Non-anastomotic biliary strictures after liver transplantation
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Dearterialization of the liver causes intrahepatic cholestasis due to reduced bile transporter expression

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ABSTRACT

Background Bile duct injury after hepatic artery thrombosis (HAT) in liver transplantation is believed to be caused by ischemia predominantly. We aimed to define the involvement of bile secretory dysfunction in the pathogenesis of liver injury after HAT. Methods In a murine model, the main hepatic artery, the extrahepatic peribiliary plexus or both arterial connections to the liver were interrupted (n=5 for each group). After 1, 14 or 28 days, hepatobiliary function was assessed by analysis of bile transporter expression, serum bile acids and bilirubin, and hepatic ATP-content. In addition, cellular injury was assessed by light microscopy and biochemical markers. Results There were no signs of hepatobiliary dysfunction or injury in sham-operated animals or in mice with interruption of the hepatic artery or the extrahepatic peribiliary plexus alone. However, as early as 24 hours after complete dearterialization, bile transporter expression was significantly reduced and intrahepatic cholestasis started to progress the following weeks. Histological studies at 28 days after complete dearterialization showed severe hepatobiliary injury. Conclusions This study indicates that arterial blood supply is critical for normal bile secretion. Bile duct injury after complete arterial deprivation is preceded by a loss of bile secretory function and subsequent intrahepatic cholestasis.
INTRODUCTION

Hepatic artery thrombosis (HAT) after orthotopic liver transplantation (OLT) is a devastating complication. HAT, a common complication after liver transplantation, occurs in about 3 to 9% of adults and children and is associated with high morbidity and mortality (1). Outside the setting of transplantation, HAT appears to have limited consequences for the liver. For example in the treatment of multiple hepatic metastases, occlusion of the hepatic artery is well tolerated. This can largely be explained by the presence and rapid development of arterial collaterals, replacing the occluded artery (2, 3). In this scenario, perfusion is maintained by portal venous and hepatic arterial input. The transplanted liver, however, differs from the native livers in that arterial blood supply from accessory arteries and collateralization is completely interrupted during hepatectomy. As a consequence of HAT, the transplanted liver is perfused by the portal vein alone.

Arterial blood supply is closely associated with biliary structures before entering the sinusoids. Arterial blood is supplied to the bile ducts through a network of arterioles and capillaries, called the peribiliary plexus (PBP), coming from the hepatic arteries (4). Due to this distribution and its greater oxygen content, complete loss of arterial blood supply to the liver is generally followed by severe morbidity, mainly from biliary damage. This condition is often referred to as ischemic cholangiopathy. Of interest, hepatic artery blood flow seems to be critical for the recovery of bile secretory function after OLT (5). Ex vivo completely dearterialized porcine livers and perfused by the portal vein alone, showed diminished choleresis carried on phospholipids (6). In human, phospholipids are secreted into bile via the concerted action of the multidrug resistance 3 MDR3 P-glycoprotein (gene symbol ABCB4), located in the canalicular membrane of hepatocytes (7). Recently, we demonstrated in mice heterozygous for the disruption of the Mdr2 gene (a homologue of human MDR3), that endogenous bile salts act cytotoxic after OLT, due to intrahepatic cholestasis and intracellular bile salt retention (8). Intrahepatic cholestasis originates from altered bile transporter expression/function and altered secretory responses induced by proinflammatory cytokines (9).

This introduces the possibility that the observed hepatobiliary injury can be attributed in part to the inability to maintain bile secretion. When the graft loses arterial blood supply, metabolic capacity of hepatocytes may be limited. Hepatocellular damage may in turn induce pro-inflammatory cytokines TNF-alpha and IL1-beta. However,
direct evidence for such cholestatic injury is still lacking. To confirm the critical involvement of bile secretory function in the pathogenesis of hepatobiliary injury, arterial perfusion by the hepatic artery or extrahepatic PBP alone was compared to complete dearterialization of the liver in a mouse model. Hepatobiliary injury and hepatocyte transporter expression, responsible for sinusoidal uptake (Ntcp) and canalicular secretion of bile salts (Bsep) and phospholipids (Mdr2) were assessed after one, fourteen and twenty eight days. In this study, evidence is provided for a pivotal role of endogenous bile salts in the pathogenesis of hepatocellular injury after loss of arterial blood supply.

