Bile salt toxicity aggravates cold ischemic injury of bile ducts after liver transplantation in Mdr2\(^{+/-}\) mice


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ABSTRACT

Background Non-anastomotic strictures (NAS) of the bile duct are a serious type of biliary complication after orthotopic liver transplantation (OLT). We aimed to define the role of endogenous bile salt toxicity in the pathogenesis of bile duct injury after OLT. Methods Livers from wild-type mice and mice heterozygous for disruption of Mdr2 gene (Mdr2+/-) were transplanted into wild-type recipient mice. Mdr2+/- mice secrete only 50% of the normal amount of phospholipids into their bile, leading to an abnormally high bile salt/phospholipid ratio. In contrast to homozygous Mdr2-/- mice, the Mdr2+/- mice, however, have normal liver histology and function under normal conditions. Two weeks after OLT, bile duct injury and cholestasis were assessed by light and electron microscopy, as well as through molecular and biochemical markers. Results There were no signs of bile duct injury or intrahepatic cholestasis in liver grafts from wild-type donors. Liver grafts from Mdr2+/- donors, however, had enlarged portal tracts with cellular damage, ductular proliferation, biliostasis, and a dense inflammatory infiltrate after OLT. Parallel to this observation, recipients of Mdr2+/- livers had significantly higher serum transaminases, alkaline phosphatase, total bilirubin, and bile salt levels, as compared to recipients of wild-type livers. In addition, hepatic bile transporter expression was compatible with the biochemical and histological cholestatic profile found in Mdr2+/- grafts after OLT. Conclusions This data indicates that toxic bile composition, due to a high biliary bile salt/phospholipid ratio, acts synergistically to cold ischemia in the pathogenesis of bile duct injury after transplantation.
INTRODUCTION

Bile duct complications are an important cause of morbidity and graft loss after orthotopic liver transplantation (OLT) in humans, occurring between 10% and 30% of the patients (1, 2). A common type of biliary complication are non-anastomotic strictures (NAS) with dilatation of the intrahepatic bile ducts, found in up to in 20% of the patients (3). Prolonged cold ischemia time is an independent risk factor for this type of injury (2-6). However, NAS are also observed in absence of long cold ischemia or other identifiable risk factors. Experimental studies in pigs suggested that bile salts may contribute to hepatic injury during cold ischemia and after liver transplantation (7-9). Bile salts have potent detergent properties, and may damage cells by affecting the integrity of cellular membranes. In addition, bile salts are cytotoxic via intracellular processes, such as mitochondria-mediated toxicity (10). Normally, these toxic effects are prevented by neutralization of bile salts by phospholipids and the formation of mixed micelles in bile. Phospholipids are secreted into bile in human via the concerted action of the multidrug resistance 3 MDR3 P-glycoprotein (gene symbol ABCB4), located in the canalicular membrane of hepatocytes, and canalicular bile salts. Mutations in the MDR3 gene have been associated with diseases characterized by intrahepatic cholestasis, biliary sludge formation and injury, and high serum gamma-glutamyltransferase activity, as seen in the progressive familial intrahepatic cholestasis (PFIC) type 3 and intrahepatic cholestasis associated to pregnancy (11, 12). Mice homozygous for the disruption of the Mdr2 gene (Abcb4), a homologue of human MDR3, completely lack phospholipids in their bile and develop progressive bile duct injury and cholestasis early in life (13, 14). Based on the development of intrahepatic biliary strictures, these animals have been proposed as a model for sclerosing cholangitis (15, 16), and also share many features of the NAS observed after OLT.

Of interest, bile duct injury occurring after human OLT was recently found to correlate with the formation of toxic bile early after OLT, characterized by a high bile salt/phospholipid ratio (17). Whether bile salt toxicity actively contributes to hepatic injury or is an epiphenomenon could not be identified in this clinical study. Therefore, we designed a study using a model of arterialized OLT in mice that are heterozygous for the disruption of Mdr2 gene (Mdr2-/-) to determine the impact of bile contents on biliary injury after OLT. These Mdr2-/- mice disclose approximately half of the normal phospholipid concentration in bile, but in contrast to their homozygous (Mdr2-/-)
littermates, they do not develop biliary injury and intrahepatic strictures under normal conditions (13). We hypothesized that the high bile salt/phospholipid ratio in Mdr2−/− contributes to bile duct injury related to cold storage and subsequent reperfusion during OLT. Livers from wild-type or Mdr2−/− mice were transplanted into wild-type recipients after a short period of cold ischemic storage. The type and degree of hepatocellular and biliary injury was assessed at 14 days after OLT using established markers of injury. This study provides novel insights into the molecular mechanism of bile duct injury after OLT. Moreover, evidence is provided for a pivotal role of endogenous bile salts in the pathogenesis of bile duct injury.

