Molecular changes in hepatobiliary function and injury after human liver transplantation
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SUMMARIZING DISCUSSION
INTRODUCTION

The liver plays an important role in the metabolism of carbohydrates, fats and several proteins. It is the main producer of plasma proteins such as albumin and coagulation factors (1). Next to uptake and synthesis, the liver excretes a range of compounds like bile salts, phospholipids, cholesterol, bilirubin and xenobiotics, via bile (2). Bile formation is an important function of the liver. Bile functions as an escape route of excess, waste and toxic compounds and is necessary for efficient digestion and absorption of fats and fat-soluble vitamins in the intestine (3,4). Bile flow mainly depends on the active secretion of bile salts into bile canaliculi by the hepatocytes (5-7). Bile salts are synthesized in hepatocytes from cholesterol (8,9). The secretion of bile salts and cholesterol into bile is under normal circumstances accompanied by the excretion of phospholipids (10,11). Together, bile salts, phospholipids and cholesterol form mixed micelles in bile. Bile salts are potent detergents that keep hydrophobic compounds, such as cholesterol in solution and aid in their secretion or absorption (12). The detergent properties of bile salts in turn, are neutralized by phospholipids (13). Secretion of bile salts and phospholipids into bile is an active process, mediated by the ATP binding cassette (ABC)-transporter proteins BSEP (bile salt export pump) and MDR3 (multidrug resistance protein type 3), respectively. These transporters are located in the canalicular hepatocyte membrane that faces the bile (14-17). Several animal experiments have suggested that the ABC half-transporters Abcg5 and Abcg8 are responsible for the biliary secretion of cholesterol (18-21). Cholesterol fulfills an essential role in the body. It is the precursor of steroid hormones and bile salts, and forms an important constituent of (intra) cellular membranes. Cholesterol is obtained through dietary intake and endogenous synthesis, the latter being the major source in humans (22). A well balanced cholesterol homeostasis is essential. High plasma cholesterol concentrations, especially in the Low-Density-Lipoprotein (LDL) fraction (the so-called "bad cholesterol"), are associated with an increased risk for the development of atherosclerosis and may eventually lead to ischemic vascular disease. The removal of excess cholesterol from the body is performed by a complex network of tightly regulated pathways. For the biliary excretion of cholesterol, hepatic Abcg5/Abcg8 transporters are believed to be important mediators.

Several viruses, autoimmune diseases, drugs and inherited intrahepatic cholestatic syndromes can affect normal liver physiology and cause hepatic failure and disease. Patients with end-stage liver disease may ultimately require an orthotopic liver transplantation (OLT). OLT is associated with ischemia/reperfusion (I/R)
which represents a continuum of harmful processes that may culminate into compromised donor liver function. It is increasingly recognized that cells respond to I/R by activation of various cytoprotective pathways such as the hemoxynase-1 (HO-1) system. HO-1 has been proposed as a graft survival gene \(^{(22)}\), and is suggested to exert four major protective properties: (i) antioxidant function, (ii) maintenance of microcirculation, (iii) modulatory function upon the cell cycle, and (iv) anti-inflammatory function \(^{(22)}\). HO-1 forms the rate-limiting step in the enzymatic conversion of heme into CO, Fe\(^{2+}\) and bilirubin \(^{(28)}\). Protective properties of HO-1 are thought to result from the elimination of heme, and the function of its downstream biologically active mediators \(^{(29,30)}\). Although the downstream molecules have cytoprotective properties, at high concentrations all of them are cytotoxic \(^{(31-34)}\).

Liver transplantation may postoperatively be accompanied by acute cellular rejection of the graft. Acute cellular rejection is characterized by the infiltration of granzyme B-positive cytotoxic T lymphocytes (CTLs) into the transplanted liver that cause inflammation with subsistent hepatobiliary damage \(^{(35)}\). CTLs can induce injury via the granule exocytosis route in which the perforin/granzyme-B molecules collaborate to induce target cell apoptosis (programmed cell death). In this pathway, perforin creates transmembrane pores through which granzyme-B can enter the target cell, and cause target cell demise \(^{(36)}\). Activity of granzyme-B can be blocked by the endogenous protease inhibitor-9 (PI-9) \(^{(37,38)}\). It is suggested that an overexpression of PI-9 in the graft could resist a fatal attraction of CTLs, and provide an explanation for the phenomenon of subclinical rejection in human kidney transplantation \(^{(39)}\).