MATERIALS AND METHODS

Animals
In this study FVB/N mice (25–30 g) were used (n=5 for each group) and obtained from Harlan (Zeist, The Netherlands). Mice were housed in a light- and temperature-controlled facility. Food and water were available ad libitum. Mice were maintained on standard laboratory chow (Provimi Kliba AG, Kaiseraugst, Switzerland). Animals received human care according to guidelines of the University Hospital of Zurich. The Cantonal Veterinary Office of Zurich approved the study protocol.

Surgical procedures
A murine model of hepatic arterial deprivation was used. Livers were disconnected from the main hepatic artery, the extrahepatic PBP or both. In sham operated animals no additional vascular intervention was performed. In summary: after midline laparotomy, all oesophageal collateral vessels and ligaments to the liver were dissected. The main hepatic artery was ligated using a double suture just above the level of the bifurcation of the gastroduodenal and celiac artery. Ligation of the extrahepatic PBP was established by transsection of the extrahepatic bile duct. The reconnection of the bile duct was subsequently established by using a polyethylene stent (inner diameter 0.28 mm, outer diameter 0.61 mm; SIMS Portex, Kent, UK), which was secured with two circular ties. After 1, 14 and 28 days respectively, the animals (n=5, for each time point, per group) underwent relaparotomy. Blood was collected from the inferior vena cava and the liver was rapidly excised and processed for further analysis. All serum samples were snap-frozen and stored at -80 oC.
For determination of baseline values, a separate group of mice (n=5) was used to obtain samples of serum and liver tissue.

**Analysis of serum**

Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and total bilirubin levels were measured using the Ektachem DT60 II System and DTSC II Module (Johnson & Johnson Clinical Diagnostics Inc., Rochester, NY). Serum total bile salt concentrations were measured spectrophotometrically using 3α-hydroxysteroid dehydrogenase (10).

**Histology and immunohistochemistry**

Liver tissues were immersion-fixed in 4% PBS-buffered formalin, embedded in paraffin, sectioned, and stained with haematoxylin-eosin and sirius red using standard histological techniques. In addition, slides were immunostained for cytokeratin (polyclonal rabbit antibody; DakoCytomation) and Ki-67 (monoclonal rabbit clone SP6; NeoMarkers) using the Ventana Discovery automated staining system with an iView DAB kit (Ventana, Tucson, AZ). All histological assessments were performed by a single pathologist who was unaware of other study data.

**Real-time quantitative PCR**

Total RNA was extracted from 50 mg of liver tissue using TRIzol reagent (Invitrogen, Paisley, UK) and 5 µg of RNA was reverse transcribed using ThermoScript RT-PCR System (Invitrogen). Quantitative real-time polymerase chain reaction amplification and data analysis were performed using an ABI Prism 7000 Sequence Detector System (PE Applied Biosystems, Rotkreuz, Switzerland). TaqMan gene expression assay (PE Applied Biosystems) for Ntcp, Bsep, Mdr2, TNF-alpha (assay ID Mm00443258_m1) and IL1-beta (assay ID Mm00434228_m1) were used to quantify mRNA expression after normalisation to the house-keeping gene 18S (TaqMan ribosomal RNA control reagents, PE Applied Biosystems) (8). The results shown represent percentage induction in sham operated animals, and livers that were perfused by either the hepatic artery or extrahepatic PBP and complete dearterialized livers, compared to baseline levels.
Membrane isolation and western blotting

Total liver membrane fractions were prepared according to Trauner et al. (11), with a minor modification (single 39,000 RPM centrifugation step of gauze filtered liver homogenates). Membrane fractions were re-suspended in 300 mM Sucrose, 10 mM HEPES at pH 7.5, and snap-frozen in liquid nitrogen. Protein concentrations were determined with the bicinchoninic acid method (Pierce, Rockford, IL) (12). Total liver membranes (40 µg) were separated using a 7.5% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis, and transferred to nitrocellulose. The blots were probed with rabbit antisera raised against Ntcp (13) and Bsep (14) at appropriate dilutions. Immune detection was assessed by using the ECL chemiluminescent detection system (Amersham, UK). For quantitative analysis of expression levels, autoradiographs were scanned with an AlphaImagerTM 1220 gel documentation system (Witec AG, Luzern, Switzerland).

