocytes in the spleen at 1 month and 6 months. However, the authors concluded that the observed improvement in encephalopathy could not be attributed to the transplant (51). Fisher and Strom described a series of 7 patients with liver cirrhosis of varying degrees and etiologies (5, 46, 52, 53). They demonstrated engraftment and long-term survival of allogenic hepatocytes in the spleen. Four patients were successfully bridged to organ transplantation, and a subset of patients showed improvements in encephalopathy and blood ammonia. However, the results were inconclusive due to the small patient number and the lack of controls.

1.1.4.2 Acute liver failure

In acute liver failure, hepatocyte transplantation is performed to provide temporary liver support to bridge patients until liver transplantation or until native liver regeneration (54). Unlike liver transplantation, hepatocyte transplantation has the advantage that it can be performed with readily available cryopreserved hepatocytes. Additionally, hepatocyte transplantation is reversible and, thus, immunosuppression can be discontinued upon recovery of the native liver. To date, 46 patients with acute liver failure of various etiologies have been treated with hepatocyte transplantation at 11 different centers worldwide (5, 36, 45, 46, 52, 53, 55-60). These results have been reported as either case reports or case series. Some patients were treated with the intention to bridge to liver transplantation, while others were not considered candidates for liver transplantation due to underlying medical contraindications. Of the hepatocyte transplanted patients, 27 patients died within 52 days, 10

patients were successfully bridged to organ transplantation, and 9 patients recovered without organ transplantation. The investigators reported improvements in encephalopathy, cerebral edema, coagulopathy, and blood ammonia after hepatocyte transplantation. These results are encouraging and are confirmed in animal experiments. However, as these trials were performed in small numbers of patients and without adequate controls, it is difficult to draw firm conclusions.

1.1.4.3 Inherited metabolic liver diseases

The most promising clinical hepatocyte transplantation results have been obtained in patients with metabolic liver diseases. Inherited metabolic liver diseases are characterized by the deficiency of a single hepatic enzyme or protein, which results in hepatic or extrahepatic disease. Replacing 5–20% of the missing enzyme is often sufficient to reverse the disease state. Preclinical and clinical experience with hepatocyte transplantation for treatment of inherited metabolic liver diseases, as well as future challenges of this procedure are summarized in the supplemental publication (9).

1.2 LIPOPROTEINS AND APOLIPOPROTEIN E

Lipoproteins are macromolecules that emulsify hydrophobic lipids, thus enabling their transportation in the hydrophilic plasma environment (61). They comprise an outer hydrophilic layer and an inner hydrophobic core. The outer layer is composed of amphiphatic phospholipids and apolipoproteins, whereas the core consists of triglycerides and cholesteryl esters (CE)(62). In addition to their structural function, apolipoproteins serve a metabolic function as cofactors of enzymes or as ligands for cell surface receptors, and can be exchanged among the lipoprotein particles (61). Apolipoproteins can be divided into five main types (A, B, C, D, and E) as well as several subtypes (e.g., A-I, A-II, A-IV; and C-I, C-II, and C-III) (63). Lipoproteins can be classified according to their density, size, or their apolipoproteins (Table 1).

	CM	VLDL	IDL	LDL	HDL
Density (g/mL)	< 0.95	0.95–1.006	1.006–1.019	1.019–1.063	1.063–1.210
Diameter (nm)	75–1200	30–80	25–35	18–25	5–12
Major apolipoproteins	B48,A-I,A-IV, E,CI,CII,CIII	B100, E, CI,CII,CIII	B100,E, CI,CII,CIII	B100	A-I,A-II,CI, CII,CIII,E

Table 1 Characteristics of plasma lipoproteins (61). Abbreviations: CM, chylomicron; HDL, high-density lipoprotein; IDL, intermediate-density lipoprotein; LDL, low-density lipoprotein; VLDL, very low- density lipoprotein.

Apolipoprotein E (ApoE) is a 34-kDa apolipoprotein mainly found on chylomicrons, very low-density lipoproteins (VLDL), and their remnants, as well as on high-density lipoproteins (HDL) (64). This apolipoprotein is mainly produced by hepatocytes but can also be expressed by other tissues and cells, with about 20% of serum ApoE produced by extrahepatic sources (65, 66). ApoE binds to the LDL receptor (LDLR) and the LDL-receptor-related protein (LRP), facilitating hepatic uptake and degradation of its associated lipoprotein (64). Humans express three different isoforms of ApoE—E2, E3, and E4—which are the product of three alleles (ε2, ε3, and ε4) that each differ by only a single amino acid substitution. Thus, six phenotypes are possible—E3/3, E4/3, E3/2, E4/4, E4/2, and E2/2—which occur with varying frequencies in different populations (67). This gene polymorphism has several functional consequences, including an influence on the level of gene product, e.g., ε2 is associated with higher ApoE serum concentrations compared to ε4 (63). The different isoforms also vary in their binding capacity to the LDLR, with E2 showing less than 2% of the capacity of E3 and E4, resulting in decreased hepatic uptake of its lipoprotein. Consequently, ε2 allele carriers exhibit slower clearance of dietary lipids from their circulation (68). Homozygosity for ε2 is strongly associated with type III hyperlipoproteinemia, which is characterized by increased triglyceride and cholesterol levels, accumulation of chylomicrons and VLDL remnants, and premature atherosclerosis (69).