In chapter 1 an overview is given of the current knowledge of several ABC transporters that play an important role in the recovery of normal liver physiology after transplantation, and of the cytoprotective molecules HO-1 and PI-9 which may protect the graft against the detrimental effects of I/R and for graft infiltrating CTLs during the development of early acute rejection.

In humans, alternative pathways of hepatobiliary cholesterol secretion exist that are not controlled by ABCG5/ABCG8

The molecular mechanisms that are involved in biliary cholesterol secretion have been studied extensively \(^{(40,41)}\). Nowadays, it is assumed that Abcg5 and Abcg8 are critically involved in this process \(^{(42,43)}\). Animal experiments have shown that Abcg5 and Abcg8 are coordinately regulated and dimerize into a functional transporter \(^{(44-49)}\). Strong relationships between hepatic Abcg5/Abcg8 mRNA levels and biliary cholesterol secretion rates in (genetically-modified) mice, have indicated
a high degree of control exerted by this transporter pair \(^{(50-55)}\). In the study described in chapter 2 we have examined the relationship between hepatic ABCG5/ABCG8 expression and biliary cholesterol secretion in OLT patients to assess the role of these transporters in biliary cholesterol secretion in the human situation. Postoperatively, bile samples were collected daily during the first two postoperative weeks to determine bile composition. ABCG5 and ABCG8 gene expression was assessed by real time PCR in liver biopsies that were collected during and one week after transplantation. Hepatic ABCG5 and ABCG8 mRNA levels were strongly correlated, supporting the concept of a coordinated regulation of both genes and heterodimerization of both proteins into a functional transporter. After transplantation, biliary cholesterol secretion continuously increased. Hepatic mRNA levels of ABCG5 and ABCG8 however, remained unaffected in time. Surprisingly, we could not find a correlation between hepatic expression of ABCG5/ABCG8 and biliary cholesterol secretion rates. On basis of these findings we suggested that, although the biliary secretion of cholesterol may be mediated by ABCG5/ABCG8, this step does not seem to be rate-controlling in the overall process. Moreover, it may very well be that alternative, ABCG5/ABCG8-independent processes are involved. Recent studies in mice showed that cholesterol secretion in bile might be facilitated by PXR target genes \(^{(56,57)}\). PXR target genes may be involved in the modulation of the activity of ABCG5/G8 transporters or could constitute an alternative cholesterol secretion pathway. In this respect it is important to note that, while in Abcg5\(^{-/-}\)/Abcg8\(^{-/-}\) mice the biliary cholesterol levels are extremely low compared to wild type mice \(^{(58)}\), the secretion of cholesterol and other sterols in bile is reduced by only ~50% in patients with sitosterolemia compared to healthy individuals \(^{(59)}\). This residual biliary sterol secretion in sitosterolemic patients \(^{(60)}\), and the absence of a relationship between hepatic ABCG5/G8 expression and biliary cholesterol secretion in OLT patients, supports the concept of the presence of additional cholesterol transport mechanisms that may operate in parallel to ABCG5 and ABCG8.

**Rapid recovery of bile salt secretion after liver transplantation induces bile duct injury**

After OLT, graft function gradually recovers as is reflected by an increase of bile formation during the first two postoperative weeks \(^{(61-63)}\). Although hepatocellular injury rapidly diminishes after the operation, serum γ-glutamyltransferase (γ-GT) and alkaline phosphatase (ALP) levels, reflecting biliary injury, typically continue to increase through the second postoperative week and then progressively decline \(^{(64,65)}\). Based on the pathophysiology of the disease Progressive Familial
Intrahepatic Cholestasis type 3 (PFIC3), as well as that of Mdr2\(^{-/-}\) (homologue of MDR3) knockout mice, we hypothesized that changes in bile composition early after OLT, could be involved in the origin of bile duct injury \((66-68)\). Histological and biochemical evidence of bile duct injury was studied in a group of 28 liver transplant patients in relation to MDR3 and BSEP expression and bile composition. The findings of this study are explicated in detail in chapter 3. We found that, immediately after transplantation, bile salt secretion increased more rapidly than phospholipid secretion, resulting in high BS/PL ratios early postoperative. In parallel with this, mRNA levels of BSEP increased significantly after transplantation, whereas MDR3 mRNA levels remained unchanged. The release of \(\gamma\)-GT and ALP enzymes into bile, which was used as a biochemical measure of actual cellular injury over time, strongly related to biliary bile salt secretion rates. On histological examinations, intrahepatic bile duct damage, ductular proliferation and cholestasis were seen more often in association with high biliary BS/PL ratios. This study demonstrated that endogenous bile salts may play a role in the pathogenesis of early intrahepatic bile duct injury which invariably occurs during the first 2 to 3 weeks after liver transplantation. We therefore speculated that an enhanced canalicular transport of bile salts in proportion to phospholipids into bile could possibly also explain some of the incomprehensive forms of biliary lesions occurring at more prolonged intervals after OLT.

