Ischemia – reperfusion injury in human liver transplantation

Mechanisms and effects on graft function

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ISBN 978-952-92-4668-7 (paperback)
ISBN 978-952-10-5083-1 (PDF)
http://ethesis.helsinki.fi
Helsinki 2008, Yliopistopaino

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MINNA ILMAKUNNAS

Academic dissertation

To be presented, with the permission of the Faculty of Medicine, University of Helsinki, for public examination in the Faltin hall, Surgical Hospital, Kasarmikatu 11–13, Helsinki, on December 20th, 2008, at 12 noon.

HELSINKI 2008

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Abstract

Liver transplantation is an established therapy for both acute and chronic liver failure. Despite excellent long-term outcome, graft dysfunction remains a problem affecting up to 15-30% of the recipients. The etiology of dysfunction is multifactorial, with ischemia-reperfusion injury regarded as one of the most important contributors.

This thesis focuses on the inflammatory response during graft procurement and reperfusion in liver transplantation in adults. Activation of protein C was examined as a potential endogenous anti-inflammatory mechanism. The effects of inflammatory responses on graft function and outcome were investigated.

Seventy adult patients undergoing liver transplantation in Helsinki University Central Hospital, and 50 multiorgan donors, were studied. Blood samples from the portal and the hepatic veins were drawn before graft procurement and at several time points during graft reperfusion to assess changes within the liver. Liver biopsies were taken before graft preservation and after reperfusion. Neutrophil and monocyte CD11b and L-selectin expression were analysed by flow cytometry. Plasma TNF-α, IL-6, IL-8, sICAM-1, and HMGB1 were determined by ELISA and Western-blotting. HMGB1 immunohistochemistry was performed on liver tissue specimens. Plasma protein C and activated protein C were determined by an enzyme-capture assay.

Hepatic IL-8 release during graft procurement was associated with subsequent graft dysfunction, biliary in particular, in the recipient. Biliary marker levels increased only 5–7 days after transplantation. Thus, donor inflammatory response appears to influence recipient liver function with relatively long-lasting effects.

Hepatic phagocyte activation and sequestration, with concomitant HMGB1 release, occurred during reperfusion. Neither phagocyte activation nor plasma cytokines correlated with postoperative graft function. Thus, activation of the inflammatory responses within the liver during reperfusion may be of minor clinical significance. However, HMGB1 was released from hepatocytes and were also correlated with postoperative transaminase levels. Accordingly, HMGB1 appears to be a marker of hepatocellular injury.

During reperfusion, marked hepatic protein C uptake occurred without APC release from the graft. Protein C uptake correlated with decreased hepatic phagocyte activation. The results likely reflected intrahepatic APC production and consumption in anticoagulant and/or anti-inflammatory activity.

In human liver transplantation, hepatic inflammatory responses in the donor, but not those occurring during graft reperfusion in the recipient, appear to be associated with graft dysfunction in the recipient. Activation of protein C may attenuate hepatic inflammatory responses during reperfusion.

Original Publications

I Ilmakunnas M, Höckerstedt K, Mäkisalo H, Siitonen S, Repo H, Pesonen EJ. Hepatic IL-8 release during graft procurement is associated with impaired graft function after human liver transplantation. Submitted 2008.

II Ilmakunnas M, Petäjä J, Höckerstedt K, Mäkisalo H, Fernandez JA, Griffin JH, Jansson S-E, Repo H, Pesonen EJ. Activation of protein C during reperfusion in clinical liver transplantation. *Transplantation* 2003;75: 467–472.

III Ilmakunnas M, Pesonen EJ, Höckerstedt K, Mäkisalo H, Fernandez JA, Griffin JH, Repo H, Siitonen S, Petäjä J. Graft protein C entrapment is associated with reduced phagocyte activation during reperfusion in human liver transplantation. *Crit Care Med* 2006;34:426–32.

IV Ilmakunnas M, Tukiainen EM, Rouhiainen A, Rauvala H, Arola J, Nordin A, Mäkisalo H, Höckerstedt K, Isoniemi H. HMGB1 as a marker of hepatocellular injury in human liver transplantation. *Liver Transpl* 2008;14:1517–1525.

In the text, the original publications are referred to by their roman numerals. The articles are reproduced with the kind permission from the copyright holders. Additionally, some unpublished data are presented.

Abbreviations

ALF, acute liver failure
ALP, alkaline phosphatase
ALT, alanine aminotransferase
AP-1, activator protein-1
APC, activated protein C
AST, aspartate aminotransferase
ATP, adenosine triphosphate
BMI, body mass index
CD, cluster of differentiation
CIT, cold ischemic time
CRP, C-reactive protein
DAMPs, damage-associated molecular patterns
DMSO, dimethyl sulfoxide
EDTA, ethylenediaminetetraacetic acid
ELISA, enzyme linked immunosorbent assay
ELTR, European liver transplant registry
EPCR, endothelial protein C receptor
FACS, fluorescence-activated cell sorting
FITC, fluorescein isothiocyanate
GGT, γ-glutamyl transferase
HAT, hepatic artery thrombosis
HBV, hepatitis B virus
HCC, hepatocellular carcinoma
HCV, hepatitis C virus
HIV, human immunodeficiency virus
HMGB1, high mobility group box 1
HSPs, heat shock proteins
HUCH, Helsinki University Central Hospital
I/R, ischemia-reperfusion
ICAM-1, intercellular adhesion molecule-1
sICAM-1, soluble intercellular adhesion molecule-1
ICH, intracranial hemorrhage
ICU, intensive care unit
ICU stay, intensive care unit length of stay
IL, interleukin
INR, international normalized ratio

IQR, interquartile range
LPS, lipopolysaccharide
MCP-1, monocyte chemotactic protein-1
MELD, Model for End-Stage Liver Disease
MHC, major histocompatibility complex
MMF, mycophenolate mofetil
MMPs, matrix metalloproteinases
MTT, methylthiazolyldiphenyl-tetrazolium bromide
NADPH, nicotinamide adenine dinucleotide phosphate
NF-κB, nuclear factor kappa B
NK cells, natural killer cells
NKT cells, T-cell expressing NK molecules
NO, nitric oxide
PAF, platelet-activating factor
PAMP, pathogen-associated molecular pattern
PAR-1, protease activated receptor-1
PBC, primary biliary cirrhosis
PBS, phosphate buffered saline
PDF, primary dysfunction
PE, phycoerythrin
PMNs, polymorphonuclear neutrophils
PNF, primary non-function
PRRs, pattern-recognition receptors
PSC, primary sclerosing cholangitis
PSGL-1, P-selectin glycoprotein ligand-1
PT, prothrombin time
RAGE, receptor for advanced glycation end-products
RFU, relative fluorescence unit
ROS, reactive oxygen species
SDS-PAGE, sodiumdodecylsulphate-polyacrylamide gel electrophoresis
SOD, superoxide dismutase
TF, tissue factor
TM, thrombomodulin
TNF-α, tumor necrosis factor- α
TLR, toll-like receptor
UNOS, United Network for Organ Sharing
UW, University of Wisconsin organ preservation solution
VCAM-1, vascular adhesion molecule-1

Introduction

During the past 40 years, liver transplantation has evolved from a highly experimental procedure into the treatment of choice for acute liver failure (ALF), end-stage liver disease, and liver tumors of limited size and number. The first human liver transplantation was performed in 1963 in Denver, USA, by Thomas Starzl (Starzl *et al.* 1963). In Europe it was pioneered by Sir Roy Calne, who in 1968 performed the first liver transplantation in Cambridge, UK. Finland set up a liver transplantation program in 1982; the first Nordic country to do so. The number of transplanted patients has since increased rapidly. To date, over 75,000 livers have been transplanted in Europe (European Liver Transplant Registry, ELTR, 2007), with 725 livers transplanted into 658 patients (by July 2008) in Helsinki University Central Hospital (HUCH), the only transplant centre in Finland.

Patient outcome has improved along with advances in patient selection, organ preservation, surgical techniques, perioperative anesthetic and intensive care, infection control, and immunosuppression. Currently in Europe, the overall 1-year survival is 82% and 10-year survival 61%, with the best outcome in recipients with chronic liver disease (ELTR 2007). During the first postoperative year, recipients who had ALF have the worst outcome, whereas long-term survival is unfavorable for those who had malignancy (ELTR 2007). From 1982 to the present time, the overall 1-year survival in Finland is 89% and 10-year survival 72%, and currently the results for ALF are equal to those for chronic liver disease (Isoniemi, personal communication). As the survival has improved, emphasis has shifted to detection and treatment of long-term complications, such as late allograft dysfunction, and medical problems related to immunosuppression. However, graft function early after transplantation still continues to influence significantly the long-term outcome.

Several events at the time of transplantation can influence initial graft function and thereby the outcome. The liver may suffer damage already in the donor by prepreservation injury. Brain death and the following period of intensive care induce alterations in hemodynamic and metabolic regulation, and also inflammatory responses. These factors contribute to liver viability (Pratschke *et al.* 1999, Pratschke *et al.* 2004). Cold preservation of the graft leads to impaired cellular metabolism, non-parenchymal cell injury, and disturbances in microcirculation. During implantation into the recipient, the graft is exposed to rewarming ischemia, a period deleterious to hepatocytes. When the liver blood flow is restored, the graft sustains ischemia-reperfusion (I/R) injury, characterized by the activation of the Kupffer cells, neutrophil recruitment into the liver, tissue destruction by reactive oxygen species (ROS) and proteases, and also the amplification of the inflammatory response by cytokines (Strasberg *et al.* 2005).

In a clinical setting, the inflammatory responses and their effects on graft function remain poorly defined in both the liver donor and recipient. This study explored the hepatic inflammatory responses during graft procurement and also reperfusion. In addition, endogenous anti-inflammatory mechanisms, specifically the protein C – activated protein C (APC) pathway, were examined. Finally, the effect of these events on graft function and outcome was investigated.

Review of literature

LIVER TRANSPLANTATION

Indications

Currently, indications for liver transplantation are increasing in number, whereas absolute contraindications are becoming infrequent. Any patient with a failing liver, and who is expected to survive perioperatively and adhere to postoperative medical care, should be considered for transplantation. The main indications are cirrhosis due to various reasons, cholestatic diseases, metabolic or genetic disorders, vascular abnormalities, ALF, and primary malignant liver diseases (Mehrabi *et al.* 2008). In Europe, the most common indications in adult patients include cirrhosis 58%, cancers 12%, and cholestatic diseases 11% (including primary biliary cirrhosis, PBC; and primary sclerosing cholangitis, PSC) (ELTR 2007). In Finland, the respective figures are 22%, 9%, and 33%. In other European countries, virus related cirrhosis is the foremost cause for transplantation, whereas in Finland only 3% of transplantations are due to hepatitis B (HBV) or C (HCV). In contrast, in Finland the most common single indication is ALF (19%), followed by PBC (17%) and PSC (14%), and alcoholic cirrhosis (9%) (Isoniemi, personal communication). In children, biliary atresia is the most prevalent disease leading to liver transplantation in Finland.

The contraindications include a lack of patient compliance, continuous alcohol and substance abuse, untreatable advanced cardiovascular or pulmonary disease, extrahepatic malignancy, sepsis, and multiorgan failure (Strasberg *et al.* 1994, Markmann *et al.* 2001, Mehrabi *et al.* 2008) Although there is no formal age limit, older patients have reduced survival (Collins *et al.* 2000, ELTR 2007), and patients over 70 years of age are transplanted only in selected cases. Until recently, HIV infection was considered a contraindication, but improved survival related to antiretroviral therapy has also made HIV patients eligible for liver transplantation (Verdonk *et al.* 2007).

Organ allocation

In Finland, liver allocation is based on recipient medical urgency, ABO blood group compatibility, time on the waiting list, weight compatibility (estimated donor liver size), and donor–recipient age compatibility. There are no uniform allocation criteria in Europe, although the Model for End-Stage Liver Disease (MELD) is used increasingly (Mehrabi *et al.* 2008). Based on international normalized ratio (INR), and serum creatinine and bilirubin levels, MELD is a scoring system by which the patient mortality risk determines organ allocation, instead of the time on the waiting list (Kamath and Kim 2007).

Organ donors

In Finland, all liver grafts are obtained from brain dead multiorgan donors. Grafts are harvested in donor hospitals by an organ procurement team, which includes an anesthesiologist, from HUCH. Donor care in the intensive care unit and during organ retrieval is standardized according to national guidelines (Ohjeet elintenluovuttajan hoidosta ja elinten talteenotosta, HUCH, Department of Surgery 2004).

Donor selection. An ideal donor is under 50 years old with stable hemodynamics, normal liver and kidney function, and without malignancy or systemic infection (Fondevila and Ghobrial 2005, Mehrabi *et al.* 2008). As there is a continuously growing shortage of suitable donor organs, livers from compromised "extended criteria" donors are commonly accepted, with an increased risk of initial graft dysfunction (Strasberg *et al.* 1994, Busuttil and Tanaka 2003). Donor specific predictors of graft dysfunction include age, graft macrovesicular steatosis >30%, prolonged intensive care, hypotension with high dose vasoactive support, and acid-base and electrolyte imbalances, specifically peak sodium over 155 mmol/l (Mor *et al.* 1992, Markmann *et al.* 2001, Briceno *et al.* 2002, Cuende *et al.* 2005, Nissen and Colquhoun 2005). Importantly, all these factors have a cumulative deleterious effect on the graft (Pokorny *et al.* 2005, Fischer-Fröhlich and Lauchart 2006). Absolute contraindications for organ donation remain active malignancy and transmissible infections, such as HIV, HBV, HCV, and sepsis (Mehrabi *et al.* 2008).

Alternative graft sources. To overcome the organ shortage, livers can, in addition to the brain dead donors, be obtained from living donors or non-heart-beating donors (donation after cardiac death). Further, the liver from a cadaveric donor can be split into a right and left liver graft to be transplanted into two recipients. However, the use of these alternative grafts is associated, in addition to the risks to the living donor, with increased rate of primary dysfunction, postoperative surgical complications, and recipient mortality (Deshpande and Heaton 2006, Foster *et al.* 2007).

Donor medical management. The main objective in the donor's care is to maintain an optimal physiological environment for the organs until their procurement (Ohjeet elintenluovuttajan hoidosta ja elinten talteenotosta, HUCH, Department of Surgery 2004). Systolic blood pressure is kept above 100 mmHg with colloid and hypotonic (0.45%) saline infusions. If unresponsive to volume resuscitation, then vasoactive drugs, dopamine (2–10 µg/kg/min) as the primary choice, are initiated to treat donor hypotension. Diuresis is maintained 0.5 ml/kg/h at a minimum with fluids, and desmopressin (0.5–1 µg) administered for polyuria. Target plasma sodium level is below 145 mmol/l. Ventilator settings are adjusted to achieve normoxia and normocapnia. All donors receive 250 mg methylprednisolone at the beginning of the organ procurement operation.

Liver Procurement. After dissection of the graft and preparation of the vessels for cannulation, anticoagulation is induced by 25,000 U of heparin. After cross-clamping and cannulation of the aorta or the common iliac artery, and superior mesenteric vein, the liver is flushed *in situ* first with 1000 ml of +4°C Ringer solution via the portal vein, and then perfused with 30 ml/kg (body weight) of +4°C University of Wisconsin (UW) solution via the portal vein and approximately 50 ml/kg via the aorta. The graft is then explanted and preserved at +4°C in UW solution.

Surgery

The technique of liver transplantation varies to some extent between transplant centres. In general, the procedure involves three phases; dissection of the recipient native liver, the anhepatic phase, and finally orthotopic implantation and reperfusion of the graft.

Hepatectomy. The aim during the dissection of the native liver is to preserve enough length in the hilar structures (hepatic artery, portal vein, biliary tract) for the implantation and revascularizarion of the new liver. Most of the bleeding during surgery occurs during this period due to impaired blood clotting, portal hypertension and collaterals in cirrhotic patients, and the presence of adhesions in patients with previous abdominal surgery (Eghtesad *et al.* 2005).

Anhepatic phase. At the beginning of the anhepatic phase, the hepatic artery, portal vein, and both supra- and infrahepatic caval vein, are crossclamped. The native liver is then removed. During the anhepatic period, complete occlusion of both portal and inferior caval veins can result in considerable hemodynamic instability particularly in patients without porto-systemic collaterals. This can be overcome by veno-venous bypass or caval vein sparing (Piggyback) surgery. Both techniques are associated with reduced blood loss, improved hemodynamic stability, and protection of renal venous outflow and function (Eghtesad *et al.* 2005). In veno-venous bypass, blood is diverted from the portal and inferior caval veins (via femoral vein) to the superior caval vein (to axillary or internal jugular vein) through the extracorporeal circuit. The veno-venous bypass approach is not used in HUCH. Veno-venous bypass can be avoided by preservation of the recipient's retrohepatic caval vein (Piggyback technique). In the conventional Piggyback technique (Tsakis *et al.* 1989, Eghtesad *et al.* 2005), the liver is dissected separated from the retrohepatic caval vein, and a cuff formed from the hepatic veins is anastomosed to the donor suprahepatic caval vein end-to-side. In HUCH, a modified Piggyback (Belghiti *et al.* 1992) is performed with cavo-caval anastomosis side-to-side. In this thesis, Piggyback was used in two patients (included in analysis, studies III,IV) with significant hemodynamic instability during tentative inferior caval vein crossclamping.