1.2.1 Apolipoprotein E knock out mouse

Apolipoprotein E-deficient (−/−) mice were simultaneously developed in two different laboratories with the purpose of generating a mouse model of atherosclerosis (70, 71). Murine cholesterol metabolism substantially differs from that in humans, with mice showing lower serum cholesterol concentrations (2.2 mmol/L) that mainly comprise HDL particles. In humans, normal blood cholesterol ranges from 4–8 mmol/L and mainly comprises VLDL and LDL particles (72). Moreover, due to their rapid hepatic LDL clearance, wild-type mice do not develop atherosclerosis even with higher cholesterol ingestion and synthesis (73). However, ApoE deficiency results in a murine serum cholesterol concentration of ~13 mmol/L on chow diet and a further four-fold increase on a high-fat Western-type diet. These mice show a lipoprotein profile characterized by cholesterol accumulation in VLDL and chylomicron remnants. ApoE (−/−) mice develop spontaneous atherosclerosis on chow diet early in life, and are the most commonly used animal model in atherosclerosis-related research (74). Heterozygous ApoE (+/−) mice display normal cholesterol levels and do not develop atherosclerosis (70). ApoE replacement in the ApoE (−/−) mouse by gene therapy, bone marrow transplantation, or infusion of recombinant ApoE protein results in lipoprotein profile normalization and prevention or reversal of atherosclerosis (75-77). Mouse atherosclerosis is most commonly evaluated either by quantifying plaque cross-sectional area in aorta sections or by measuring cholesteryl ester content in the aorta as an index of lesion development (78).

2 AIMS

The general aim of the present thesis was to improve results in clinical hepatocyte transplantation. The specific aims of the studies were as follows:

I. To evaluate strategies for short-term storage of human hepatocytes before transplantation.

II. To establish a hepatocyte transplantation model in the ApoE knockout mouse using serum apolipoprotein E as a marker of graft function.

III. To study whether hepatocyte transplantation can correct hypercholesterolemia in the ApoE knockout mouse.

IV. To study the safety and efficacy of partial hepatectomy preconditioning and HLA antibody formation in clinical hepatocyte transplantation.

3 METHODOLOGY

3.1 ETHICS

All studies were approved by the regional ethics committee and informed consent from the patients and/or patient´s parents were obtained. Animal studies were approved by the ethical Committee for Animal Experiments at the Swedish Board for Agriculture.

3.2 HUMAN HEPATOCYTE ISOLATION

Human hepatocytes were isolated from liver tissue obtained from deceased donors or patients undergoing liver transplantation or liver surgery (Paper I & IV). Isolation was performed by classical three step collagenase perfusion according to Strom et al. (23). In brief, major hepatic vessels were cannulated and perfused with three different solutions. The first solution contains ethylene glycol tetraacetic acid (EGTA), an iron chelator, to remove calcium disrupting intercellular connections. The second solution consists of an electrolyte solution including a buffer to flush out the remaining EGTA as calcium is needed for proper function of collagenase. The third solution contains collagenase disrupting the extracellular matrix. In this study two different collagenases were used. For in vitro research purposes Collagenase XI (Sigma) and for clinical transplant purposes a good manufacturing practice (GMP) approved enzyme was used (CIzyme™,Vitacyte, Indianapolis). For optimal function of the collagenase the liver was placed in a water bath at 37°C. After 15 – 35 min of digestion the liver capsule was cut open and the cell suspension released into ice-cold media solution. Thereafter hepatocytes were filtered, washed and purified by low speed centrifugation. Hepatocyte viability was assessed by trypan blue exclusion test. Hepatocyte yield is expressed as the number of viable hepatocytes divided by weight of liver tissue.

3.3 CULTURE OF HUMAN HEPATOCYTES

Hepatocytes were cultured in Williams E medium supplemented with insulin (10^{-7} M), dexamethasone (10^{-7} M), and antibiotics-antimycotics. 7.5-15 x 10^5 viable hepatocytes were seeded per well of a six-well culture plate precoated with type I rat collagen (Paper I & IV). Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 : 95% air.

3.4 HEPATOCYTE CHARACTERIZATION

3.4.1 Plating efficiency

Plating efficiency measures the capability of isolated hepatocytes to adhere to an extracellular matrix. It is expressed as percentage of total cells seeded per well. 7.5 x 10^5 viable hepatocytes were seeded per well of a six-well culture plate. Two hours after plating, medium from 3 wells was exchanged with fresh medium and 3 wells were left untouched. After overnight culture wells with exchanged media were washed again to remove all unattached cells. Plating efficiency was defined as the percentage of protein content in washed wells of the protein content in unwashed wells. Protein content was quantified by Bradford assay (Bio-Rad Protein Assay kit).

