Ischemia reperfusion of the hepatic artery induces the functional damage of large bile ducts by changes in the expression of angiogenic factors

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Short title: Ischemia reperfusion modulation of cholangiocyte growth

Abbreviations: Ang = angiopoietin; BDL = bile duct ligation; BSA = bovine serum albumin; cAMP = cyclic adenosine 3', 5'-monophosphate; CK-19 = cytokeratin-19; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; HAIR = hepatic artery ischemia reperfusion; PCNA = proliferating cell nuclear antigen; PVIR = portal vein ischemia reperfusion; SR = secretin receptor; TUNEL = terminal deoxynucleotidyl transferase biotin-dUTP nick-end labeling; VEGF = vascular endothelial growth factor.

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ABSTRACT

Background: Liver transplantation and cholangiocarcinoma induce biliary dysfunction following ischemia reperfusion (IR). The function of the intrahepatic biliary tree is regulated by both autocrine and paracrine factors. The aim of the study was to demonstrate that IR-induced damage of cholangiocytes is associated with altered expression of biliary angiogenic factors. Methods: Normal and BDL rats underwent 24 hr sham or hepatic reperfusion after 30 minutes of transient occlusion of the hepatic artery (HAIR) or portal vein (PVIR) before collecting liver blocks and cholangiocyte RNA or protein. We evaluated liver histology, biliary apoptosis, proliferation and expression of VEGF-A/C, VEGFR-2/3, Ang-1/2 and Tie-1/2 in liver sections and isolated small and large cholangiocytes. Normal rat intrahepatic cholangiocyte cultures (NRICC) were maintained under standard conditions in normoxic or under a hypoxic atmosphere for 4 hr and then transferred to normal conditions for selected times. Subsequently, we measured changes in biliary proliferation and apoptosis and the expression of VEGF-A/C and VEGFR-2/3. Results: In vivo, HAIR (but not PVIR) induced damage of large bile ducts and decreased proliferation and secretin-stimulated cAMP levels. HAIR-induced damage of large bile ducts was associated with increased expression of VEGF-A/C, VEGFR-2/3, Ang-1/2 and Tie-1/2. In vitro, under hypoxic conditions, there was increased apoptosis and reduced proliferation of NRICC concomitant with enhanced expression of VEGF-A/C and VEGFR-2/3. Conclusion: The functional damage of large bile ducts by HAIR and hypoxia is associated with increased expression of angiogenic...
factors in small cholangiocytes, presumably due to a compensatory mechanism in
response to biliary damage.

