Administration of r-VEGF-A prevents hepatic artery ligation-induced bile duct damage in bile duct ligated rats

Eugenio Gaudio, Barbara Barbaro, Domenico Alvaro, Shannon Glaser, Heather Francis, Antonio Franchitto, Paolo Onori, Yoshiyuki Ueno, Marco Marzioni, Giammarco Fava, Julie Venter, Ramona Reichenbach, Ryun Summers, and Gianfranco Alpini

Administration of r-VEGF-A prevents hepatic artery ligation-induced bile duct damage in bile duct ligated rats. Am J Physiol Gastrointest Liver Physiol 291: G307–G317, 2006. First published March 30, 2006; doi:10.1152/ajpgi.00507.2005.—The hepatic artery, through the peribiliary plexus, nourishes the intrahepatic biliary tree. During obstructive cholestasis, the nutritional demands of intrahepatic bile ducts are increased as a consequence of enhanced proliferation; in fact, the peribiliary plexus (PBP) displays adaptive expansion. The effects of hepatic artery ligation (HAL) on cholangiocyte functions during cholestasis are unknown, although ischemic lesions of the biliary tree complicate the course of transplanted livers and are encountered in cholangiopathies. We evaluated the effects of HAL on cholangiocyte functions in experimental cholestasis induced by bile duct ligation (BDL). By using BDL and BDL + HAL rats or BDL + HAL rats treated with recombinant-vascular endothelial growth factor-A (r-VEGF-A) for 1 wk, we evaluated liver morphology, the degree of portal inflammation and periductular fibrosis, microcirculation, cholangiocyte apoptosis, proliferation, and secretion. Microcirculation was evaluated using a scanning electron microscopy vascular corrosion cast technique. HAL induced in BDL rats J) the disappearance of the PBP, 2) increased apoptosis and impaired cholangiocyte proliferation and secretin-stimulated ductal secretion, and 3) decreased cholangiocyte VEGF secretion. The effects of HAL on the PBP and cholangiocyte functions were prevented by r-VEGF-A, which, by maintaining the integrity of the PBP and cholangiocyte proliferation, prevents damage of bile ducts following ischemic injury.

cAMP; ductal secretion; intrahepatic biliary epithelium; mitosis; microcirculation; secretin

Cholangiocytes, the epithelial cells lining the intrahepatic biliary epithelium (6), modify bile, originally secreted at the bile canaliculus (43), by a series of absorptive and secretory events regulated by a number of factors including gastrointestinal hormones/peptides, bile salts, and nerve receptor agonists (4–7, 11, 31, 38). The gastrointestinal hormone secretin increases ductal secretion by interaction with specific receptors (expressed only by cholangiocytes) (10), an interaction that induces an increase in intracellular adenosine 3',5'-monophosphate (cAMP) levels (4, 7, 25, 26, 31, 38). Increased intracellular cAMP levels induce activation of the CFTR Cl⁻ channels (9, 31) and Cl⁻/HCO₃⁻ exchanger activity (7, 11, 31), a series of events that leads to secretin-stimulated bile and bicarbonate secretion (5, 26).

In normal liver, cholangiocytes have low basal DNA synthesis (4, 51). However, cholangiocytes proliferate in a number of experimental models of cholestasis including bile duct ligation (BDL) (4, 5, 12, 25, 37, 38), Cholangiocyte proliferation in the course of cholangiopathies compensates for the loss of injured ducts, and, in fact, proliferating cholangiocytes display enhanced basal and secretin-stimulated ducital secretory activities (4–6, 26, 31, 38). A number of studies in rats (3–6, 8–10, 25, 36–40) and humans (45) have shown that changes in cholangiocyte proliferation are associated with parallel modifications in secretin receptor gene expression, secretin-stimulated CAMP levels, and secretin-induced bile and bicarbonate secretion. For example, we have shown in rats that in pathological conditions associated with increased cholangiocyte proliferation (e.g., following BDL or partial hepatectomy) (4, 5, 25, 38), there is increased secretin receptor gene expression, augmented basal and secretin-stimulated CAMP levels and bile and bicarbonate secretion. On the other hand, reduced cholangiocyte proliferation or enhanced cholangiocyte loss (e.g., following vagotomy, acute administration of CCl₄ or depletion of endogenous bile acid pool) (3, 36, 39) is coupled with decreased basal and secretin-stimulated CAMP levels and bile and bicarbonate secretion. In humans, an impaired response to secretin was observed in cholestatic conditions (45).

Cholangiocyte proliferation is regulated by neuropeptides, hormones, and growth factors including vascular endothelial growth factor (VEGF) (6, 21, 37). Rat cholangiocytes express the protein for VEGF-A, secrete VEGF, and express the VEGF receptor subtypes VEGFR-2 and VEGFR-3 but not VEGFR-1 (21). VEGF secretion is enhanced in proliferating cholangiocytes from BDL rats, where it stimulates, by autocrine mechanisms, cholangiocyte proliferation (21).

