Studies on injury and repair of donor bile ducts after liver transplantation
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Regeneration of human extrahepatic biliary epithelium: 
the peribiliary glands as progenitor cell compartment.

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ABSTRACT

Background
Although regeneration of intrahepatic bile ducts has been extensively studied and intrahepatic progenitor cells have been identified, few studies have focussed on the extrahepatic bile duct (EHBD). We hypothesized that local progenitor cells are present within the EHBD of humans. Human EHBD specimens (n = 17) were included in this study.

Methods
Specimens of normal EHBD tissue were obtained from healthy donor livers (n = 6), mildly injured EHBD from patients with cholangitis (n = 6) and severely injured EHBD from patients with ischemic type biliary lesions (n = 5). Double immunostaining for K19 and the proliferation marker Ki-67 was performed to identify and localize proliferating cells. In addition, immunofluorescent doublestaining using antibodies against K19 and c-Kit was performed to identify and localize cholangiocytes co-expressing putative progenitor cell markers.

Results
In normal EHBD, few Ki-67+ cells were detected, whereas large numbers of Ki-67+ were found in the diseased EHBD. In EHBD affected by cholangitis, Ki-67+ cells were mainly located in the basal layer of the lumen. EHBD specimens from patients with ischemic type biliary lesions displayed histological signs of epithelial cell loss and large numbers of Ki-67+ cells were observed in the peribiliary glands. C-Kit expression was localized throughout the EHBD wall and immunofluorescent doublestaining identified a few K19+/c-Kit+ cells in the luminal epithelium of the EHBD as well as in the peribiliary glands.

Conclusions
These findings support the hypothesis that progenitor cells exist in the EHBD and that the peribiliary glands can be considered a local progenitor cell niche in the human EHBD.
INTRODUCTION

The liver and its various cell types, including hepatocytes and biliary epithelial cells (or cholangiocytes), have a well-described regenerative capacity in response to different types of injury and cellular loss. While mild-to-moderate hepatocellular injury or depletion is considered a stimulus for the replication of mature hepatic cells, more severe acute and chronic types of injury have been associated with a regenerative response that includes the recruitment of hepatic progenitor cells (1, 2). Accumulating evidence suggests that there are four possible intrahepatic stem/progenitor cell niches: the canals of Hering, intralobular bile ducts, periductal mononuclear cells, and peribiliary hepatocytes (3, 4). Hepatic progenitor cells have been shown to have bi-potent capacities, allowing them to differentiate into either hepatocytes or cholangiocytes (5).

Most studies on hepatic regeneration alluding to these progenitor cells dealt with replenishment of hepatocytes and intrahepatic bile ducts. Data of regeneration of the cholangiocytes lining the extrahepatic bile ducts (EHBD) are sparse. The biliary epithelial lining of the EHBD consists of simple columnar epithelial cells, which are continuously exposed to bile flow and the cytotoxic properties of bile salts. In addition, toxins and pathogens may cause cellular injury in pathological situations, such as in ascending bacterial cholangitis. Therefore, it is conceivable that biliary epithelium of the EHBD has an endogenous regenerative capacity, which may include a local niche of progenitor cells (6). Because the previously mentioned intrahepatic progenitor cell niches are thought to be closely related to hepatocytes and because of the lack of hepatocytes in the EHBD it is conceivable that a different source of progenitor cells exists in the proximity of the EHBD.

The intrahepatic bi-potent progenitor cells have been characterized by the expression of hematopoietic markers such as c-Kit (7-10). The expression of these progenitor cell markers in combination with the biliary markers keratin 7 (K7) and keratin 19 (K19) has been used to identify sublineages of the bi-potent progenitor cells differentiating into cholangiocytes. Experiments in mice have suggested that the EHBD contains local progenitor cells that are c-Kit positive (11). In addition, Cohen et al. and Nakanuma et al. hypothesized that the peribiliary glands of the EHBD might be the compartment which harbours regenerative cells, but formal evidence for this is still lacking (6, 12).

The aim of the current study was to identify possible site(s) where epithelial regeneration may be initiated in the human EHBD and to study the possible role of local progenitor cells
in this process. We have examined tissue specimens from normal and diseased human EHBD varying from mild cellular injury (as in cholangitis/cholecystitis) to severe epithelial injury with cholangiocyte loss, as can be seen in post-ischemic cholangiopathy, i.e. ischemic type biliary lesions (ITBL), after orthotopic liver transplantation.