ATP extraction and measurement

Liver samples were immediately frozen in liquid nitrogen until the extraction procedure. The frozen tissue was powdered and homogenized in 1 mL of 0.6 N ice-cold HCLO4 and incubated for 10 minutes. The precipitated proteins were removed by centrifugation (10,000 x g for 10 minutes), and 300 µL of supernatant was neutralized by 1 N KHCO3. Forty microliters of supernatant were pipetted into the wells of a white non-phosphorescent microplate, placed in a luminometer (Labsystems Luminoscan 1.2-0), and processed by addition of 150 µL of ATP monitoring reagent. ATP concentrations were calculated from a calibration curve constructed at the same time by means of standard ATP dissolved in the appropriate solution for each experiment.

Statistics

Values are expressed as mean ± SD. Data was analyzed using SPSS software version 11.5 for Windows (SPSS Inc., Chicago, Il). A non-paired Mann-Whitney U-test and analysis of variance (ANOVA) were used for the comparison between the groups, respectively. Differences were considered statistically significant when P was ≤ 0.05.
RESULTS

**Complete arterial deprivation of the liver in mice.**
The arterial anatomy was carefully evaluated in every animal. In none of the animals collateral vessels were observed between the gastroduodenal artery and extrahepatic PBP. The main hepatic artery normally arises from the celiac artery. Retrospectively during harvesting, in rare cases an adjuvant hepatic artery was patent, arising from the main hepatic artery or left gastric artery, supplying the right and left hemi-liver, respectively. These animals (n=5, 3.5% of all operated animals) were excluded from the experiment. Patency of the arterial ligation was checked in all mice that underwent ligation, either from the main hepatic artery, extrahepatic PBP or both connections, by transection of the arteries. No animal was found to have an open artery, when this was meant to be. To exclude the possibility that obstruction of the biliary stent anastomosis contributed to the cholestatic changes, all animals that underwent relaparotomy, patency of the main bile duct was checked. Ligation of extrahepatic PBP using a stent anastomosis was not associated with an increased risk of bile duct occlusion compared with sham-operated animals.

There were no technical complications.

**Does hepatic arterial deprivation have an impact on animal survival?**
All animals that underwent an interruption of the arterial blood flow from either the hepatic artery or extrahepatic PBP, and maintained arterial perfusion survived 28 days. Two out of five animals that underwent complete dearterialization of the liver, died between 21 and 28 days after surgery.

**Does hepatic arterial deprivation induce hepatocyte injury and cholestasis?**
Biochemical analysis included both serum and bile measurements. Serum AST levels were used as markers of hepatocyte injury. Serum levels of ALP, total bile salts and bilirubin served as markers of cholestasis. There were no significant differences in serum AST, ALP, total bilirubin, and bile salts between sham operated animals, and livers that were perfused by either the hepatic artery or extrahepatic PBP. However, in animals with complete dearterialized livers, serum AST, ALP, total bilirubin and total bile salts were increased significantly (Figure 1).
Serum (A) aspartate aminotransferase (AST), (B) alkaline phosphatase, (C) total bilirubin and (D) bile salt levels. Data shown are mean +/- SD. There were no significant differences for AST, alkaline phosphatase, total bilirubin and bile salts between sham (▲) operated animals and livers that were perfused by either the hepatic artery (■) or peribiliary plexus (♦) alone. However, in animals with completely dearterialized livers (●) serum AST, alkaline phosphatase, total bilirubin and bile salts were increased significantly (*P≤0.025) indicating liver injury and cholestasis.