The peribiliary plexus (PBP) stems from the hepatic artery, nourishes the biliary tree, and sustains a countercurrent of
substances reabsorbed from bile toward hepatocytes (23). A true microvascular plexus vascularizes larger ducts, whereas around the smaller ducts the plexus gets progressively simpler (up to a single capillary) and thinner (23). In normal rats, where cholangiocytes are in a quiescent status (39), ligation of the main hepatic artery on its own is not sufficient to induce bile duct damage, suggesting that accessory arteries, collateral vessels, or anastomosis between the PBP and the portal system may overcome the interruption of arterial flow in the main hepatic artery (16, 17). Consistently, interruption of blood flow to intrahepatic bile ducts (by short-term ligation of the hepatic artery of normal guinea pigs) does not alter cholangiocyte secretion (50). The extrahepatic and intrahepatic PBP in the normal liver has been the subject of a number of studies using three-dimensional observations (22–24). Changes in intrahepatic bile duct mass are always associated with changes of the PBP architecture (22, 23). After BDL, the increase in intrahepatic bile duct mass is followed by a parallel growth of the PBP (23), which is fundamental in sustaining the enhanced nutritional and functional demands of proliferating cholangiocytes (4–6). Nevertheless, the proliferation of the PBP occurs only after the hyperplasia of the intrahepatic biliary epithelium (23). This finding suggests a cross-talk mechanism between cholangiocytes and endothelial cells, an interaction that mediates the adaptive changes of these cells during liver damage. However, limited information exists on the role of blood supply through the hepatic artery in pathological conditions characterized by cholangiocyte proliferation/loss (14, 48). This concept has clinical implications since ischemic bile duct lesions are considered possible causes of cholestatic disorders, in particular after liver transplantation, hepatic surgery, and intra-arterial chemotherapy (13, 15).

MATERIAL AND METHODS

Materials. Reagents were purchased from Sigma (St. Louis, MO) unless otherwise indicated. The substrate for γ-glutamyltransferase (γ-GT), N-γ-(L-glutamyl)-4-methoxy-2-naphthylamide, was purchased from Polysciences (Warrington, PA). Porcine secretin was purchased from Peninsula (Belmont, CA). The antibodies against TGF-α, VEGF-A, and the VEGF receptor subtypes VEGFR-1, VEGFR-2, and VEGFR-3 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The recombinant mouse VEGF (r-VEGF-A) was purchased from Leinco Technologies (St. Louis, MO).

Animal models. Male Fischer 344 rats (150–175 g) were purchased from Charles River (Wilmington, MA). The animals were kept in a temperature-controlled environment (22°C) with a 12:12-h light-dark cycle and fed an ad libitum diet. The studies were performed in normal donors and rats + HAL (for evaluation of cholangiocyte apoptosis and proliferation in liver sections), 2) 1-wk BDL (for isolation of cells) (4, 5) or bile duct uncannulated (BDL, for bile collection) (5) rats, and 3) rats that (immediately after BDL or BDL + HAL) were treated intraperitoneally using implanted Alzet osmotic minipumps with 0.2% bovine serum albumin (BSA) or r-VEGF-A (2.5 nmol kg−1h−1 with 0.2% BSA) for 1 wk. The dose (nM range) of r-VEGF-A administered to BDL + HAL rats was chosen according to the concentration (nM range) of VEGF found in the serum of rats and human in other studies (12, 52). The group of BDL + HAL rats was studied, since we observed impaired secretion of VEGF in BDL cholangiocytes after HAL. Since we observed impaired secretion of VEGF in BDL cholangiocytes after HAL, the group of BDL + HAL + VEGF rats was chosen to evaluate whether the effects of HAL observed in BDL rats are prevented by chronic administration of r-VEGF-A. BDL and BDI were performed as described (5). HAL was performed as described (29). Before each procedure, animals were anesthetized with pentobarbital sodium (50 mg/kg body wt ip). Study protocols were performed in compliance with the institution guidelines. All studies involving animals were performed with approval from the Institutional Animal Care and Use Committee.

Isolation of hepatocytes and cholangiocytes. Hepatocytes were isolated as described (2). Cholangiocytes (97–100% pure by γ-GT histochemistry) (46) were purified by immunofluorometry separation (4, 28). Cell number and viability (greater than 97%) was assessed by Trypan blue exclusion.

Body weight, liver morphology, necrosis, inflammation, and periductular fibrosis. We evaluated the effect of BDL, HAL, and HAL + r-VEGF-A administration to BDL + HAL rats on body weight, liver morphology, necrosis, inflammation, and periductular fibrosis. We evaluated, by hemotoxylin and eosin (H&E) staining of paraffin-embedded liver sections (4 μm thick, six slides evaluated for each group), the degree of portal inflammation (18), necrosis, and lobular morphology (disarrangement of hepatocytes). At least 10 different portal areas were evaluated. Following H&E staining, liver sections were examined in a coded fashion with an Olympus BX-40 (Tokyo, Japan) microscope equipped with a camera. We evaluated, by Masson's trichrome staining of paraffin-embedded liver sections (4 μm thick, six slides evaluated for each group), the degree of fibrosis around proliferating ducts from BDL, BDL + HAL, and BDL + HAL + r-VEGF-A-treated rats. At least six different microscopic fields (×10 and ×20) for each slide were analyzed in a coded fashion with an Olympus BX-51 microscope equipped with a video camera (Spot Insight; Diagnostic Instrument, Sterling Heights, MI) and processed with an image analysis system (IAS; Delta Sistemi, Rome, Italy). Periductular fibrosis was measured as the volume fraction of the entire liver tissue specimen (percent volume fraction of the green-stained collagen fibers less volume fraction occupied, respectively, by portal triads and by the parenchyma).