INTRODUCTION
Cholangiocytes line the intrahepatic biliary epithelium, a complex network of
interconnecting ducts of different sizes and functions (5, 35). In humans, cholangiocytes
are the target cells in a number of biliary disorders characterized by dysregulation
between biliary growth/loss (23). Normally, cholangiocytes have low replicative activity,
but proliferate in experimental models that mimic human pathologies, such as ligation of
the extrahepatic bile duct (BDL) and acute carbon tetrachloride administration (CCl4) (3,
32). The human and rodent biliary epithelium is morphologically and functionally
heterogeneous (5, 21, 27, 32, 35, 38). In rat liver, isolated small cholangiocytes lining
smaller ducts (< 15 µm in diameter), are mitotically dormant and exert their functions by
activation of the D-my-o-inositol 1,4,5-trisphosphate (IP₃)/Ca²⁺/calmodulin-dependent
protein kinase I signaling pathway (1, 13, 35). On the other hand, isolated large
cholangiocytes lining larger ducts (> 15 µm in diameter) secrete and proliferate by
activation of cyclic adenosine 3′,5′-monophosphate (cAMP)-dependent signaling (2, 5,
21, 27, 35).
Several experimental models have shown that ischemia reperfusion (IR) injury plays a
role in liver pathophysiology (43). Orthotopic liver transplantation (OLT) has been shown
to be a successful treatment choice for patients with end-stage chronic or acute liver
failure. However, biliary complications remain a significant source of morbidity (51, 52).
Most of these complications consist of anastomotic or non-anastomotic strictures (NAS),
biliary necrosis or leakage (48). Therefore, biliary complications represent major causes
of morbidity, graft failure as well as mortality after liver transplantation (26, 34). Hepatic
IR elicits hepatic tissue repair, which is characterized by the proliferation of hepatocytes,
removal of necrotic tissue, and restoration of the hepatocellular and hepatic
microvascular architecture (44). Several mediators, including cytokines and angiogenic
factors, regulate the mechanisms underlying hepatocellular regeneration. The function of
the intrahepatic biliary epithelium is linked to its vascular supply sustained by the
peribiliary arterial plexus (PBP) (15, 47), since alterations of intrahepatic bile duct mass
are associated with architectural changes in the PBP(14). The PBP stems from the
hepatic artery, nourishes the biliary tree, and sustains a countercurrent of substances
reabsorbed from bile toward parenchymal cells (17). After BDL, the increase in
intrahepatic bile duct mass is followed by a parallel growth of the PBP, which is
fundamental in sustaining the enhanced nutritional and functional demands of the
proliferating biliary epithelium (16, 22). Nevertheless, the proliferation of the PBP occurs
only after the hyperplasia of the biliary epithelium (17), suggesting a cross-talk
mechanism between cholangiocytes and endothelial cells, an interaction that mediates
the adaptive changes of these cells during liver damage (14, 45).
Vascular endothelial growth factor (VEGF) is a family of related growth factors including
VEGF-A, -B, -C, -D, and -E and placenta growth factor (11). VEGF is secreted by a
number of epithelia including cholangiocytes where it modulates functions by autocrine
and paracrine mechanisms by interacting with VEGFR-2 and VEGFR-3 (14, 15). Other
important angiogenic factors are the Angiopoietins (Ang-1 and Ang-2), which bind to the
tyrosine kinase receptor Tie-1/2 (41). Angiopoietins have opposite effects on their
receptors: Ang-1 activates Tie-2 by tyrosine phosphorylation, whereas Ang-2
antagonizes the Ang-1/Tie-2 binding (40). In primary biliary cirrhosis the enhanced
expression of VEGF-A, Ang-1, Ang-2, and Tie-2 receptor by endothelial cells and
periportal hepatocytes is responsible for the angiogenesis occurring in close proximity to
the damaged bile ducts (40), which may contribute to recruit the inflammatory cells worsening the pathology. Reduced portal fibrosis and hypertension was observed in cholestatic rats by inhibition of VEGFR-2 (39).

In this study, we evaluated the impact of the ischemia/reperfusion of hepatic artery and portal vein on biliary functions and the role of the angiogenic factors during these conditions.

MATERIALS AND METHODS

Materials

All reagents were purchased from Sigma Chemical (St. Louis, MO) unless otherwise indicated. The primary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The selected rat primers were purchased from SABiosciences (Qiagen, Valencia, CA) and designed using sequences with the following NCBI GenBank Accession numbers: PCNA, NM_022381; Bax NM_017059; VEGF-A NM_031836; VEGF-C NM_053653; VEGFR2 NM_013062; VEGFR3 NM_053652; angiopoietin 1 NM_053546; angiopoietin 2 NM_134454; Tie-1 NM_053545; and Tie-2 NM_001105737. The RNeasy Mini Kit to purify total cholangiocyte RNA was purchased from Qiagen Inc, Valencia, CA. The RIA kits for the measurement of intracellular cAMP (cAMP $[^{125}\text{I}]$ Biotrak Assay System, RPA509) were purchased from GE Healthcare (Little Chalfont, Buckinghamshire, UK).

In Vivo and In Vitro Models

Male 344 Fischer rats (150–175 gm) were purchased from Charles River (Wilmington, MA) and kept in a temperature-controlled environment (22°C) with 12:12 hr light/dark cycles. Animals were fed ad libitum and had free access to drinking water. The studies
were performed in normal rats and rats that after 1 wk of BDL underwent 30 minutes of transient occlusion of the hepatic artery (HAIR) or the portal vein (PVIR) followed by 24 hr of hepatic reperfusion or sham surgery (28). BDL was performed as described (3).

From these groups of animals, we collected liver blocks and cholangiocytes. Before each procedure, animals were injected with euthasol following the regulations of the panel of euthanasia of American Veterinarian Medical Association and protocols approved by Baylor Scott & White IACUC. The \textit{in vitro} studies were performed in our normal rat intrahepatic cholangiocyte line (NRICC) that displays morphological and functional phenotypes similar to freshly isolated cholangiocytes (4).

\textbf{Evaluation of Liver Histology}

We measured necrosis, and the degree of portal inflammation in H&E-stained paraffin-embedded liver sections (4–5 μm thick, 3 sections evaluated per group of animals) from the animal groups of Table 1. At least 10 different portal areas (from three different sections) were evaluated for each parameter. Liver sections were examined in a coded fashion by BX-51 light microscopy (Olympus, Tokyo, Japan) equipped with a camera (49).