MATERIALS AND METHODS

Animals

Mice heterozygous for disruption of the Mdr2 gene (Inbred FVB.129P2-Acbb<sup>tm1Bar</sup>) were obtained from The Jackson Laboratory (Maine, USA). These animals were bred at the animal facility of the University Hospital of Zurich. Male wild-type (Mdr2<sup>+/+</sup>) and heterozygous (Mdr2<sup>−/+</sup>) littermates (25–30 g) were used in this study. The mice were housed in a light- and temperature-controlled facility. Standard laboratory chow was available ad libitum. Animals received humane care according to guidelines of the University Hospital of Zurich. The study protocol was approved by the Federal Veterinary Office of Zurich.

Surgical procedures

A murine model of arterialized OLT was used. Donor procedure, back-table preparation and recipient procedure were performed as described by Tian et al. (18). In summary: after midline laparotomy, all vessels and ligaments to the liver were dissected in the donor. In situ perfusion of the liver was performed, using cold (4°C) Ringer’s solution. The liver was subsequently freed from its retroperitoneal attachments and removed. The graft was stored in cold (4°C) Ringer’s solution for 60 minutes until implantation in the recipient. After hepatectomy of the native liver in the recipient, the donor liver was implanted in an orthotopic position. Anhepatic time in the recipient was consistently kept below 20 minutes. After completing the anastomosis between the suprahepatic inferior vena cava of the recipient and donor, the portal vein was reconstructed and the liver was reperfused. Arterial recirculation was established by an end-to-side anastomosis.
between the recipient aorta and an aortic segment attached to the hepatic artery of the graft. Antibiotic prophylaxis was provided by a single subcutaneous injection of 5 mg of cefazolin.

After 14 days, the animals (n=5 for each group) underwent relaparotomy. The grafted liver was dissected free from its surrounding connective tissue and patency of the hepatic artery was checked. The bile duct was cannulated to drain (20-30 minutes) and collect bile, using a polyethylene catheter (inner diameter 0.28 mm, outer diameter 0.61 mm; SIMS Portex, Kent, UK). Subsequently, blood was collected from the inferior vena cava and the liver was rapidly excised and processed for further analysis. In addition, 3 animals in each group were sacrificed at one day after OLT to collect bile samples for analysis of bile salt and phospholipid concentration. All bile and serum samples were snap-frozen and stored at -80 °C.

Separate groups of wild-type and heterozygous (Mdr2−/−) mice (n=5 for each group) were used to obtain samples of bile, serum and liver tissue for determination of baseline values.

**Histology and immunohistochemistry**

Liver tissues were immersion-fixed in 4% PBS-buffered formalin, embedded in paraffin, sectioned, and stained with haematoxylin-eosin, Sirius red or for chloroacetate esterase using standard histological techniques. In addition, slides were immunostained for CD3 (monoclonal rabbit antibody; Neomarkers, Fremont, CA), CD45R (monoclonal rat clone RA3-6B2; BD Biosciences Pharmingen), myeloperoxidase (polyclonal rabbit antibody; Neomarkers), cytokeratin (polyclonal rabbit antibody; DakoCytomation) and Ki-67 (monoclonal rabbit clone SP6; Neomarkers) using the Ventana Discovery automated staining system with the DAB Map kit (Ventana, Tucson, AZ). All sections were counterstained with hematoxylin. Quantification of CD3+ T-lymphocytes, CD45R+ B-lymphocytes, myeloperoxidase-positive myeloid cells and cytokeratin-positive cells was performed in 10 portal tracts with portal veins measuring 50-150 µm in diameter.

Double immunofluorescence labeling for Cytokeratin 18 (Progen Biotechnik GmbH, Heidelberg, Germany) and Laminin (E-Y Laboratories, San Mateo, CA) was performed using cryosections. Secondary antibodies were FITC and Cy3-labelled antibodies (Milan Analytica AG, La Roche, Switzerland). Nuclear DNA was stained by adding 4-6-Diamidino-2-phenylindole from Sigma Chemicals.
All histological assessments were performed by a single pathologist who was unaware of the genotype of the animals and of the other study data.