Evaluation of liver microcirculation. Following anesthesia, the abdomen of the selected animal was opened, and a cannula (Inpharven, diameter 1.4 mm; Inpharval) was inserted into the aorta and fixed with two silk ties. Before flushing the vascular bed with heparinized saline solution (23), the thorax was opened, and the right atrium was incised to allow the efflux of the perfusate. When the outflow fluid appeared clear of blood, Mercox CL2R resin, diluted with methyl methacrylate monomer (4:1) (34) and mixed with a standard amount of its catalyst (up to 2 ml catalyst per 20 ml of base compound), was injected at room temperature. A constant pressure control was maintained (by an electronic manometer; Conel, Rome, Italy) through the lateral port of the cannula's injection valve until resin polymerization was visible. The animals were left at room temperature for 24 h, and after the polymerization of the resin, the livers were removed and macerated in 20% NaOH solution at room temperature. After rinsing in distilled water, the liver casts were placed in 5% trichloroacetic acid solution to free the cast from tissue remnants. The casts were then glued onto stubs by means of silver dag and gold coated in an SIS0 sputterer (Edwards, London, UK). The prepared casts were examined with a field emission scanning electron microscope (model S4000 Hitachi; Tokyo, Japan) operating at 5–8 kV (23).

Evaluation of cholangiocyte VEGF protein expression and secretion. Protein expression for VEGF-A, VEGFR-1, VEGFR-2, and VEGFR-3 was evaluated by immunohistochemistry in liver sections (5 μm thick, six slides evaluated per group) mounted on glass slides coated with 0.1% poly-L-lysine. Following staining, sections were analyzed in a coded fashion with an Olympus BX-51 microscope equipped with a video camera (Diagnostic Instrument) and processed with an IAS image analysis system (Delta Sistemi). The intensity and distribution of immunostaining were assessed in a coded fashion.
Following isolation, hepatocytes or cholangiocytes (1 × 10^6) were incubated at 37°C for 0 or 6 h and centrifuged at 1,500 rpm for 10 min, and the supernatant (100 µl) was transferred to a tube and analyzed for VEGF concentration by ELISA (Peninsula Laboratories, San Carlos, CA). VEGF secretion (ng/l × 10^6 cells) was calculated as the difference between the amount of VEGF detected at 6 h and the amount detected at time 0.

**Cholangiocyte apoptosis and proliferation.** We evaluated cholangiocyte and hepatocyte apoptosis by terminal deoxyuridine nick end labeling (TUNEL) analysis (39) in liver sections (three slides evaluated for each group, 5 µm thick) from the selected group of animals. Following counterstaining with hematoxylin solution, liver sections were examined by light microscopy with an Olympus BX-40 microscope equipped with a camera. Approximately 100 cells per slide were counted in a coded fashion in 10 nonoverlapping fields.

Cholangiocyte and hepatocyte proliferation was evaluated by measuring the number of PCNA- and hepatocyte-positive cholangiocytes. Cholangiocyte growth was also evaluated by measuring the percent of CK-19- and γ-GT-positive ducts in liver sections (three slides evaluated for each group of animals, 5 µm thick) (39). Sections were counterstained with hematoxylin and examined in a random, blinded fashion with an Olympus BX-51 light microscope. Data were expressed as 1) number of PCNA-positive cholangiocytes or hepatocytes per each 100 cells measured and 2) percent of CK-19-positive duct area evaluated in 10 different fields (×10 or ×20) of slide taken from each six blocks randomly taken from medial lobe. Histochemistry for γ-GT-positive ducts (46) was performed in frozen sections (5 µm thick, three slides evaluated for each group).

**Measurement of basal and secretin-stimulated ductal secretion.** At the functional level, cholangiocyte proliferation was evaluated by measurement of basal and secretin-stimulated cAMP levels (32, 39) in purified cholangiocytes and measurement of bile and bicarbonate secretion (5) in bile fistula rats, two functional indices of cholangiocyte proliferation (4, 5, 20, 25, 26, 36–39).

For the measurement of cAMP levels, purified cholangiocytes were incubated for 1 h at 37°C (32) and incubated for 5 min at room temperature (32) with 0.2% BSA (basal) or 100 nM secretin with 0.2% BSA. Intracellular cAMP levels were measured by commercially available RIA kits (Amersham Life Science) (32).