\textbf{Evaluation of Cholangiocyte Apoptosis}

Apoptosis in small and large cholangiocytes was evaluated by: (i) quantitative terminal-deoxynucleotidyl-nick end-labeling (TUNEL) analysis (Apoptag; Chemicon International, Billerica, MA) in liver sections; and (ii) immunoblots (12) for Bax expression in isolated cholangiocytes from the selected groups of animals. Sections were analyzed in a coded manner using BX-51 light microscopy (Olympus, Tokyo, Japan) with a video cam (Spot Insight; Diagnostic Instrument, Inc., Sterling Heights, MI) and processed with an Image
Analysis System (Delta Sistemi, Rome, Italy). At least 10 different portal areas (from three different sections) were evaluated. Bax expression was performed in protein (10 μg) from whole cell lysates from purified cholangiocytes. Immunoblots were normalized by β-actin. The intensity of the bands was determined by scanning video densitometry using the phospho-imager, Storm 860 (GE Healthcare, Piscataway, NJ, USA) and the ImageQuant TL software version 2003.02 (GE Healthcare, Little Chalfont, Buckinghamshire, UK) (8).

Evaluation of Cholangiocyte Proliferation

Cholangiocyte proliferation was studied in liver sections by PCNA immunohistochemical expression. Immunohistochemistry was performed in 3–4 μm thick sections. Sections were deparaffinized and endogenous peroxidase activity was blocked by a 30-min incubation in methanolic hydrogen peroxide (2.5%). Later, the endogenous biotin was blocked by a biotin blocking system (code X0590; Dako, Copenhagen, Denmark) according to the instructions supplied by the vendor. Sections were then hydrated in graded alcohol and rinsed in 1X phosphate-buffered saline (PBS, pH 7.4) before applying the selected primary antibody. Sections were incubated overnight at 4°C with PCNA polyclonal antibodies (Santa Cruz Biotechnology, Milan, Italy). The following day, samples were rinsed with PBS for 5 min, incubated for 20 min at room temperature with secondary biotinylated antibody (LSAB Plus system; Dako, Milan, Italy), then with Dako ABC (LSAB Plus system; Dako, Milan, Italy), and finally developed with 3,3’-diaminobenzidine. To confirm the specificity of immunoreaction, negative controls were performed for all immunoreactions. We measured the percentage of PCNA-positive
small and large cholangiocytes (36). At least 10 different portal areas (from three
different sections) were evaluated. Intrahepatic bile duct mass (BDM) was evaluated by
determining the area fraction of liver parenchyma occupied by bile ducts using BX-51
light microscopy (Olympus, Tokyo, Japan) with a video cam (Spot Insight; Diagnostic
Instrument, Inc., Sterling Heights, MI) and processed with an Image Analysis System
(Delta Sistemi, Rome, Italy) (20). BDM was expressed as percentage of area occupied
by bile ducts with respect to the total liver parenchyma. Proliferation was also evaluated
by measurement of PCNA protein expression by western blots using specific antibody
and normalized by β-actin as seen previously.

Expression of Angiogenic Factors in Cholangiocytes

The immunohistochemical expression of VEGF-A, VEGF-C, VEGF-R2, VEGF-R3, Ang-
1, Ang-2, Tie-1 and Tie-2 (Santa Cruz Biotechnology, Milan, Italy) in small and large
cholangiocytes was evaluated in liver sections. Immunohistochemistry for these proteins
was performed as described above for PCNA staining. We measured the percentage of
cholangiocytes expressing the selected angiogenic factors (36). At least 10 different
portal areas (from three different sections) were evaluated for each parameter. Real time
PCR analysis was performed using specific primers designed against rat VEGF-A,
VEGF-C, VEGFR-2, VEGFR-3, Ang-1, Ang-2, Tie-1 and Tie-2 genes. A delta delta of the
threshold cycle (ΔΔCT) analysis was performed using normal cholangiocytes as the
control sample; as housekeeping, we used GAPDH (8).

Measurement of cAMP Levels in Purified Cholangiocytes

We measured basal and secretin-stimulated cAMP levels, a functional marker of
cholangiocyte proliferation (24, 31), in purified cholangiocytes from the selected groups
of animals. Following incubation for 1 h at 37°C (5, 29), cholangiocytes (1 x10^5 cells) were stimulated at room temperature for 5 min with 0.2% BSA (basal), or secretin (100 nmol/l in 0.2% BSA). Intracellular cAMP levels were assessed with commercially available kits (31, 32).