Graft implantation. Supra- and infrahepatic caval vein anastomoses are performed

in an end-to-end fashion, followed by portal anastomosis. Next, hepatic arterial anastomosis is completed, the site of anastomosis depending on the arterial anatomy. Finally, the biliary tract is reconstructed. Graft cholecystectomy is performed before reconstruction of the bile ducts, which is performed as duct-to-duct anastomosis without the T-tube (Koivusalo *et al.* 1996, Scatton *et al.* 2001). In patients with PSC, the biliary tract is reconstructed by choledochojejunostomy to a Roux-en-Y intestinal limb (Eghtesad *et al.* 2005).

Ischemia and reperfusion. Graft cold preservation begins at explantation from the donor and ends when the recipient portal circulation is restored. The cold ischemic time (CIT), is kept as short as possible, preferably under 8 h (Furukawa *et al.* 1991, Briceno *et al.* 2002). During implantation, the graft is ambiently exposed to body temperature, a period of rewarming ischemia. The anhepatic time (time from clamping of the native hepatic vessels to restoration of portal circulation) reflects the duration of rewarming ischemia. Graft reperfusion comprises three phases (Figure 1). First, the liver is rinsed with 1000 ml of Ringer solution (pH 7.4, +20°C) via the portal vein, followed by flushing with 400 ml of portal venous blood. Second, these fluids are washed out through the untied infrahepatic caval vein anastomosis (caval effluent, wasted except for research samples). Third, portal and suprahepatic caval veins are declamped, and the liver is connected to the systemic circulation. Fourth, caval blood flow is restored after the completion of infrahepatic caval anastomosis and declamping, either concomitantly with or a few minutes after portal declamping. Finally, the arterial circulation is typically restored 45 minutes after initial reperfusion.

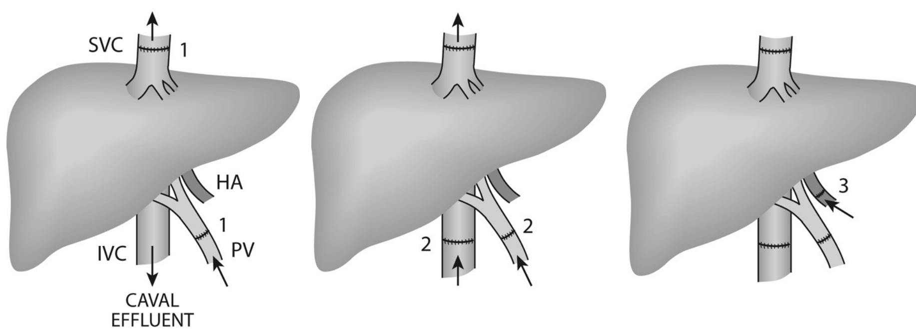


Figure 1. Phases of liver reperfusion.
1. Initial reperfusion (graft flushing with portal blood) is followed by the suprahepatic caval vein (SVC) declamping. During initial reperfusion, blood samples were drawn from the portal vein and caval effluent.
2. Declamping of the infrahepatic caval vein (IVC) and portal vein (PV). Blood samples were drawn at 5 to 10 min after portal vein declamping from the portal and hepatic veins.
3. Declamping of the hepatic artery (HA). Blood samples were drawn at 10 min after hepatic artery declamping from the portal and hepatic veins.

Immunosuppression

A calcineurin inhibitor, either cyclosporine or tacrolimus, is indicated as the the basis for immunosuppression. Both calcineurin inhibitors are associated with significant adverse effects such as nephrotoxicity, hypertension, dyslipidemia, and neurotoxicity. Both cyclosporine and in particular tacrolimus are also diabetogenic (Sethi and Stravitz 2007, Mehrabi *et al.* 2008). Corticosteroids are an integral part of the immunosuppressive regimen. They have dose-dependent side-effects, including hypertension, dyslipidemia, diabetes, and osteoporosis. Therefore tapering of the dose is usually attempted during the first year after transplantation (Verdonk *et al.* 2007, Mehrabi *et al.* 2008). In addition to calcineurin inhibitors and corticosteroids, antimetabolites, either azathioprine or mycofenolate mofetil (MMF), are frequently used. When administered concomitantly with antimetabolites, the calcineurin inhibitor dose can be reduced to limit the side-effects (Verdonk *et al.* 2007). In HUCH, liver recipients generally receive a standard triple immunosuppression with cyclosporine, MMF (azathioprine until 2004), and methylprednisolone.

Outcome

Initial graft function. Within the first days postoperation, a patient with an optimally functioning liver graft demonstrates stable hemodynamics, decreasing liver enzyme levels, improving coagulation profile, preserved renal function, and an absence of encephalopathy.

Graft dysfunction varies from a potentially reversible dysfunction to a failure necessitating urgent retransplantation. Graft dysfunction can be divided into primary dysfunction (PDF) and primary non-function (PNF) (Strasberg *et al.* 1994). Although uniform definitions for PDF are lacking, alanine aminotransferase (ALT) and/or aspartate aminotransferase (AST) levels over 1500 U/l within the first three days are usually considered to indicate PDF (Strasberg *et al.* 1994). PDF is reversible with improving graft condition, whereas recipients with PNF progressively deteriorate (Nissen and Colquhoun 2005). PNF manifests as rapidly increasing transaminase levels resulting from hepatocellular necrosis, an absence of bile production, coagulopathy, lactic acidocis, and multiorgan failure, in recipients with patent vascular anastomoses. The United Network for Organ Sharing (UNOS) proposed criteria for PNF as being: AST over 5000 U/l, and INR over 3.0 or the presence of acidocis (pH <7.3 or lactate level >2×normal) within 10 days of transplantation (Nissen and Colquhoun 2005). It is worth noting that ELTR defines PDF as a graft loss or patient death due to impaired liver function in excess of 7 days, and PNF as those same events within 7 days after transplantation. In international series, the incidence of PDF is estimated to be 15–30% and PNF 6% (Mehrabi *et al.* 2008). In contrast, the incidence on PNF is only 0.6% in Finland (Isoniemi, personal communication).

The risk factors for graft dysfunction, in addition to the extended donor criteria, include CIT >12 h (Furukawa *et al.* 1991, Briceno *et al.* 2002, Burroughs *et al.* 2006), a prolonged rewarming ischemia (Strasberg *et al.* 1994), and increased blood product requirements during transplantation (Markmann *et al.* 2003). Further, such recipient conditions as ALF, high MELD score, and a degree of renal dysfunction before transplantation predispose to PDF and PNF (Nissen and Colquhoun 2005).

Surgical complications. Vascular and biliary complications are fairly common and significantly contribute to the outcome. Hepatic artery thrombosis (HAT) is the most common vascular complication with an incidence of up to 5%, and often necessitates retransplantation. Risk factors for HAT are long CIT and surgical problems with anastomosis (Settmacher *et al.* 2000). HAT can occur either early (within 30 days of transplantation) or late, and usually presents with elevated transaminases, followed by biliary strictures, cholangitis, and septic infections (Drazan *et al.* 1996, Settmacher *et al.* 2000). Postoperative venous complications, such as portal vein stenosis and thrombosis, or hepatic outflow obstruction, are relatively rare.

The incidence of biliary complications, the most typical of which are biliary leakage and biliary tract strictures, is 10-15% (Eghtesad *et al.* 2005, Mehrabi *et al.* 2008). Anastomotic leakage occurs early, whereas strictures appear later after transplantation. Strictures can be divided into anastomotic and non-anastomotic. Anastomotic strictures are usually due to surgical difficulties and fibrous healing of the anastomosis (Verdonk *et al.* 2006). The most common reason for non-anastomotic strictures, for both hilar and intrahepatic ducts, is ischemia of the biliary epithelium caused by impaired arterial blood flow, HAT, or I/R injury (Sanchez-Urdazpal *et al.* 1992, Buis *et al.* 2006). Elevations in bilirubin levels and canalicular enzyme levels and cholangitis episodes are characteristic of biliary strictures (Eghtesad *et al.* 2005).

Long term outcome. During the period of this study (registry data over the 2000–2005 period), the patient survival at 1-year was 94% and at 5-years 87% (Isoniemi, personal communication). Late complications can be either related to graft function, or metabolic derangements. Acute rejection is characterized by lymphocytic infiltration in the portal tracts, and is typically diagnosed within 30 days. However, it can occur any time after transplantation. The incidence of acute rejection varies from 20% to 60% (Fisher *et al.* 1995, Wiesner *et al.* 1998, Pfitzmann *et al.* 2008). In contrast, chronic rejection, which is characterized by a loss of bile ducts and cholestasis is a rare phenomenon. The most common cause of late graft dysfunction is the recurrence of the original disease, commonly HCC, HBV, HCV, and the autoimmune diseases PBC, PSC, and autoimmune hepatitis (Wiesner and Menon 2001). Common medical causes for late morbidity and mortality include renal dysfunction and cardiovascular diseases. Their risk factors include hypertension, hyperlipidemia, and post-transplant diabetes (Sethi and Stravitz 2007). Further, *de novo* malignancy after

transplantation is relatively common (Haagsma *et al.* 2001). All these complications can be attributed mainly to the immunosuppressive agents.

DONOR INFLAMMATORY RESPONSE

Brain death alters cardiovascular and neuroendocrine functions. The severe physiological derangements contribute to the viability of organs to be transplanted. Brain stem herniation leads to a catecholamine surge. This "autonomic storm" is characterized by extreme vasoconstriction and, despite concomitantly elevated blood pressure, tissue hypoperfusion. Subsequently, a loss of autonomic tone leads to impaired vascular autoregulation and hypotension, potentially worsening the tissue ischemia. Regarding neuroendocrine functions, hypothalamic-pituitary dysfunction can lead to endocrine failure. In addition, an antidiuretic hormone deficiency leads to hypovolemia and electrolyte disturbances. The loss of normal thyroid function can result in mitochondrial dysfunction and impaired cellular energy production, whereas glucocorticoid deficiency debilitates normal control of inflammation (Novitzky 1997, Pratschke *et al.* 1999).

Brain death elicits both systemic and organ-specific inflammatory responses. Although little understood, activation of these responses is multifactorial. Hemodynamic instability leads to periods of tissue ischemia and reperfusion in the donor (Pratschke *et al.* 2004). Catecholamine release during the autonomic storm may also directly activate intracellular apoptotic and proinflammatory pathways (Novitzky 1997). The loss of neural regulatory mechanisms, including both neuroendocrine stress response and autonomic nervous system functions, also contributes to the uncontrolled activation of innate immune responses (Sternberg 2006).

Systemic inflammation is manifested as elevated plasma levels of cytokines tumor necrosis factor-α (TNF-α) and interleukin-6 and -8 (IL-6, IL-8) in human brain dead organ donors (Palombo *et al.* 1994, Strangl *et al.* 2001, Weiss *et al.* 2007, Murugan *et al.* 2008), whereas inflammatory responses at the end organ level are best defined in experimental models.

Induction of brain death leads to Kupffer cell activation in the liver (Olinga *et al.* 2005) and also enhanced hepatic TNF-α, IL-1, and IL-6 expression (Takada *et al.* 1998, Okamoto *et al.* 2000). Upregulation of adhesion molecule expression on the sinusoidal endothelium is followed by hepatic neutrophil and monocyte infiltration (van der Hoeven *et al.* 1999, van der Hoeven *et al.* 2000, Okamoto *et al.* 2000). Irrespective of the systemic hemodynamic status, liver microcirculation becomes impaired after brain death (Okamoto *et al.* 2000, Golling *et al.* 2003). These events directly contribute to reduced graft survival from brain dead donors. Cold preservation and reperfusion of the graft further potentiate the adverse effects of brain death,

resulting in greater hepatocellular damage and further reduced survival (van der Hoeven *et al.* 2001).

LIVER ISCHEMIA-REPERFUSION INJURY

The liver receives 70% of its perfusion from the splanchnic area via the portal vein, and the remaining 30% from the hepatic artery. However, for oxygen delivery the hepatic artery accounts for 50%. Both portal and arterial blood ultimately drain into the sinusoids, where liver cells are exposed to nutritive and toxic substances, foreign antigens, microbial components, and inflammatory mediators. Hepatocytes, constituting approximately 70% of the liver mass, are responsible for the metabolic and detoxification functions of the liver. In addition to the hepatocytes, the liver consists of non-parenchymal cells. These include sinusoidal endothelial cells, Kupffer cells, stellate cells, and lymphocytes. Non-parenchymal cells stimulate hepatocyte proliferation and control extracellular matrix turnover, regulate sinusoidal blood flow, and participate in immune responses.

Ischemia

Liver I/R injury can occur in several situations, such as low-flow states (*e.g.* distributive or hypovolemic shock), during liver resection with inflow occlusion (Pringle maneuver), and in liver transplantation. The liver can be subjected to either warm (normothermic) or cold (+4ºC) ischemia. In the clinical setting, warm ischemia is fairly common, whereas cold ischemia occurs almost exclusively during preservation of the graft for transplantation.

Ischemia is a state where inadequate or interrupted blood flow leads to intracellular oxygen depletion and a subsequent decrease in oxidative phosphorylation and adenosine triphosphate (ATP) generation. ATP depletion leads to a loss of cell membrane integrity, followed by intracellular swelling and derangement in cellular calcium homeostasis. Increased cytosolic calcium activates intracellular phospholipases and proteases, leading to cell membrane disruption and activation of apoptotic and necrotic pathways (Nieuwenhuijs *et al.* 2006).

Warm ischemia. Warm ischemia is associated with profound mitochondrial dysfunction (Townsend *et al.* 1987). Warm ischemia primarily affects hepatocytes, with extensive liver enzyme release from necrotic cells (Ikeda *et al.* 1992). Most of the studies on hepatic I/R injury used experimental models of warm segmental or total hepatic ischemia with relatively long ischemic times lasting for up to 90 minutes. Such a setting does not directly apply to I/R injury in liver transplantation.

Cold ischemia. As compared to warm ischemia, cold ischemia is associated with a reduced oxidative phosphorylation and an increased anaerobic metabolism (Church-

ill *et al.* 1994, Gillispie *et al.* 2007). Cold ischemia mainly affects non-parenchymal cells, particularly sinusoidal endothelial cells, Kupffer cells, and biliary epithelial cells (McKeown *et al.* 1988, Ikeda *et al.* 1992, Noack *et al.* 1993). Although hepatocytes appear relatively resistant to cold ischemia, Vajdová *et al.* (2000) have demonstrated that cold preservation sensitizes hepatocytes to rewarming ischemia, which in turn contributes to the loss of hepatocyte viability.

Reperfusion

While ischemia primes the cells for damage, the actual injury usually manifests after the restoration of blood flow and tissue oxygenation. The key mechanism of tissue injury is the intense and excessive inflammatory response to reperfusion. Although initially considered a condition mediated by innate immune responses, I/R injury also triggers adaptative immunity and liver regeneration, thus influencing late graft function.

The effects of reperfusion seem to depend on the type of ischemia. After warm ischemia, I/R injury occurs in a biphasic manner; in that an initial phase of Kupffer cell activation is followed by hepatic neutrophil infiltration after several hours have elapsed (Jaeschke *et al.* 1990). In contrast, in response to cold ischemia, hepatic neutrophil sequestration occurs early after reperfusion and concomitantly with Kupffer cell activation (Shibuya *et al.* 1997, Kataoka *et al.* 2002).

In general, ROS generation and complement activation occur early during reperfusion. In addition to direct cytotoxic effects, these mediators prime, activate, and recruit neutrophils into the liver. Activated Kupffer cells express cytokines and chemokines, thereby leading to further neutrophil activation and recruitment. Neutrophils inflict tissue damage on the liver through the generation of ROS and the release of proteolytic enzymes.

"No reflow" ischemia

Despite the restoration of circulation, the liver may still suffer from hypoxia. Microcirculatory disturbances related to endothelial I/R injury can cause uneven tissue perfusion, resulting in "no-reflow" ischemia. Kupffer cells and sinusoidal endothelial cells express tissue factor (TF) after reperfusion, thereby activating intravascular coagulation (Kobayashi *et al.* 1998). The activation of coagulation cascades lead to fibrin and platelet deposition within the sinusoids during reperfusion (Kakizoe *et al.* 1990, Cywes *et al.* 1993). In addition to thrombus formation, sinusoid plugging can be due to adherent leukocytes and sloughed endothelial cells (McKeown *et al.* 1988, Vollmar *et al.* 1994). Further, endothelial injury is associated with increased production of vasoconstrictor molecules (platelet-activating factor, PAF; endothelin-1, thromboxane A_2,) and a decrease in the vasodilator molecules (nitric oxide, NO; prostacyclin), lead-

ing to vascular dysregulation (Clemens *et al.* 1997). Accordingly, Puhl *et al.* (2005) have suggested that both mechanical obstruction and vasoconstriction impair hepatic microcirculation, thereby causing hepatocellular damage even after reperfusion.