EXPERIMENTAL METHODS

Source of human EHBD specimens

All procedures and use of (anonymized) tissue specimens were performed according to recent national guidelines. A total of 17 human EHBD specimens were included in this study. Specimens of normal EHBD tissue were obtained from six healthy donor livers that had been retrieved from DCD (donation after cardiac death) organ donors. These samples were obtained during the backtable procedure prior to transplantation of these organs. Specimens of mildly injured EHBD were obtained from six patients with cholangitis/cholecystitis who underwent cholecystectomy. Specimens of severely injured EHBD were obtained from five patients undergoing a retransplantation of the liver due to ITBL, in whom hepatic artery thrombosis was excluded by either Doppler ultrasound or CT angiography. All specimens were fixed in 10% buffered formalin, embedded in paraffin and 4-μm thick sections were used for immunohistochemical analysis.

Immunohistochemistry

To determine the number and location of proliferating cholangiocytes within the EHBD, double immunostaining using monoclonal antibodies against keratin 19 (rabbit anti-keratin 19, Abcam, Cambridge, UK, dilution of 1/100) and Ki-67 (mouse anti-Ki-67, DAKO, Glostrup, Denmark, dilution of 1/100) were performed. In short, after deparaffinization through a graded alcohol series, antigen retrieval was performed with 0.1M Tris/HCl buffer (pH = 9) for 15 min at 98°C in a microwave oven. Endogenous peroxidase was blocked with H2O2 for 30 min. Thereafter, sections were incubated with Ki-67 for 1 h at room temperature, followed by incubation with peroxidase-labelled rabbit anti-mouse antibody (dilution 1/100) and goat anti-rabbit antibody (dilution 1/100). The staining reaction was developed using diaminobenzidin (DAB). Sections were then washed in a glycin/HCl solution (pH = 2) for 45 min, followed by incubation with K19 for 1 h at room temperature. This was followed by peroxidase-labelled goat anti-rabbit antibody (dilution 1/100) and rat anti-goat antibody (1/100) and the staining reaction was developed with 3-amino-9-ethyl-carbazole (AEC). Sections were counterstained with haematoxylin. Histological evaluation was performed by a single pathologist (A. S. H. G).
Immunofluorescent double stainings

Immunofluorescent double staining using monoclonal antibodies against K19 (mouse anti-K19, Abcam, Cambridge, UK, dilution of 1/100) and polyclonal antibodies against c-Kit (rabbit anti-c-Kit, DAKO, C7244, Glostrup, Denmark) was performed on 4 μm paraffin sections. In short, after deparaffinization antigen retrieval was performed with 1 mmol/L ethylene diamine tetra acetate (EDTA) buffer (pH = 8) for 15 min at 98°C in a microwave oven. Thereafter, sections were incubated with antibodies against c-Kit for 1 h at room temperature, followed by incubation with antibodies against K19 for 1 h at room temperature. Thereafter, slides were incubated in the dark with a mixture of secondary fluorescent antibodies Alexa Fluor 568-conjugated goat anti-rabbit IgG antibody (dilution 1/50) and Alexa Fluor 488-conjugated goat anti-mouse IgG antibody (dilution 1/50) for 1 h at room temperature. Sections were counterstained with 4’,6-diaminido-2-phenylindole (DAPI) dilution 1/1000) for 5 min at room temperature in the dark and then mounted with Vectashield H 1000 (Vector Laboratories, Burlingame, CA, USA). Microscopy images were captured using a Leica DMLB microscope (Leica Microsystems, Rijswijk, the Netherlands) equipped with a Leica DC300F camera and Leica QWin 2.8 software.

RESULTS

Cholangiocyte damage in normal and diseased EHBD

Examples of the microscopic appearance of healthy and diseased EHBD specimens are presented in Figure. 5.1. Samples of EHBD obtained from donor livers had a fully preserved epithelial lining, without any other histological abnormalities in the bile duct wall. In contrast, specimens from patients with cholangitis showed variable grades of inflammation but a preserved epithelial lining. EHBD specimens from patients with ITBL showed complete or partial denudation of the biliary epithelial lining.
Healthy and diseased EHBD sections were double stained for the proliferation marker Ki-67 and the biliary epithelial marker K19. K19 positive cholangiocytes were detected lining the bile ducts and the peribiliary glands. In the healthy EHBD, a few isolated cells expressing both Ki-67 and K19 (K19+/Ki-67+ cells) were identified at the basal layer of the lumen and no expression of Ki-67 was detected in the peribiliary glands (Figure 5.2).

**Figure 5.1.** Overview of the different pathological features of the extrahepatic bile ducts (EHBD) examined. Representative haematoxylin-eosin staining of surgical specimens of a normal EHBD without pathological signs of injury (A), an EHBD from a patient with cholangitis, expressing signs of inflammation without disruption of the epithelial layer (B) and an EHBD suffering from ischemic type biliary lesions (ITBL) with severe cholangiocyte injury indicated by loss of the continuity of the cholangiocyte layer (C). L; lumen, PBG; peribiliary glands.