3.4.2 Drug metabolism studies

Drug metabolism studies were performed to characterize hepatocyte specific function of isolated hepatocytes in suspension or after a period of culture. Evaluation of hepatocyte specific functions included ammonia metabolism assay, Cyp 450-Glo assays, 7-Ethoxyresorufin-O-Deethylase (EROD) assay, Testosterone hydroxylation assay, Resorufin conjugation.

3.4.3 Ammonia metabolism

Ammonia metabolism in hepatocytes was evaluated by direct colorimetric quantification of ammonia according to Okuda et al. (79). Hepatocytes were exposed to 1mM ammonium chloride (NH_4Cl) for 2 hours. After deproteinization with 5% Sodium Tungstate solution samples were incubated with color reagent solution (4% phenol, 0.015% Sodium Nitroprusside, 5% KOH, 28% K_2CO_3, 3% Sodium Hypochlorite) for 25 min at 37°C and read on a spectrophotometer at 560nm. Results were quantified on a standard curve and expressed as nmol/min normalized to total protein content. Ammonia metabolism was evaluated in hepatocytes in suspension as well as in culture.

3.4.4 Cyp 450-Glo

Cytochrome P450 enzyme activity for CYP 1A2, 2C9, 3A7, and 3A4 were analyzed with a commercially available assay according to the manufacturer´s instructions with minor modifications according to Gramignoli et al. (CYP-GLO™, Promega)(80). The assay is based on pro-luminescent substrates that are converted by specific CYP enzyme subtypes into a luciferin product, which can be quantified by a second reaction with added luciferase. The amount of light is proportional to CYP activity. Luminescence was read with a luminometer. Results are expressed as luminescence counting units (LCU)/min and

normalized to double-stranded DNA (dsDNA) content. DsDNA was quantified Quant-iT^{TM} PicoGreen dsDNA staining kit according to manufacturer´s instructions (Molecular Probes, Invitrogen). CYP 450-Glo was used for cells in suspension and in culture with and without induction by rifampicin and phenobarbital.

3.4.5 7-Ethoxyresorufin-O-Deethylase (EROD) assay

EROD assay measures the activity of CYP1A1/2 by the conversion of 7-ethoxyresorufin to resorufin. Resorufin is fluorescent and can be quantified on a fluorescent spectrometer. Hepatocytes in culture or in suspension were incubated with 7-ethoxyresorufin for 1 hour at 37°C. Additionally, to study CYP induction hepatocytes in culture cells were treated for three days with beta-naphthoflavone (BNF) or vehicle control (DMSO). After incubation media was collected and read on a fluorescent spectrometer at excitation wavelength 535 nm and emission wavelength of 581 nm. Concentrations were extrapolated from a standard curve and expressed as picomole per min normalized to total protein content.

3.4.6 Resorufin conjugation

Phase II activity was assessed by the conjugation of resorufin. Resorufin was added to hepatocytes in suspension and culture for 30min. Media and cells were collected and analyzed with the same conditions described for the EROD assay. Resorufin was quantified by the decrease in the fluorescent signal interpolated from a standard curve expressed as picomole per min normalized to total protein content.

3.4.7 Apoptosis analysis

3.4.7.1 Caspase 3/7 Activity

Caspase 3/7 Activity was evaluated with a luminescent, commercially available assay according to the manufacturer´s instructions (Caspase-Glo 3/7, Promega). Hepatocytes in suspension were incubated with Caspase-Glo reagent for 30 min at room temperature. Luminescence was read with a luminometer and results expressed as LCU/min normalized to a million of viable hepatocytes.

3.4.7.2 Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) Staining

TUNEL staining was performed on liver tissue and isolated hepatocytes. ApopTag® Peroxidase In Situ Apoptosis Detection Kit was used according to manufacturer´s instructions (Millipore). TUNEL staining detects DNA fragmentation, a result of apoptosis,

by using the enzyme terminal deoxynucleotidyl transferase (TdT) adding labeled Deoxyuridine triphosphate (dUTP) to nicks in the DNA. After TUNEL staining slides were counterstained with hematoxylin and evaluated under the microscope. Results are expressed as percentage of TUNEL-positive hepatocytes of the total number of hepatocytes.

3.4.8 Immunocytochemistry

Immunocytochemistry was performed on smears of hepatocytes to quantify contamination of the hepatocyte graft with non-parenchymal cells. Cell smears were prepared using standard cytology techniques. Slides were postfixed with 4% formaldehyde. All reactions were performed on an automated Leica Bond-III immunostainer. The following primary antibodies were used: CD45 (hematopoietic cell marker), CD31 (marker of endothelial cells), CK18 (marker of hepatocytes), and CD68 (macrophages/Kupffer cells). Quantification was performed under a light microscope by counting more than 800 cells on each slide.