In Vitro Effect of Normoxia and Hypoxia on Apoptosis, Proliferation and Expression of VEGF-A/-C and VEGFR-2/-3 in NRICC

The in vitro experiments were performed in our NRICC (4). NRICC were maintained in culture with DMEM-F-12, MEM nonessential amino acids solution, Insulin-Transferrin-Selenium-X, chemically defined lipid concentrate, MEM vitamin solution, L-glutamine, Pen/Strep, Gentamicin, Bovine pituitary extract, Dexamethasone, 3,3’,5-triiodo-L-thyromine, EGF, FBS and forskolin. NRICC were maintained under standard conditions in normoxic atmosphere of 21% O_2, 74% N_2 and 5% CO_2 to reflect physiologic conditions or under a hypoxic atmosphere of 5% O_2, 90% N_2 and 5% CO_2 for 4 hr and transferred to normal conditions for different times (1 - 3 hr) with or without pre-incubation with an antibody anti-VEGF-A or anti-Angiopoietin-1. Cells were plated at 80% confluence 1 day before the incubation under hypoxic or normoxic conditions. The cells were seeded in Petri dishes and maintained in their medium. Then, cells were placed in the incubator at 37°C and exposed to hypoxia in a modular incubator chamber (C-Chamber Hypoxia chamber) with continuous monitoring and automated adjustments to maintain the several parameters stable (ProOx controller, Biospherix, Redfield, NY). After, we extracted from NRICC proteins and total RNA to measure changes in: (i) proliferation and apoptosis by immunoblotting and real-time PCR for PCNA and Bax, respectively; and (ii) mRNA expression of VEGF-A/C and VEGFR-2/3 by real-time PCR.
(12). A delta delta of the threshold cycle (ΔΔCT) analysis was performed using NRICC maintained at normal levels of oxygen for 7 hr as the control sample.

RESULTS

Effect of HAIR and PVIR on liver histology and biliary apoptosis and proliferation

There were no significant histological differences between the experimental groups of normal rats and normal rats plus HAIR or PVIR (not shown). On the contrary, both HAIR and PVIR induced lobular damage and areas of focal necrosis and portal inflammation in normal compared to BDL rats. In both normal and BDL rats, HAIR (and at lower degree PVIR): (i) induced apoptosis of large bile ducts as evidenced by TUNEL in liver sections (Figure 1A and Table 1); and (ii) decreased proliferation of large cholangiocytes (by reduced PCNA expression in liver sections) (Figure 1B and Table 1). There were increased Bax and reduced PCNA expression in purified cholangiocytes from the selected groups of animals (Figure 1A-B, lower graphs).

Expression of Angiogenic Factors in Cholangiocytes

In BDL rats, there were: (i) enhanced expression of VEGF-A/C, VEGFR-2/3, Ang-1/2 and Tie-1/2 in small bile ducts (in liver sections) (see Table 2) and purified pooled cholangiocytes (that include both small and large cholangiocytes) (5) compared to normal rats; and (ii) decreased expression of VEGF-A/C, VEGFR-2/3, Ang-1/2 and Tie-1/2 in large bile ducts (Figure 2A-B and Table 2). In BDL rats, HAIR (and at lower degree PVIR) determined: (i) a further increased expression of VEGF-A/C, VEGFR-2/3, Ang-1/2 and Tie-1/2 in small bile; and (ii) decreased expression of VEGF-A/C, VEGFR-2/3, Ang-1/2 and Tie-1/2 in large bile ducts compared to BDL sham rats (Figure 2A-B and Table 2).
there was low expression of these factors in hepatocytes (Figure 2A-B). The increase in the aforementioned growth factors/receptors was lower in BDL + PVIR rats compared to BDL + HAIR rats (Figure 2A-B and Table 2). The same results were obtained by real-time PCR in purified pooled cholangiocytes, where angiogenic factors and receptors were present at higher levels after HAIR both in normal and BDL rats compared to control animals (Figure 2C).

Measurement of Basal and Secretin-stimulated cAMP Levels
Consistent with previous findings (19), basal cAMP levels of cholangiocytes from BDL rats were higher than cAMP levels of normal cholangiocytes (Figure 3). In BDL (but not BDL + HAIR) rats, secretin increased cAMP levels of purified pooled cholangiocytes (Figure 3).