Innate immunity

The innate immune system provides the first line of defense against pathogens and non-infectious tissue injury, and initiates adaptative immune responses. Non-parenchymal cells, particularly the Kupffer cells and sinusoidal endothelial cells are important innate immune effectors in the liver.

Pathogen recognition or tissue damage initiates acute inflammatory responses. Innate immune cells detect specific microbial structures, pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS) and peptidoglycan. Moreover, endogenous substances released from distressed cells, so called alarmins, activate innate immunity responses. Alarmins include, among others, the high mobility group box 1 (HMGB1) protein (Park *et al.* 2004), heat shock proteins (HSP) (Vabulas *et al.* 2002), and extracellular matrix components released by proteases at sites of tissue damage (Johnson *et al.* 2002). Collectively, PAMPs and alarmins are called damage-associated molecular patterns (DAMPs).

Pattern-recognition molecules and receptors (PRRs) recognize DAMPs. PRRs can be divided into three categories: secreted, membrane-bound, and phagocytic. Hepatocytes secrete PRRs, or acute phase proteins, in response to cytokine stimulation. Secreted PRRs include complement proteins and C-reactive protein (CRP), that activate the complement and opsonize microbes for phagocytosis (Gao *et al.* 2008). Membrane-bound PRRs, such as Toll-like receptors (TLRs), are expressed in all cell types in the liver (Schwabe *et al.* 2006). TLR signalling leads to the expression of cytokines, cellular adhesion molecules, and acute phase proteins through the activation of transcription factor nuclear factor kappa B (NF-κB) (Medzhitov *et al.* 1997, Muzio *et al.* 1998). Phagocytic PRRs, expressed by Kupffer cells, the sinusoidal endothelium, and neutrophils, directly bind to opsonized microbes and macromolecules on the cell surface, followed by phagocytosis. Phagocytic PRRs include complement, scavenger, mannose, and Fc -receptors (Gao *et al.* 2008).

Kupffer cells

Monocytes in the blood are relatively inactive. However, upon tissue migration, monocytes transform into actively phagocytosing tissue macrophages. Kupffer cells, the liver residing macrophages, constitute over 80% of tissue macrophages in the body and over 30% of non-parenchymal cells in the liver (Bilzer *et al.* 2006). The Kupffer cells reside within the sinusoids, partly adherent on the endothelial surface. The Kupffer cells are primarily located in the periportal areas and therefore in continuous contact

with the portal blood from the gastrointestinal tract. These are involved in the clearance of defective erythrocytes and activated neutrophils. They are also involved in the elimination of microbes and microbial components, such as LPS (Bilzer *et al.* 2006). In addition to directly phagocytosing microbes, Kupffer cells introduce extracellularly bound microbes to the neutrophils, which then ingest and kill the microbes (Gregory *et al.* 2002). Further, Kupffer cells are capable of antigen presentation through major histocompatibility complex (MHC) class I and II molecules (Lohse *et al.* 1996).

Kupffer cells (Figure 2) can be activated by the following: By DAMPs through TLR (Su *et al.* 2000), by complement factors through specific complement receptors (Hinglais *et al.* 1989), by cell surface structures through mannose and scavenger receptors (Gao *et al.* 2008), and by cytokines through specific receptors (Hoffmann *et al.* 1994). According to Schemmer *et al.* (1998), simple physical manipulation of the liver also directly activates Kupffer cells. In human liver transplantation, Kupffer cells become activated already during cold ischemia and further during reperfusion (Carles *et al.* 1994).

Activated Kupffer cells promote both local and systemic inflammation after liver

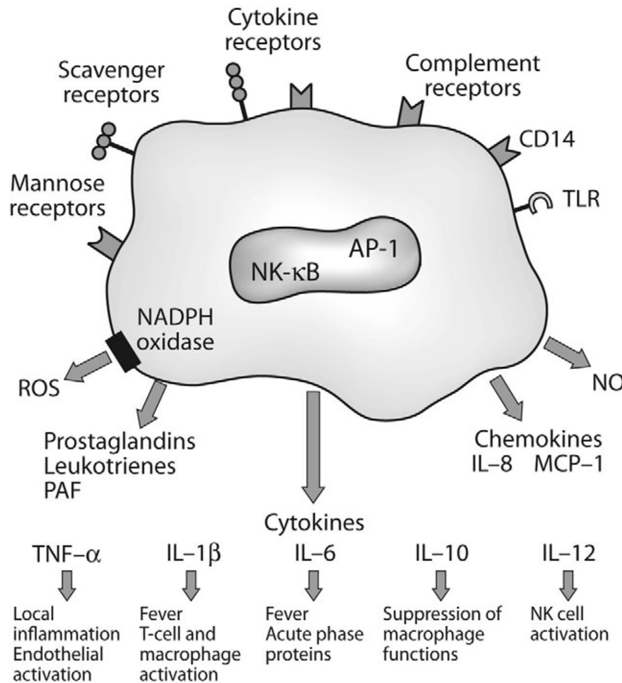


Figure 2. Kupffer cell functions.

I/R injury by producing an array of inflammatory mediators, including cytokines, chemokines, ROS, NO, and bioactive lipids (Decker 1990, Wanner *et al.* 1996). These agents activate the endothelium and increase vascular permeability, activate neutrophils, activate lymphocytes, and induce acute-phase protein synthesis in hepatocytes (Figure 2).

In an experimental setting, Kupffer cell inhibition prior to cold ischemia improves graft microcirculation after reperfusion, in addition to improving graft survival after transplantation (Kukan *et al.* 1997, Schemmer *et al.* 1998).

Neutrophils

Polymorphonuclear neutrophils (PMNs) are the major effector cells of the innate immune system. Neutrophils are the most prevalent leukocytes in circulating blood, but are not present in tissues under normal conditions. Upon acute inflammation, activated neutrophils rapidly extravasate and migrate into the tissues (Figure 3).

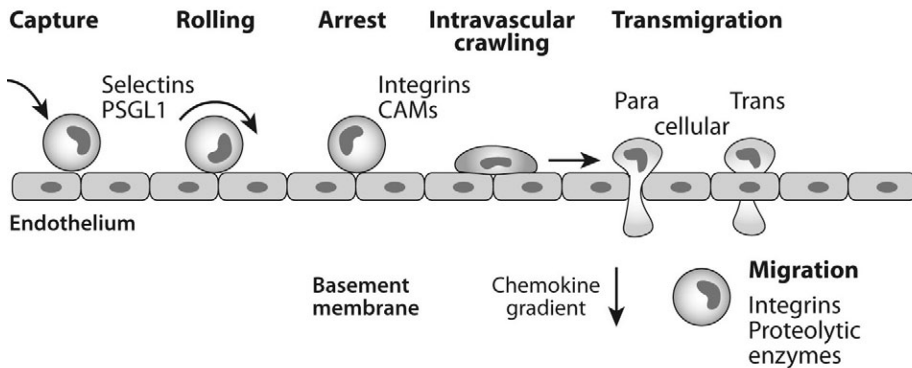


Figure 3. Neutrophil adhesion cascade.

Granules. Neutrophil functions are dependent on controlled exocytosis of cytoplasmic granules. These granules are formed sequentially during neutrophil differentiation from myeloblasts, and are degranulated in a reverse sequence during neutrophil activation. Granules can be classified as azurophilic (primary), specific (secondary), gelatinase (tertiary), and secretory vesicles. Azurophilic granules containing myeloperoxidase and serine proteases undergo only limited exocytosis and mainly fuse with the intracellular phagosome, thereby participating in killing and the subsequent degradation of micro-organisms and cell debris. Specific granules contain antibiotic substances such as lysozyme and lactoferrin, whereas gelatinase granules contain mainly matrix degrading enzymes (Faurschou and Borregaard 2003). Secretory vesicles constitute an important reservoir for membrane-bound receptors, such as CD11b/CD18, CD14, complement receptors, and also components of nicotinamide adenine dinucleotide

phosphate (NADPH) oxidase. These are upregulated rapidly in the initial phases of neutrophil-mediated inflammatory responses (Calafat *et al.* 1993).

Activation. Neutrophil activation status varies from resting, to primed, and then to fully activated. During the priming phase, inflammatory mediators lower the threshold for neutrophil activation and enhance the capacity for degranulation and that of ROS formation through respiratory burst (Edwards 1995, Condliffe *et al.* 1998). Several factors trigger neutrophil priming and activation. These include the macrophage-derived cytokines (TNF-α, IL-1β) and chemokines (IL-8), activated complement proteins (C3a, C5a), bioactive lipids (prostaglandins, PAF), and bacterial components. Activating signals are transmitted through various neutrophil cell surface PRRs and specific receptors (Edwards 1995).

Extravasation. Neutrophil recruitment into sites of inflammation is an essential step in the innate immune response (Figure 3). Extravasation typically takes place in the postcapillary venules, and is mediated by three different types of cell adhesion molecules (Ley *et al.* 2007). The selectins are membrane glycoproteins that interact with P-selectin glycoprotein ligand-1 (PSGL-1) and other glycosylated ligands. P-selectin and E-selectin are expressed by the activated endothelium, whereas L-selectin is present in most leukocytes. The integrins are heterodimeric proteins expressed on leukocytes. The key integrins in neutrophil extravasation include CD11a/CD18 and CD11b/CD18. The third type of adhesion molecules belong to the immunoglobulin superfamily and are expressed on the activated endothelium. These molecules include intercellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1).

Inflammatory mediators released by activated monocytes and macrophages stimulate the endothelial cells to rapidly translocate P-selectin onto their luminal surfaces from intracellular stores. Endothelial E-selectin synthesis and ICAM-1 upregulation are also stimulated by TNF-α and IL-1β. Neutrophil rolling on the endothelium is mediated by the selectins. This interaction triggers mobilization of neutrophil secretory vesicles, resulting in concomitant cell surface CD11b upregulation and L-selectin shedding (Kishimoto *et al.* 1989, Borregaard *et al.* 1994). Neutrophil arrest during rolling is triggered by chemokines and subsequently mediated by CD11b. Chemokines increase CD11b affinity for ICAM-1, thus favoring strong adhesion. Integrins also mediate neutrophil crawling along the endothelial surface to suitable sites for transmigration. Transmigration occurs either through the endothelial cell junctions (paracellular route) or directly through endothelial cells at thin parts of the endothelium (transcellular route). Diapedesis, migration through the basement membrane and underlying tissue, is directed along the chemotactic gradient and is facilitated by integrins and proteolytic enzymes degrading the extracellular matrix (Ley *et al.* 2007).

In the liver, neutrophils can extravasate in the sinusoids or post-sinusoidal venules

(Vollmar *et al.* 1994, Fox-Robichaud and Kubes 2000). In the post-sinusoidal venules, neutrophils rolling, adherence, and transmigration occur as described above (Jaeschke and Smith 1997). However, neutrophil extravasation takes place most commonly in the sinusoids. The occurrence of neutrophil trapping within the sinusoids is mainly due to mechanical constraints, such as Kupffer cell processes, endothelial cell swelling, and vasoconstriction (MacPhee *et al.* 1992, Jaeschke and Smith 1997). Neutrophil adhesion onto the sinusoidal endothelial surface is independent of selectins (Wong *et al.* 1997), and despite constitutive ICAM-1 expression is mostly independent of ICAM-1 (Jaeschke *et al.* 1996). However, transmigration requires CD11b–ICAM-1 interaction between the neutrophil and endothelium (Sakamoto *et al.* 1997). When extensive inflammatory injury to the endothelium occurs, neutrophils have direct access to the liver parenchyma and hepatocytes expressing ICAM-1 (Jaeschke and Smith 1997, Kobayashi *et al.* 2001).

The inhibition of neutrophil adhesion limits neutrophil-mediated tissue injury in experimental models. Although neutrophil recruitment into the liver is partly independent of selectins, soluble PSGL-1 reduces both warm and cold liver I/R injury (Dulkanchainun *et al.* 1998). Similarly, anti-ICAM-1 antibodies also attenuate both warm and cold liver I/R injury (Farhood *et al.* 1995, Nishimura *et al.* 1996). Moreover, anti-CD11b/CD18 antibodies are efficient in reducing hepatic neutrophil sequestration and hepatocellular injury after warm I/R injury (Jaeschke *et al.* 1993).

Cell killing. Microbe or cell destruction by neutrophils is mediated through two principal means; ROS and proteolytic enzymes. Neutrophils phagocytose opsonized material, an event followed by ROS generation within the phagosome and intracellular granule coalescence into the phagosome.

Neutrophils produce ROS by the action of membrane-bound NADPH oxidase that catalyzes the superoxide anion (O_2^{-}) formation from oxygen (O_2). The superoxide is then converted into hydrogen peroxide (H_2O_2) both spontaneously and catalyzed by superoxide dismutase (SOD). Myeloperoxidase reacts with H_2O_2 to induce hypochloric acid (HOCl) formation. H_2O_2 can also be converted into toxic hydroxyl radicals (OH^{-}) in the presence of metal ions. ROS induce cell damage through protein and DNA oxidation, subsequent degradation, and also by lipid peroxidation. Several different antioxidants have beneficial effects on liver I/R injury (Glantzounis *et al.* 2005).

The most important proteolytic enzymes are the serine proteases and matrix metalloproteinases (MMPs). The serine proteases include proteinase-3, cathepsin G, and elastase, that are stored in the azurophilic granules in their active form. They exhibit proteolytic capacity against collagens and proteoglycans (Owen and Campbell 1999). Neutrophil gelatinase granules contain MMP-8 (collagenase) and MMP-9 (gelatinase B) in their inactive proforms, which undergo proteolytic activation upon exocytosis.

Generally, MMPs can degrade all extracellular matrix protein components (Owen and Campbell 1999). In addition to proteolysis, proteolytic enzymes regulate innate immune responses by activating macrophages (Hubbard *et al.* 1991), and by activating or inactivating cytokines and the cell signaling receptors (van den Steen 2000, Wiedow and Meyer-Hoffert 2005). Both elastase and MMP-9 inhibition has proved effective, in addition to I/R injury after warm liver ischemia, against I/R injury in experimental liver transplantation (Soejima *et al.* 1999, Defamie *et al.* 2008).

Reperfusion in clinical liver transplantation. Hepatic neutrophil activation occurs early during reperfusion. This is indicated by the upregulation of CD11b expression and intracellular H_2O_2 production across the hepatic circulation during initial reperfusion. Neutrophils are sequestered into the graft concomitantly, and proteolytic enzymes such as elastase and MMP-9 are released from the graft (Himmelreich *et al.* 1994, Thiel *et al.* 1998, Kiuchi *et al.* 1998, Pesonen *et al.* 2000, Upadhya and Strasberg 2000, Marti *et al.* 2004).

Lymphocytes

Resident lymphocytes in the liver are dominated by innate immune cells, including natural killer (NK) cells and T-cells expressing NK molecules (NKT cells). They predominantly reside along the portal tracts. These lymphocytes express antigen receptors of limited variability, recognize cell membrane glycoproteins and lipid components, and exhibit rapid cytokine production and cytotoxic activity (Doherty and O'Farrelly 2000).

Lymphocytes, specifically the CD4 positive T-cells, are rapidly recruited into the liver after reperfusion (Zwacka *et al.* 1997). The CD4 positive T-cells can be activated independently of antigen presentation by ROS, cytokines released by the Kupffer cells, and complement factors. These T-cells may also undergo antigen-dependent activation as both sinusoidal endothelial cells and hepatic dendritic cells activated during ischemia are capable of antigen presentation (Huang *et al.* 2007). In addition to initiating adaptative immune responses, T-cells enhance hepatic neutrophil recruitment, thereby amplifying tissue injury. In a murine warm hepatic I/R injury model, depletion of CD4 positive cells reduces inflammation and hepatocellular injury (Zwacka *et al.* 1997).

Soluble mediators

The innate immune system induces inflammatory responses through soluble mediators. Cytokines are small proteins (<30 kDa) produced by various cells in response to pattern recognition, or stimulation by other cytokines. They induce responses in target cells through specific receptors, share common signal transduction cascades, and are involved in both the innate and adaptative immune responses. Chemoattract-

ant cytokines (chemokines) are released early during inflammation. They recruit and guide inflammatory cells into the affected tissues, and activate their target cells. In addition to cytokines and chemokines, the complement fragments (C3a, C5a), lipid mediators formed from degraded membrane phospholipids (prostaglandins, leukotrienes, PAF), and adhesion molecules shed from the endothelium, are all engaged in the regulation and amplification of inflammatory responses.