Following anesthesia, rats were surgically prepared for bile collection as described by us (5). One jugular vein was cannulated with a plastic cannula to infuse either Krebs-Ringer-Henseleit (KRH) or secretin (100 nM) dissolved in KRH. Biliary bicarbonate concentration (measured as total CO₂) was determined by an ABL 520 blood gas system (Radiometer, Copenhagen, Denmark).

**Statistical analysis.** Values are means ± SE. Differences between groups were analyzed by the Student’s unpaired t-test when two groups were analyzed and analysis of variance (ANOVA) when more...
were treated by minipumps with control or r-VEGF-A (absence of a PBP) (Fig. 2). The microvascular pattern observed in BDL rats prevented the HAL-induced microvascular modification to that of the BDL rat (see Fig. 1, A and Table 1). The degree of necrosis, portal inflammation, and percent volume fraction were similar in BDL rats and rats that were treated by minipumps with control or recombinant-vascular endothelial growth factor-A (r-VEGF-A). There were no significant differences in the extent of lobular damage in liver sections (4 μm thick) from the different groups of animals. In BDL + HAL rats, the degree of necrosis, portal inflammation, and periductular fibrosis decreased compared with BDL rats. Following the administration of r-VEGF-A to BDL + HAL rats, the degree of necrosis, portal inflammation, and periductular fibrosis were similar to those in the BDL rat. Values are means ± SE of 17 cumulative evaluations from the evaluation of 3 slides for each group. *P < 0.05 vs. all the other groups.

RESULTS

Effects of HAL and r-VEGF-A on body weight, liver morphology, necrosis, inflammation, and periductular fibrosis. No changes in body weight were observed among BDL (161.7 ± 4.0 g), BDL + HAL (172.3 ± 7.0 g), and BDL + HAL + r-VEGF-A (165.6 ± 5.6 g) rats. There were no differences in the amount of lobular damage among BDL rats and rats that (immediately after BDL + HAL) were treated with 0.2% BSA or r-VEGF-A for 1 wk (see Fig. 1A and Table 1). The degree of necrosis, portal inflammation, and percent volume fraction of periductular fibrosis observed in BDL rats was reduced in BDL + HAL rats compared with the value of BDL rats (Fig. 1, A and B, and Table 1). Following the administration of r-VEGF-A to BDL + HAL rats, the degree of necrosis and portal inflammation and percent volume fraction were similar to that of the BDL rat (see Fig. 1, A and B, and Table 1).

Effect of HAL and r-VEGF-A on the PBP and VEGF expression. The PBP, observed in BDL rats (23), was not demonstrated in BDL + HAL rats by scanning electron microscopy corrosion cast technique. We did not observe the PBP in BDL + HAL rats, because the PBP is nourished by the hepatic artery. In normal rats, the PBP was observed more easily in large portal tracts (not shown) (23). In the small portal tracts, the PBP was characterized by a single layer of capillaries or even by a single capillary; in BDL + HAL we do not observe a PBP. Administration of r-VEGF-A to BDL + HAL rats prevented the HAL-induced microvascular modification (absence of a PBP) (Fig. 2). The microvascular pattern observed after administration of r-VEGF-A to BDL + HAL rats demonstrated the presence of a PBP (Fig. 2) with similar characteristics previously described in BDL rats (23). In vascular corrosion casts, we observe only a vascular tree because the tissue is completely digested. Therefore, in Fig. 2, we did not observe a bile duct (completely digested) but only the absence of the PBP.

Immunohistochemistry in BDL liver sections shows that bile ducts express VEGF-A, VEGFR-2, and VEGFR-3 (Fig. 3, A and B, and Table 2). HAL induced a decrease in the number of cholangiocytes positive for VEGF-A, VEGFR-2, and VEGFR-3 receptors compared with liver sections from 1-wk BDL rats (Fig. 3, A and B, and Table 2). Following the administration of r-VEGF-A to BDL + HAL rats, the expression of VEGF-A, VEGFR-2, and VEGFR-3 was similar to or higher than that of the BDL rat (Fig. 3, A and B, and Table 2). VEGFR-1 was not expressed by cholangiocytes (Table 2). VEGF-A was predominantly expressed by hepatocytes of the centrolobular zone (Table 3). Hepatocyte VEGF-A protein expression did not increase significantly in BDL liver sections compared with normal sections, decreased after HAL, and returned to values similar to those of BDL rats (Table 3).

Normal rat hepatocytes and cholangiocytes secrete VEGF (Fig. 4). Following BDL, there was an increase in VEGF secretion in cholangiocytes (Fig. 4, top). VEGF secretion significantly decreased in BDL hepatocytes compared with normal hepatocytes (Fig. 4, bottom). In cholangiocytes and hepatocytes from BDL + HAL rats, there was decreased VEGF secretion (Fig. 4) compared with cholangiocytes and hepatocytes from BDL rats (Fig. 4 bottom). Administration of r-VEGF-A prevented the decrease of cholangiocyte VEGF secretion in BDL + HAL rats (Fig. 4 bottom). VEGF secretion in cholangiocytes was not affected by r-VEGF-A (Fig. 4 bottom). The increase in VEGF secretion in BDL + HAL + r-VEGF-A rats compared with BDL + HAL rats was not significant (Fig. 4 bottom). The increase in VEGF secretion in BDL + HAL + r-VEGF-A rats compared with BDL + HAL rats was not significant (Fig. 4 bottom).