3.5 ANIMALS

For hepatocyte transplantation experiments female C57bl/6J mice were used as hepatocyte donors and female apoE knock out mice on a C57bl/6 background (apoE -/-; B6.129P2-Apoetm1 Unc N11) were used as recipients (Paper II and III). Animals received normal chow diet if not specified otherwise. Blood samples were taken from the tail vein under isoflurane anesthesia. Animals were euthanized under isoflurane anesthesia by blood collection from the inferior vena cava and organ removal.

3.6 MOUSE HEPATOCYTE ISOLATION

In contrast to human hepatocyte isolation mouse hepatocyte isolation was performed in situ. The inferior vena cava or the portal vein was used for perfusion of the liver. Perfusion solutions were the same as for human hepatocyte isolation except that collagenase IV (Sigma) was used instead. After digestion cells were filtered and washed by low speed centrifugation.

3.7 MOUSE HEPATOCYTE TRANSPLANTATION

Mouse hepatocytes were resuspended in saline solution containing dextran and heparin. 1 – 2 million hepatocyte resuspended in 100-200 µl solution were injected into the spleen or directly into the liver.

3.8 APOE ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

A sandwich ELISA was established to quantify mouse ApoE (Paper II &III). 96 well plates were incubated over night with capture antibody (polyclonal goat anti-ApoE, Calbiochem 178479). Plates were incubated with diluted mouse serum samples. After washing plates were incubated with detection antibody (polyclonal rabbit anti-ApoE, PA1- 46367, Thermo Scientific Pierce Products). As secondary antibody ALP conjugated goat anti-rabbit IgG was used. ALP substrate pNPP (p-nitrophenyl-phosphate) was added before absorbance was measured at 405 nm. ApoE concentration was extrapolated from a standard curve with serial dilutions of wild type serum. Results are expressed as percentage of wild type.

3.9 APOE GENE EXPRESSION ANALYSIS

Quantitative reverse transcription polymerase chain reaction (RT-PCR) was used to quantify mouse ApoE gene expression in various organs after transplantation. Tissue was homogenized and total RNA extracted with Trizol reagent. cDNA was synthesized using reverse transcriptase and random hexamer primers. Quantitative PCR was performed with TaqMan technique with mouse ApoE and mouse cyclophilin specific primers spanning over exon-exon junction. ApoE levels were normalized to cyclophilin expression and expressed as percentage of wild type expression.

3.10 LIPID ANALYSIS

Cholesterol content in serum lipoprotein fractions was analyzed by size exclusion chromatography according to Parini et al. (81). Serum lipoproteins were separated over a Superose gel column using a pump. The separated lipoproteins were then mixed with cholesterol detecting reagents (Roche/Hitachi Diagnostic) and absorbance was measured at 500nm. Data were collected every 20 seconds. Results were either presented as lipoprotein profiles with arbitrary values or the area under the curve was used to calculate the cholesterol concentration expressed as mmol/L.

3.11 APOE RNA IN SITU HYBRIDIZATION

RNA in situ hybridization was used to detect engrafted wild type hepatocytes in ApoE (-/-) liver. RNA in situ hybridization uses complementary labeled RNA probes to detect mouse ApoE mRNA. In this thesis OCT mounted liver cryosections from transplanted ApoE (-/-) mice and controls were evaluated with RNAscope® assay with a customized mouse ApoE probe (ID#313271, Advanced Cell Diagnostics, Hayward, CA) according to manufacturer´s instructions. Detection was performed by the conversion of DAB (3,3'-Diaminobenzidine) by peroxidase labeled probes resulting in a brown color.

3.12 HUMAN HEPATOCYTE TRANSPLANTATION

Human hepatocytes were transplanted by infusion into the portal vein. Access to the portal vein was established by open cannulation of the umbilical vein or a mesenteric vein. The tip of an intravascular catheter was advanced to the bifurcation of the main portal vein. Correct position was verified by fluoroscopy with injection of intravenous contrast. To induce liver regeneration patients underwent resection of liver segments 2 and 3 before infusion of hepatocytes. Liver resection was only performed with the first hepatocyte transplantation. To minimize surgical injury the resection line was placed 1 cm left of the falciform ligament and hilus structures were left untouched. Liver parenchyma was transected by cavitron ultrasonic surgical aspirator. Blood vessels and bile ducts were divided along the transection lane. In this study only fresh and not cryopreserved hepatocytes were used. Hepatocytes were infused in an electrolyte solution with addition of 4% human albumin at a concentration of 1-2x 10^7 hepatocytes/ml. Infusion was performed with a pump at a flow rate of 1-3ml/min. During the infusion portal pressure was continuously measured and flow rate was adjusted accordingly. Heparin was given with the hepatocyte graft and thereafter intravenously to obtain an activated partial thromboplastin time (aPTT) of 70 – 140s. Doppler ultrasound was performed regularly after transplantation to rule out portal thrombosis. Immunosuppression consisted of 20mg basiliximab on day 0 and 4 and 500 mg of i.v. methylprednisolone on day 0. Thereafter steroids were tapered to 5mg prednisolone daily. Tacrolimus was given to maintain trough concentrations of 10-13ng/ml the first month and 6-8ng/ml thereafter.