**Transmission electron microscopy**

Tissues were immersion-fixed in 2.5% buffered glutaraldehyde for 60 minutes, buffered in PBS and embedded in Epon in accordance to the guidelines of the Electron Microscopy Centre of the University of Zurich and studied in a MC12 Philips electron microscope (19).

**Biochemical analysis of serum and bile**

Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and total bilirubin levels were measured using the Ektachem DT60 II System and DTSC II Module (Johnson & Johnson Clinical Diagnostics Inc., Rochester, NY). Conjugated bile salts in serum were measured using a radioimmunoassay ^125^I RIA-Kit (MP Biomedicals, Eschwege, Germany). In bile samples, total bile salt concentration was measured spectrophotometrically using 3α-hydroxysteroid dehydrogenase (20). Biliary phospholipid concentration was analyzed using a commercially available enzymatic method (Wako Chemicals GmbH, Neuss, Germany).

**Isolation and processing of membrane fractions for western blotting**

Liver membrane fractions (microsomes) were prepared according to Trauner et al. (21), with a minor modification (single 39,000 RPM centrifugation step of gauze filtered liver homogenates). Membrane fractions were resuspended and protein concentrations were determined with the bicinchoninic acid method (Pierce, Rockford, IL) (22). Microsomes (40µg) were separated using a 7.5% SDS polyacrylamide gel electrophoresis, and transferred to nitrocellulose. The blots were probed with rabbit antisera raised against the sinusoidal bile salt transporter Ntcp (23) and the canalicular bile salt export pump Bsep (24) at appropriate dilutions. In addition, blots were probed with an anti-beta-actin antibody (Abcam, Cambridge, UK) to confirm equal protein loading and the specificity of the changes in transporter protein levels. Immune detection was assessed by using the ECL chemiluminescent detection
system (Amersham, UK). For comparison of expression levels, autoradiographs were scanned with an AlphaImager™ 1220 gel documentation system (Witec AG, Luzern, Switzerland).

**Expression of mRNA determined by Real-time quantitative PCR**

Total RNA was isolated from frozen mouse liver using TRIzol reagent and reverse transcription was performed using random hexamer (Invitrogen life technologies, Basel, Switzerland), according to the manufacturer’s instructions. Real-time quantitative PCR to quantify mRNA coding for Ntcp, Bsep and Mdr2, was performed on cDNA samples as described by Assay-by DesignSM Service, Applied Biosystems (Rotkreuz, Switzerland) using ABI Prism 7000 Sequence Detector, SDS software version 1.1 (Applied Biosystems). Unlabeled PCR primers and TaqMan® MGB probes (FAMTM dye labelled) were obtained as singleplex PCR reaction mix from Applied Biosystems (Table 1). A probe against ribosomal 18S-RNA was used as internal control (Applied Biosystems).

**Statistics**

Values are expressed as mean ± SD. Data was analyzed using SPSS software version 11.5 for Windows (SPSS Inc., Chicago, Il). Differences within and between groups were compared using a paired and non-paired Mann-Whitney U-test, respectively. All P-values were two-tailed and considered as statistically significant at a level of less than 0.05.

**Table 1. Sequences of primers and probes used for RT-PCR**

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*Note: Table 1 sequences are for Ntcp, Bsep, and Mdr2 cDNA with corresponding Accession numbers.*
RESULTS
Is there any histopathological evidence of hepatobiliary injury in wild-type and Mdr2\(^{-/-}\) mice after OLT?