Tumor necrosis factor-α. TNF-α is derived primarily from monocytes and macrophages activated by DAMPs. TNF-α is expressed as a membrane bound protein that is enzymatically cleaved into a soluble form. Both membrane bound and soluble forms exhibit biological activity (Bradley 2008). TNF-α itself is short-lived (Selby *et al.* 1987), though it stimulates the expression of secondary inflammatory mediators such as IL-6 and IL-8 and thereby propagates inflammation. In addition, TNF-α induces inflammatory changes in the endothelium including increased permeability, adhesion molecule expression, and local activation of blood coagulation cascade through TF expression. In the liver, TNF-α appears to promote hepatocyte proliferation during liver regeneration both directly and through enhanced IL-6 release from Kupffer cells (Bradham *et al.* 1998). During the reperfusion in clinical liver transplantation, TNF-α is released from the graft (Steininger *et al.* 1994, Tange *et al.* 2001).

Interleukin-6. IL-6 is produced rapidly in response to TNF-α and IL-1β stimulation mainly by macrophages and T-cells. IL-6 regulates hematopoiesis and induces lymphocyte proliferation and activation. It also contributes to the defense against infection and tissue trauma by inducing fever, adrenocorticotropic hormone release, and acute phase protein synthesis in the liver (Song and Kellum 2005). Although IL-6 is generally a marker of proinflammatory response (Waage *et al.* 1989), it also has protective functions in the liver. For example, IL-6 regulates hepatic inflammatory response (Clavien *et al.* 1996), hepatocyte apoptosis (Kovalovich *et al.* 2001), and induces liver regeneration (Cressman *et al.* 1996, Camargo *et al.* 1997).

Interleukin-8. IL-8 (also known as CXCL8) is a chemotactic cytokine (chemokine) expressed by virtually all nucleated cells, including hepatocytes (Thornton *et al.* 1990), though it is mainly expressed by monocytes and macrophages (Remick 2005). TNF-α and IL-1β induce the secretion of IL-8 early during the inflammatory response and unlike the other chemokines, the IL-8 response persists for days. Thus it ensures ongoing recruitment of inflammatory cells into damaged tissue (DeForge *et al.* 1992). Neutrophils are the main target for IL-8 mediated chemotaxis. IL-8 bound to the endothelium (Middleton *et al.* 1997) and extracellular matrix (Webb *et al.* 1993) directs neutrophil migration into sites of infection and tissue damage along a chemotactic gradient. Further, IL-8 primes neutrophils for respiratory burst and degranulation (Edwards 1995). In the liver, IL-8 may also promote acute phase protein synthesis (Wigmore *et al.* 1997), and it is also released from the liver during reperfusion in clini-

cal liver transplantation (Mueller *et al.* 1996, Pesonen *et al.* 2000).

High mobility group box 1 protein. HMGB1 (amphoterin) is an abundant nuclear protein involved in DNA bending and transcription regulation in all nucleated cells (Lotze and Tracey 2005). Extracellular HMGB1 (Figure 4) can be released either passively from necrotic cells (Scaffidi *et al.* 2002) or actively secreted primarily by immune cells of myeloid origin (Wang *et al.* 1999, Lotze and Tracey 2005) and by endothelial cells (Mullins *et al.* 2004). Further, Tsung *et al.* (2007) reported that hepatocytes can secrete extracellular HMGB1 in response to ischemia. Bacterial components, cytokines, and ROS are also known inducers of active HMGB1 secretion (Wang *et al.* 1999, Rouhiainen *et al.* 2004, Tang *et al.* 2007).

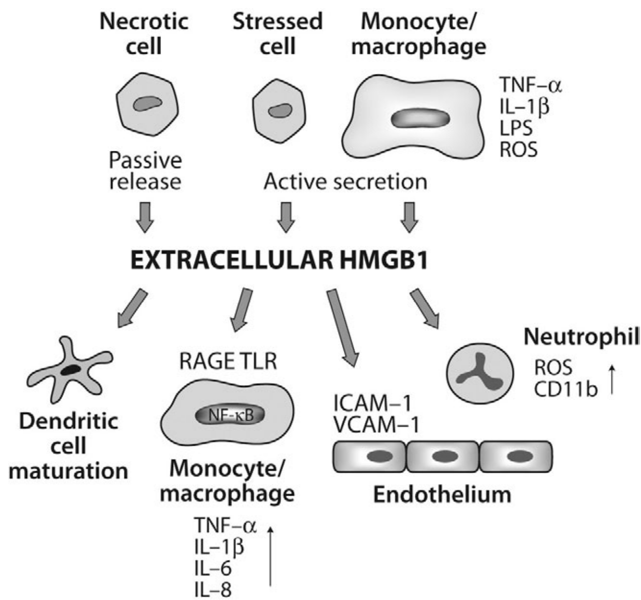


Figure 4. Extracellular HMGB1 as a mediator of inflammation.

Extracellular HMGB1 acts as an alarmin that induces innate immune cell activation, fever, increased vascular and intestinal permeability, and hypotension. HMGB1 exerts its effects on target cells through receptor for advanced glycation end-products (RAGE) and TLRs (Lotze and Tracey 2005). HMGB1 stimulates monocyte and neutrophil activation, in addition to the release of multiple cytokines and chemokines (Andersson *et al.* 2000, Park *et al.* 2003). HMGB1 also induces endothelial cell ac-

tivation and upregulation of adhesion molecules (Fiuza *et al.* 2003, Treutiger *et al.* 2003). It also mediates monocyte and neutrophil recruitment and migration into the sites of inflammation (Rouhiainen *et al.* 2004, Orlova *et al.* 2007, Palumbo *et al.* 2007).

In sepsis and endotoxemia, HMGB1 is a late inflammatory mediator: HMGB1 release is delayed for several hours after initial TNF-α expression (Wang *et al.* 1999, Yang *et al.* 2004, Rouhiainen *et al.* 2004). Further, according to Levy *et al.* (2007), extracellular HMGB1 initiates systemic inflammation and injury at the end organ level after locally confined tissue injury.

In the murine model of warm hepatic I/R injury, HMGB1 acts as an early mediator of inflammation and organ damage. Inhibition of HMGB1 activity with neutralizing antibody decreases hepatocellular injury (Tsung *et al.* 2005a). Moreover, treatment with ethyl pyruvate, an antioxidant with direct inhibitory activity against HMGB1, is hepatoprotective in an experimental setting (Tsung *et al.* 2005b).

Soluble intercellular adhesion molecule-1. sICAM-1 represents a circulating form of ICAM-1. TNF-α and IL-1β stimulate ICAM-1 expression on and also sICAM-1 release from the endothelium (Witkowska and Borawska 2004). In addition, sICAM-1 originates from monocytes (Rothlein *et al.* 1991) and hepatocytes (Thomson *et al.* 1994), among others. Generally, sICAM-1 is considered to reflect endothelial activation and damage. It is shed from the endothelial surface by the action of proteases (Champagne *et al.* 1998), but also appears on membranes of microparticles released from disturbed endothelial cells (Jy *et al.* 2002). It has been suggested that sICAM-1 may modulate the inflammatory process by acting as a chemotactic factor, and by competing with membrane-bound ICAM-1 to prevent leukocyte adhesion to the endothelium (Rothlein *et al.* 1991).

Graft function

Hepatocellular injury. Although hepatocytes are resistant to cold ischemia, they are sensitive to rewarming ischemia (Vajdová *et al.* 2000), and prolonged rewarming ischemia is also a risk factor for graft dysfunction in clinical liver transplantation (Strasberg *et al.* 1994). According to Lehmann *et al.* (1998), hepatocyte function can be impaired, even in the absence of morphological changes and microcirculatory disturbances. Rewarming hinders the restoration of optimum hepatocyte ATP levels, thereby disturbing cell volume and electrolyte homeostasis, in addition to disruptions to synthetic and metabolic functions. Moreover, complement activation and neutrophil sequestration within the liver during reperfusion inflict direct damage on hepatocytes (Kakizoe *et al.* 1990, Scoazec *et al.* 1997). During uneventful recovery, hepatocellular integrity and synthetic function, as assessed by transaminase and coagulation factor levels, generally normalize within two to three days after transplantation.

Biliary function. ATP degradation during ischemia leads to biliary epithelial microvilli dysfunction, dilatation of bile canaliculi, and decreased bile flow through the activation of intracellular proteases (Cutrin *et al.* 1996). Upon reperfusion, neutrophils infiltrate into the bile ducts, thus intensifying the ischemic injury (Carrasco *et al.* 1996, Busquets *et al.* 2001). Cholestasis and impaired secretion of hydrophobic bile salts can directly damage both biliary cells and hepatocytes (Sokol *et al.* 1995). Compared to that in the hepatocytes, recovery of the biliary epithelium is prolonged after I/R injury. Markers of biliary function (bilirubin; γ-glutamyl transferase, GGT; alkaline phosphatase, ALP) peak as late as 10 to 15 days after transplantation (Cutrin *et al.* 1996).

Long term outcome. In a clinical setting, liver I/R injury is generally graded according to postoperative hepatocellular damage, as indicated by transaminase levels. A significant rise in transaminases is associated with an increasing incidence of acute rejection, PNF, 1-year patient survival, and a late recurrence of the original liver disease (Howard *et al.* 1990, Devlin *et al.* 1995, Rosen *et al.* 1998, Watt *et al.* 2006).

PROTEIN C – ACTIVATED PROTEIN C PATHWAY AND INFLAMMATION

The protein C pathway plays a critical role in linking coagulation and inflammation. Protein C, which is synthesized in the liver, is a major endogenous anticoagulant. Protein C is converted into activated protein C (APC) on the endothelial surface by thrombin-thrombomodulin (TM) complex (Figure 5). This conversion is augmented by protein C binding to the endothelial protein C receptor (EPCR). APC exhibits direct anticoagulant activity by irreversible proteolytic inactivation of the coagulation factors Va and VIIIa. APC also enhances fibrinolysis. In addition to maintaining coagulation homeostasis, APC has anti-inflammatory properties. APC reduces thrombin formation and fibrin deposition, and thereby indirectly attenuates vascular permeability and reduces phagocyte recruitment into the sites of vascular damage. Furthermore, APC has direct cytoprotective properties that are independent of its anticoagulant effects (Mosnier *et al.* 2004).

When mediating cytoprotective effects, APC binds to EPCR activates protease activated receptor 1 (PAR-1) on the cell surface (Figure 5). In addition to the endothelium, both EPCR and PAR-1 are expressed in monocytes and neutrophils (Calligan *et al.* 2001, Kannan 2002, Colognato *et al.* 2003, Sturn *et al.* 2003). PAR-1 activation by APC results in the inhibition of inflammatory gene expression. Moreover, APC blocks the expression of cytokines, particularly that of TNF-α, in monocytes and also the endothelial cells (Grey *et al.* 1994, White *et al.* 2000, Joyce *et al.* 2001, Yuksel *et al.* 2002, Franscini *et al.* 2004). According to Yamaji *et al.* (2005), APC also suppresses

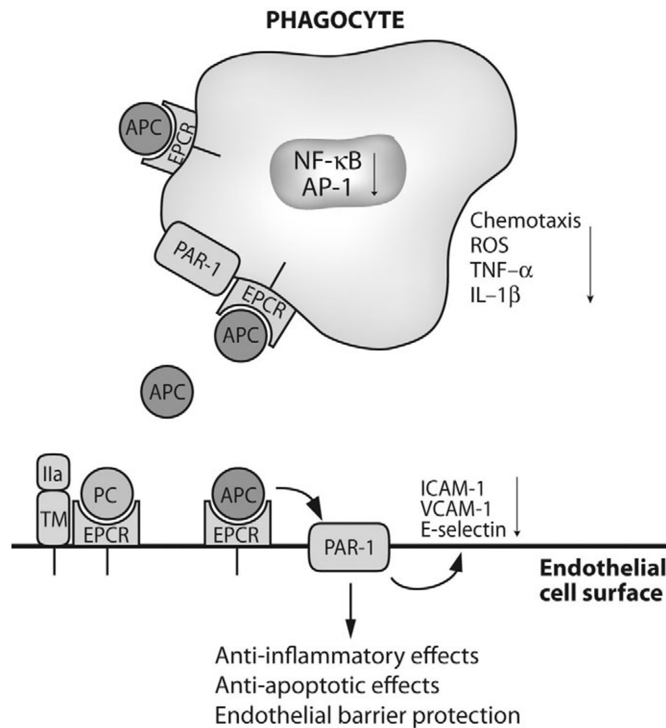


Figure 5. Cytoprotective effects of activated protein C (APC).
Modified from Mosnier et al. 2007.

ROS production in macrophages and has direct antioxidant properties through the inhibition of lipid peroxidation.

The APC molecule modulates phagocyte recruitment into sites of inflammation (Figure 5). Although endothelial expression of several chemokines is upregulated in response to APC (Hooper *et al.* 1998, Riewald *et al.* 2002, Franscini *et al.* 2004), APC bound to EPCR effectively inhibits phagocyte chemotaxis (Sturn *et al.* 2003, Nick *et al.* 2004). Furthermore, APC down-regulates the expression of endothelial adhesion molecules, thereby reducing endothelial phagocyte adhesion (Joyce *et al.* 2001, Franscini *et al.* 2004). A soluble form of EPCR binds to CD11b/CD18 on neutrophils, which may further interfere with neutrophil adherence onto the endothelium (Kurosawa *et al.* 2000). In addition to its anti-inflammatory activity, APC maintains the endothelial barrier function and normal permeability under inflammatory conditions (Zeng *et al.* 2004, Feistritzer and Riewald 2005). It also inhibits endothelial cell and monocyte apoptosis (Cheng *et al.* 2003, Stephenson *et al.* 2006).

In vivo, APC supplementation has beneficial effects both on coagulopathy and

inflammation under pulmonary inflammation and injury, and septic infections. In addition, APC prevents LPS induced pulmonary injury by inhibiting the inflammatory responses in the lungs in both rats and humans (Murakami *et al.* 1996, Nick *et al.* 2004). Likewise, in experimental and human severe sepsis, APC limits inflammation (Bernard *et al.* 2001, Hoffmann *et al.* 2004) and reduces mortality (Taylor *et al.* 1987, Bernard *et al.* 2001). Further, APC improves microcirculation in experimental endotoxemia (Hoffmann *et al.* 2004, Iba *et al.* 2005). Other actions of APC include neuroprotection and promotion of wound healing and angiogenesis (Mosnier *et al.* 2007).

Exogenous APC also has anti-inflammatory effects on experimental I/R injury of liver (Yamaguchi *et al.* 1997) and kidney (Mizutani *et al.* 2000), among others. Moreover, APC attenuates remote organ injury after local I/R injury (Teke *et al.* 2008). Recently, Kuriyama *et al.* (2008) demonstrated that APC treatment was associated with multiple cytoprotective effects both through anti-inflammatory and anticoagulant functions in an experimental model of warm hepatic I/R injury. APC decreased hepatocellular injury and apoptosis. Further, APC attenuated neutrophil and monocyte activation and hepatic infiltration, in addition to hepatic TNF-α expression. Improvements in microcirculation by the inhibition of coagulation and sinusoidal endothelial cell injury were also mediated by APC.

Aims of the study

The overall purpose of this thesis was to explore the inflammatory response and endogenous regulatory mechanisms in clinical liver transplantation. The main emphasis of the research was on graft reperfusion, particularly those changes occurring in the transplanted liver.

The specific objectives were:

1. To evaluate the hepatic inflammatory response in the multiorgan donor at the time of liver procurement and its subsequent effects on early postoperative graft function and outcome (I).

2. To describe the kinetics of protein C – APC pathway both during and after transplantation, to explore the potential anti-inflammatory effects of protein C – APC pathway during graft reperfusion, and to evaluate the effect of protein C – APC pathway on early postoperative graft function and outcome (II,III).

3. To describe the kinetics of HMGB1 both during and after transplantation, and to evaluate HMGB1 in relation to early postoperative graft function (IV).

Patients and methods

PATIENTS AND GENERAL STUDY DESIGN

Seventy adult patients undergoing liver transplantation, regardless of the indication, participated the studies (Table 1). In addition, blood samples were obtained from a total of 50 multiorgan donors (I and IV), 30 of whom were also evaluated regarding their clinical variables (I; Table 2). The studies were conducted in the Transplantation and Liver Surgery Clinic of the Surgical Hospital, HUCH. The patients were recruited during the 2000-2001 period and also 2006. Laboratory samples were assayed in the Department of Bacteriology and Immunology, Haartman-Institute, University of Helsinki (I, II, and III); in the Department of Clinical Chemistry, HUCH and HUSLAB (I, II, and III); in the Department of Molecular and Experimental Medicine, Scripps Research Institute, La Jolla, CA, USA (II and III); and in the Neuroscience Center, University of Helsinki (IV).