3.13 IMMUNOLOGICAL INVESTIGATION

3.13.1.1 Evaluation of HLA antibodies

Complement-dependent cytotoxicity- (CDC) and flow cytometry crossmatch (FACS) were performed by the routine clinical immunology laboratory before each hepatocyte transplantation. Patients were evaluated before and every 3-4 months after hepatocyte transplantation for HLA-antibodies using Luminex-based LABScreen® PRA and Single Antigen assay (One Lambda, Inc.). Detection of complement binding anti-HLA antibodies was performed by C1q assay with single-antigen flow bead assays (C1qScreenTM, one Lambda). Reactivity was normalized for background and expressed as mean fluorescence intensity (MFI). MFI values above 1000 were considered positive.

3.13.1.2 Evaluation for autoantibodies

Patient serum was analyzed by the clinical immunology laboratory for presence of the following autoantibodies: Antinuclear antibodies (ANA) against dsDNA, centromere B, nucleosomes, Jo1, Ribosomal P, RNP 68, RNP A, Scl-70, Sm, SmRNP, SS-A52, SS-A60, SS-B were analyzed by indirect immunofluorescence assay (Immunoconcepts) and multiplex ANA assay (BioPlex2200, BioRad). Anti-mitochondrial and Liver specific autoantibodies

against SLA/LP, M2, M2-3E, gp210, Sp100, PML, RO52, LKM-1, and LC-1 were analyzed by line immunoassay (Euroimmune). Anti-smooth muscle antibodies were evaluated by indirect immunofluorescence assay (Kallestad, BioRad).

3.13.1.3 Evaluation of UGT1A1 antibodies

Patient serum was analyzed for the presence of antibodies against UGT1A1 by ELISA according to Seppen et al (82)

3.14 ANALYSIS OF BILIRUBIN CONJUGATES IN BILE

Bile samples were collected from the patient before hepatocyte transplantation by direct puncture of the gallbladder during surgery. After transplantation duodenal bile was collected by a nasal duodenal tube and stimulation of bile flow by a meal containing high content of protein and fat. Bilirubin conjugates were quantified by HPLC according to Seppen et al (83). Bilirubin and bilirubin conjugates were separated on a HPLC column (84). Absorbance of the eluate was measured at 450 nm. The identity of the peaks was confirmed by examining the spectra. The percentage of bilirubin conjugates was calculated by the area under the curve.

3.15 ANALYSIS OF GROWTH FACTORS AND CYTOKINES

Serum concentration of hepatocyte growth factor (HGF) was quantified by ELISA (HGF Quantikine). Serum epidermal growth factor (EGF), tumor necrosis factor alpha (TNF-alpha) and interleukin 6 (IL-6) were analyzed by Luminex (Human cytokine magnetic kit, Merck Millipore).

4 RESULTS AND DISCUSSION

4.1 PAPER I

At most centers performing hepatocyte transplantation, the hepatocytes are cold stored in a preservation solution after isolation until transplantation. This period commonly lasts 1–3 days, as only a limited number of hepatocytes can be infused during one transplantation event due to the increased portal pressure and the risk of portal thrombosis. The method of cold storage in UW solution is adopted from preservation of organs for transplantation and has its limitations as hepatocytes undergo apoptosis due to loss of cell anchorage (85). Thus, we hypothesized that hepatocyte viability would be better preserved by storing several pieces of liver tissue and performing repeated hepatocyte isolations rather than storing isolated hepatocytes.

In the current study, we compared traditional cold storage of isolated hepatocytes in UW solution to storage of liver tissue with repeated hepatocyte isolations on hepatocyte viability and function. Liver tissue was divided into two pieces (Fig 1, Paper I). From one piece hepatocytes were isolated directly. These hepatocytes were either characterized immediately (Fresh) or analyzed after 48h cold storage in UW (UW cells). The other liver tissue piece was stored in UW and hepatocytes were isolated and analyzed after 48h (UW tissue cells).

Our results show that hepatocyte viability, plating efficiency and CYP activity decreases during cold storage of isolated hepatocytes whereas these hepatocyte specific functions are better preserved in UW tissue cells. We found significantly higher viability and plating efficiency in UW tissue cells compared to UW cells. The UW cells and UW tissue cells did not significantly differ with regard to CYP activity, phase II activity, or ammonia metabolism. However, apoptosis analysis revealed significantly higher TUNEL staining and Caspase 3/7 activity in UW cells compared to UW tissue cells.

This study confirms previous evidence that cold storage of isolated hepatocytes decreases viability and function. Our results further suggest the storage of liver tissue as a simple method of improving hepatocyte viability and function during cold storage for clinical transplantations. Based on these findings, we changed our protocol for clinical hepatocyte transplantation. We currently schedule our isolations to minimize hepatocyte suspension time, and perform repeated isolations when the size and anatomy of the liver tissue is suitable. Repeated isolations involve additional cost and labor, but this is compensated by improved cell quality. Although not shown in this study the total yield is not decreased by this procedure.