Table 1. H&E staining of liver sections from 1-wk BDL rats and rats that (immediately after BDL + HAL) were treated by minipumps with control or r-VEGF-A

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Portal Inflammation</th>
<th>Necrosis</th>
<th>Lobular Damage</th>
<th>Periductular Fibrosis, % volume fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>BDL 1 wk</td>
<td>1.7 ± 0.1</td>
<td>1.3 ± 0.1</td>
<td>1.5 ± 0.1</td>
<td>12.8 ± 0.8</td>
</tr>
<tr>
<td>BDL + HAL + 0.2% BSA 1 wk</td>
<td>1.2 ± 0.1*</td>
<td>0.7 ± 0.1*</td>
<td>1.2 ± 0.1</td>
<td>7.9 ± 0.7*</td>
</tr>
<tr>
<td>BDL + HAL + r-VEGF-A 1 wk</td>
<td>1.4 ± 0.1</td>
<td>1.0 ± 0.1</td>
<td>1.3 ± 0.1</td>
<td>14.1 ± 1.0</td>
</tr>
</tbody>
</table>

Hematoxylin and eosin staining of liver sections from 1-wk bile duct ligated (BDL) rats and rats that [immediately after BDL + hepatic artery ligation (HAL)] were treated by minipumps with control or recombinant-vascular endothelial growth factor-A (r-VEGF-A). There were no significant differences in the extent of lobular damage in liver sections (4 μm thick) from the different groups of animals. In BDL + HAL rats, the degree of necrosis, portal inflammation, and periductular fibrosis decreased compared with BDL rats. Following the administration of r-VEGF-A to BDL + HAL rats, the degree of necrosis, portal inflammation, and periductular fibrosis were similar to those in the BDL rat. Values are means ± SE of 17 cumulative evaluations from the evaluation of 3 slides for each group. *P < 0.05 vs. all the other groups.

Fig. 2. Scanning electron microscopy vascular corrosion cast from rats (immediately after BDL + HAL) were treated by intraperitoneally implanted Alzet osmotic minipumps with 0.2% BSA or r-VEGF-A with 0.2% BSA for 1 wk. Observe in BDL + HAL rats the presence of a sinusoidal network (S) and portal vein (P) and the absence of the peribiliary plexus (PBP) (original magnification ×50); differently, in BDL + HAL + r-VEGF-A rats the PBP that runs together with the portal tract was observed (Original magnification ×100).
secretion induced by HAL in cholangiocytes and hepatocytes (Fig. 4).

Cholangiocyte apoptosis and proliferation. Parallel to previous studies (41), TUNEL analysis showed a few apoptotic bodies in the liver sections of normal (results not shown) and BDL rats (Fig. 5). The number of cholangiocytes undergoing apoptosis increased in liver sections from BDL + HAL rats compared with BDL rats (Fig. 5). HAL had no effect on cholangiocyte apoptosis of normal rats (not shown). Administration of r-VEGF-A prevented the increase in cholangiocyte apoptosis induced by HAL in BDL rats (Fig. 5). The number of apoptotic hepatocytes was not changed in the different groups.

Table 2. Immunohistochemical evaluation of cholangiocyte VEGF-A, VEGFR-1, VEGFR-2, and VEGFR-3 expression in liver sections from 1-wk BDL rats and rats that (immediately after BDL + HAL) were treated by minipumps with control or r-VEGF-A

<table>
<thead>
<tr>
<th>Treatment</th>
<th>VEGF Expression, % positive cholangiocytes</th>
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<tbody>
<tr>
<td></td>
<td>VEGF-A</td>
</tr>
<tr>
<td>BDL 1 wk (n = 6)</td>
<td>78.3±3.9</td>
</tr>
<tr>
<td>BDL + HAL + 0.2% BSA 1 wk (n = 6)</td>
<td>23.1±2.7*</td>
</tr>
<tr>
<td>BDL + HAL + r-VEGF-A 1 wk (n = 6)</td>
<td>74.9±2.9#</td>
</tr>
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</table>

The immunohistochemical cholangiocyte expression of VEGF-A, VEGFR-2, and VEGFR-3 decreased in liver sections from BDL + HAL rats compared with BDL rats and BDL + HAL rats treated with r-VEGF-A. Chronic administration of r-VEGF-A prevented HAL-induced loss of cholangiocyte expression of VEGF-A, VEGFR-2, and VEGFR-3, values that were similar or higher. Cholangiocytes did not express VEGFR-1. *P < 0.05 vs. all the other groups. #P < 0.05 vs. corresponding value of 1-wk BDL rat liver sections.
of animals (BDL = 2.87 ± 0.13, BDL + HAL = 3.47 ± 0.16, BDL + HAL + r-VEGF-A = 3.39 ± 0.16).