**Cholangiocyte turnover in normal and diseased EHBD**

Healthy and diseased EHBD sections were double stained for the proliferation marker Ki-67 and the biliary epithelial marker K19. K19 positive cholangiocytes were detected lining the bile ducts and the peribiliary glands. In the healthy EHBD, a few isolated cells expressing both Ki-67 and K19 (K19+/Ki-67+ cells) were identified at the basal layer of the lumen and no expression of Ki-67 was detected in the peribiliary glands (Figure 5.2).
Figure 5.2. The pattern of cholangiocyte proliferation after different types of biliary injury. Immunohistochemical stainings showed that the number of proliferating cholangiocytes is very low in normal extrahepatic bile ducts (EHBD) (A). Cholangiocyte proliferation predominantly occurred in the luminal lining of the EHBD in the case of cholangitis (B) with low proliferation in the peribiliary glands (C). However, in EHBD suffering from ischemic type biliary lesions (severe injury with partial denudation of the cholangiocytes lining the lumen), proliferation was mainly located in the peribiliary glands with only low numbers of proliferating cholangiocytes with in the luminal cholangiocyte lining (D). Cholangiocytes are stained in red (keratin 19), proliferating cells are stained in brown (Ki-67). L; lumen, PBG; peribiliary glands.
In EHBD from patients with cholangitis, with mild-to-moderate biliary epithelial injury, the intact cholangiocytes were K19 positive and a substantial number of K19+/Ki-67+ cells were detected in the basal layer of the lumen of the EHBD, whereas K19+/Ki-67+ cells were detected only sporadically in the peribiliary glands (Figure 5.2). In contrast, EHBD of patients with ITBL and severe biliary epithelial injury, characterized by a marked loss of lining cholangiocytes on the luminal side, exhibited a high number of K19+/Ki-67+ cells in the peribiliary glands, whereas only a few double positive cells were observed at the lumen of the bile duct (Figure 5.2).

**Cellular localization of c-Kit**

We next examined whether cells expressing c-Kit could be detected in the EHBD specimens. Expression of c-Kit was detected in individual cells throughout the bile duct wall. Cells positive for c-Kit were located at the basal epithelial layer of the lumen, in the connective tissue of the bile duct, and a few positive cells were detected in the peribiliary glands. No differences in number and localization between normal, mildly and severely injured EHBDs were present.

To determine if a subpopulation of c-Kit positive cells also express K19, double immunofluorescence staining was performed. A few K19+/c-Kit+ cells were observed at the epithelial lining of the lumen (Figure 5.3) of the EHBD as well as in the peribiliary glands (Figure 5.4). The number and localization of these cells did not differ between normal and diseased EHBD.

**DISCUSSION**

In this study, we provide evidence that there are two distinct sites in the human EHBD where regeneration of cholangiocytes can be identified. In addition, our data suggest that biliary progenitor cells reside within these sites.

In specimens with mild-to-moderate injury of the EHBD, proliferating cells with cholangiocyte characteristics were primarily present in the epithelial lining of the EHBD lumen, whereas proliferating cholangiocyte-like cells were primarily present in the peribiliary glands in specimens with more severe damage showing loss of cholangiocytes at the luminal side of the EHBD. In line with this finding, we have also demonstrated K19-positive putative progenitor cells within both the lining of the bile duct lumen and the peribiliary glands.
Figure 5.3. Evaluation of c-Kit expression by cholangiocyte-like cells at the luminal compartment. Immunofluorescent double staining with c-Kit (red) and keratin 19 (K19) (green) showed that, although in small numbers, K19+/c-Kit+ cells are present in the epithelial lining of the extrahepatic bile ducts (EHBD) lumen (indicated by an arrow). These K19+/c-Kit+ were found in normal and diseased states (cholangitis or ischemic type biliary lesions) of the EHBD. This population of double positive cells is in contrast with other type of cells that are positive for c-Kit but do not express K19 (indicated by arrowheads).

Figure 5.4 Evaluation of c-Kit expression by cholangiocyte-like cells in the peribiliary glands. Immunofluorescent double staining with c-Kit (red) and keratin 19 (K19) (green) showed that, small numbers of K19+/c-Kit+ cells are present in the peribiliary glands of the extrahepatic bile duct (EHBD) wall (indicated by an arrow). These K19+/c-Kit+ were found in normal and diseased states (cholangitis or ischemic type biliary lesions) of the EHBD. This population of double positive cells is in contrast with other type of cells that are positive for c-Kit but do not express K19 (indicated by arrowheads).
Several studies on intrahepatic regeneration of hepatocytes and cholangiocytes have suggested c-Kit as a useful marker of biliary progenitor cells (8, 13). However, it is also expressed by other cell types inside the liver leading to a less unambiguous interpretation of c-Kit stainings. In the current study, we found c-Kit expression in small cholangiocytes at the epithelial lining of the lumen as well as in the epithelial lining of the peribiliary glands. This finding strongly suggests that both the luminal epithelial lining and the peribiliary glands contain cholangiocyte progenitors that are involved in repair of the biliary epithelial lining following injury.