Studies I, II, and III were approved by the Ethics committee in the Surgical Hospital and for study IV by the Ethics committee, Department of Surgery, Hospital District of Helsinki and Uusimaa. Informed consent was obtained from the patients prior to their transplantations. For patients with significant encephalopathy, the consent was obtained from their next of kin.

Study I comprised 30 consecutive multiorgan donors in Finland and their respective liver recipients. One donor-recipient pair was excluded from the final data analysis due to the recipient's excessive perioperative bleeding of 75,000 ml. During graft procurement, plasma IL-6, IL-8, and sICAM-1 levels, and adhesion molecule expression in circulating neutrophils were measured across the hepatic circulation. The effect of hepatic inflammatory response at the time of procurement on the postoperative liver function and outcome were investigated. Immunosuppression consisted of cyclosporine, azathioprine, and steroids.

Study II comprised 10 consecutive patients, in whom plasma protein C and APC were measured during liver transplantation and postoperatively up to day 6. Adhesion molecule expression in circulating neutrophils and monocytes was also measured. Immunosuppression consisted of cyclosporine, azathioprine, and steroids.

Study III comprised 50 consecutive patients, including those from study II. Three patients were excluded from the final data analysis. Of these, two exclusions were due to technically different reperfusion (portal vein and hepatic artery declamped simultaneuously), and one exclusion due to excessive perioperative bleeding. Plasma protein C and APC, and adhesion molecule expression in circulating neutrophils and monocytes were measured during graft reperfusion across the hepatic circulation. The effect of protein C activation on postoperative liver function and outcome were in-

Table 1. Patient characteristics

	I	II	III	IV
Patients, n	30	10	47	20
Age, yrs	49 (22–64)	38 (24–61)	44 (20–64)	50 (22–66)
Sex, male/female	18/11	6/4	28/19	14/6
Acute liver failure, n (total)	5	2	8	3
nonA-E hepatitis	4	2	6	2
Toxic liver injury	1		1	
Acute autoimmune hepatitis			1	
Angiosarcoma				1
Chronic liver disease, n (total)	21	8	36	17
PBC	2	1	9	3
PSC	4	2	8	1
Alcoholic cirrhosis	5	2	7	4
Cryptogenic cirrhosis	2	1	3	3
Chronic rejection	2	1	2	
Familial biliary cirrhosis	1		2	
Idiopathic portal hypertension	1	1	1	
Liver fibrosis	1		1	
Chronic active hepatitis			1	
Post-hepatitic cirrhosis			1	
Subacute liver failure			1	
NAFLD				2
Polycystic liver disease				1
Chronic autoimmune hepatitis				1
Budd-Chiari				1
Hepatitis B				1
Hepatocellular carcinoma, n (total)	3		3	*

PBC, primary biliary cirrhosis; PSC, primary sclerosing cholangitis;
NAFLD, non-alcoholic fatty liver disease.
For subacute liver failure, the origin was unknown.
* Histology revealed incidental carcinoma in 4 patients with cirrhosis.

vestigated. Patients received either cyclosporine (n=31) or tacrolimus (n=16) based immunosuppression. The effect of immunosuppression was not evaluated.

Study IV comprised 20 consecutive patients, in whom plasma HMGB1, IL-6, and TNF-α were measured during transplantation and postoperatively up to 48 h. In addition, plasma HMGB1 was measured in the donors before organ procurement. The associations between HMGB1, other cytokines, and postoperative liver function were evaluated. The cellular HMGB1 expression was determined by immunohistochemistry. Patients received either cyclosporine (n=7) or tacrolimus (n=13) based immuno-

Table 2. Donor characteristics

Age (yrs)		43 (14–59)
Gender (M/F)		24/19
Body mass index (kg/m^2)		23 (19–31)
History of hypertension (yes/no)		7/36
Cause of BD (n)		
	trauma	11
	intracranial bleeding	27
	other	5
Donor cardiac arrest (yes/no)		4/39
ICU length of stay (days)		1 (0–9)
Time from BD to liver perfusion (hrs)[a]		10.6 (4.9–25.2)
Hypotensive events (yes/no)[b]		15/28
Vasoactive support (yes/no)		41/2
	dopamine alone (n)	24
	dopamine + other (n)[c]	14
	norepinephrine alone (n)	3
	dopamine dose (μg/kg/min)	5.9 (2.1–15.9)
Desmopressin (yes/no)		34/9
Liver graft steatosis (%)[d]		0 (0–80)
	< 30 % (n)	36
	> 30 % (n)	7

Continuous data are expressed as median (range).
BD = brain death, ICU = intensive care unit
[a] Time from BD declaration to perfusion;
[b] Systolic blood pressure < 90 mmHg > 15 min
[c] Norepinephrine, epinephrine, or phenylephrine;
[d] Macrovesicular

suppression. As no difference in study parameters or outcome between the immunosuppression groups were found (data not shown), the groups were combined.

CLINICAL ASSESSMENT

Donors

The data analysis donor variables included: gender, age, body mass index (BMI), history of hypertension, cause of brain death (trauma vs. intracranial hemorrhage, ICH), donor cardiac arrest, ICU length of stay (ICU stay; days), time from brain death diagnosis to liver graft perfusion, hypotensive events (defined as systolic blood pressure <90 mmHg for >15 minutes) (Mor *et al.* 1992, Fernandez-Merino *et al.* 2003) during intensive care and organ procurement, and vasoactive agents used. For vasoactive drugs, mean dose (µg/kg/min) over the 24 hours prior to organ procurement was calculated. The need for desmopressin, and plasma sodium levels were recorded. Other laboratory values measured were hemoglobin level, leukocyte counts, ALT, GGT, ALP, bilirubin, prothrombin time (PT), and CRP, all taken as a part of screening of the donors. Graft steatosis was assessed from biopsies taken prior to graft perfusion during procurement. Only macrovesicular steatosis was taken into account in data analysis (Fishbein *et al.* 1997).

Recipients

Perioperative variables analyzed were anhepatic time, graft CIT, and bleeding. All postoperative laboratory tests were performed as a part of routine clinical follow-up. Plasma ALT, ALP, GGT, bilirubin, and PT, were measured daily during the first postoperative week, and the peak (the lowest for PT) values were recorded (I, III). The galactose elimination half-life (t½) was determined at day 7 postoperation (III). In studies I, II, and III, patients were followed up to one year after transplantation at our centre. In study IV, the follow-up period was three months. Complications and outcome, including biliary and vascular complications, rejection episodes, patient survival, and graft survival, were recorded (I, II, and III). Patient follow-up was discontinued at graft loss or death.

In study IV, peak ALT and INR values were measured within 72 hours of reperfusion, and those of bilirubin on day 7, were included in data analysis. The outcomes within three months were recorded.

RESEARCH SAMPLES

Blood samples

Donor. Blood samples were collected before donor surgery in study IV. In contrast,

blood samples were collected at the beginning of donor surgery and before perfusion and explantation of the liver graft in study I. The first blood sample was drawn via an arterial cannula. Before perfusion, samples were taken simultaneously by puncture from the portal and hepatic veins.

Recipient. Blood samples were collected (Figure 1): after the induction of anesthesia but before surgery (II, III, and IV); immediately before the anhepatic period (II); immediately before reperfusion (II, III, and IV); during initial reperfusion of the graft with portal blood (II-IV); at 5 min (II, III) or at 10 min (IV) after portal vein declamping; and at 10 min after hepatic artery declamping (II, IV). After surgery, samples were collected on 1, 2, 4, and 6 postoperative days (II). In study IV, samples were collected at 8 h, 24 h, and 48 h after reperfusion.

Before surgery and reperfusion, blood samples were drawn from the radial arterial cannula. Before the anhepatic period, samples were drawn by puncture from the portal and hepatic veins. During initial reperfusion, samples were drawn from the portal vein and the inferior caval vein stump of the graft (caval effluent). After portal vein and hepatic artery declamping, samples were drawn from the portal and hepatic veins. In study IV blood samples were drawn, in addition to hepatic circulation, from radial arterial cannula. Postoperative samples were collected either from radial artery or by venipuncture.

Sample handling. Blood samples (volume 10 ml) were drawn into pyrogen-free syringes. In order to minimise inflammatory activation *ex vivo*, samples were immediately divided into aliquots into pre-cooled tubes, and these tubes were placed in thawing ice (+0ºC) until further handling.

For analysis of phagocyte adhesion molecule expression (I, II, and III), a 1 ml quantity of the sample was transferred into a polystyrene tube (Falcon No 2054, Becton Dickinson Labware, Lincoln Park, NJ, USA) supplemented with 170 µl of pyrogen-free citrate (Baxter Health Care, Norfolk, England). The tube was then stored in thawing ice (+0ºC) until cell labelling.

For analyses of protein C and APC (II, III), a 1.8 ml quantity of the sample was mixed with 0.2 ml of 0.3 mol/l benzamidine (a reversible thrombin inhibitor; Sigma-Aldrich, St. Louis, MO, USA) in 0.109 mol/l sodiumcitrate. Within 30 minutes of sampling, the plasma was separated by centrifugation (at 1900 g for 15 min), and thereafter stored at -70ºC until analyses.

For analyses of soluble mediators, in study I a 3 ml quantity or the whole sample in study IV was transferred into a sodium citrate anticoagulated tube (Vacutainer Systems Europe, Becton Dickinson, France). The plasma was separated by centrifugation (at 1900 g for 15 min, study I; and at 2000 g for 10 min, study IV), and thereafter stored at -70 to -80ºC until assayed.

The remaining blood was transferred into an EDTA-tube (Vacutainer) and stored at room temperature until analysis of leukocyte differential counts (II, III) (Advia 120, Bayer, Terrytown, NY, USA).

Liver biopsies

In study IV, two biopsies were obtained from each graft; the first was taken during liver procurement immediately before graft perfusion and the second biopsied at the end of liver transplantation prior to skin closure. The liver biopsies were fixed in formalin and then embedded in paraffin.

LABORATORY METHODS

Cellular markers CD11b and L-selectin

Adhesion molecule expression in circulating neutrophils and monocytes were determined by flow cytometry (Repo *et al*. 1993). All steps of cell staining were carried out on thawing ice (+0°C) with ice-cold reagents.

Cell labelling. All monoclonal antibodies were purchased from Becton Dickinson (Becton Dickinson Europe, Temse, Belgium). For the analyses of neutrophil CD11b and L-selectin (CD62L) expression, an aliquot of 25 µl of whole blood was double-stained with saturating concentrations of anti-CD11b-phycoerythrin (PE) and anti-CD62L-fluorescein isothiocyanate (FITC). In the analyses of monocyte CD11b and L-selectin expression, two 25 µl aliquots of whole blood were double stained with saturating concentrations of anti-CD14-FITC and anti-CD11b-PE, or anti-CD14-PE and anti-CD62L-FITC, respectively. After staining, the samples were lysed twice in 1:10 diluted FACSLysing solution (Becton Dickinson) to remove contaminating erythrocytes. Leukocytes were then fixed with 1.35% formaldehyde in FACSFlow solution (Becton Dickinson), with a final concentration of 0.5%. Samples were stored on thawing ice (+0°C) until flow cytometry analysis was carried out within 24 h.

Flow cytometry. A FACSort flow cytometer (Becton Dickinson) and CellQuest software (Becton Dickinson) were used for the acquisition and analysis of the data. Their light scattering properties enabled data representing 5000 neutrophils to be collected. Adhesion molecule expression was evaluated by creating fluorescence histograms for both CD11b and L-selectin. Data for 1000 monocytes were collected from a gate in a side scatter/CD14 fluorescence dot plot, and fluorescence histograms were created for CD11b and L-selectin. Adhesion molecule expression is given in relative fluorescence units (RFU), *i.e.* as the mean channel number of the positively fluorescent cell population.

Soluble intercellular adhesion molecule-1

Plasma sICAM-1 levels were determined using Quantikine Human sICAM-1 Immunoassay (R&D Systems, Abington, UK), with a detection limit of 0.35 ng/ml.

Tumor necrosis factor-α

Plasma TNF-α was measured using a WEHI-13VAR cell assay (Khabar et al. 1995, Tange et al. 2001). Briefly, 40 000 WEHI-13VAR cells per microwell were cultured overnight. The incubation medium was then changed to one containing Actinomycin D (Sigma-Aldrich). A 100 µl quantity of the diluted plasma samples (25% and 50% plasma in phosphate buffered saline, PBS) or recombinant TNF-α standards (0-1000 pg/ml, Pierce-Endogen, Rockford, IL, USA) was added to wells and cultured overnight. After culturing in a medium containing methylthiazolyldiphenyl-tetrazolium bromide (MTT, Sigma-Aldrich), the medium was replaced with 150 µl of dimethyl sulfoxide (DMSO, Riedel-de Haen, Seelze, Germany), each plate was mixed for 10 min, and the absorbance read at 595 nm. A standard curve was derived from TNF-α standards, and plasma TNF-α concentrations were calculated from the standard curve. All samples were measured twice in separate assays and the respective mean of each sample was calculated.

Interleukin-6 and -8

Plasma IL-6 was determined using Quantikine Human IL-6 Immunoassay (R&D Systems, Abington, UK) with a detection limit of 0.70 pg/ml in study I, or by using Human IL-6 Instant ELISA (Bender MedSystems) immunoassay with a detection limit of 0.92 ng/ml in study IV. Plasma IL-8 was determined using Quantikine Human IL-8 Immunoassay (R&D Systems, Abington, UK), with a detection limit of 10 pg/ml.

High mobility group box 1 protein

Western-blotting. Plasma HMGB1 levels were determined by combined heparin-Sepharose precipitation and Western-blotting methods (Sunden-Cullberg *et al.* 2005). Plasma was added to heparin-Sepharose slurry in PBS. Heparin-Sepharose bound proteins were eluted by SDS-PAGE sample buffer, electrophoresed by SDS-PAGE, then analysed in a Western-blot probed with chicken anti-HMGB1 IgY (Rouhiainen *et al.* 2004) and peroxidase conjugated anti-chicken IgY (Zymed Laboratories, San Francisco, CA, USA). Optical densities of immunoreactive 30 kDa bands were quantified as described (Rouhiainen *et al.* 2004, Sunden-Cullberg *et al.* 2005). Normal plasma samples containing known amounts of recombinant HMGB1 were analysed equally in each gel, and a standard curve was plotted to calculate sample HMGB1 levels. The sensitivity of the assay was 30 ng/ml.

Enzyme linked immunosorbent assay. Plasma HMGB1 in donor samples was determined by HMGB1 ELISA Kit II (Shino-Test Corporation, Kanagawa, Japan) immunoassay with a detection limit of 1.0 ng/ml.

Immunohistochemistry. Tissue HMGB1 is mainly in oxidized form (Rouhiainen *et al.* 2007), and therefore a monoclonal antibody recognizing the oxidized form was used. Sections of 4 µm were cut from paraffin-embedded liver biopsies, deparaffinized in xylen, and rehydrated in a series of graded alcohols. The endogenous peroxidase activity was inhibited with 0.1% H_2O_2 in PBS. The sections were then blocked and incubated overnight with anti-HMGB1 (clone KS1, MBL International Woburn, MA, USA) in blocking solution. Horseradish peroxidase conjugated anti-mouse IgG (GE Healthcare, Little Chalfont Buckinghamshire, UK) was diluted 1:400 in blocking buffer and added to the sections. After incubation overnight, the sections were washed. Bound antibodies were detected with 1% 3,3′-diaminobenzidine tetrahydrochloride hydrate (Sigma-Aldrich) in 0.0003% H_2O_2 50 mM Tris-HCl-buffer, and after color development the sections were washed. Counterstaining was performed with Toluidine blue.

HMGB1 immunostaining was evaluated using a semiquantitative numerical scoring system, with 0 representing completely negative staining, 1 occasional positive, 2 intermediate, and 3 intense positive staining. Slides were scored independently by a pathologist blinded to the blood HMGB1 levels, and also by two members of the research group.

Protein C and activated protein C

Plasma protein C and APC were determined by an enzyme capture assay (Gruber and Griffin 1992). An immunoaffinity-purified monoclonal antibody against protein C was immobilized in microplates, and the surface was blocked using 1% casein in buffer. For the capture of APC and protein C antigen, plasma samples supplemented with benzamidine were diluted 1:20 in dilution buffer in the microplate, followed by incubation. Then the plates were washed to remove sample constituents and benzamidine. The amidolytic activity of the captured APC was measured using chromogenic substrate S-2366 (Chromogenix AB, Mölndal, Sweden), with substrate hydrolysis measured at 405 nm. Assays were run in duplicate, and a noncommercial plasma pool was used as a standard. The sensitivity of the assay was 5 pmol/l, corresponding to 13% of the normal mean plasma level of APC.