**Hemoglobinase-1 expression before liver transplantation correlates with graft injury and function early after transplantation**

In chapters 4 and 5 we studied the role of endogenous HO-1 expression in human liver transplants in relation to early postoperative graft injury and function. Hepatic HO-1 expression was measured (i) at the end of cold ischemic preservation of the liver (before transplantation), (ii) 3 hours after onset of reperfusion of the graft and (iii) at one week after OLT. Before transplantation, median HO-1 mRNA levels were 3.4-times higher (range: 0.7-9.3-fold) than in normal control livers. To be able to identify donor variables that are associated with HO-1 induction, and to study the possible impact of HO-1 on I/R injury and graft viability after transplantation, we divided liver grafts into two groups based on the median upregulation of HO-1 before transplantation (i.e. lower or higher than a 3.4-fold upregulation compared to controls). Several donor variables and laboratory values were analyzed in an attempt to explain the wide variation in HO-1 expression levels before OLT. Differences in HO-1 expression, could not simply be explained by a larger number of compromised donors in the group with high HO-1 expression. Moreover, factors that point out major hemodynamic alterati-
ons in the donor and several surgical variables, were similarly distributed among the two donor groups. The variation in initial HO-1 expression also could not be explained by a well described (GT)n dinucleotide repeat length polymorphism in the promoter region of the HO-1 gene. However, in an additional study we have later shown that another polymorphism of the HO-1 promoter, the A(-413)T single nucleotide polymorphism (SNP), is the only factor that may discriminate livers with initial high HO-1 mRNA levels from livers with initial low HO-1 expression levels (chapter 5). Others have recently suggested that HO-1 is upregulated as a consequence of brain death (69,70). Brain death is a known stress factor able to induce an inflammatory response in various target organs via yet incompletely understood mechanisms (71). Whether increased HO-1 expression is a direct result of brain death, or a secondary event in response to the inflammation evoked by brain death, is also unknown (72,73). In our study, all donor livers were obtained from brain death, multi-organ donors. Although the exact mechanisms of HO-1 induction are yet undefined, we suggest that the degree of HO-1 upregulation in donor livers may be affected by the A(-413)T SNP polymorphism of the HO-1 promoter.

Although there was no relationship between HO-1 expression and serum aspartate aminotransferase (AST) levels in donors, we found strong positive correlations between serum AST levels on postoperative day 1 and HO-1 expression levels before OLT in recipients. Surprisingly, liver grafts with an initial high (>3.4-fold) HO-1 expression before transplantation exhibited more I/R injury and showed poorer hepatobiliary function after transplantation than grafts with an initial low (<3.4-fold) HO-1 expression. Although downstream mediators of HO-1 have protective properties, at high concentrations each of them are detrimental (74-78). We therefore assume that the exaggerated HO-1 activity in the initial high HO-1 expression group before transplantation increased hepatic injury through detrimental effects of downstream HO-1 "effector" molecules, resulting in a higher susceptibility to I/R injury.

Interestingly, we found that, in the initial low HO-1 expression group, HO-1 mRNA levels significantly increased after reperfusion of the graft, while in the initial high HO-1 expression group, HO-1 mRNA levels significantly decreased after recirculation. To study possible effects of HO-1 induction upon reperfusion, we also categorized groups based on the ability to increase HO-1 expression during reperfusion of the liver graft. In this analysis, serum AST levels immediately after OLT were significantly lower in the group with HO-1 induction compared to livers without upregulation of HO-1 upon reperfusion. This suggests that the ability to induce HO-1 expression at the time of graft reperfusion may still confer protec-
Further research will be necessary to determine what is more important: a low expression of HO-1 before OLT, or the ability to induce HO-1 at the time of graft reperfusion.