Following HAL, the number of PCNA-positive cholangiocytes and the percent of CK-19 and γ-GT-positive ducts decreased compared with liver sections from BDL rats (Table 4 and Fig. 6 showing the percent of CK-19-positive ducts in liver sections). Administration of r-VEGF-A prevented the inhibitory effect of HAL on the number of PCNA-positive cholangiocytes and the percent of CK-19 and γ-GT-positive ducts, values that were similar to those of BDL rats (Table 4 and Fig. 6). HAL did not alter the number of PCNA-positive cholangiocytes and the percent of CK-19 and γ-GT-positive ducts in normal liver sections (not shown). The proliferation of hepatocytes (PCNA positive) was similar in the different groups of animals (BDL = 3.44 ± 0.19, BDL + HAL = 3.04 ± 0.16, BDL + HAL + r-VEGF-A = 3.68 ± 0.14).

Basal and secretin-stimulated cAMP levels and ductal secretion. In agreement with previous studies (25), secretin increased intracellular cAMP levels of cholangiocytes from BDL rats (Fig. 7). HAL significantly reduced basal cholangiocyte cAMP levels and inhibited secretin-stimulated cAMP levels of cholangiocytes compared with cholangiocytes from BDL rats (Fig. 7). Administration of r-VEGF-A prevented the inhibition of basal and secretin-stimulated cAMP levels induced by HAL (Fig. 7).

Secretin stimulated bile flow and bicarbonate concentration and secretion in BDI rats (Table 5). In HAL + BDI rats, secretin did not increase bile flow and bicarbonate concentration and secretion (Table 5). Administration of r-VEGF-A prevented the inhibition of secretin-stimulated bile and bicarbonate concentration and secretion, which were effects similar to that in BDI rats (Table 5).

**DISCUSSION**

This study demonstrates that in BDL rats, HAL 1) induced the absence of the PBP; 2) decreased the immunolocalization of VEGFR-2 and VEGFR-3 receptors and VEGF-A in liver sections and VEGF secretion in purified cholangiocytes and hepatocytes; 3) induced the loss of intrahepatic bile ducts in BDL but not normal rats, caused by both increased apoptosis and decreased cholangiocyte proliferation; no changes in hepatocyte apoptosis and proliferation were observed in BDL rats and rats that (immediately after BDL + HAL) were treated by intraperitoneally implanted Alzet osmotic minipumps with 0.2% BSA or r-VEGF-A for 1 wk; and 4) decreased basal and secretin-stimulated cAMP levels of cholangiocytes from BDL rats (Fig. 7). Administration of r-VEGF-A prevented the inhibition of secretin-stimulated bile and bicarbonate concentration and secretion, which were effects similar to that in BDI rats (Table 5).

**Fig. 4.** VEGF secretion in primary cultures (6 h) of cholangiocytes (top) or hepatocytes (bottom) from normal, 1-wk BDL rats, and rats that (immediately after BDL + HAL) were treated by intraperitoneally implanted Alzet osmotic minipumps with 0.2% BSA or r-VEGF-A (2.5 nmol·kg⁻¹·h⁻¹ with 0.2% BSA) for 1 wk. Primary cultures (i.e., 6 h) of normal rat hepatocytes and cholangiocytes secrete VEGF. Following BDL, there was an increase in VEGF secretion in primary cultures of cholangiocytes. VEGF secretion significantly decreased in BDL hepatocytes compared with normal hepatocytes. In both cholangiocytes and hepatocytes from BDL + HAL rats, there was a marked decrease of VEGF secretion compared with cholangiocytes and hepatocytes from control 1-wk BDL rats. Administration of r-VEGF-A prevented the decrease of cholangiocyte VEGF secretion induced by HAL in cholangiocytes and hepatocytes. Values are means ± SE of 7 (cholangiocytes) and 4 (hepatocytes) experiments. Top: *P < 0.05 vs. VEGF secretion from normal rats. Bottom: #P < 0.05 vs. VEGF secretion from BDL rats. *P < 0.05 vs. VEGF secretion of normal hepatocytes.

**Table 3. Measurement of hepatocyte VEGF-A protein expression in liver sections from normal and 1-wk BDL rats and rats that (immediately after BDL + HAL) were treated by minipumps with control or r-VEGF-A**

<table>
<thead>
<tr>
<th>Treatments</th>
<th>VEGF-A, % positive hepatocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (n = 6)</td>
<td>20.3 ± 1.7</td>
</tr>
<tr>
<td>BDL 1 wk (n = 6)</td>
<td>24.5 ± 2.1</td>
</tr>
<tr>
<td>BDL + HAL + 0.2% BSA 1 wk (n = 6)</td>
<td>13.2 ± 1.6*</td>
</tr>
<tr>
<td>BDL + HAL + r-VEGF-A 1 wk (n = 6)</td>
<td>28.5 ± 2.5</td>
</tr>
</tbody>
</table>

Hepatocyte VEGF-A protein expression did not increase significantly in BDL liver sections compared with normal sections, decreased after HAL, and returned to values similar to those in BDL rats and BDL + HAL rats treated with r-VEGF-A. *P < 0.05 vs. corresponding value of liver sections from 1-wk BDL rats and BDL + HAL rats.
secretin-induced intracellular cAMP synthesis and impaired basal and secretin-stimulated bile flow and bicarbonate secretion. The adverse effects of HAL on the PBP, VEGF expression and secretion (in cholangiocytes and hepatocytes), and on cholangiocyte proliferative and secretory activities in BDL rats were all prevented by chronic administration of r-VEGF-A to BDL + HAL rats.