Total protein C was determined by activating the bound protein C in the immunocaptured samples by a snake venom activator enzyme (Protac, American Diagnostica, Greenwich, Conn, USA). This was followed by measuring the amidolytic activity on the chromogenic substrate S-2366. As APC was less than 1% of the total protein C, the amidolytic activity observed after Protac activation essentially equaled the to-

tal protein C value. Protein C antigen was assayed by an Asserachrom Protein C kit (Diagnostica Stago, Parsippany, NJ, USA). Assays were run in duplicate, and pooled normal human plasma (Precision Biologicals, Dartmouth, Nova Scotia, Canada) was used as a standard.

The results of APC and total protein C were expressed as the percentage of the plasma pool defined as 100%. In study II, APC/protein C-ratio was calculated to evaluate the magnitude of protein C activation.

STATISTICAL ANALYSIS

Data were analysed with different versions (9.0-12.0.1) of SPSS for Windows (SPSS Inc., Chicago, IL, USA). As the sample size was small in study II or when the data distribution was skewed according to Kolmogorov Smirnov test in studies I, III, and IV, non-parametric tests were used. Time-dependent changes were evaluated by Friedman's test with *post hoc* Wilcoxon signed rank test with Holm's correction for multiple comparisons (Holm 1979). To assess changes across the liver, either transhepatic gradient (I, II, and IV) or ratio (II, III) was calculated. Transhepatic gradient was calculated as the portal vein value subtracted from the hepatic vein or caval effluent value, where 0 denotes no change, <0 a decrease, and >0 an increase across the liver. Transhepatic ratio was calculated as the hepatic vein or caval effluent value divided by the portal vein value, where 1 denotes no change, <1 a decrease, and >1 an increase across the liver. The significance was tested using the Wilcoxon signed rank test. Bivariate correlations were evaluated by the Spearman rank correlation. The α-level was set at 0.05 for all tests. The data are presented as medians with interquartile ranges (IQR; I, IV) or ranges (II, III), as indicated.

Results

PATIENT OUTCOME

Graft characteristics and the overall outcomes are presented in Table 3. Three patients in study III died during the follow up. The causes of death were: multiorgan failure (day 5 after transplantation), subarachnoid hemorrhage (day 9), and septic shock (day 15). Retransplantation was performed ON two patients due to graft necrosis resulting from graft size disparity (day 5; study III), hepatic artery thrombosis (2 months; the same patient in studies II and III), and persistent graft dysfunction (4 months; study III). Postoperative liver function was good in general (Table 3). Graft steatosis and CIT did not correlate with postoperative laboratory values (data not shown).

Table 3. Graft characteristics and outcome during 1-year follow-up

	I	II	III	IV[a]
Patients, n	30	10	47	20
Cold ischemic time, hrs	5.6 (2.5–14.2)	5.5 (3.2–8.1)	5.5 (2.5–14.2)	5.1 (3.8–10.5)
Anhepatic time, min	54 (40–120)	57 (44–110)	58 (40–120)	56 (35–69)
Graft steatosis[b], %	0 (0–80)	0 (0–80)	0 (0–80)	0 (0–30)
Bleeding, l	7.0 (1.9–20.0)	7.2 (1.9–19.8)	5.8 (1.7–20.0)	4.5 (1.5–13.0)
Postoperative liver function[c]				
ALT, U/l	238 (53–1441)	202 (53–769)	241 (53–10,870)	267 (64–1160)
ALT > 500 U/l, n (%)	7 (23)	2 (20)	12 (26)	5 (25)
ALP, U/l	243 (101–700)	162 (101–307)	287 (101–1407)	-
GGT, U/l	201 (44–1260)	184 (44–400)	329 (47–1438)	-
Bilirubin, µmol/l	57 (12–420)	41 (20–142)	101 (31–529)	34 (12–156)
PT, %	37 (21–61)	34 (27–50)	42 (21–81)	-
Galactose t½, min	-	-	13 (9.5–30)	-
INR	-	-	-	1.5 (1.2–3.6)
Biliary complications, n	5	5	6	0
Vascular complications, n	3	2	3	0
Rejection rate, %	55	80	55	40
Graft survival, %	90	90	87	100
Patient survival, %	93	100	94	100

[a] Follow-up 3 months in study IV, [b] Macrovesicular, [c] During the first postoperative week.
Data are expressed as median (range).
ALT, alanine aminotransferase; ALP, alkaline phosphatase; GGT, gammaglytamyltransferase;
PT, prothrombin time; INR, international normalized ratio

DONOR INFLAMMATORY RESPONSE

Donor characteristics in study I are shown in Table 2. Donor intensive care was associated with both an inflammatory response and liver dysfunction. Donors experiencing hypotensive events had higher IL-8 levels both in their portal and hepatic venous blood as compared to hemodynamically stable donors (see Results in study I). Donor ICU stay correlated with donor CRP and IL-6 levels, and with both donor and recipient GGT levels (Table 4). On the other hand, donor GGT, correlated with recipient peak GGT, ALP, and bilirubin levels (Table 4).

Cytokine and adhesion molecule expression levels are presented in Table 5. In 17 out of 29 donors, hepatic IL-8 release was observed immediately before graft perfusion, *i.e.* IL-8 levels were higher in hepatic venous blood than in portal blood (posi-

Table 4. Correlations between donor and recipient variables

		R	P
Donor ICU stay	Donor CRP	0.472	0.013
	Donor IL-6	0.419	0.026
	Donor GGT	0.478	0.016
	Recipient GGT	0.432	0.019
Donor GGT	Recipient GGT	0.633	0.001
	Recipient ALP	0.422	0.035
	Recipient bilirubin	0.654	<0.001

ICU = intensive care unit, CRP = C-reactive protein, GGT = gamma-glutamyl transferase, ALP = alkaline phosphatase. R,P from Spearman rank correlation.

Table 5. Donor inflammatory response during liver procurement

	Baseline	Portal vein	Hepatic vein
IL-6 (ng/mL)	235 (61–614)[a]	636 (228–1875)	490 (127–1611)
IL-8 (ng/mL)	31 (28–34)[b]	45 (36–61)	47 (39–61)
Soluble ICAM-1 (ng/mL)	219 (174–234)[b]	160 (145–184)	158 (142–176)
PMN CD11b (RFU)	485 (256–673)	387 (264–645)	487 (349–598)
PMN L-selectin (RFU)	93 (76–142)[c]	149 (84–171)	110 (76–158)

Data are expressed as median (interquartile range). PMN = neutrophil, RFU = relative fluorescence unit
[a] $P < 0.01$, [b] $P < 0.001$, [c] $P < 0.05$ baseline vs. portal vein; Wilcoxon signed rank test

tive transhepatic gradient). Similar subgroups could be created according to hepatic IL-6 release and neutrophil CD11b expression, but only hepatic IL-8 release was associated with liver dysfunction. Before organ procurement, GGT levels were elevated in donors in whom the transhepatic IL-8 gradients were subsequently positive (Figure 6). After transplantation, both ALP and bilirubin levels were higher in those recipients who received a graft with a positive transhepatic IL-8 gradient (Figure 6). A similar trend was found for ALT and GGT levels (Figure 6). Five recipients suffered from postoperative biliary complications (anastomotic strictures or leakage). Their occurrence was not associated with hepatic cytokine release in the donor.

INFLAMMATORY RESPONSE DURING LIVER TRANSPLANTATION

Systemic inflammation

In studies II and III, CD11b and L-selectin expression in the circulating neutrophils and monocytes increased throughout surgery (Table 6; see also Table 2 in study III). In study IV, TNF-α levels in systemic circulation decreased before reperfusion, but

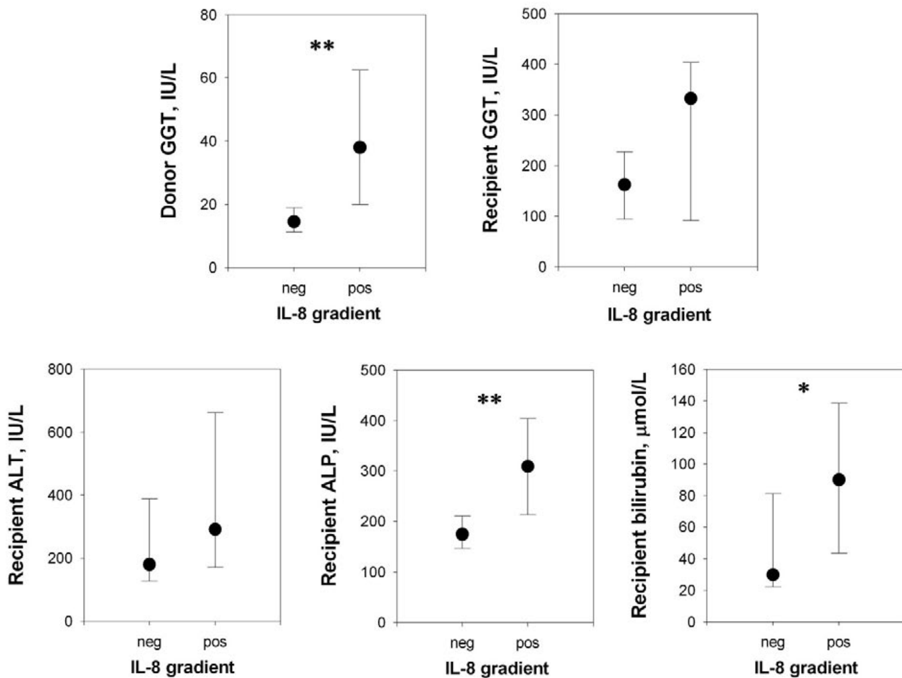


Figure 6. Graft IL-8 release and donor and recipient liver function.
Data are medians with IQR. Transhepatic gradient was calculated as portal vein value subtracted from hepatic vein value. Positive (pos) gradient denotes efflux from the liver, and negative (neg) gradient denotes influx into the liver. * $P < 0.05$, ** $P < 0.01$ pos vs. neg.

returned close to the preoperative levels after reperfusion (Figure 7). In contrast to decreasing TNF-α levels, IL-6 levels had already increased during the recipient hepatectomy. After reperfusion, IL-6 levels remained high throughout surgery, but decreased quickly postoperatively (Figure 7). Unlike TNF-α and IL-6, HMGB1 was undetectable in the systemic circulation before reperfusion. However, HMGB1 levels rapidly increased upon reperfusion and peaked at 10 min after portal vein declamping. Thereafter, HMGB1 levels declined, and at 8 h after reperfusion it was only detectable in six patients (Figure 8).

Table 6. Neutrophil and monocyte adhesion molecule expression during surgery

	0	A1	A2	R1	R2	R3
CD11b Expression, RFU						
Neutrophil	160 (57–443)	244 (64–422)	237 (128–504)	357 (149–470)	309 (168–478)	331 (181–595)
Monocyte	201 (129–556)	216 (93–3286)	198 (108–662)	194 (146–2274)	370 (185–2860)	254 (154–781)
L-selectin Expression, RFU						
Neutrophil	159 (79–305)	206 (100–301)	199 (158–380)[a]	190 (158–380)[a]	214 (166–342)[a]	211 (164–361)[a]
Monocyte	178 (59–285)	295 (167–517)[a]	348 (162–469)[a]	275 (154–543)[a]	266 (174–605)[a]	347 (201–461)[a]

Time points: before surgery (0), before anhepatic period (A1), before reperfusion (A2), during initial reperfusion (R1), 5 min after portal vein declamping (R2), 10 min after hepatic artery declamping (R3). RFU, relative fluorescence unit. [a]$p < 0.05$ vs. 0

Table 7. Hepatic neutrophil and monocyte activation during reperfusion

	R1			R2		
	portal vein	graft caval effluent	transhepatic ratio	portal vein	hepatic vein	transhepatic ratio
CD11b expression, RFU						
Neutrophil	224 (28–502)	364 (29–1048)	1.5 (0.2–4.8)[a]	235 (28–548)	306 (27–886)	1.2 (0.1–2.5)[a]
Monocyte	194 (22–2274)	724 (33–2190)	3.0 (0.2–10.0)[a]	345 (26–3885)	690 (64–2776)	1.6 (0.5–9.8)[a]
L-selectin expression, RFU						
Neutrophil	243 (83–620)	205 (100–441)	0.9 (0.4–1.8)[a]	255 (86–633)	249 (87–628)	0.9 (0.5–1.8)[a]
Monocyte	311 (123–764)	235 (66–568)	0.8 (0.3–1.6)[a]	261 (110–605)	230 (105–589)	0.9 (0.4–1.4)[a]

[a] all $P < 0.001$, graft caval effluent or hepatic vein vs. porta.
Time points: R1 = initial reperfusion, R2 = 5 min after portal vein declamping. RFU = relative fluorescence unit

Hepatic inflammation

During reperfusion, hepatic neutrophil and monocyte activation, as indicated by CD11b upregulation with concomitant L-selectin shedding across the hepatic circulation, was evident (Table 7; see also Results in study II). Graft neutrophil sequestration occurred during initial reperfusion and at 5 min after portal vein declamping (see Results in studies II and III), whereas graft monocyte entrapment was not detected.

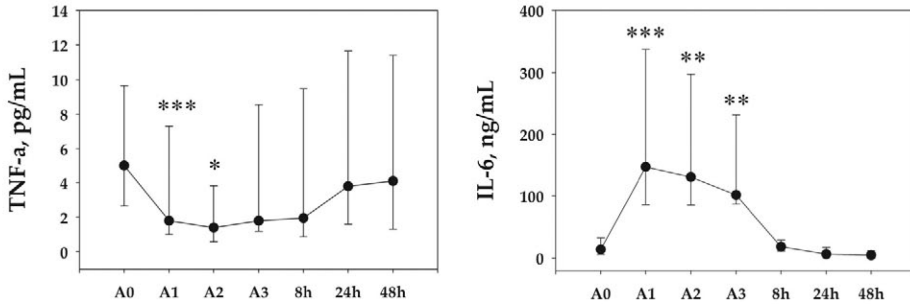


Figure 7. Plasma TNF-α, and IL-6 levels in systemic circulation during and after transplantation. Time points: before surgery (A0), immediately before initial reperfusion (A1), 10 min after portal vein declamping (A2), and 10 min after hepatic artery declamping (A3), and at 8, 24, and 48 hours after reperfusion (8h, 24h, and 48h, respectively). Data are as medians with IQR. *P<0.05, **P<0.01, ***P<0.001 vs. before surgery.

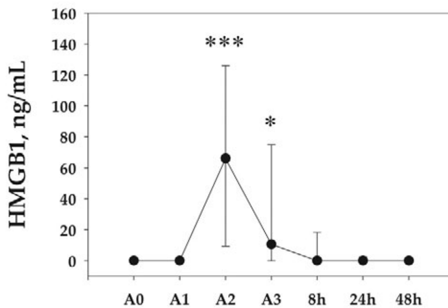


Figure 8. Plasma HMGB1 levels in systemic circulation during and after transplantation. Time points and detailed explanations, see the legend in Figure 7. *P<0.05, ***P<0.001 vs. before surgery.

In study IV, TNF-α levels were comparable across the hepatic circulation at all time points (Table 8). During initial reperfusion, graft IL-6 uptake occurred, as IL-6 levels were higher in portal venous blood than in the caval effluent. A few moments later, at 10 min after portal vein declamping, a slight graft IL-6 release was evident (Table 8).

During initial reperfusion, HMGB1 levels (study IV) were undetected in the portal venous blood. In contrast, HMGB1 levels were remarkably high in the caval effluent, indicating substantial graft HMGB1 release. After reperfusion, ongoing HMGB1 release from the graft was demonstrated as HMGB1 levels remained significantly higher in the hepatic venous blood than in the portal venous blood (Figure 9). Hepatic HMGB1 expression increased during graft preservation and reperfusion in the tissue biopsies. Moreover, HMGB1 was predominantly expressed in the cytoplasm of hepatocytes located close to the central veins (Figure 3 in study IV), with only occasional neutrophil staining. The sinusoidal endothelium and portal structures were HMGB1 negative. The HMGB1 expression in biopsies taken both before and after reperfusion correlated with HMGB1 levels in the caval effluent.

Table 8. TNF-α and IL-6 levels during reperfusion

	Initial reperfusion			10 min portal vein declamping			10 min hepatic artery declamping		
	A	P	H	A	P	H	A	P	H
TNF-α, pg/mL	1.7 (1.0–4.8)	1.9 (0.7–4.9)	1.7 (0.6–3.2)	1.3 (0.5–3.3)	1.2 (0.5–2.1)[a]	1.3 (0.2–4.5)	1.8 (1.0–6.9)	2.1 (0.7–7.3)	2.5 (1.2–6.9)
IL-6, ng/mL	147 (86–337)	153 (81–361)	135 (60–248)[b]	124 (85–184)	122 (77–264)	121 (67–222)[b]	96 (86–224)	113 (80–242)	97 (58–222)

Data are expressed as median (interquartile range). A, systemic artery; P, portal vein; H, hepatic vein (caval effluent at initial reperfusion)
[a] $P < 0.05$, systemic artery vs. portal vein; [b] $P < 0.05$, portal vs. hepatic vein.