In Vitro Effect of Normoxia and Hypoxia on Apoptosis, Proliferation and Expression of VEGF-A/C and VEGFR-2/3 in NRICC
To verify if the in vivo findings were specific to cholangiocytes, we performed in vitro studies in NRICC during hypoxic (4 hr) conditions using antibodies against VEGF-A and Ang-1 or controls (non-immune serum). Consistent with previous studies showing increased apoptosis during hypoxic conditions (46, 53), there was increased Bax expression when NRICC were incubated under hypoxia; this effect was higher after 4 hr of hypoxia and 2 hr of normoxia, but it was increased after 3 hr of reoxygenation if we pre-incubated cells with the antibodies for anti-angiogenic factors (Figure 4A-B). NRICC also displayed a lower proliferative rate (by PCNA expression) following hypoxic conditions that was enhanced using an anti-VEGF-A or an anti-Ang-1 antibody (Figure 4A-B). Recovery of the normoxic conditions led to restoration of cholangiocyte proliferation and prevention of biliary apoptosis in a time-dependent manner (Figure 4A-
B). During hypoxic conditions, there was a significant increase in mRNA expression of VEGF-A, VEGF-C, VEGFR-2 and VEGFR-3 (measured as ratio to GAPDH mRNA) (Figure 5). These increased values returned to normal levels following restoration of normoxic conditions for 1, 2 or 3 hr (Figure 5).

DISCUSSION

Our study has shown that in normal and BDL rats, HAIR (and to lesser extent PVIR) induced a functional damage of bile ducts as demonstrated by increased apoptosis of large bile ducts in liver sections and Bax expression in purified pooled cholangiocytes. In BDL rats, HAIR decreased proliferation of large cholangiocytes in liver sections, and PCNA expression and secretin-stimulated cAMP levels of pooled cholangiocytes compared to BDL sham rats. In BDL rats, HAIR and PVIR induced lobular damage and focal areas of necrosis compared to normal and BDL rats without HAIR or PVIR. HAIR-induced damage of large bile ducts was coupled with increased expression of VEGF-A/C, VEGFR-2/3, Ang-1/2 and Tie-1/2 in small bile ducts and decreased expression of VEGF-A/C, VEGFR-2/3, Ang-1/2 and Tie-1/2 in large bile ducts compared to BDL sham rats. The increased expression of angiogenic factors in small cholangiocytes is likely due to a compensatory mechanism due to the damage of large BDL cholangiocytes by HAIR. 

In vitro, during hypoxia/normoxia conditions there was increased apoptosis and reduced proliferation of NRICC concomitant with enhanced expression of VEGF-A/C and VEGFR-2/3.

The liver is susceptible to ischemia-reperfusion (I/R) injury that often occurs after conditions such as shock, trauma, surgical hepatectomy and transplantation. Hepatic I/R leads to an acute inflammatory response, causing significant hepatocellular damage and liver dysfunction. The mechanisms of hepatic I/R injury are complex and involve multiple
mechanisms (50). For example, I/R-induced apoptosis of bile ducts may contribute to
the pathogenesis of biliary complications after liver transplantation (9). To begin to
understand the mechanisms of I/R-induced biliary disorders, we used, in vivo, an
experimental model of BDL rats that underwent 24 hr of hepatic reperfusion immediately
following sham or 30 m of transient occlusion of HAIR or PVIR. The in vitro model
consisted of NRICC that were exposed to normoxic or hypoxic conditions for 4 hr and
then to normoxia for 1 to 4 hr.