After BDL, the intrahepatic biliary epithelium undergoes cholangiocyte proliferation (4–6, 25), which leads to bile duct mass expansion, which is followed by an adaptive proliferation of the PBP (23). Proliferating bile ducts are characterized by enhanced cholangiocyte secretory and proliferative activities (5). Therefore, the adaptive proliferation of the PBP (and its circulating factors including VEGF) is fundamental to sustain the enhanced functional and nutritional demands of the proliferating biliary tree. Since proliferation of the PBP follows, in order of time, the proliferation of bile ducts (23), it is reasonable to suppose that proliferating cholangiocytes modulate the adaptive response of the vascular bed. Consistently, proliferating cholangiocytes express VEGF-A and secrete VEGF (21), which modulates cholangiocyte proliferation by autocrine mechanisms. The fact that 1) hepatocytes express and secrete VEGF (35, 49) and 2) hepatocyte VEGF secretion is decreased by HAL and maintained by administration of r-VEGF-A raises the possibility that cholangiocyte growth may also be regulated by a paracrine mechanism. However, we have demonstrated

Table 4. Measurement of cholangiocyte proliferation by quantitative measurement of the number of PCNA-positive cholangiocytes and the percent of CK-19- and γ-GT-positive ducts in liver sections from 1-wk BDL rats and rats that (immediately after BDL + HAL) were treated with control or r-VEGF-A

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PCNA-positive cholangiocytes, per portal tract (n = 12)</th>
<th>% CK-19-positive ducts, per portal tract (n = 10)</th>
<th>% γ-GT-positive ducts, per portal tract (n = 14)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BDL 1 wk</td>
<td>16.4±1.2</td>
<td>5.6±0.6</td>
<td>7.2±0.5</td>
</tr>
<tr>
<td>BDL + HAL + 0.2% BSA 1 wk</td>
<td>4.6±0.05*</td>
<td>3.7±0.4*</td>
<td>1.1±0.1*</td>
</tr>
<tr>
<td>BDL + HAL + r-VEGF-A 1 wk</td>
<td>11.7±0.6</td>
<td>6.4±0.6</td>
<td>6.5±0.5</td>
</tr>
</tbody>
</table>

Following HAL, the number of proliferating cellular nuclear antigen (PCNA)-positive cholangiocytes and the percent of CK-19 and γ-GT-positive ducts decreased in liver sections compared with liver sections from BDL rats and BDL + HAL rats treated with r-VEGF-A. Chronic administration of r-VEGF-A prevented the inhibitory effect of HAL on the number of PCNA-positive cholangiocytes and the percent of CK-19 and γ-GT-positive ducts, values that were not statistically different from those of 1-wk BDL rats. Values are means ± SE. *P < 0.05 vs. all other groups.
that 1) hepatocyte VEGF secretion decreases following BDL and is much lower than cholangiocyte VEGF secretion and 2) the proliferation/apoptosis of hepatocytes is similar in BDL rats and in rats that (immediately after BDL/H11001 HAL) were treated by intraperitoneally implanted Alzet osmotic minipumps with 0.2% BSA or r-VEGF-A (2.5 nmol·kg⁻¹·h⁻¹ with 0.2% BSA) for 1 wk. Following HAL, the percent of CK-19-positive ducts decreased in liver sections compared with liver sections from 1-wk BDL rats. Chronic administration of r-VEGF-A prevented the inhibitory effect of HAL on the percent of CK-19-positive ducts, a value that was not statistically different from that of 1-wk BDL rats. For statistical evaluation of the percent of CK-19-positive ducts see Table 4. Arrows indicate CK-19-positive cholangiocytes.

We submitted BDL rats to hepatic artery ligation and evaluated the pathophysiology of the intrahepatic bile duct system at the morphological and functional levels compared with BDL and BDL + HAL rats. Interestingly, VEGF-A expression and secretion in cholangiocytes was decreased by HAL. On the basis of this finding, we evaluated whether the effects of HAL on BDL rats are prevented by chronic in vivo administration of r-VEGF-A. The dose of VEGF used in our studies is similar to the concentration of VEGF found in the serum of rats and human (12, 52).