**Does arterial deprivation lead to liver damage?**

Histological analysis of liver parenchyma and large bile ducts was performed after sham operation, ligation of the extrahepatic PBP, the hepatic artery, or both. No morphological abnormalities were observed in the portal tracts and lobular parenchyma after sham operation, ligation of the hepatic artery or extrahepatic PBP at 1, 14 and 28 days after surgery (fig.2A-D; data not shown). In contrast, ligation of both hepatic artery and extrahepatic PBP was associated with portal inflammation and ductular reaction at 14 and 28 days after surgery (fig.2A,B). Lobular parenchyma was maintained in most of these livers, but areas of ischemic necrosis and bile infarcts were present.
in the liver parenchyma of some of these mice (fig. 2C). Ki-67 staining of complete
dearterialized livers revealed increased hepatocyte proliferation at 14 and 28 days after
surgery in comparison to livers after sham operation, ligation of the hepatic artery or
extrahepatic PBP, suggesting increased hepatocyte turnover (fig. 2D). After 28 days, large
bile ducts showed purulent cholangitis in mice with ligation of both hepatic artery and
extrahepatic PBP (fig. 2E).

**Does arterial deprivation change expression of key hepatocyte transporters?**

To evaluate the molecular mechanisms underlying the observed cholestatic phenotype,
we measured the expression of hepatocellular transporters responsible for sinusoidal
uptake (Ntcp) and canalicular secretion of bile salts (Bsep) and phospholipids (Mdr2).
There were no significant differences in Ntcp, Bsep and Mdr2 mRNA levels between
sham operated animals, and livers that were perfused by either the hepatic artery or
extrahepatic PBP alone. However, Ntcp mRNA levels were significantly reduced in
completely dearterialized livers at all time points. Bsep mRNA levels showed a tendency
to increase at day 14, however, this did not reach significance, whereas Mdr2 mRNA
levels were significantly increased at day 14 (Figure 3). In contrast, Ntcp and Bsep
protein levels were significantly reduced in completely dearterialized livers at all time
points (Figure 4).

**Is reduced bile secretory function associated with ATP depletion?**

To investigate whether a reduced energy state of the hepatic tissue, might be responsible
for the decrease of (ATP-dependent) hepatocyte transporter synthesis, ATP levels were
measured in liver tissue at day 1, 14 and 28. There were no significant differences in
ATP levels between sham operated animals, and livers that were perfused by either the
hepatic artery or extrahepatic PBP alone at day 1 and 14. Completely dearterialized
livers, however, had significantly lower ATP levels at 24 hours which were restored after
14 days (Figure 5).
Figure 2
Histological analysis of liver parenchyma 28 days after sham operation, ligation of the hepatic artery (HA), extrahepatic peribiliary plexus (PBP), or complete dearterialization (HA+PBP).
(A) Inflammation and ductular reaction in the portal tracts after complete dearterialization (H&E, original magnification 200x). (B) Ductular reaction after dearterialization revealed by cytokeratin immunostaining (original magnification 200x). (C) Maintained lobular liver parenchyma in all experimental groups (original magnification 200x). (D) Increased numbers of Ki-67 positive hepatocytes and inflammatory cells after complete dearterialization (original magnification 200x). (E) Purulent cholangitis of large bile ducts after complete dearterialization (original magnification 100x).
Figure 3
Transcript levels of (A) Ntcp, (B) Bsep and (C) Mdr2.