This study has several limitations. The tissue used for hepatocyte isolation does not meet the criteria for clinical use, as a majority of the donors were older, showed higher degrees of steatosis, and had received neoadjuvant chemotherapy—which are all factors that affect

hepatocyte quality after isolation (86-88). Furthermore, additional analysis after 1 day and 3 days would have been preferable, but the number of analysis time-points was limited by the tissue sample sizes. Finally, our study does not address cryopreservation as an alternative method of hepatocyte storage. Cryopreservation has the advantage of enabling long-term storage, thus allowing more extensive quality testing and scheduled transplantations. However, recent experiences from other centers indicate that cryopreservation and thawing cause severe hepatocyte damage, substantially reducing viability and function (27, 89, 90).

4.2 PAPER II

This study aimed to establish a hepatocyte transplantation model in the ApoE knockout mouse, with the long-term goal of using this model to study different pretreatment modalities to improve donor hepatocyte engraftment and liver repopulation. The ApoE knockout mouse was selected because ApoE is mainly produced by hepatocytes. The mouse model is a prototype of a metabolic liver disease in which deficiency of a single liver-specific protein results in extrahepatic disease (74). It has been used extensively in cardiovascular research and is a very well-characterized model. We hypothesized that serum ApoE concentration, hepatic ApoE expression, and changes in hypercholesterolemia and lipoprotein profiles could be used as graft function markers. We used this model to evaluate whether early inflammatory injury—a recognized factor for graft loss during cell transplantation—could be modified by peritransplant treatment with methylprednisolone, anakinra, or a combination of both (91-94).

Wild-type hepatocytes isolated from C57bl/6 mice were transplanted into ApoE knockout mice by infusion into the spleen. All animals displayed donor hepatocyte engraftment as measured by ELISA, and hepatic ApoE mRNA expression at 1–2% of wild-type levels. Hepatic ApoE mRNA expression correlated with serum ApoE measurements. We detected no difference between the different treatment groups: control, methylprednisolone, anakinra, and anakinra + methylprednisolone. In all groups a small decrease in VLDL cholesterol was observed, but lipoprotein profiles remained unchanged with accumulation of cholesterol in VLDL and LDL particles.

These results showed that serum ApoE can be used as a marker for donor hepatocyte engraftment. Furthermore, the system was shown to be very sensitive as ELISA detected serum ApoE levels of less than 0.1%. Our findings also have implications for clinical hepatocyte transplantation. A reliable marker of graft function could substantially improve clinical hepatocyte transplantation. Moreover, as there are three different isoforms of human ApoE, ApoE isoform-specific detection could be used in case of a mismatch between donor and recipient.

4.3 PAPER III

In study II, the observed engraftment rates were low (1–2%) as no strong pretreatment was used. Although we detected a slight decrease in VLDL cholesterol, hypercholesterolemia and lipoprotein profiles were not corrected. In study III, we evaluate whether higher engraftment rates would lead to hypercholesterolemia normalization. ApoE (−/−) mice were pretreated with retrorsine to give the donor hepatocytes a selective growth advantage. Retrorsine is an alkylating agent that induces DNA damage, resulting in long-lasting inhibition of hepatocyte cell division. It has been shown to give donor hepatocytes a growth advantage in hepatocyte transplantation in rats; however, its efficacy in mice has been questioned in previous studies (95). In study III, ApoE (−/−) mice received three intraperitoneal injections of 70 mg/kg retrorsine 1 week apart. After 6 weeks, the mice underwent partial hepatectomy followed by splenic infusion of wild-type hepatocytes. Control mice were sham transplanted or transplanted without retrorsine pretreatment. Animals were monitored weekly with blood sampling, and sacrificed 12 weeks after transplantation.

All retrorsine-pretreated mice showed a continuous increase of serum ApoE levels, reaching 3.5–55% of wild-type levels after 12 weeks, suggesting donor hepatocyte proliferation. Hepatic ApoE mRNA expression correlated with serum ApoE concentrations. In situ hybridization of liver cryosections revealed a high degree of donor cell repopulation in multiple clusters throughout the liver. All retrorsine-treated mice showed continuous improvements in lipoprotein profiles and hypercholesterolemia. After 5–7 weeks, hypercholesterolemia and lipoprotein profiles were normalized in retrorsine-treated animals, except that HDL cholesterol remained significantly lower compared to wild-type mice. We found significantly lower cholesteryl ester in the thoracic aorta among retrorsine-pretreated animals compared to controls, suggesting that hepatocyte transplantation also prevented atherosclerosis.

Paper III shows that hepatocyte transplantation can cure the metabolic disease in ApoE (−/−) mice. Our findings confirm that retrorsine is effective in mice for increasing repopulation of recipient liver. Interestingly, a serum ApoE concentration of 3.5% was sufficient to normalize hypercholesterolemia.