Using immunofluorescence microscopy, we could identify Kupffer cells as the predominant HO-1 expressing cells. Through morphometrical analysis, we observed that all Kupffer cells were recruited to express HO-1 after reperfusion of the graft. It has been suggested that Kupffer cells may serve as sensor cells detecting local hemodynamic changes in sinusoids, which invariably occurs in OLT. By means of increasing HO-1 activity Kupffer cells can produce the vasorelaxing CO, and maintain microvascular blood flow in the liver\(^{(79-81)}\). Despite these properties, it is well known that Kupffer cells play a critical role in the pathogenesis of I/R injury through the production of reactive oxygen species (ROS) and cytokines\(^{(82,83)}\). Our finding that high HO-1 expression before OLT is associated with increased hepatic injury after OLT could, therefore, possibly be explained by a preexisting activation of Kupffer cells.

Although there is a large body of evidence obtained from transplant experiments in animals suggesting that exogenously upregulated HO-1 confers protection against I/R injury\(^{(84-86)}\), our findings caution against an uncontrolled exogenous upregulation of HO-1 in human donors as a measure to reduce I/R injury. We assume that an exogenous induction of HO-1 in post-mortem organ donors will further increase an already elevated HO-1 expression, leading to more cell damage instead of less. Pharmaceutic interventions should, therefore, rather be aimed at induction of HO-1 during transplantation, and not at upregulation of HO-1 prior to transplantation.

Hyperexpression of PI-9 during transplantation may protect liver grafts against early acute cellular rejection

In chapter 6 we have studied the expression of the cytoprotective molecules PI-9 and HO-1 in the development of early subclinical rejection of the graft. Although graft infiltrating granzyme B-positive CTLs induce hepatic injury during early acute cellular rejection, in early subclinical rejected livers such cytotoxic cell infiltrates do not cause overt graft rejection. Patients with early subclinical rejected livers do not show clinical and biochemical signs of rejection-associated graft injury. Apparently during the development of subclinical rejection the activity of graft infiltrating CTLs is kept silent. Based on the known potent immune-modulating properties of either the granzyme B-inhibitor PI-9 and the stress-responsive molecule HO-1, we hypothesized that both molecules play a role in the protection of early subclinical rejected liver grafts\(^{(87-93)}\). Expression of perforin, gran-
zyme B, PI-9 and HO-1 was assessed by real-time PCR in liver biopsies that were collected during and 1 week after transplantation. The diagnosis acute rejection was based on histological findings in liver samples according to Banff criteria\(^{(94)}\). Patients with subclinical rejection of the liver demonstrated histopathological characteristics of graft rejection without biochemical or clinical evidence of liver graft rejection. No changes in immunosuppressive treatment protocols were made in patients with early subclinical liver graft rejection. All patients with early acute rejection were treated with either an increased dose of calcineurin inhibitor or boluses of 1 gram methylprednisilone for 3 days.

Perforin and granzyme B expression levels demonstrated a strong positive linear correlation, supporting a close coupling between the pore-forming perforin and the pro-apoptotic granzyme B and suggesting a similar mode of regulation. Granzyme B mRNA levels were significantly increased in both early clinical and subclinical rejected liver grafts (6.2 and 3.6-times, respectively), indicating an equal potential of cytotoxic T cell infiltrates to damage the graft. Interestingly, we observed that in liver grafts developing early subclinical rejection, PI-9 expression levels were strongly increased (about 3.9-times) during the transplantation operation. We speculate that these initial high expression levels of PI-9 during OLT may have protected liver grafts against the deleterious activities of granzyme B-positive cytotoxic cell infiltrates during the first postoperative week. This may mean that initial hyperexpression of PI-9 could be a mechanism that leads to early subclinical rejection of otherwise early rejected liver grafts. In early subclinical and clinical acute rejection, HO-1 expression levels were substantially increased (1.9 and 3.1-times, respectively). Whether the induced HO-1 expression in both early subclinical and rejected liver grafts has been protective is unclear. Given the described cytoprotective properties of HO-1 at relative low induced expression levels\(^{(95)}\), the enhanced HO-1 expression in both early subclinical and rejected liver grafts may have limited graft injury.
CONCLUSIONS AND PERSPECTIVES