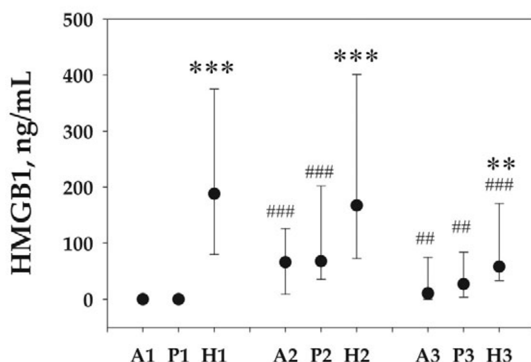
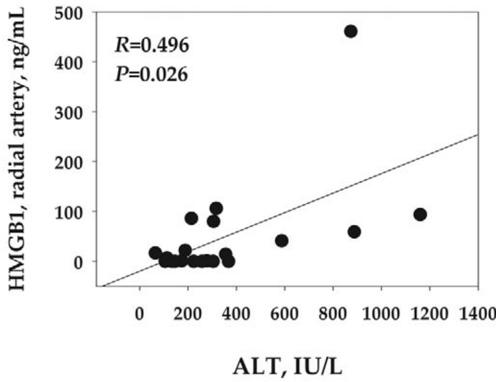
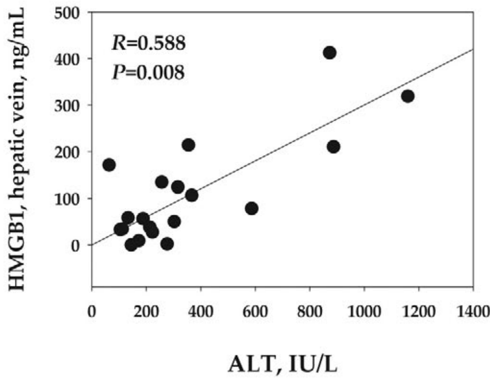


Figure 9. Plasma HMGB1 levels during reperfusion. A, systemic artery; P, portal vein; H, hepatic vein (caval effluent during initial reperfusion). Time points and detailed explanations, see the legend in Figure 7. **P<0.01, ***P<0.001 portal vs. hepatic vein. ##P<0.01, ###P<0.001 vs. previous time point

Graft function

In contrast to those of other cytokines or markers of neutrophil and monocyte activation (data not shown), HMGB1 levels were associated with subsequent graft function. At 10 min after hepatic artery declamping, HMGB1 levels in both the systemic arterial and hepatic venous blood correlated with peak postoperative ALT levels (Figure 10). Further, graft steatosis correlated with HMGB1 levels in hepatic venous blood at 10 min after hepatic artery declamping (Figure 10).





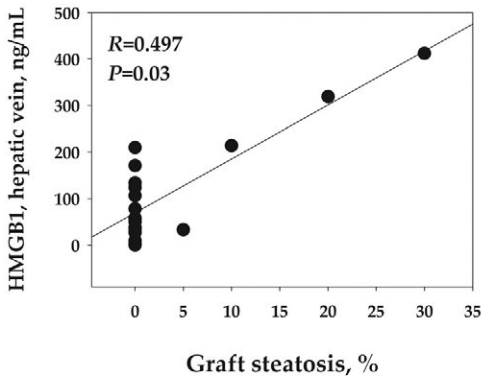


Figure 10. Correlations between HMGB1 levels, ALT, and graft steatosis. For HMGB1, levels represent those measured from radial arterial and hepatic venous blood at 10 min after hepatic artery declamping.

PROTEIN C – ACTIVATED PROTEIN C PATHWAY IN LIVER TRANSPLANTATION

Before surgery, preoperative protein C levels were low, and decreased slightly during surgery in studies II and III. In contrast, APC levels increased constantly during surgery, and peaked at 5 min after portal vein declamping (Figure 11; see also Figure 1 in study III). Calculated APC/protein C-ratio in study II increased during surgery, indicating an increased APC formation despite decreasing protein C levels (Figure 11). The postoperative protein C levels increased markedly with a concomitant decrease in APC levels close to those observed preoperatively (Figure 11).

Protein C levels were lower in the caval effluent as compared to portal venous blood during initial reperfusion , but not thereafter, indicating substantial graft protein C uptake (Figure 12; see also Figure 2 in study II). In study III, graft protein C

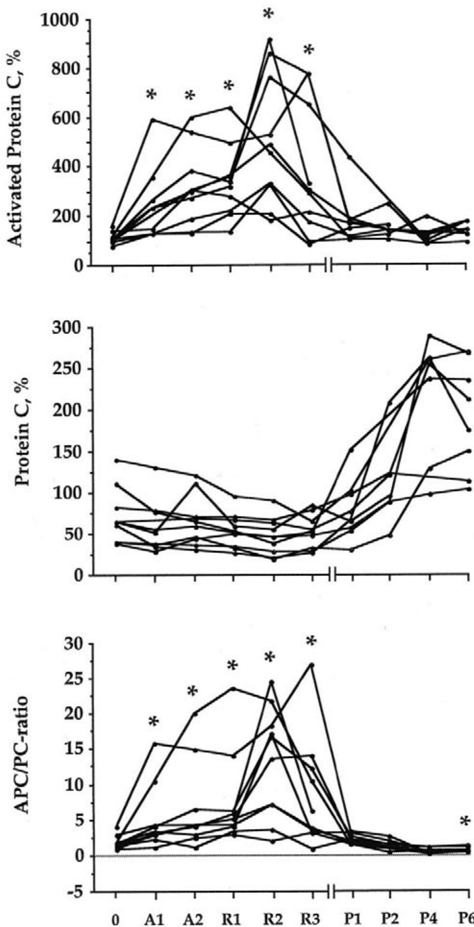


Figure 11. Plasma activated protein C (APC), and protein C (PC) levels, and APC/PC ratio during and after transplantation. Time points: before surgery (0), before anhepatic period (A1), before reperfusion (A2), during initial reperfusion (R1), 5 min after portal vein declamping (R2), 10 min after hepatic artery declamping (R3). P1-P6 indicate postoperative days. *$P<0.05$ vs. before surgery.

entrapment occurred in all but two patients (n=45). Despite graft protein C uptake, APC levels were comparable across the hepatic circulation, suggesting that APC was not released concomitantly from the graft with protein C uptake (Figure 12).

Transhepatic protein C ratio (III) correlated with neutrophil and monocyte CD11b expression levels. This indicated that graft protein C uptake was associated with weaker hepatic neutrophil and monocyte activation during initial reperfusion (Figure 13).

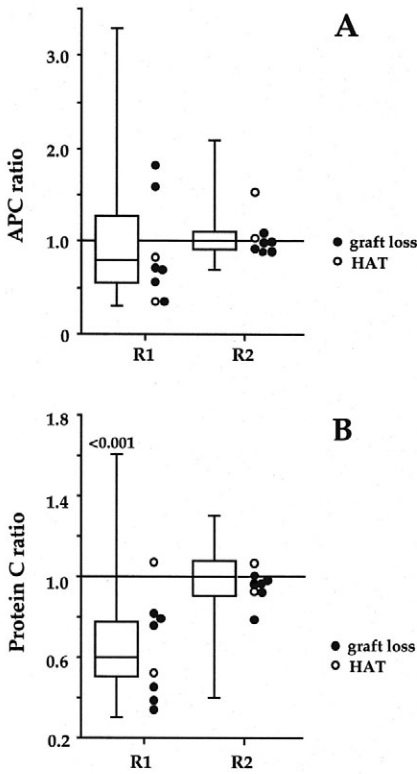


Figure 12. Protein C uptake within the liver graft during reperfusion. Transhepatic ratios of APC and protein C. Transhepatic ratio was calculated as hepatic venous or graft caval effluent value divided by portal venous value. Time points: during initial reperfusion (R1), 5 min after portal vein declamping (R2). Values for the whole study population are expressed as box-whisker plots. Boxes indicate median with IQR. Vertical bars denote the range. P value is given for caval effluent vs. portal vein.

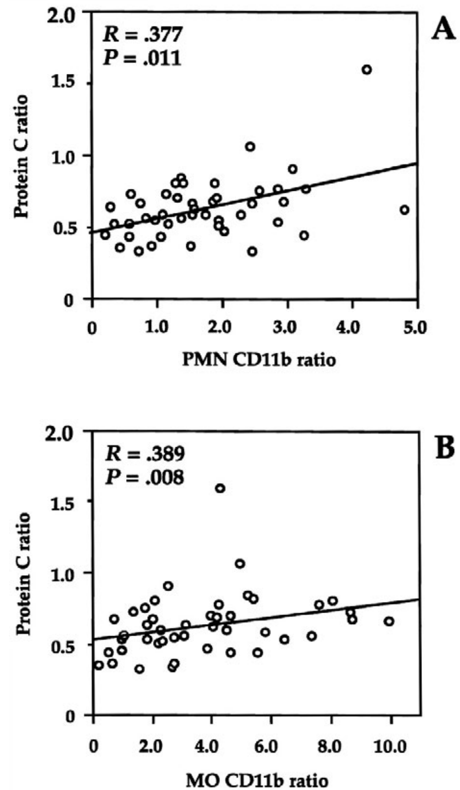


Figure 13. Correlation between transhepatic protein C ratio and transhepatic neutrophil (PMN) and monocyte (MO) CD11b ratio during initial reperfusion. Transhepatic ratio was calculated as graft caval effluent value divided by portal venous value.

Discussion

METHODOLOGY

The sample sizes of each study were mainly constrained by the availability of patients, *i.e*. the annual liver transplantation rate in HUCH that varies from 40 to 60 per year. All studies comprise consecutive patients. Nevertheless, the sample sizes are similar or even large compared to previous observational studies on the inflammatory response in clinical liver transplantation. Further, in study II with only 10 patients, a similar trend in study parameters were observed in all patients, implying a true biological phenomenon.

The patient population was heterogenous because both patients with ALF and those with chronic liver disease were included. This may have influenced the results to a certain extent, as ALF is characterized by systemic inflammatory response with elevated plasma cytokine levels (Muto *et al*. 1988, Sekiyama *et al*. 1994). Further, in experimental liver transplantation, Wang *et al*. (2001) demonstrated that the proinflammatory state of the recipient potentiates I/R injury. On the other hand, the numbers of ALF patients included in these studies were small (Table 1), and any resulting strong influence on the results is therefore unlikely.

Immunosuppression varied in studies III and IV, with either cyclosporine or tacrolimus based regimens being used. In addition to inhibiting T-cell proliferation and activation, tacrolimus and cyclosporine have similar effects on the innate immune system. These immunosuppressants *in vitro* inhibit Kupffer cell activation (Tojimbara *et al*. 1996) in addition to downregulating hepatic TNF-α expression and inhibit neutrophil tissue infiltration in experimental liver I/R injury (Suzuki *et al*. 1993, Ishii *et al*. 1994, Matsuda *et al*. 1998). Thus, presumably the effect of immunosuppression on the inflammatory response was concordant regardless of the drug used. Although the effect of immunosuppression was not evaluated in study III, in contrast there was no difference in cytokine levels between patients administered with either cyclosporine or tacrolimus in study IV.

Systemic blood samples during reperfusion were not taken in studies II and III, instead only blood samples from the portal and hepatic veins were collected. During portal venous clamping (the anhepatic phase) the splanchnic region suffers from stagnant blood flow and hypoperfusion, followed by reperfusion upon restoration of the portal blood flow. In an experimental setting, portal vein ligation induces activation of coagulation cascades in the intestinal circulation (Nakao and Tagaki 1993). On the other hand, reperfusion in clinical liver transplantation is associated with inflammatory activation in the splanchnic region (Pesonen *et al*. 2000). It is possible that protein C and/or APC are utilized in anticoagulant and anti-inflammatory activity within

the splanchnic microvascular beds during liver reperfusion. Therefore, this may have influenced protein C and APC levels in the portal venous blood, and thus the levels of these substances within the liver. Although protein C and APC levels in systemic circulation might have been higher than those in the portal venous blood, the main focuses of studies II and III were on the liver, and the study setting depicts the actual situation within the liver during reperfusion.

DONOR INFLAMMATORY RESPONSE

Plasma HMGB1 levels in study IV were already slightly elevated in organ donors before graft procurement. This plausible reflects the systemic proinflammatory response induced by brain death (Palombo *et al.* 1994, Strangl *et al.* 2001, Weiss *et al.* 2007, Murugan *et al.* 2008).

Hepatic IL-8 release observed in study I reflects the end result of several contributing factors. In human organ donors, liver cytokine expression is already upregulated at the beginning of donor laparotomy (Kuecuek *et al.* 2005, Weiss *et al.* 2006), suggesting that circumstances before organ procurement influence graft quality. Both brain death and the following donor intensive care can contribute to inflammatory responses in the liver. In a clinical setting, it is impossible to investigate the effects of brain death independently of donor intensive care. However, in experimental models, brain death alone leads to enhanced cytokine expression in Kupffer cells, hepatocytes, and sinusoidal endothelial cells (Takada *et al.* 1998, Okamoto *et al.* 2000). Nonetheless, donor intensive care seems to aggravate the hepatic inflammation already present. Jassem *et al.* (2003) reported that donor infection, mechanical ventilation, and vasoactive support were associated with the activation of inflammatory cascades in the liver. Moreover, organ procurement surgery itself possibly activates directly through the Kupffer cell activation (Schemmer *et al.* 1998), hepatic cytokine expression during donor laparotomy (Weiss *et al.* 2006). In the current study, the relative impact of brain death, intensive care, and surgery on the graft IL-8 release could not be determined. Even so, blood samples across the hepatic circulation were collected immediately before graft perfusion, thereby reflecting the cumulative effects of these factors upon graft viability.

Hepatic IL-8 release during graft retrieval correlated with higher recipient ALP and bilirubin levels during the first postoperative week. This was accompanied by a trend towards higher ALT and GGT levels. These data suggest that donor inflammatory response predisposes to liver dysfunction after transplantation. Weiss *et al.* (2006) demonstrated more intense inflammatory activation and I/R injury (defined as elevated transaminase levels) in grafts from brain dead donors, compared to those from living donors. In this study, instead of the immediate hepatocellular damage af-

ter reperfusion, hepatic IL-8 release during procurement was associated with apparent biliary dysfunction a few days after transplantation. The biliary epithelium is sensitive to cold ischemia and reoxygenation (Noack *et al*. 1993). In addition, long CIT is also associated with biliary complications after transplantation (Sanchez-Urdazpal *et al*. 1992). Interestingly, hepatic IL-8 release in the donor was not only associated with recipient liver function, but also with elevated donor GGT levels. Moreover, donor GGT levels correlated with recipient postoperative GGT, ALP, and bilirubin levels. These findings suggest that damage to the biliary epithelium may already have begun before graft preservation.

Systemic inflammation, hemodynamic instability, and mechanical ventilation are known risk factors for liver dysfunction in patients in intensive care (Brienza *et al*. 2006). In addition to changes caused by brain death, donor intensive care may contribute to graft quality. Indeed, a prolonged donor ICU stay is reported to be a risk factor for poor graft function and outcome (Briceno *et al*. 2002, Cuende *et al*. 2005). In this study, even short donor ICU stay (median one day) correlated with both elevated GGT levels in the donor and the recipient. These data underline that factors related to donor intensive care, although essential for organ donation, can also contribute to liver dysfunction subsequent to tranplantation.

INFLAMMATORY RESPONSE DURING LIVER TRANSPLANTATION

Systemic inflammation

During recipient hepatectomy, surgical stress had already induced systemic inflammation. Both CD11b and L-selectin expression on neutrophils and monocytes increased in studies II and III, indicating the activation of circulating phagocytes (Kishimoto *et al*. 1989) and mobilization of new cells from the bone marrow (van Eeden *et al*. 1995). Further, IL-6 levels in systemic circulation increased during hepatectomy, and remained high throughout surgery in study IV.

After transplantation, both IL-6 and HMGB1 levels decreased rapidly and were detectable in only a few patients at 8 h after reperfusion in study IV. In clinical sepsis, elevated IL-6 and HMGB1 levels persist for days, indicating ongoing inflammation (Sunden-Cullberg *et al*. 2005). The rapidly declining IL-6 and HMGB1 levels observed in this study argue against prolonged systemic inflammatory response after liver transplantation. However, in this study patient recovery was uneventful in most cases and graft function remained good. Possibly, elevated IL-6 and HMGB1 levels could be detected in the event of postoperative complications. Indeed, Mueller *et al*. (1996) reported that prolonged elevation of IL-6 postoperative levels seems to indicate impaired graft function in human liver transplantation.