4.4 PAPER IV

The aim of study IV was to establish a clinical hepatocyte transplantation program at the Karolinska University Hospital to evaluate the safety and feasibility of partial hepatectomy preconditioning. We also monitored the immune response to the hepatocyte graft via serum analysis for HLA antibodies.

Two patients with Crigler-Najjar type I were evaluated at our center. Patient 1 was a 13-year-old male of Middle Eastern descent, and Patient 2 was an 11-year-old female of mixed European and Latin American descent. Both patients had been diagnosed based on elevated

unconjugated serum bilirubin, identification of a *UGT1A1* mutation, lack of bilirubin glucuronides in bile, and non-responsiveness to phenobarbital treatment. Each patient had received daily phototherapy of 7–10 hours since birth. At presentation, their serum bilirubin concentration ranged from 400–450 μmol/L. These patients were evaluated and scheduled for hepatocyte transplantation after receiving informed consent from both the patients and their parents.

Hepatocytes were isolated from liver tissue obtained from deceased organ donors at the Livercell laboratory VECURA, Karolinska University Hospital. The isolated hepatocytes showed excellent viability and function, as determined by trypan blue staining and analysis of Cyp P450 enzyme activity. Portal vein catheters were introduced by cannulation of the umbilical vein or a mesenteric vein. Before the first hepatocyte infusion, resection of liver segments 2 and 3 was performed to induce liver regeneration and thus repopulation with donor hepatocytes. Liver resection was only performed with the first transplantation in each patient.

In both cases, liver resection and hepatocyte transplantation were uncomplicated. Portal pressure increased during infusion, but repeated Doppler ultrasound of the liver revealed no signs of portal thrombosis. Patient 1 underwent two transplantations from two different donors, receiving a total of 11.5×10^9 viable hepatocytes corresponding to 4.2% of the theoretical liver mass. Patient 2 underwent one transplantation from one donor, receiving a total of 5.3×10^9 viable hepatocytes corresponding to 3.3% of the total liver mass. After transplantation, both patients exhibited an initial increase of serum bilirubin, followed by a decline to 50% of pretransplant concentrations. Graft function was further verified by detection of bilirubin diglucuronides in bile.

Patient 1 experienced two serious adverse events. At day 7 after transplantation, he presented with mycophenolate intoxication with abdominal pain, vomiting, and diarrhea. Then at 212 days after transplantation, he presented with crusted scabies (norvegica), a severe form of scabies seen in immunosuppressed patients. Patient 2 experienced no serious adverse events. Serum analysis for growth factors and cytokines revealed increased levels of hepatocyte growth factor (HGF) and epidermal growth factor (EGF) after liver resection and hepatocyte transplantation, suggesting that liver resection induced expression of mitogenic signals. Interestingly, Patient 1 also exhibited a temporal increase of HGF after the second transplantation without liver resection.

Immunological evaluation of both patients before transplantation showed no HLA antibodies. Before transplantation, complement-dependent cytotoxicity and FACS cross-match was found to be negative for all donors. Every 3–4 months after transplantation, each patient was monitored for HLA antibodies using a Luminex-based panel-reactive antigen (PRA) and a Single Antigen assay. At day 342 after transplantation, Patient 1 exhibited high levels of HLA antibodies, a subset of which were donor specific. This antibody formation was induced by discontinuation of immunosuppression due to recurring scabies infection. Patient 2 showed high levels of HLA antibodies on day 670 while receiving adequate

immunosuppressive therapy. In both patients, HLA antibodies development was temporally associated with graft loss as determined by increase in total serum bilirubin.

This study presents two novel observations. Firstly, we demonstrate the safe application of partial hepatectomy to improve liver repopulation with donor hepatocytes—a method that has been extensively used in animal models to improve engraftment. Secondly, it is the first report of de novo donor-specific HLA antibodies after hepatocyte transplantation in association with graft loss. The obvious limitation of this study is that only two patients are evaluated. Inborn metabolic liver diseases suitable for hepatocyte transplantation are rare and, to date, only 10 patients have received hepatocyte transplantation for Crigler-Najjar type I. Thus, this report represents an important contribution to the scant literature regarding clinical hepatocyte transplantation. Furthermore, although we note an association between DSA emergence and graft loss, no causal association can be determined since we were unable to perform histopathological evaluation of liver biopsies, which may have shown antibody-mediated rejection. Nevertheless, HLA antibodies have not been investigated and pre-transplant cross-matches are not routinely performed in clinical hepatocyte transplantation. Our study emphasizes the need for additional studies to elucidate the role of HLA antibodies in human hepatocyte transplantation.

CONCLUDING REMARKS AND FUTURE CHALLENGES

In conclusion, hepatocyte transplantation is still a technology under development. Results presented in this thesis have shed light on some limitations and possible solutions to improve outcome in human hepatocyte transplantation:

I. Short-term cold storage of liver tissue with repeated hepatocyte isolations preserves hepatocyte viability and function better than cold storage of isolated hepatocytes.