As reported previously (13, 25), no histological abnormalities, especially no portal tract inflammation, bile duct injury, ductular proliferation or cholangitis of large bile ducts, was observed in livers obtained from Mdr2\(^{-/-}\) and wild-type mice prior to transplantation (Figures 1A-B). At 14 days after transplantation, no major histological abnormalities were found in wild-type livers. However, in contrast with this, Mdr2\(^{-/-}\) liver grafts revealed signs of small bile duct damage such as irregular configuration of bile ducts, uneven spacing and loss of biliary epithelial cells (Figure 1D). Furthermore, the number of bile ducts was increased in Mdr2\(^{-/-}\) liver grafts suggesting ductular reaction. To quantify ductular reaction, the number of cytokeratin-positive cells per portal tract was determined using immunohistochemistry (Figure 1E-F). Portal tracts in Mdr2\(^{-/-}\) liver grafts showed a two-fold increase in the number of cytokeratin-positive cells (Figure 1G). Double-immunofluorescence labeling for laminin and cytokeratin 18 revealed no basal membrane disruptions of small bile ducts in wild-type or Mdr2\(^{-/-}\) liver grafts (Figure 1H-I). Portal tracts of Mdr2\(^{-/-}\) grafts were also enlarged and contained a mixed inflammatory infiltrate composed of neutrophils, plasma cells and lymphocytes (Figure 1D). To further characterize and quantify portal inflammation, sections were stained with immunohistochemical markers for B lymphocytes (CD45R), T lymphocytes (CD3) and myeloid cells (myeloperoxidase, MPO). In comparison to wild-type liver grafts 14 days after transplantation and to wild-type and Mdr2\(^{-/-}\) grafts prior to transplantation, Mdr2\(^{-/-}\) grafts showed a significant increase in myeloid, B and T cells (Figure 1J). There was no fibrosis or development of fibrous septa in both wild-type and Mdr2\(^{-/-}\) liver grafts (Figure 1K-L). Histological analysis of intermediate and large bile ducts revealed purulent cholangitis in Mdr2\(^{-/-}\) grafts at 14 days after transplantation. The periductal stroma of intermediate and large bile ducts demonstrated myxoid transformation and infiltration by neutrophils (Figure 2B-D). Neutrophils were also present within the bile duct epithelium and duct lumina, indicating cholangitis. Furthermore, the epithelial outline of intermediate and large bile ducts in Mdr2\(^{-/-}\) was irregular with increased proliferation as revealed by Ki-67 staining (Figure 2F).
Figure 1

Hoekstra et al.

Keratin-positive cells per portal tract

<table>
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<tr>
<th></th>
<th>WT</th>
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a,b
Figure 1

Histological analysis of wild-type and Mdr2<sup>−/−</sup> liver grafts before and 14 days after OLT.

(A-B) Normal portal tract morphology in wild-type (A) and Mdr2<sup>−/−</sup> (B) liver grafts before OLT. (H&E). Original magnification: x200.

(C-D) In comparison to wild-type controls (C), inflammation, signs of bile duct damage and ductular reaction in portal tracts of Mdr2<sup>−/−</sup> liver grafts (D) 14 days after transplantation. Arrowheads indicate small bile ducts. Note: bile duct damage presents as uneven spacing of biliary epithelial cells, nuclear irregularity and loss of epithelial cells (H&E). Original magnification: x200.

(E-F) Ductular reaction revealed by immunohistochemical staining for cytokeratin in Mdr2<sup>−/−</sup> liver grafts (F) in comparison to wild-type controls (E) 14 days after transplantation. Original magnification: x200.

(G) Quantification of cytokeratin-positive cells in portal tracts of Mdr2<sup>−/−</sup> and wild-type liver grafts before and 14 days after transplantation, *p ≤ 0.05 compared to WT at the same time point, **p ≤ 0.05 compared to pre-OLT.

(H-I) Double-immunofluorescence labeling for cytokeratin 18 and laminin revealed no basal membrane disruptions of small bile ducts in wild-type (H) or Mdr2<sup>−/−</sup> (I) liver grafts. Original magnification: x1000.
(J) Quantification of CD3-, CD45R- and myeloperoxidase-positive cells in portal tracts of Mdr2+/− and wild-type liver grafts 14 days after transplantation, *p ≤ 0.05 compared to WT at the same time point, †p ≤ 0.05 compared to pre-OLT.

(K, L) Absence of portal fibrosis in Mdr2+/− (L) in comparison to wild-type (K) liver grafts 14 days after transplantation (Sirius red). Original magnification: x200.

Figure 2
Cholangitis of large bile ducts in Mdr2+/− liver grafts 14 days after OLT. In contrast to wild-type controls (A, C, E), intermediate and large bile ducts of Mdr2+/− grafts (B, D, F) display myxoid transformation of the periductal stroma (B), infiltration by neutrophils (arrow heads) as revealed by chloroacetate esterase staining (D) and increased proliferation of biliary epithelial cells (Ki-67 staining) (F) 14 days after transplantation. bd: bile duct. (A, B: H&E). Original magnification: x150 (A, B), x300 (C-F).
Are there differences in ultrastructural cell morphology between wild-type and Mdr2+/- mice on transmission electron microscopy?