Previous studies have shown that 45 minutes of ischemia in cholestatic rats resulted in
extensive damage during 24 hr of reperfusion, leading to the premature death of most of
these animals (30). Therefore, for our experiments, ischemia time was reduced to 30
minutes to achieve a nonlethal I/R injury model. In our model, the reperfusion time was
extended to 24 hr, resulting in hepatic necrosis and an inflammatory response. Another
study in mice has demonstrated that cholestasis protected against I/R injury (18). The
study suggested that hyperbilirubinemia induces decreased inflammatory response and
subsequent reduced injury (42). As an alternative protective mechanism, the study
suggested that cholestatic mice failed to activate nuclear factor-κB and tumor necrosis
factor-α synthesis, two mediators of post-ischemic liver inflammation (18). In our study a
marked inflammatory response persisted in the presence of cholestasis, possibly
indicating a species difference in the inflammatory response during cholestasis. In
addition, we also evaluated during I/R damage the role of selected angiogenic such as
VEGF, which upon multiple stimuli, is produced by endothelial cells, macrophages,
activated T cells, and cholangiocytes (22, 36). VEGF plays important roles in both
physiologic as well as pathologic vasculogenesis and angiogenesis (11, 37). Because
ischemia is a potent stimulus for VEGF synthesis, it has been suggested to play a role
during I/R-induced liver injury (33). Also, a recent study has shown that endogenous
VEGF in the liver is expressed and released after transplantation, and that the administration of neutralizing VEGF antibody during reperfusion attenuated liver damage in a cold I/R injury model (6). Among other angiogenic factors, Ang-1 is a strong vascular protective agonist of the Tie-2 receptor responsible for suppressing vascular leakage, maintaining endothelial cell survival, and inhibiting vascular inflammation. Ang-2 promotes in a dose-dependent manner destabilization, vessel leakage, and inflammation (7, 10). In the kidney, I/R induces a dysregulation in Ang-2/Ang-1, which is accompanied by a loss of endothelial cells, proliferation of pericytes, and development of fibrosis.

With regard to the expression/role of vascular factors on the biliary epithelium after I/R injury, we propose that small cholangiocytes express higher levels of VEGF after HAIR most likely due to a compensatory mechanism to maintain biliary homeostasis following the injury of large cholangiocytes following I/R. In fact, the in vitro study with NRICC treated in hypoxic conditions displayed a lower proliferative rate (by PCNA expression) following the first two hours of normoxic conditions, but proliferation began to increase after 3 hours of normoxia. An effect blocked by the pre-incubation with antibodies against VEGF-A and Ang-1. In fact, cholangiocyte growth is inhibited to confirm the key role of these angiogenic factors in the recovery after hypoxic damage. In fact, it has been shown that: (i) angiogenic factors modulate cell growth by autocrine and paracrine mechanisms in liver; and (ii) overexpression in small cholangiocytes, together with the inhibitory effects of the antibodies in cholangiocyte proliferation, show that under hypoxic conditions, VEGFs and Angiopoietins may contribute to hepatic repair. On the other hand, we also found that the biliary apoptosis is controlled by angiogenic factors as protective factors, since the pre-incubation with an anti-VEGF-A or an anti-Ang-1 induces an increase in cholangiocyte apoptosis after the first hours of reoxygenation.
Supporting this finding, a number of studies support the concept that small cholangiocytes are more resistant than large cholangiocytes to hepatic injury (32, 35). The higher resistance of small cholangiocytes to damage may be due to their undifferentiated nature, whereas large cholangiocytes are more differentiated and more susceptible to injury (25). The locally produced VEGF may be an early mediator promoting hepatic I/R injury. Therefore, the blockade of this endogenous VEGF may have a cytoprotective effect. On the other hand, exogenous VEGF may also exert cytoprotection during I/R injury. Additional studies need to be performed to clarify the underlying mechanisms, but the manipulation of the small cholangiocyte compartment may be the key in the regulation of the expression/secretion of angiogenic factors and for the management of I/R induced biliary injury.


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LEGENDS

**Figure 1** In BDL rats, HAIR increased apoptosis by TUNEL analysis [A] and decreased the proliferation by reduced PCNA expression [B] of large cholangiocytes in liver sections; orig. magn., x20, scale bar= 60 µm (for semiquantitative analysis see Table 1). [C] There was increased Bax expression [D] and reduced PCNA presence in purified cholangiocytes from normal and BDL rats by immunoblots. *p<0.05 vs. BDL rats.

**Figure 2** In BDL rats, there was enhanced expression of VEGF-A/C, VEGFR-2 and R3 [A] and Ang-1/2 and Tie-1/2 [B] in biliary epithelium compared to normal rats by immunohistochemistry. In BDL + HAIR rats there was increased expression of VEGF-A/C, VEGFR-2 and R3 [A], Ang-1/2 and Tie-1/2 [B] in bile ducts and purified cholangiocytes compared to BDL rats by real time PCR [C]. The increase in the aforementioned growth factors/receptors was smaller in BDL + PVIR rats compared to BDL + HAIR rats. Orig. magn., x20, scale bar= 60µm (for semiquantitative analysis see Table 2).

