Studies on injury and repair of donor bile ducts after liver transplantation
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Recipient-derived cells are present in the human extrahepatic bile duct after sex-mismatched liver transplantation.

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ABSTRACT

Background
Cholangiocytes are particularly vulnerable to ischemic damage, and ischemia-related damage to the bile duct is a significant contributor to adverse outcomes for orthotopic liver transplant (OLT) recipients. The mechanisms of regeneration of cholangiocytes after OLT are poorly understood. The origin of the cells in the extrahepatic bile duct (EHBD; donor vs recipient) that might contribute to bile duct regeneration after OLT are unknown.

Methods
The presence of recipient-derived cholangiocytes was determined in ten EHBD samples from male recipients transplanted with a female liver. Recipient-derived cells were detected using X and Y chromosome-specific fluorescence in situ hybridization (FISH) probes. The specificity and sensitivity of the FISH assay were determined using corresponding controls.

Results
No Y-positive cells were detected in female-to-female grafts, while 22% of nucleated cells contained a detectable Y chromosome in male-to-male positive controls. Recipient-derived cholangiocytes were found in four of the ten EHBDs examined. In three cases, Y chromosome-positive cells were located in the luminal epithelium of the EHBD, while in three cases Y chromosome-positive cells were located in cholangiocytes lining the peribiliary glands. In six cases no recipient-derived cholangiocytes were detected.

Conclusions
Recipient-derived cholangiocytes are present in the EHBD of human sex-mismatched liver transplantations. These findings suggest that recipient-derived cells can repopulate the extrahepatic biliary epithelium.
INTRODUCTION

Orthotopic liver transplantation (OLT) remains the only effective therapy for patients with end-stage liver failure. Different surgical techniques are available to restore biliary enteric continuity during liver transplantation, with duct-to-duct anastomosis the gold standard (1). In this technique an anastomosis is made between the donor bile duct and the remnant of the extrahepatic bile duct (EHBD) in the recipient.

Although graft and patient survival outcomes after OLT are acceptable, damage to the bile duct frequently occurs and the bile duct is therefore considered to be the ‘Achilles heel’ of liver transplantation (2, 3). One explanation for the high incidence of biliary complications after a technically successful liver transplantation is that the biliary epithelial cells, or cholangiocytes, are especially vulnerable to periods of hypoxia and subsequent reoxygenation (known as ischemia/reperfusion injury), which is an inevitable consequence of liver transplantation (4). Hence it is likely that there is always a degree of cholangiocyte damage and regeneration after transplantation that can either be subclinical with no adverse effects, or result in biliary complications.

Previous studies have primarily focused on the basis of regeneration of intrahepatic biliary epithelium and the mechanisms of extrahepatic biliary regeneration are poorly understood. Intrahepatic cholangiocytes regenerate via different mechanisms after biliary damage, namely: (1) proliferation of resident adult cholangiocytes, (2) repopulation via bipotent progenitor cells residing in the Canals of Hering, and (3) repopulation by bone marrow-derived cells (5-8). Recent reports have suggested that EHBD epithelium responds to injury not only by proliferation of adult epithelial cells within the EHBD epithelium (as occurs in intrahepatic regeneration), but from cells residing in the peribiliary glands (PBG). PBG glands are located in the wall of the large bile ducts and EHBD, are lined by a layer of biliary epithelium, and are thought to be the regenerative compartments of the EHBD (9-11).

There are several studies investigating the contribution of recipient-derived cells and the presence of chimerism in intrahepatic cholangiocytes and hepatocytes (8, 12, 13). However, similar studies on the biliary epithelium of the EHBD are lacking. The aim of this study is to investigate whether recipient-derived cells are present in the biliary epithelium of the human EHBD after sex-mismatched OLT.
PATIENTS AND METHODS

Tissue from the donor segment of the EHBD (EHBD proximal to the biliary anastomosis towards the liver hilum) in livers obtained from adult male patients who received a female full-sized liver graft and subsequently underwent retransplantation were analyzed using fluorescence in situ hybridization (FISH) of X and Y chromosomes. In our center the liver explants, including the EHBD after duct-to-duct anastomosis are routinely biopsied and stored for analysis. Positive and negative controls were obtained from male-male and female-female transplant recipients who underwent retransplantation. All procedures and the use of tissue specimens were performed according to recent national guidelines.

EHBD specimens

Formalin-fixed, paraffin-embedded EHBD were cut into 1.5-μm thick sections. Ultra thin sections were used to prevent overlay of cells.