To further characterize injury at the cellular level, transmission electron microscopy was performed. Similar to light microscopy, there were no differences in ultrastructural cell morphology in livers from wild-type or Mdr2+/- mice before transplantation (Figure 3A-B). 14 days after transplantation, however, striking differences in hepatocyte and bile duct epithelial cell injury were observed between the two groups. Hepatocellular and bile duct epithelial cell injury in the Mdr2+/- group was characterized by a variation in nucleus size, loss of nuclei, destruction of mitochondria, and loss of ribosomes (Figure 3D-F). In addition, there were signs of loss of microvilli and dilatation of the bile ducts and primary bile canaliculi (Figure 3D-F). There were no signs of basal membrane destruction or loss of tight junctions.

Do the histopathological changes in Mdr2+/- grafts correlate with biochemical markers of hepatobiliary injury and function?

Biochemical analysis included both serum and bile measurements, as indicators for liver injury and cholestasis. Before transplantation, there were no significant differences in serum transaminases, ALP, bilirubin, and conjugated bile salts between the two groups (Table 2). In the wild-type group, no significant changes in these variables were observed at 14 days after transplantation. However, in recipients of Mdr2+/- livers, serum transaminases, ALP, and conjugated bile salts increased significantly at this time point, reflecting cellular injury and cholestasis. Serum total bilirubin was also higher in the Mdr2+/- group, but this did not reach statistical significance (Table 2).

Analysis of bile samples revealed no differences in biliary bile salt concentration before transplantation in the two groups. However, as expected, biliary phospholipid concentration in Mdr2+/- donors was significantly lower than in wild-type animals, resulting in a 1.9-fold higher bile salt/phospholipid ratio (Table 3). Analysis of bile collected at one day after transplantation confirmed that the bile salt/phospholipid ratio in bile produced by Mdr2+/- liver grafts remained abnormally high (Table 3). In accordance with observations in humans (17), an increase in bile salt/phospholipid ratio was observed in recipients of wild-type livers, although ratios remained significant lower
than in recipients of Mdr2<sup>−/−</sup> livers. At 14 days after transplantation, biliary bile salt and phospholipid concentrations had returned to pre-transplant values in the wild-type group. In the Mdr2<sup>−/−</sup> group, biliary bile salt concentration decreased significantly and was 2.4-times lower than the preoperative values, which is compatible with the overall phenotype of intrahepatic cholestasis (Table 3).

**Figure 3**
Transmission electron microscopy of bile canaliculi and small bile ducts. Ultrastructural cell morphology in livers from wild-type (A) and Mdr2<sup>−/−</sup> (B) mice before transplantation versus wild-type (C) and Mdr2<sup>−/−</sup> (D) mice 14 days after OLT. Note: damaged mitochondria and ribosomes, loss of microvilli and canalicular dilation in Mdr2<sup>−/−</sup> (D) mice. In comparison with wild-type (E), bile ducts of Mdr2<sup>−/−</sup> (F) grafts display cell damage 14 days after OLT. bd: bile duct, m: mitochondria; rer: ribosomes; arrowheads point to the microvilli. Original magnification: x8800 (A-D), x5600 (E-F).
Table 2. Serum Transaminases, Alkaline Phosphatase, Bile Salts, and Total Bilirubin

Transaminases, alkaline phosphatase and bile salt levels were significant higher in Mdr2<sup>++/-</sup> mice 14 days after transplantation (OLT), <sup>a</sup>p ≤ 0.05 compared to WT at the same time point, <sup>b</sup>p ≤ 0.05 compared to pre-OLT. WT: wild-type.

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<td></td>
<td>WT</td>
<td>Mdr2&lt;sup&gt;++/-&lt;/sup&gt;</td>
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<tr>
<td>aspartate aminotransferase (U/L)</td>
<td>121±31</td>
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<td>alanine aminotransferase (U/L)</td>
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<td>alkaline phosphatase (U/L)</td>
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<td>126±16</td>
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<td>conjugated bile salts (umol/L)</td>
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<td>1.2±0.9</td>
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<tr>
<td>total bilirubin (mg/dL)</td>
<td>0.5±0.3</td>
<td>0.8±0.9</td>
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</table>

NOTE. Values are mean ± SD from n = 5 per group

Do the histopathological changes in Mdr2<sup>++/-</sup> grafts correlate with biochemical markers of hepatobiliary injury and function?