**Figure 3** Basal cAMP levels of cholangiocytes from BDL rats were higher than cAMP levels of normal cholangiocytes. In BDL (but not BDL + HAIR) rats, secretin increased cAMP levels in purified cholangiocytes. Data are mean ± SEM of 6 experiments. *P < 0.05 versus the corresponding basal value. # ns, not significant vs. the corresponding basal value.
Figure 4  [A] Panel above: Evaluation of cholangiocyte apoptosis (by immunoblotting analysis for Bax) in NRICC maintained in condition of normoxia or hypoxia for 4 hours and transferred to normal conditions for different times (1 - 3 hours) with or without pre-incubation with an antibody anti-VEGF-A or anti-Ang-1. Bax expression was increased when NRICC were incubated under hypoxia; this effect was higher after 4hr of hypoxia followed by 2hr of normoxia, but it decreases after 4hr of hypoxia followed by 3hr of normoxia. These effects were reversed administering an antibody anti-VEGF-A or anti-Ang-1, where the levels of Bax expression were enhanced after 4hr of hypoxia + 3hr of normoxia. Panel below: Measurement of cholangiocyte proliferation (by immunoblotting analysis for PCNA) in the same samples. Without the pre-incubation with the antibodies anti-VEGF-A and anti-Ang-1, PCNA expression was increased only after 3hr of normoxic conditions. Conversely, administration of the antibodies increased proliferation in the first hours of normoxia but inhibited it after 3 hours. Data are means ± SE of 6 blots. *P < 0.05 vs. its corresponding control value. #P < 0.05 vs. normoxia control value.

[B] Panel above: Cholangiocyte apoptosis was also assessed by real time PCR for Bax in the several treatments of NRICC. The apoptotic degree was lower in the last sample, where the cells returned to the normoxic conditions for 3 hours after 4 hours of hypoxia. Instead, pre-incubation with an anti-VEGF-A or an anti-Ang-1 enhanced the mRNA expression of Bax. Panel below: Cholangiocyte growth was investigated by real time PCR for PCNA in the same in vitro samples. PCNA mRNA expression was significantly increased after 3 hours of reoxygenation, while it was worsened with the administration of the antibodies, in particular after treatment with an anti-Ang-1. Data are means ± SE of 6 blots. *P < 0.05 vs. its corresponding control value. #P < 0.05 vs. normoxia control value.
value.

Figure 5  Treatment in hypoxic conditions induced a significant increase in the message expression of VEGF-A, VEGF-C, VEGFR-2 and VEGFR-3 (measured as ratio to GAPDH mRNA). This increase returned close to normal values proportionally following restoring of normoxia for 3 hr. Data are mean ± SEM of 6 experiments. *P < 0.05 versus the corresponding basal value.
Fig. 3

CAMP levels (pmol/1x10^5 cells)