mRNA coding for Ntcp, Bsep and Mdr2 were quantified by RT-PCR (n=5 for each group) and the number of transcripts normalized to baseline. Data shown are mean +/- SD. There were no significant differences in mRNA levels for Ntcp, Bsep and Mdr2 between sham (▲) operated animals and livers that were perfused by either the hepatic artery (■) or peribiliary plexus (♦) alone. However, mRNA levels for Ntcp of complete dearterialized livers (●) were significantly (*p≤0.036) reduced at all time points. In contrast to Mdr2, which was significantly (*p<0.01) increased at day 14, Bsep showed a tendency to increase at day 14, however this did not reach significance.
Figure 4
Expression for Ntcp and Bsep.
(A) Western blot analysis of 50 kDa Ntcp (top row) and 160 kDa Bsep (bottom row) from liver extracts (n=3 for each group) in control, sham and completely dearterialized (HA+PBP) livers, at day 1, 14 and 28. The levels were normalized to control animals and set to 100%. Samples shown are representative for 3 individual total liver membrane preparations. (B-C) No clear differences in protein expression of Ntcp and Bsep were observed between control and sham operated animals. However, in complete dearterialized livers Ntcp and Bsep expression were decreased significantly (*P≤0.05).
Liver ATP levels.

ATP concentrations from liver extracts (n=3 for each group) in sham and completely dearterialized (HA+PBP) livers, at day 1, 14 and 28. ATP levels (nMoles/mg) were significant (*P=0.05) reduced in complete dearterialized livers at day 1, which might explain the early decline in bile salt transporter translation. ATP levels turned normal at 14 and 28 days.

Can intrahepatic cholestasis maintained by induced proinflammatory cytokines?

To study whether intrahepatic cholestasis was associated with the induction of proinflammatory cytokines that may aggravate the injury, TNF-alpha and IL1-beta mRNA levels were determined. There were no significant differences in mRNA levels for TNF-alpha and IL1-beta between sham operated animals and livers that were perfused by either the hepatic artery or peribiliary plexus alone, at all time points. Complete dearterialized livers, however, showed significant induction of TNF-alpha and IL1-beta mRNA levels reaching significance at 14 and 28 days, respectively (Figure 6).
Figure 6

Transcript levels of (A) TNF-alpha and (B) IL1-beta.

mRNA coding for TNF-alpha and IL1-beta were quantified by RT-PCR (n=5 for each group) and the number of transcripts normalized to baseline. Data shown are mean +/- SD. There were no significant differences in mRNA levels for TNF-alpha and IL1-beta between sham (▲) operated animals and livers that were perfused by either the hepatic artery (■) or peribiliary plexus (♦) alone. Completely dearterialized livers (●), however, showed significant (*p≤0.025) induction of TNF-alpha and IL1-beta mRNA levels after 14 and 28 days, respectively.
DISCUSSION

Our aim was to investigate the consequences of arterial deprivation in the mouse liver. Impaired arterial perfusion induced by ligation of either the hepatic artery or the extrahepatic PBP alone did not induce marked histological, biochemical or molecular changes in the liver. This suggests that both the hepatic artery and the extrahepatic PBP have the capacity to complement for each other. However, complete loss of arterial blood supply to the liver resulted in hepatobiliary injury, with two out five animals dying between 21 and 28 days after surgery. These results are in contrast to those of Beaussier et al., who showed that completely dearterialized rat livers recover from ischemia-induced liver damage within six weeks (2), and in contrast to the situation after non-arterialized OLT in rats, in which omission of the hepatic artery is not associated with significant negative effects and poor outcome of the procedure (15-17). However, our results are consistent with the previous observation that arterialized mouse OLT is associated with less liver injury and better survival in comparison to OLT without rearterialization (18). Taken together, these findings suggest that the mouse model, rather than the rat model, resembles more closely the consequences of hepatic blood supply after human OLT.

Complete arterial deprivation in mice may serve as a model to study the pathogenesis of graft damage after HAT in human OLT. Wheatley et al. have indicated that hepatic arterial blood flow is critical for the recovery of hepatocyte bile secretory function after OLT (5). In the absence of bile secretion, bile salts can accumulate and act cytotoxic (8). This introduces the possibility that the observed hepatobiliary injury can be attributed, in part, to decreased bile secretory function of hepatocytes after the graft loses arterial blood supply. However, formal evidence for such cholestatic injury after loss of arterial blood supply is lacking. Therefore, we analyzed the effects of complete loss of hepatic arterial blood flow on liver histology, serum parameters of cholestasis and the expression of hepatocyte transporters. The effects were compared to those after ligation of either the hepatic artery or the extrahepatic PBP alone.