Paper I identifies hepatocyte suspension time as a potentially important factor influencing hepatocyte quality. In this study, only one time-point could be investigated. Additional studies have been started with the aim of describing the decline of hepatocyte viability and function over time, as well as the actual impact on post-transplantation outcome. The broader goal of this and future studies is to increase the quality and number of hepatocytes available for transplantation.

Another means of accomplishing this goal is by improving the hepatocyte isolation process. Although hepatocyte isolation techniques have advanced over the years since their introduction, the current procedure is still based on the original principles. Isolation success as determined by viability and yield varies tremendously from day to day, collagenase to collagenase, and liver to liver. During harvest and isolation, liver tissue is subjected to several periods of cooling and reheating, which negatively impacts hepatocyte quality. The use of a suitable enzyme or other method of digestion that could allow temperature to remain constant from liver donation throughout isolation and transplantation would certainly improve outcome.

Another challenge is the limited pool of donor tissue. In our clinical program, we addressed this issue by splitting donor liver tissue and using the left lateral segment for hepatocyte isolation and the extended right lobe for liver transplantation. We are currently investigating whether neonatal donors can be used as an alternative source that would not compete with whole organ transplantation. Other studies from our group have also shown that liver explants from patients with metabolic liver diseases undergoing liver transplantation can be used as “domino donors” that yield a new source of good quality hepatocytes (96). Current and planned research further aims to evaluate alternative cell sources of hepatocyte-like cells, e.g., progenitor cells, amniotic epithelial cells, induced pluripotent stem cells, and embryonic stem cells (97, 98).

II. Serum ApoE is a sensitive marker of engrafted wild-type hepatocytes in the ApoE knockout mouse.

III. Hepatocyte transplantation with retrorsine pretreatment in ApoE knockout mice results in robust liver repopulation, normalization of cholesterol metabolism, and atherosclerosis prevention.

Clinically relevant animal models are an invaluable tool for studies of hepatocyte engraftment, cell loss during transplantation, and techniques to improve engraftment and long-term function of transplanted hepatocytes. Papers II and III illustrate the stem cell-like potential of adult hepatocytes. In physiological liver regeneration, damaged liver mass is regenerated by proliferation of adult hepatocytes, not stem or progenitor cells (10, 97, 99, 100). Due to their tremendous proliferative potential, transplanted hepatocytes with a growth advantage have the ability to repopulate the entire recipient liver. In animal models, such a growth advantage can exist due to an underlying disease—as seen in the fumarylacetoacetate hydrolase (FAH)-deficient mouse—or can be induced by chemical or radiation pretreatment that blocks the proliferative potential of the native hepatocytes (13, 101-104). Successful clinical translation of this concept will revolutionize clinical hepatocyte transplantation, potentially leading to hepatocyte transplantation replacing liver transplantation for the treatment of metabolic liver diseases. Clinical trials have already started to evaluate preconditioning with local hepatic irradiation, and the preliminary results are promising (105).

IV. Partial hepatectomy combined with hepatocyte transplantation is safe in patients with Crigler-Najjar type I, and induces a robust release of hepatocyte growth factor. HLA immunization occurs after hepatocyte transplantation, and donor-specific HLA antibodies might be involved in hepatocyte allograft rejection.

Paper IV describes the first clinical application of partial hepatectomy preconditioning, a technique that improves hepatocyte engraftment and repopulation in animal models (106-111). We show that partial hepatectomy induces a robust release of hepatocyte growth factor, and that transplantation of less than 5% of the liver mass induces a strong clinical effect. However, additional patients must be evaluated before conclusions can be drawn regarding the efficacy of this procedure compared to traditional hepatocyte transplantation.

Paper IV also addresses HLA immunization, with the results suggesting a possible impact of donor-specific HLA antibodies on hepatocyte allograft rejection. HLA antibodies are associated with inferior outcome following kidney, heart, liver, and islet transplantation, but their role has not previously been addressed in the context of hepatocyte transplantation. HLA antibody screening or pretransplant cross-matches are rarely reported for hepatocyte

transplantations, and there is no consensus on how to manage a pretransplant positive cross-match, as the relevance of preexisting or de novo HLA antibodies to the hepatocyte allograft is not known.

Since only a limited number of clinical hepatocyte transplantations have been performed to date, we plan to conduct a retrospective multicenter study to screen for HLA antibodies in stored serum samples of patients who have undergone hepatocyte transplantation. The aim of this study is to generate a comprehensive description of HLA antibody formation and of the possible associated factors—e.g., prevalence of pretransplant and de novo HLA antibodies, relation to transplantation, graft loss, HLA mismatch, numbers of donors, or immunosuppressive treatment. In addition to HLA antibodies, future prospective studies should routinely examine cellular immunity against the hepatocyte allograft. Ideally, liver biopsies should also be performed to learn more about the mechanisms of hepatocyte allograft rejection, which could potentially lead to improved immunosuppression protocols in clinical hepatocyte transplantation.

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