- Normal
- Normal + HAIR
- BDL
- BDL + HAIR

- Basal
- Secretin
Fig. 5

VEGFs relative mRNA expression

GAPDH

VEGFA  VEGFC  VEGFR2  VEGFR3

- NRC normoxia
- NRC hypoxia 4+1h
- NRC hypoxia 4+2h
- NRC hypoxia 4+3h
Table 2 Measurement of the % cholangiocytes positive for VEGF-A/C, VEGFR-2/3, Angio-1/2 and Tie-1/2 in liver sections.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normal</th>
<th>Normal + HAIR</th>
<th>Normal + PVIR</th>
<th>BDL</th>
<th>BDL + HAIR</th>
<th>BDL + PVIR</th>
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<tbody>
<tr>
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<td>Large</td>
<td>Small</td>
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<td>Small</td>
<td>Large</td>
</tr>
<tr>
<td>VEGF-A (%)</td>
<td>5.5 ± 0.4</td>
<td>10.2 ± 0.8</td>
<td>9.9 ± 0.3*</td>
<td>13 ± 0.5*</td>
<td>8.5 ± 0.2*</td>
<td>10.5 ± 0.2</td>
</tr>
<tr>
<td>VEGF-C (%)</td>
<td>5 ± 0.5</td>
<td>8.1 ± 0.6</td>
<td>7.6 ± 0.2*</td>
<td>10.8 ± 0.7</td>
<td>6.9 ± 0.2</td>
<td>10.6 ± 0.2</td>
</tr>
<tr>
<td>VEGF-R2 (%)</td>
<td>2.1 ± 0.3</td>
<td>6.5 ± 0.6</td>
<td>5.2 ± 0.2*</td>
<td>7.9 ± 0.3*</td>
<td>5.6 ± 0.2</td>
<td>18.5 ± 0.6</td>
</tr>
<tr>
<td>VEGF-R3 (%)</td>
<td>2.3 ± 0.3</td>
<td>4.2 ± 0.6</td>
<td>4.3 ± 0.3*</td>
<td>6.1 ± 0.2*</td>
<td>5.5 ± 0.1*</td>
<td>18.6 ± 0.8</td>
</tr>
<tr>
<td>Ang-1 (%)</td>
<td>2.7 ± 0.3</td>
<td>4.2 ± 0.6</td>
<td>4.5 ± 0.3*</td>
<td>5.5 ± 0.2</td>
<td>3.2 ± 0.3*</td>
<td>13.2 ± 0.4</td>
</tr>
<tr>
<td>Ang-2 (%)</td>
<td>1.3 ± 0.3</td>
<td>6.1 ± 0.2</td>
<td>4.9 ± 0.2*</td>
<td>6.7 ± 0.1</td>
<td>3.3 ± 0.3*</td>
<td>12.1 ± 0.2</td>
</tr>
<tr>
<td>Tie-1 (%)</td>
<td>1.3 ± 0.4</td>
<td>6.3 ± 0.5</td>
<td>4.8 ± 0.2*</td>
<td>6.4 ± 0.4</td>
<td>3.5 ± 0.3*</td>
<td>13.6 ± 0.2</td>
</tr>
<tr>
<td>Tie-2 (%)</td>
<td>2.1 ± 0.4</td>
<td>5.7 ± 0.3</td>
<td>5.2 ± 0.3*</td>
<td>6.9 ± 0.1*</td>
<td>6.4 ± 0.4</td>
<td>12.4 ± 0.2</td>
</tr>
</tbody>
</table>

Semiquantitative analysis of the expression of VEGF-A/C, VEGF-R2/3, Ang-1/2 and Tie-1/2 in small and large cholangiocytes in liver sections. Data are expressed as mean ± SEM.
* P < 0.05 versus the number of small bile ducts (positive for VEGF-A/C, VEGF-R2/3, Ang-1/2 and Tie-1/2) from the corresponding normal and BDL control rats. # P < 0.05 versus the number of large bile ducts (positive by TUNEL and PCNA) from the corresponding normal and BDL control rats.
Table 1  Measurement of biliary apoptosis by TUNEL assay and proliferation by PCNA expression in small and large bile ducts.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normal</th>
<th>Normal + HAIR</th>
<th>Normal + PVIR</th>
<th>BDL</th>
<th>BDL + HAIR</th>
<th>BDL + PVIR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Small</td>
<td>Large</td>
<td>Small</td>
<td>Large</td>
<td>Small</td>
<td>Large</td>
</tr>
<tr>
<td>Biliary apoptosis (%)</td>
<td>1.89 ± 0.2</td>
<td>2.92 ± 0.3</td>
<td>3.15 ± 0.8</td>
<td>8.79 ± 0.2*</td>
<td>2.97 ± 0.6</td>
<td>6.24 ± 0.4</td>
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<tr>
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<td>7.64 ± 0.3</td>
<td>14.42 ± 0.5</td>
<td>7.78 ± 0.2</td>
<td>17.15 ± 0.6*</td>
<td>7.44 ± 0.4</td>
<td>14.94 ± 0.4</td>
</tr>
<tr>
<td>PCNA expression</td>
<td>2.06 ± 0.4</td>
<td>9.74 ± 0.2</td>
<td>3.3 ± 0.1</td>
<td>5.64 ± 0.3*</td>
<td>3.13 ± 0.1</td>
<td>4.81 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>29.1 ± 0.6</td>
<td>29.1 ± 0.6</td>
<td>23.1 ± 0.5</td>
<td>23.1 ± 0.5</td>
<td>23.1 ± 0.5</td>
<td>23.1 ± 0.5</td>
</tr>
</tbody>
</table>

Small bile ducts = <15 μm diameter; large bile ducts = >15 μm diameter. Apoptosis of small and large bile ducts was measured by TUNEL analysis in liver sections. Proliferation of small and large bile ducts was measured by evaluating the expression of PCNA positive cholangiocytes in liver sections. Data are expressed as mean ± SEM. * P < 0.05 versus the number of large bile ducts (positive by TUNEL and PCNA) from the corresponding normal and BDL control rats. # P < 0.05 versus the number of small bile ducts (positive by TUNEL and PCNA) from the corresponding normal and BDL control rats.