Biochemical analysis included both serum and bile measurements, as indicators for liver injury and cholestasis. Before transplantation, there were no significant differences in serum transaminases, ALP, bilirubin, and conjugated bile salts between the two groups (Table 2). In the wild-type group, no significant changes in these variables were observed at 14 days after transplantation. However, in recipients of Mdr2<sup>++/-</sup> livers, serum transaminases, ALP, and conjugated bile salts increased significantly at this time point, reflecting cellular injury and cholestasis. Serum total bilirubin was also higher in the Mdr2<sup>++/-</sup> group, but this did not reach statistical significance (Table 2).

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**Table 3. Biliary Bile Salt and Phospholipid Concentrations**

Before and after transplantation of wild-type (WT) and Mdr2<sup>−/−</sup> livers. Mdr2<sup>−/−</sup> livers showed a significant higher bile salt/phospholipid ratio before and 24 hours after transplantation (OLT), which normalized at day 14, due to significant lower bile salt levels, *p ≤ 0.05* compared to WT at the same time point, *p ≤ 0.05* compared to pre-OLT.

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<td>Biliary total bile salts (mmol/L)</td>
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<td>Biliary phospholipids (mmol/L)</td>
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<td>Bile salt/phospholipid ratio</td>
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<td>21.1±0.6*</td>
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NOTE. Values are mean ± SD from n = 5 per group (*n = 3 per group)

**Is protein expression of hepatic bile transporters compatible with the histological and biochemical cholestatic profile of Mdr2<sup>−/−</sup> grafts after OLT?**

To characterize the molecular mechanisms underlying the observed cholestatic phenotype and changes in bile composition, we next studied protein expression of hepatocellular transporters responsible for sinusoidal uptake (Ntcp) and canalicular secretion of bile salts (Bsep) at 14 days after transplantation. There were no major differences in the expression of the most important bile salt transporters Bsep and Ntcp between livers obtained from wild-type and Mdr2<sup>−/−</sup> mice before transplantation (Figure 4). No significant changes in Bsep and Ntcp protein expression were found after transplantation in wild-type livers. However, in Mdr2<sup>−/−</sup> liver grafts protein levels of both Ntcp and Bsep decreased significantly, which was in accordance with the reduction in biliary bile salt secretion (Figure 4).
**Figure 4**

Expression of Ntcp and Bsep. Western blot analysis of Ntcp (top row) and Bsep (middle row) from liver extracts (n=3 for each group) before and 14 days after OLT. Changes for Ntcp and Bsep were standardized for beta-actin protein (bottom row). No clear differences in protein expression of Ntcp (50 kDa) and Bsep (160 kDa) were observed between wild-type (WT) and Mdr2+/− mice before OLT. However, Ntcp and Bsep were significantly decreased in Mdr2+/− mice at 14 days after transplantation, *p ≤ 0.05 compared to WT at the same time point, ‡p ≤ 0.05 compared to pre-OLT.
Transcript levels of Ntcp, Bsep and 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 the wild-type (WT) levels before OLT. Mdr2 mRNA expression was 2-times lower in Mdr2+/− livers before transplantation. 14 days after OLT, however, Ntcp mRNA levels were 1.9-times decreased and Bsep 2.3-fold increased in Mdr2+/− liver grafts. A similar increase (1.8-fold) was observed for Mdr2 expression levels in Mdr2+/− liver grafts, *p ≤ 0.05 compared to WT at the same time point, †p ≤ 0.05 compared to pre-OLT.