During the past 10 to 15 years, our understanding of hepatic molecular biology has progressed importantly. Because of rapid developments in this field, we nowadays better understand the pathophysiological events occurring in liver transplantation. In the research studies that formed the basis of this thesis, we focussed on the recovery of hepatobiliary function as well as the role of potential endogenous protective genes and pathways in human liver transplants. The discovery of endogenous cytoprotective molecules like the HO-1 and PI-9 system, are of great importance. Knowledge of such proteins may be useful for the generation of future prevention or therapeutic interventions to counteract I/R injury or acute rejection after OLT. The research described in this thesis focuses on such postulated novel targets genes, and may provide a basis for pharmacological intervention studies. One postulated novel target in reducing I/R injury is the HO-1 system. Many animal experiments have advocated HO-1 induction prior to transplantation as an important measure to decrease I/R injury. In the clinical situation, however, we found that HO-1 is already upregulated before OLT. Surprisingly, high HO-1 expression was associated with even worse postoperative outcome. In our opinion, upregulation of HO-1 prior to transplantation could therefore even enhance detrimental effects of I/R instead of conferring cytoprotection. Since less hepatic injury was found when HO-1 increased during transplantation, we believe that beneficial effects may be achieved when HO-1 is pharmacologically induced during the transplantation procedure. Considering our observations, we caution against an uncontrolled use of HO-1 promotors. Further investigations on the HO-1 system in transplantation will be needed before pharmaceutical interventions are applicable in clinical situations.

A new target gene in the prevention of the development of early acute cellular rejection may be the cytosolic serpin PI-9. Several studies in rodents and cell-lines have shown the effective inhibitory actions of PI-9 in granzyme B-positive T cell mediated cytotoxicity. The initial hyperexpression of PI-9, which we have observed in subclinically rejecting liver grafts suggest that PI-9 could offer a protective route against the development of early acute cellular rejection. Further investigations on the PI-9 system will be needed to unravel its role in transplantation.

After transplantation, we found that the recuperation of bile salt secretion, an important liver function, paradoxically amplifies biliary I/R injury. Although bile salt secretion generates bile flow, we suggest that it also provokes bile duct injury. We have shown that postoperative recovery of bile salt secretion occurred fas-
ter than phospholipid secretion. The presence of unantagonized endogenous bile salts in bile presumably leads to the formation of bile with a detrimental composition and is responsible for additional biliary damage. Differences between phospholipid and bile salt secretion rates are likely due to delayed postoperative recovery of MDR3 activity compared to BSEP transporter function. Based on our findings, we hypothesize that endogenous bile salts could also play a role in the pathogenesis of non-anastomotic biliary strictures (NAS), which comprise a collection of clinical complications, characterized by intrahepatic bile duct strictures and dilatations, with or without cast formation, occurring at more prolonged intervals after OLT. Future research should focus on transporter expression and bile composition in patients suffering from NAS.

Next to bile salts and phospholipids, cholesterol is an important bile constituent. In the last decade, research has focussed on canalicular transmembrane transport of cholesterol. Several animal studies have suggested that Abcg5 and Abcg8 are in tight control of the biliary cholesterol secretion. Although we observed a strong correlation between the expression of ABCG5 and ABCG8 in humans, we could not find a direct relation between ABCG5/ABCG8 expression and hepatobiliary cholesterol secretion after OLT. Our findings suggest that in humans different or perhaps parallel mechanisms of hepatobiliary cholesterol secretion may be operational which are not controlled by ABCG5/ABCG8. Clearly, hepatic cholesterol secretion routes in man remains indefinite. More research therefore is needed to define alternative molecular pathways of biliary cholesterol secretion in man.

In conclusion, studies on the molecular changes in hepatobiliary function and injury after OLT have provided us important new insights in the pathophysiological processes associated with liver transplantation. Better understanding of these processes not only enables us to identify novel preventive and therapeutic strategies to improve liver graft function after OLT, but also provides important knowledge that can be extrapolated beyond the field of transplantation. In this respect it remains important to realize that pathophysiological processes in humans may differ substantially from that in rodents, as shown in two of the studies described in this thesis. Patient oriented research therefore remains the ultimate proof of concepts and disease mechanisms relevant for advancing modern health care.
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