Protein expression of the bile salt transporters Ntcp and Bsep was decreased significantly in completely dearterialized livers. As a consequence, the serum conjugated bile salt levels increased. The basolateral bile salt transporter Ntcp was down-regulated rapidly on both mRNA and protein levels in complete dearterialized livers. In general, changes in basolateral bile salt transporter (Ntcp) expression have been interpreted largely as
secondary and adaptive phenomena, in an attempt to protect the hepatocyte from bile acid overload under cholestatic conditions (19). In parallel, Bsep levels were also decreased rapidly in complete dearterialized livers. Conversely, Bsep and Mdr2 transcript levels were 2-fold up-regulated in complete dearterialized livers at day 14. Up-regulation of canalicular transport systems is known to be an initial response of cholestatic hepatocytes. It is presumed to be a mechanism to overcome bile salt overload and diminish the extent of liver injury produced by bile salt retention (20-22).

Our results suggest that endogenous bile salts may actively contribute to liver injury after loss of arterial blood supply of the liver graft after OLT. In our hypothesis, the putative insult to the liver is an interaction between ischemic bile duct injury and retention of endogenous bile salts. Although the bile duct is affected by ischemia predominantly, hepatocellular injury is an invariable feature of cholestasis, associated with accumulation of bile salts in the liver and blood (23). As a consequence of early limited metabolic capacity of hepatocytes (ATP depletion) bile transporter expression and function are reduced. Subsequently, bile salts and other bile compounds accumulate, as indicated by increased serum alkaline phosphatase levels, total bilirubin and bile salt levels increase and the animals become cholestatic. Bile salt retention in the liver causes hepatocellular injury and hepatocyte proliferation, perpetuates the initial inflammatory response, leading to chronic portal inflammation and ductular proliferation (24, 25). At the same time, increased pro-inflammatory mediators (TNF-alpha and IL1-beta) maintain the altered secretory responses (9).

HAT after OLT has been shown to result in ischemic injury of the bile ducts (26, 27). We can not exclude that some of the changes observed in the liver parenchyma after complete arterial deprivation in mice are due to ischemic damage of large bile ducts. Consistent with this notion, we observed purulent cholangitis of large bile ducts 28 days after complete arterial deprivation. This inflammation may induce some obstruction of large bile ducts with secondary impaired bile flow. This possibility is supported by the observation that (prolonged) decreased hepatic Ntcp and Bsep protein levels have also been observed in human cholestatic liver diseases (28).

Therapeutic strategies should therefore focus on maintaining choleresis and awaiting collateralization. Ursodeoxycholic acid is known to improve cholestatic disorders. It has been shown to stimulate canalicular transport and bile secretion (29). However, its choleretic action also increases biliary pressure, which has been shown to aggravate bile
infarcts and hepatocyte necrosis in the homozygous Mdr2-/ mice (30). Administration of fibrates or statins might provide an alternative therapeutic approach to modify hepatocellular injury after HAT (31, 32). These lipid lowering drugs have all been shown to induce biliary phospholipid secretion by the induction of Mdr2. However, fibrates and statins not only induce Mdr2, but also lower Bsep and Ntcp expression and potentially lower bile flow (33). Their use is also not without side-effects.

In conclusion, our data indicate that arterial blood flow either by the hepatic artery or extrahepatic PBP is mandatory to preserve normal liver architecture and bile secretory function. Complete loss of arterial blood supply to the liver results in a decrease in hepatobiliary secretory function and subsequent intrahepatic cholestasis. Intrahepatic cholestasis induces hepatocellular injury and aggravates ischemic injury in the presence of HAT. Therapeutic strategies in patients with HAT after OLT should, therefore, not only focus on immediate restoration of the arterial blood supply, but also aim at maintaining choleresis and avoiding intrahepatic cholestasis.

References