**Does mRNA expression of hepatic bile transporters correlate with protein expression in cholestatic Mdr2+/− grafts?**

To determine whether changes in bile transporter gene transcription may explain the observed changes in protein expression at day 14 post-OLT, we measured mRNA expression of the bile salt transporters Ntcp and Bsep. In addition, mRNA expression of the phospholipid transporter Mdr2 was studied. mRNA expression in wild-type mice, before transplantation, was considered to be 100%. Before transplantation, there were no differences in mRNA expression of Ntcp and Bsep between the two groups. As expected, Mdr2 mRNA expression before transplantation was 2-times lower in Mdr2+/− livers when compared to wild-type livers (Figure 5). After transplantation, however, the expression of Ntcp, Bsep, and Mdr2 decreased slightly in recipients of wild-type livers, but this did not reach statistical significance. In accordance with the protein expression of Ntcp in Mdr2+/− liver grafts, however, a 1.9-fold decrease in Ntcp mRNA expression
was found. In this group, expression of Bsep and Mdr2 mRNA increased 2.3 and 1.8-fold, respectively (Figure 5).

**DISCUSSION**

Our aim was to investigate the role of toxic bile salts in the origin of bile duct injury after OLT, and to obtain new insights on the pathogenesis of cholestasis after transplantation. In a recent clinical study it was suggested that an increased biliary bile salt/phospholipid ratio early after OLT, leading to toxic bile composition, may result in increased intrahepatic bile duct injury (17). However, formal evidence for a cause-effect relationship between toxic bile formation and bile duct injury after OLT could not be established by this observational study. In fact, it could not be ruled out that toxic bile composition and bile duct injury both result from the same underlying factor. By using inbred mice that are heterozygous for the phospholipid transporter Mdr2 (Mdr2+/−) or wild-type, we were able to further unravel the role of bile salt toxicity in the pathogenesis of biliary injury after OLT, without the need for immunosuppression to avoid acute rejection.

The principle finding of our study is that we could demonstrate that endogenous bile salts act synergistically to ischemia/reperfusion in the origin of bile duct injury in vivo, as shown by light and electron microscopy. In addition, our data suggest that intrahepatic cholestasis and intracellular bile salt retention may be critical mechanisms triggering hepatobiliary injury after OLT. The altered expression of the bile salt and phospholipid transporters, as well as the reduced biliary bile salt/phospholipid ratio at 14 days after transplantation, were compatible with the observed hepatobiliary injury and reflect intrahepatic cholestasis and subsequent intracellular bile salt retention.

**Aggravation of bile duct injury in Mdr2+/− liver grafts after transplantation**

Evidence for a pivotal role of bile salt-mediated hepatotoxicity, in the pathogenesis of preservation and reperfusion injury of liver grafts, has gradually emerged during the last decade. Hertl et al. have shown that intrahepatic bile ducts of pig livers are injured during cold ischemia by hydrophobic bile salts, whereas hydrophilic bile salts (such as tauroursodeoxycholate) provide protection (26). Moreover, the infusion of exogenous hydrophobic bile salts before liver procurement significantly increases hepatobiliary
injury after transplantation (7-9). Injury of the biliary tree can be prevented by infusion of hydrophilic bile salts in donor animals prior to liver procurement (25).

Our study provides for the first time direct evidence that endogenous bile salts actively contribute to bile duct injury after transplantation. Although the mechanism underlying the development of bile duct injury following transplantation is only emerging, we propose a pathogenetic pathway as it has been described earlier for other biliary diseases by Lazaridis et al. (27). In our hypothesis, the putative initial insult to biliary epithelial cells is caused by cold ischemia and subsequent reperfusion. In wild-type liver grafts the initial response after transplantation is an inflammatory reaction, which is normally resolved with no pathology left behind. In Mdr2^{-/-} donor liver grafts, however, toxic bile salts aggravate ischemia/reperfusion injury of the bile duct, perpetuating the initial inflammatory response. This may lead to chronic inflammation of the bile ducts, development of cholestasis and bile duct proliferation, as was observed in Mdr2^{-/-} liver grafts after transplantation. Ultimately this may progress to fibrosis and intrahepatic strictures like in the homozygous Mdr2^{-/-} mice. Further research in this area using experiments with long-term follow-up is indicated to establish such an effect.

Homozygous Mdr2^{-/-} mice have been proposed as a model for sclerosing cholangitis and the biliary strictures resemble that of non-anastomotic strictures after transplantation. Nevertheless, we did not observe increased paracellular permeability related to disrupted tight junctions and basement membranes as described in the Mdr2^{-/-} mice by Fickert et al. (16). However, unlike Mdr2^{-/-} livers, liver grafts of Mdr2^{-/-} donors were not exposed to such high levels of toxic nonmicellar-bound bile salts after transplantation as they still excrete some phospholipids. Moreover, the initially high biliary bile salt/phospholipid ratio in Mdr2^{-/-} liver grafts turned normal after 14 days due to cholestasis and reduced bile salt excretion.