Hepatic inflammation

During initial reperfusion, pronounced hepatic neutrophil and monocyte activation and subsequent sequestration became evident, as manisfested by a transhepatic increase in CD11b expression and a decrease in cell counts in studies II and III. Earlier, it was suggested that phagocytes have no role in the initial liver I/R injury as hepatic phagocyte infiltration was delayed for up to several hours after reperfusion (Jaeschke *et al.* 1990). However, these results were from experimental studies on liver I/R injury after long warm ischemic times. The data presented in this thesis corroborate previous studies, both experimental and clinical, which indicated that, after cold ischemia, phagocyte activation occurs immediately after restoration of blood flow (Shibuya *et al.* 1997, Pesonen *et al.* 2000, Kataoka *et al.* 2002).

As the liver is the main site for IL-6 clearance in the body (Castell *et al.* 1988), it is not surprising that IL-6 uptake by the liver occurred during initial reperfusion in study IV. This finding is in accordance with previous studies by our group (Pesonen *et al.* 2000). Although speculation, this IL-6 uptake may reflect IL-6 utilization in anti-inflammatory processes. IL-6 treatment reduces I/R injury after warm liver ischemia (Camargo *et al.* 1997), and endogenous IL-6 regulates hepatocyte apoptosis and induces liver regeneration (Clavien *et al.* 2003).

Extensive hepatic HMGB1 release occurred during initial reperfusion in study IV. Afterwards, HMGB1 levels remained significantly higher in hepatic venous blood than they did in portal blood, indicating ongoing HMGB1 release from the graft. However, both absolute HMGB1 levels in hepatic venous blood and the calculated transhepatic gradient decreased after initial reperfusion. Therefore, the HMGB1 efflux appears to reflect a wash-out of HMGB1 released as early as during graft preservation and/or immediately during initial reperfusion, *i.e.* graft flushing with portal venous blood instead of progressive inflammatory activation.

HMGB1 has been regarded as a proinflammatory cytokine that is actively secreted by neutrophils, monocytes, and macrophages (Wang *et al.* 1999, Lotze and Tracey 2005). Although transhepatic neutrophil and monocyte activation was evident during reperfusion, hepatocytes appear to be the main source of HMGB1 in clinical liver transplantation. Moreover, HMGB1 expression in hepatocytes significantly increased during graft preservation and reperfusion, whereas neutrophils infiltrating the liver, portal structures, and the sinusoidal endothelium had no or only weak HMGB1 staining. In addition to active secretion from the innate immune cells, HMGB1 is released passively from damaged cells (Scaffidi *et al.* 2002). Although hepatocytes are relatively resistant to cold preservation, they are readily damaged by the subsequent rewarming ischemia (Vajdová *et al.* 2000). Further, despite maintaining cellular integrity, hepatocyte HMGB1 expression increases quickly after noxious stimuli (Sass *et al.* 2002, Tsung *et al.* 2005a, Tsung *et al.* 2007). In this study, HMGB1 was pre-

dominantly expressed in hepatocytes located close to the central veins, *i.e.* in areas most susceptible to ischemic injury. Thus, HMGB1 appears to originate mainly from hepatocytes, either through active secretion from ischemic cells or passive release from those that are necrotic.

HMGB1 stimulates phagocytes to release proinflammatory cytokines including TNF-α, IL-1β, IL-6, and IL-8 (Andersson *et al*. 2000, Park *et al*. 2003). Interestigly, HMGB1 levels did not correlate with those of TNF-α and IL-6. Rouhiainen *et al*. (2007) have demonstrated that HMGB1 itself exhibits only weak proinflammatory activity, whereas binding to bacterial lipid components strengthens the proinflammatory effects of HMGB1 (Rouhiainen *et al*. 2007, Sha *et al*. 2008). Although li ver transplantation is known to induce systemic endotoxemia, LPS is not uniformly present in all patients (Hamilton *et al.* 1993). Indeed, in the absence of stimulating cofactors, the proinflammatory effects of HMGB1 may be of minor significance in clinical liver I/R injury.

Graft function

Surprisingly, neither hepatic phagocyte activation and sequestration, nor cytokine levels correlated with postoperative graft function (II, III, and IV). Hepatic neutrophil activation during reperfusion in particular was very strong compared to that in other organs. In clinical kidney transplantion, even modest kidney neutrophil sequestration predicts delayed graft function (Turunen *et al*. 2004). However, despite early infiltration into the graft after reperfusion in experimental liver transplantation, neutrophils may not contribute to hepatocellular injury, if the cold preservation period remains short (Kataoka *et al*. 2002). In other words, a short CIT may protect the liver from neutrophil-mediated graft injury. In our centre, CIT is generally very short (median 5 h 27 min in study III). Although undeniable as a phenomenon, the clinical significance of hepatic neutrophil activation may be questioned in human liver transplantation, at least after a short graft cold preservation.

To reiterate, HMGB1 was expressed in hepatocytes after reperfusion, whereas innate immune cells did not seem to contribute to HMGB1 release in study IV. Accordingly, HMGB1 primarily appears as a marker of hepatocellular injury. Indeed, sustained hepatic HMGB1 release was associated with postoperative graft function. Hepatic venous HMGB1 levels measured at 10 min after hepatic artery declamping (approximately 45 min after initial reperfusion) correlated both with graft steatosis and peak postoperative ALT levels. The correlation between HMGB1 release and graft steatosis suggests that even mild steatosis is associated with pronounced injury during preservation and reperfusion. Further, graft HMGB1 outflow was associated with higher peak postoperative ALT levels, supporting the role of HMGB1 as an indicator of hepatocellular injury.

PROTEIN C – ACTIVATED PROTEIN C PATHWAY IN LIVER TRANSPLANTATION

Acquired protein C deficiency contributes to postoperative thrombotic complications after liver transplantation (Harper *et al.* 1988). In this study, as expected, protein C levels were low preoperatively and decreased during transplantation in patients with liver insufficiency. However, patients were able to maintain normal to supranormal APC levels throughout surgery. This indicates that, despite protein C deficiency, protein C activation is enhanced to preserve coagulation homeostasis. Further, protein C levels normalize within a few days after a successful transplantation, which indicates normal synthetic function of the liver. These may be the key factors preventing thrombotic complications related to protein C deficiency during initial graft reperfusion. In this study, there was no association between protein C or APC levels and thrombotic complications. Three patients developed postoperative HAT, possibly due to surgical problems encountered in the arterial anastomosis (Settmacher *et al.* 2000).

Reperfusion of the liver creates an environment that favours protein C activation. TM is already upregulated on the endothelial surface as early as during graft preservation (Suehiro *et al.* 1997), and hepatic thrombin generation occurs rapidly during initial reperfusion (Himmelreich *et al.* 1994). Although not indispensable to protein C activation by thrombin-TM complex, EPCR is upregulated on the endothelium in response to thrombin formation (Gu *et al.* 2000). This probably enhances protein C activation to APC. In this study, substantial graft protein C uptake occurred within the hepatic circulation during initial reperfusion without a corresponding outflow of APC from the graft. If protein C had been simply activated without concomitant APC utilization, a significant APC release from the liver would be expected. Thus the lack of APC release suggests a demand for APC within the graft during initial reperfusion.

During initial reperfusion, extensive phagocyte activation and trapping in the liver concomitantly with graft protein C uptake became evident. Further, enhanced protein C consumption was associated with reduced hepatic phagocyte activation. These data suggest involvement of the protein C pathway in regulation of the anti-inflammatory processes *in vivo*. Therefore a direct inhibitory effect of protein C, on phagocytes is possible without preceding activation (Sturn *et al.* 2003). Alternatively, protein C uptake can non-exclusively result in significant intrahepatic activation and subsequent APC involvement in anticoagulant and anti-inflammatory activity.

Several molecular and cellular mechanisms underlie the cytoprotective actions of the protein C pathway. APC suppresses the transcription factor NF-κB expression and functional activity, in addition to inhibiting transcription factors of the activator protein-1 (AP-1) family (Joyce *et al.* 2001). Thus APC inhibits cytokine signalling

and adhesion molecule expression in both the innate immune and endothelial cells. In this study, the described phenomena occurred within only a few minutes during initial reperfusion. Therefore, mechanisms involving gene transcription and protein synthesis are unlikely to explain these findings.

The association between protein C entrapment and reduced phagocyte CD11b expression suggests that mechanisms involved in cell adhesion are important in I/R injury. *In vitro,* protein C pathway modulates leukocyte recruitment into sites of inflammation. APC inhibits phagocyte migration toward chemoattractants (Sturn *et al.* 2003, Nick *et al.* 2005, Galley *et al.* 2008). It may also inhibit both selectin and integrin mediated phagocyte adhesion. Moreover, APC impairs selectin-mediated leukocyte attachment to the endothelium (Grinnell *et al.* 1994). Soluble EPCR also binds to neutrophils via CD11b and may further interfere with phagocyte-endothelial interaction (Kurosawa *et al.* 2000). In experimental liver I/R injury, APC decreases neutrophil accumulation in the postischemic tissue (Yamaguchi *et al.* 1997, Kuriyama *et al.* 2008). This effect is likely to be mediated through cell adhesion, as APC has been observed to reduce ICAM-1 expression on the endothelium and tissue accumulation of CD11b positive neutrophils in other experimental I/R-injury models (Shibata *et al.* 2001). Moreover, previous studies on clinical I/R injury suggest that APC partly exerts its anti-inflammatory activities through the inhibition of neutrophil adhesion. In coronary bypass surgery, postischemic protein C activation is associated with decreased cardiac neutrophil accumulation (Petäjä *et al.* 2001). By comparison, in kidney transplantation, APC utilization within the graft is associated with decreased neutrophil L-selectin shedding and CD11b upregulation, in addition to reduced lactoferrin release (Turunen *et al.* 2005). Taken together, the results in the present study corroborate previous findings, and suggest that anti-inflammatory properties of protein C pathway in clinical liver transplantation are related to decreased neutrophil and monocyte activation in addition to reduced potential for endothelial adhesion.

FUTURE CONSIDERATIONS

In liver transplantation, both clinical and experimental, research has been confined to graft preservation and reperfusion, and few studies on the organ donors *per se* exist. However, organ shortage is becoming a critical problem, which has lead to the acceptance of grafts obtained from "extended criteria" donors. Therefore, instead of focusing studies on merely I/R injury, the emphasis should be directed onto the donor. Brain death itself has been shown to activate inflammatory responses in donor organs (Pratschke *et al.* 1999). Further, in a clinical setting the inevitable intensive care of the donor before organ procurement may also affect graft quality. In this thesis, hepatic

inflammatory activation already occurred during graft procurement and was associated with subsequent graft dysfunction (I). Accordingly, influencing the inflammatory responses in the donor appears tempting. In a preliminary clinical study, Kuecuek *et al.* (2005) reported that continuous steroid infusion after a loading dose leads to decreased hepatic cytokine expression in human brain dead liver donors, although the effects on subsequent graft function were not reported. Probably, limiting inflammation in the donor would result in better graft function. In the future, after resolving the ethical questions related to pre-emptive treatment of the donor, anti-inflammatory interventions may well become routine.

The clinical risk factors for graft dysfunction are quite well defined (Nissen and Colquhoun 2005). However, in individual patients these risk factors may not accurately predict the outcome of transplantation. Identifying the patients at risk of PDF or PNF during surgery would help the optimization of the postoperative treatment of these patients and, possibly avoid complications. In this thesis (IV), HMGB1 indicated hepatocellular damage. After arterial reperfusion, HMGB1 levels measured in the systemic circulation correlated with peak postoperative ALT levels. HMGB1 might therefore be useful as an early marker of graft dysfunction, although a study with sufficient statistical power is needed to evaluate the prognostic value of HMGB1 in liver transplantation. If grafts developing primary dysfunction could be identified as early as during reperfusion, possible interventions could be directed at those recipients in a timely fashion.

As I/R injury can influence subsequent graft function, interventions based on physiological anti-inflammatory agents would be useful. A growing body of evidence supports the anti-inflammatory effects of the protein C pathway in I/R injury (Levi *et al.* 2004). In rats, APC treatment has multiple cytoprotective effects that have been reported to be mediated through both anti-inflammatory and anticoagulant functions in warm hepatic I/R injury (Kuriyama *et al.* 2008). In this thesis, a rapid and marked hepatic protein C uptake occurred without a concomitant APC release in studies II and III. A shortage of APC relative to its physiologic demand and/or APC independent anti-inflammatory action of protein C during reperfusion may explain the phenomenon. Both previous experimental research and the current results, provide a rationale for future clinical intervention studies with protein C or APC in liver I/R injury. As the most marked changes were observed during initial reperfusion, *i.e.* during graft flushing with portal venous blood, supplementation during this period seems feasible. Moreover, the systemic effects on the coagulation system could be avoided, as the caval effluent is wasted and not released into the systemic circulation.

Conclusions

The following conclusions can be drawn from these studies:

1. Even a short donor ICU length of stay correlated with elevated GGT levels in the donor. Donor livers with even a subtle dysfunction in turn exhibited IL-8 release during graft procurement and subsequent graft, particularly biliary, dysfunction in the recipient. These findings suggest that the inflammatory response related to brain death and the care of the donor influenced subsequent graft function in the liver recipient (I).

2. Despite low protein C levels related to liver insufficiency, patients were able to maintain coagulation homeostasis through enhanced protein C activation, as indicated by the normal to supranormal APC levels observed. Protein C levels normalized within a few days after a successful liver transplantation. During initial reperfusion, a marked hepatic protein C uptake was evident without a concomitant APC release from the graft. Further, enhanced graft protein C uptake was associated with weaker hepatic neutrophil and monocyte activation. These data suggest that APC is utilized within the graft, at least partly, in anti-inflammatory activity (II,III).

3. HMGB1 in the systemic circulation originated from the graft. After an uneventful liver transplantation, HMGB1 levels normalized rapidly, which suggested that a prolonged inflammatory response did not occur. During reperfusion, a substantial and continuous HMGB1 release from the graft occurred. Immunohistochemistry revealed that hepatocytes, particularly those located close to the central veins were the primary source for HMGB1. Graft HMGB1 release correlated with both steatosis and peak postoperative ALT. HMGB1 therefore appears to be a marker of hepatocellular injury, and might have a prognostic value in clinical liver transplantation (IV).

Akcnowledgements

This thesis was carried out at the Transplantation and Liver Surgery Clinic, Department of Surgery; the Department of Bacteriology and Immunology, Haartman-Institute; and the Department of Anesthesiology and Intensive Care Medicine at Helsinki University Central Hospital and University of Helsinki.

I wish to express my sincere gratitude to those who have contributed to this project:

Professor Seppo Meri for the opportunity to use the facilities in the Department of Bacteriology and Immunology in Haartman-Institute. Professor Per Rosenberg for providing excellent conditions for both clinical and scientific work in the Department of Anaesthesiology and Intensive Care Medicine.

My supervisors, professor Krister Höckerstedt and Dr Eero Pesonen. Krisse's optimism and constant interest in my work helped to complete this thesis despite the occasional misfortunes. Eero guided my first steps into medical research and later encouraged to grow into an independent researcher.

Docent Heikki Repo, who placed his laboratory and, more importantly, thorough knowledge on neutrophils at my disposal during the project.

Docent Jari Petäjä for collaboration in APC-studies. It has been a pleasure to discuss science, among other aspects of life, with Jari.

Docent Helena Isoniemi, who always took the time to help whenever I needed advice or access to the registry data.

My co-authors, whose expertise in their respective fields has been of great benefit during these studies. I would especially like to thank Dr Eija Tukiainen for the smooth and dependable collaboration during the HMGB1 study and PhD Ari Rouhiainen for the pleasant moments in the laboratory in the Neuroscience Center.

Professor Risto Renkonen and docent Mikko Pakarinen, who reviewed this thesis, provided valuable comments, and proposed directions for future research.

Docent Anu Koivusalo, who introduced me to the anesthesia and intensive care of liver transplantation patients. Dr Arno Nordin and Dr Marko Lempinen for enlightening me on the surgical aspects of liver transplantation.

RN Marja Friman for the indispensable help in practical matters and sample collection. Without her this thesis simply would not exist. It has been a great pleasure to work with Marja in the OR.

Transplantation coordinators in Surgical Hospital, who collected the samples in study I, provided me with excellent files on organ donors, and all along helped to track missing patient charts.

Personnel in ELLA and in the intensive care unit in Surgical Hospital for positive attitude towards scientific ventures taking place almost exclusively at night-time.

Ms Maija Peltoperä, who taught me the principles and practice of flow cytometry.

Tulehdusklubi members Hannele Aalto, Antti Kuuliala, and Riikka and Arto Turunen, for interesting discussions on inflammation.

Ms Heli Penttinen for the layout, with cover image by courtesy of Blue Histology.

My dear sister Johanna Ilmakunnas, a dedicated scholar herself, for support and encouragement during moments of desperation, but also sharing those of joy, during these years.

Harri and Helmi.

The financial support from the Mary and Georg C. Ehrnrooth Foundation, the Wyeth Grant for Organ Transplantation, Biomedicum Helsinki Foundation, Finnish Medical Foundation, and Helsinki University Central Hospital research funds (EVO), is gratefully appreciated.

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