Fluorescent In Situ Hybridization

FISH was performed based on the protocol described previously by Boersema et al. with a few modifications (14). In brief, after deparaffinization, sections were incubated in 0.2M HCL for 20 minutes. To make DNA accessible for hybridization, sections were incubated in 8% sodium thiocyanate (NaSCN, 80°C, for 30 minutes) followed by 0.025% pepsin in 0.2 M HCL (37°C for 20 minutes). To reduce autofluorescence, sections were incubated in 100 mM copper sulfate (CuSO4, 37°C, for 1 hour) followed by 0.2% sodium borohydride (NaBH4, at room temperature, for 20 minutes). Probes were applied on dehydrated sections and simultaneously denatured (80°C, for 10 minutes). Hybridization was performed overnight in a humidified chamber at 37°C. Sections were then rinsed in 0.4 x saline sodium citrate/0.3% nonidet P-40 (NP-40, at 72°C for 2 minutes) followed by 2x saline sodium citrate/0.1% NP-40 (RT, for 1 minute) to remove excess unbound probe. Sections were then mounted in Citifluor (Agar Scientific, Stansed, UK).

Fluorescence microscopy was performed using a Leica DMLB microscope (Leica Microsystems, Rijswijk, The Netherlands) equipped with a Leica DC300F camera and Leica QWin 2.8 software. In order to obtain high resolution digitally assembled overview pictures of selected EHBD (in Figure 1A) slides were analyzed with TissueFaxes®, Zeis AxioImager Z1 Microscope System (Tissue-Gnostics GmbH, Vienna, Austria).
**Tissue analysis**

The biliary epithelium lining the lumen of the EHBD and lining the PBGs was systematically analyzed. The nuclei of all cells within the biliary epithelium containing a combination of a clear green (X chromosome-positive) and red (Y chromosome-positive) signal within the blue DAPI-stained nucleus were counted as a Y chromosome-positive cell. Red signals outside the nucleus or in between cells were not considered to be Y-chromosome positive. A specimen was considered positive for recipient derived cells if: (1) Y chromosome-positive cells were detected in the biliary epithelium lining the EHBD lumen, or (2) in the biliary epithelium lining the PBGs.

**RESULTS**

A total of 75 full-size liver retransplantations were performed in adult recipients (=>18 years) in the University Medical Center Groningen (UMCG) between January 1, 1990 and December 31, 2008. Of these patients, 40 (53%) received a gender-matched liver graft during primary liver transplantation, and 35 (47%) received a sex-mismatched liver graft. In the group that received a mismatched primary liver graft, 21 (60%) were female recipients receiving a male graft and 14 (40%) were male recipients receiving a female graft. Of the 14 female to male liver transplants, a Roux-en-Y hepatico-jejunostomy was used for biliary reconstruction in four patients and a duct-to-duct anastomosis in ten patients. Specimens from these ten patients from whom extrahepatic bile duct was available were used in this study.

The reason for retransplantation, time of biopsy, and patient and donor age are summarized in Table 6.1. Reasons leading to graft failure were chronic rejection (n=3), hepatic artery thrombosis (n=3), non-anastomotic biliary strictures (n=3), and recurrent hepatitis C infection (n=1). The mean age was 40 ± 11 years in the donor group and 38 ± 12 years in the recipient group. The mean cold ischemic time (CIT) was 567 ± 185 minutes and the mean warm ischemic time (WIT) was 58 ± 12 minutes. The median graft survival time (time between primary liver transplantation and retransplantation was 48 months (range 24 days – 129 months), as shown in Table 6.1.
The presence of Y chromosome-positive cells in the biliary epithelium

Biliary epithelium lining the EHBD lumen and PBGs was identified based on its distinct morphology on FISH-stained samples. Biliary epithelium lining the lumen and peribiliary glands was present for analysis in all specimens examined. Based on our positive male-male control specimens, the FISH efficacy was calculated at 22% for identifying the Y chromosome.

Y chromosome-positive cells were identified in 40% (4/10) of EHBDs analyzed [Patients 1, 3, 8 and 10] as single cells within the epithelium. In the other 60%, no Y chromosome-positive cells were observed. In Patients 1 and 3, Y chromosome-positive cells were found both within the biliary epithelium lining the lumen of the EHBD and also in the biliary epithelium of the PBGs. In Patient 8, Y chromosome-positive cells were only found in the biliary epithelium lining the PBGs (Figure. 6.1C and D), and in Patient 9 the Y chromosome-positive cells were only found within the biliary epithelium lining the lumen of the EHBD (Figure. 6.1B).

DISCUSSION

Here we demonstrate the existence of epithelial chimerism in the extrahepatic part of the bile duct after sex-mismatched liver transplantation. The presence of a subpopulation of recipient-derived cells within this part of the human biliary tract has not previously been described.