**How might the changes in hepatocyte transporter expression be explained?**

Like in human cholestatic liver diseases, the hepatic protein levels for the bile salt transporters Ntcp and Bsep in Mdr2^{-/-} liver grafts were significantly decreased after OLT (28). In parallel, the serum conjugated bile salt concentration was high, whereas the biliary bile salt concentration was low in Mdr2^{-/-} liver grafts after transplantation. The basolateral bile salt transporter Ntcp was down regulated on both mRNA and
protein levels in Mdr2\(^{-/-}\) liver grafts after transplantation. 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 (29). Therefore, intracellular bile salt retention is likely in these livers. Conversely, Bsep showed a differential expression of protein and mRNA levels in Mdr2\(^{-/-}\) liver grafts after transplantation, with a 2.3-fold up-regulation at the mRNA level, but not at protein level. This discrepancy between transcription and translation may be a consequence of intracellular bile salt retention in hepatocytes, acting cytotoxic and damaging mitochondria (10). The up-regulation of Bsep mRNA is in line with the observations made by Geuken et al., who showed an increase of the BSEP/MDR3 mRNA ratio as early as one week after OLT, whereas the biliary bile salt/phospholipid ratio was already decreasing at this time point (17). Apart from Bsep, we also observed an increase in Mdr2 mRNA levels in Mdr2\(^{-/-}\) mice after OLT. 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 (30-32). The induction of Mdr2 gene expression is in line with the function of biliary phospholipids to inactivate the detergent action of bile salts (33). However, biliary phospholipid concentration was unchanged in Mdr2\(^{-/-}\) liver grafts.

**The recovery of bile excretion is a key early event determining biliary injury after OLT.**

Mdr2\(^{-/-}\) liver grafts showed clear signs of intrahepatic cholestasis and intracellular bile salt retention. Cholestasis after transplantation may originate from several underlying mechanism, including 1) impairment of bile formation; 2) altered bile transporter expression/function; 3) altered secretory responses induced by inflammatory mediators; and/or, (4) rapid fibro-inflammatory obliteration of bile ducts. In addition, intracellular bile salt retention itself may promote further biliary injury, enhancing inflammation, cholestasis, and inducing ductular proliferation. Even when the primary insult occurs to the bile ducts, hepatocellular injury is an invariable feature of cholestasis, associated with accumulation of bile salts in the liver and blood (10). When the concentration of bile salts exceeds the binding capacity of the binding protein located in the cytosol of the hepatocyte, bile salts induce apoptosis and necrosis by damage to mitochondria.
Therapeutic strategies to modify intrahepatic cholestasis and prevent bile duct injury after OLT may include the hydrophilic bile salt ursodeoxycholic acid. This is known to improve cholestatic disorders. Although the exact mechanisms underlying its cyto-protective effect are not fully understood, it may reduce bile salt induced injury by replacing the toxic hydrophobic biliary bile salts. In addition, it has been shown to stimulate canalicular transport and biliary excretion enhancing bile flow and reducing the exposure time of biliary epithelium to toxic bile salts (34). However, its choleretic action also increases biliary pressure, which has been shown to aggravate bile infarcts and hepatocyte necrosis in the homozygous Mdr2⁻/⁻ mice (15). Moreover, ursodeoxycholic acid also displays a toxic character, affecting mitochondrial activity (10). Alternatively, the administration of fibrates, statins or peroxisome proliferators, which have all been shown to induce biliary phospholipid secretion by the induction of Mdr2, making bile less toxic, might pose a therapeutic approach to prevent bile duct complications after OLT (35-37). However, these compounds may induce other hepatic injury and have considerable side-effects (38).

In conclusion, our data indicate that toxic bile composition, due to a high biliary bile salt/phospholipid ratio, acts synergistically to ischemia/reperfusion injury in the origin of bile duct injury after OLT. Bile salts aggravate the cold ischemia/reperfusion injury of the bile ducts, initiating an inflammatory cell invasion, cholestasis and bile duct proliferation. Current evidence indicates that bile salt retention is a key early event that contributes to hepatobiliary injury after OLT.

References