We observed only low proliferative activity in the biliary epithelium of normal EHBD. Despite the fact that biliary epithelium is continuously exposed to bile flow and the cytotoxic effects of bile salts, cellular renewal activity is apparently low under physiological circumstances. In contrast with this, substantial proliferative activity was observed in specimens obtained from patients with cholangitis and ITBL. We found a striking difference in the pattern of proliferative activity between these two disease entities. In cholangitis, the biliary epithelial lining remains largely intact and the degree of injury can be graded as mild to moderate. In this situation, proliferating cells were observed at the luminal side of the EHBD. In contrast, in patients with ITBL and severe EHBD injury, resulting in partial or complete denudation of the epithelial lining, proliferating cells were mainly observed in the peribiliary glands and proliferation at the luminal side of the EHBD was low. These findings suggest that after a relatively mild injury of the epithelium, the necessary renewal of cholangiocytes is achieved by replication of neighbouring cholangiocytes. This repair mechanism may not suffice in the situation of severe epithelial injury when there is partial or complete loss of cholangiocytes at the lumen of the bile duct. In this situation, no actual loss of cholangiocytes was observed in the inner lining of the peribiliary glands, and recruitment seems to occur from these peribiliary glands. Altogether, these findings suggest that replication of mature cholangiocytes at the EHBD lumen is a first-line regenerative mechanism of EHBD repair. When this first-line mechanism fails, a second-line mechanism is initiated resulting in the recruitment of cells from more distant sites such as the peribiliary glands.

A similar concept is found in hepatocyte regeneration. In massive hepatic necrosis in which there is severe loss of parenchymal cells, the progenitor cell compartment is activated, whereas after partial hepatectomy these progenitor cells remain quiescent and regeneration is mainly contributed by adult hepatocytes (4, 5).
Our findings are in agreement with studies performed by Nakanuma et al., who previously suggested that the peribiliary glands may be an important reservoir of epithelial regeneration in the EHBD. Nakanuma was also the first to describe that there is a microstructural connection between the peribiliary glands and the lumen of the bile duct making it possible for replenishing cells to migrate from the peribiliary glands to the site of luminal epithelial injury (6).

To investigate the hypothesis that local progenitor cells are (in part) responsible for cholangiocyte repair during mild and more severe injury, we examined the localization of c-Kit positive cells within the EHBD. C-Kit is a putative progenitor cell maker, but is also expressed on mature cell types such as mast cells and Cajal cells. Indeed, individual c-Kit positive (but K19 negative) cells were detected in the wall of the EHBD with morphological characteristics resembling mast cells and Cajal cells. However, we found a third population of c-Kit positive/K19 positive cells located within the epithelial layer of the lumen and the peribiliary glands. These cells were found in low numbers in all sections investigated but no different pattern between normal and diseased EHBD was demonstrated. C-Kit expression by biliary epithelial cells of the human intrahepatic bile duct has been described previously by Ahmadi et al. but these investigators considered that this was attributable to non-specific immunofluorescence staining (14). In contrast, in our study we clearly show these c-Kit positive cells are present within the epithelial layer, albeit in very low numbers. Our findings that a limited number of K19 positive cells co-expressed c-Kit, is in accordance with findings of Crosby et al. who showed similar expression of c-Kit positive/K19 positive cells lining the lumen of the intrahepatic bile duct (8, 13).

We have demonstrated that a population of cholangiocytes co-expressing c-Kit resides in the luminal lining of the human EHBD and the adjacent peribiliary glands. The lack of differences between the distribution of c-Kit positive cholangiocytes in different disease states and healthy controls is supportive of the hypothesis that these are progenitor cells permanently residing in the EHBD. Progenitor cells are responsible for self-renewal instead of being the proliferating cells themselves. They give rise to a progeny of proliferating cells as indicated by our Ki-67 stainings.

In summary, in this study we have demonstrated that different patterns of cholangiocyte proliferation occur in the human EHBD. Following a mild-to-moderate type of injury (i.e. cholangitis) restoration of the epithelial lining seems predominantly provided by proliferation of (mature) cholangiocytes at the luminal lining of the EHBD. In case of severe injury and cholangiocyte loss recruitment of cells from the peribiliary glands will add to this. In addition
to this, the peribiliary glands of the EHBD could be considered as a local niche of biliary progenitor cells. More studies will be needed to further identify these progenitor cells and to examine the specific stimuli that are involved in the activation of the peribiliary glands.

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