Since recipient-derived cells are thought to contribute to intrahepatic biliary repopulation after cholangiocyte damage, the presence of cells originating from outside the liver and contributing to the intrahepatic hepatocyte and cholangiocyte population has been
Figure 6.1. Fluorescence in situ hybridization (FISH) of explanted extrahepatic bile duct (EHBD) specimen obtained from a female graft in a male recipient. Panel (A) showing an overview of the EHBD with surrounding peribiliary glands (PBG). Y-chromosome-positive cells were detected in the biliary epithelium lining the lumen (B) as well as in the PBGs (C, D). A red nuclear signal indicates Y-chromosome-positive cells and the green signal indicates X-chromosome-positive cells. Nuclei were stained with diamidino-2-phenylindole (DAPI) and appear blue.

extensively studied (8, 12, 13, 15). However, the exact contribution of intrahepatic chimerism to biliary and liver regeneration remains a topic of debate.

Studies focusing on the extrahepatic segment of the human biliary tree have been neglected probably due to the lack of availability of tissues for analysis. While it is relatively easy to obtain tissue biopsies from parenchymal liver tissue, biopsies cannot be taken from the extrahepatic segment of the human biliary tree in live patients and the only way of obtaining this material is after retransplantation, at post-mortem, or after resection of a duct-to-duct anastomosis being converted to a Roux-en-Y hepatico-jejunostomy. Therefore the availability of human EHBD is challenging, especially in the context of sex-mismatched liver transplantation, but possible in this study due to the long-period of follow-up and availability of pathological specimens for analysis. We had access to EHBDs of liver explants, which were routinely stored in our center. By selecting only the female-to-male explants, we had the unique opportunity to examine chimerism in the EHBD.
It is thought that hepatocyte chimerism is proportional to the degree and severity of hepatocyte injury (17, 18). If the same is true of biliary injury, recipient-derived repopulation would be expected to be seen more frequently or to a greater extent when severe damage to the bile duct has occurred. In this study we speculated that the most severe damage would occur in the bile ducts of patients being retransplanted for non-anastomotic biliary strictures (NAS), one of the most severe complications of OLT characterized by a combination of cholangiocyte damage, denudation with biliary fibrosis, and stricturing. Of the three patients (Patients 2, 3, and 8) examined with non-anastomotic strictures, recipient-derived cells were detected in the biliary epithelium lining the lumen in one case and in the other cases the peribiliary glands contained recipient-derived cells. This is an interesting observation that supports previous literature indicating that in cases of non-anastomotic strictures, the regenerative compartment of the EHBD is more likely to be in the peribiliary glands located at a distance from the bile duct wall and better preserved than luminal epithelium after damage (9). Unfortunately, we were limited by small numbers of specimens in this clinically heterogeneous group to make a definitive clinical correlation.

With respect to the exact origin of intrahepatic recipient-derived cells after sex-mismatched liver transplantation, it has been suggested that they originate from peripheral blood and are bone marrow-derived cells capable of differentiating into either hepatocytes or cholangiocytes (6, 19). This hypothesis is also supported by evidence from studies in which cells derived from bone marrow in bone marrow transplant recipients have been found to repopulate the liver (8, 20). Although the recipient-derived cells seen in our EHBD specimens might be bone-marrow derived, it is also plausible to hypothesize that the origin of the recipient-derived cells could be the native segment of the recipient’s bile duct derived via a process of differentiated epithelial cell proliferation and migration, a mechanism known to occur in endothelial repair of blood vessels (21). Alternatively, recipient-derived cells may originate from the recipients peribiliary glands. However, the exact origin of recipient-derived cells remains speculative and further characterization in experimental models is warranted.

A limitation of this study is our lack of quantification of recipient-derived cells. Given our FISH efficacy of 22%, we can conclude that the number of Y chromosome-positive cells is an underestimation of the actual number. However, although the specimens were a clinically heterogeneous group making quantification for comparison difficult, such tissue is difficult to acquire and these studies provide important initial insights into the presence of recipient-derived cells in EHBD. Future experimental studies should focus on
the quantitative analysis of recipient-derived cells in the extrahepatic biliary epithelium as well as the temporal (early versus late) repopulation of recipient-derived cells.

In conclusion, this is the first report to demonstrate that recipient-derived cells are present in the luminal biliary epithelium and peribiliary glands of the human EHBD after liver transplantation. Although quantification has not been performed the number of observed Y positive cells (single cells) seems low. Knowledge of the mechanisms underpinning the regeneration of extrahepatic biliary epithelium is sparse but highly relevant given their role in contributing to or preventing complications after liver transplantation.
REFERENCES