The decreased VEGF expression and secretion by BDL cholangiocytes was an unexpected finding since, in different tissues, VEGF is usually induced by ischemia (42). However, VEGF is expressed at low levels by quiescent nonproliferating cholangiocytes of normal rat liver (21) but markedly expressed and secreted by cholangiocytes proliferating following BDL (21). This suggests that interruption of hepatic artery blood supply to proliferating bile ducts, characterized by the vanishing of the PBP, as demonstrated by vascular corrosion cast, compromises VEGF protein synthesis in cholangiocytes, which is associated with impaired proliferation and with activation of apoptosis. However, that reduced VEGF expression and secretion by BDL cholangiocytes plays a role in HAL-induced impairment of proliferative and secretory activities of BDL rats was suggested by the findings that all the effects of HAL on cholangiocyte function of BDL rats were prevented by chronic in vivo r-VEGF-A administration. Furthermore, the administered dose of r-VEGF-A induced serum levels of VEGF, which was similar to control BDL rats, both being higher with respect to BDL rats submitted to HAL.

Interestingly, we found that in BDL rats, HAL induced a decrease in the degree of necrosis, portal inflammation (presumably due to decreased cholangiocyte cytokine secretion) (19), and periductular fibrosis (may be due to decreased cholangiocyte secretion of specific growth factors including platelet-derived growth factor) (27). This finding should be interpreted as a consequence of the reduced cholangiocyte proliferation following HAL, since portal inflammation and periductular fibrosis in the BDL model are triggered by cholangiocyte proliferation via secretion of a number of cytokines/chemokines able to activate stellate cells (27, 44). HAL induced no effect in the liver of normal rats, confirming findings from several previous studies (16, 17, 30). This has been attributed to accessory arteries or to anastomosis between the PBP and the portal system, which, if necessary, may overcome interruption of the blood supply through the main hepatic artery (16, 17, 30). Evidently, when the intrahepatic bile duct mass is expanded as occurs after BDL (5), blood supply through the hepatic artery becomes fundamental in...
Fig. 7. Measurement of basal and secretin-stimulated cAMP levels in purified cholangiocytes from 1-wk BDL rats and rats that (immediately after BDL + HAL) were treated by intraperitoneally implanted Alzet osmotic minipumps with 0.2% BSA or r-VEGF-A (2.5 nmol·kg⁻¹·h⁻¹ with 0.2% BSA) for 1 wk. Secretin increased intracellular cAMP levels of cholangiocytes from 1-wk BDL rats. HAL significantly reduced basal cholangiocyte cAMP levels and inhibited secretin-stimulated cAMP levels of cholangiocytes compared with cholangiocytes from 1-wk BDL rats. Chronic administration of r-VEGF-A prevented the inhibition of basal and secretin-stimulated cAMP levels induced by HAL. Values are means ± SE of 7 experiments. *P < 0.05 vs. corresponding basal value. #P < 0.05 vs. basal cAMP levels of cholangiocytes from BDL rats and BDL + HAL rats treated with r-VEGF-A for 1 wk.

sustaining the enhanced nutritional and functional demands of proliferating intrahepatic biliary epithelium (23). Regarding the deleterious effects of HAL on BDL cholangiocyte function, VEGF seems to play a role, although we cannot exclude that decreased VEGF expression and secretion is a consequence of impaired proliferation caused by decreased blood supply. However, since VEGF is a player in the complex loop of agents sustaining cholangiocyte proliferation after BDL (6, 37), the impaired synthesis and release of VEGF by cholangiocytes after HAL certainly has a role in compromising cell proliferation. In support of this, we have shown (21) that VEGF stimulates cholangiocyte proliferation. Furthermore, we found that HAL-induced impairment of proliferation is associated with decreased basal and secretin-induced bile flow and bicarbonate secretion, with all these effects being prevented by r-VEGF-A administration. This further supports the role of VEGF in mediating the effects of HAL on the functions of the intrahepatic biliary epithelium. In cholangiocytes from HAL + BDL rats, we also observed a decreased level of basal and secretin-induced cAMP levels that were normalized by r-VEGF-A in vivo administration. The second messenger cAMP plays an important role in modulating cholangiocyte growth (20, 25, 36). Furthermore, cAMP-related intracellular pathways are activated/deactivated by different agents involved in the regulation of cholangiocyte proliferation including the cholinergic system, gastrin, somatostatin, and bile salts including taurocholate and ursodeoxycholate (1, 4, 25, 36). For example, intracellular cAMP levels are elevated in BDL cholangiocytes compared with normal cholangiocytes (4, 7). Chronic in vivo administration of forskolin, a cAMP activator (33), increases cholangiocyte proliferation and secretin-stimulated ductal secretion in normal rats (20). Thus a complex loop of neuropeptides, hormones, and growth factors that potentiate the proliferation machinery, including the function of agents acting by cAMP-related pathways. Thus the decrease of cAMP levels found in HAL + BDL rat cholangiocytes is consistent with impaired proliferation and increased apoptosis. Although increased basal and secretin-stimulated cholangiocyte secretory activity is coupled with enhanced ductal hyperplasia (4, 5, 8, 10, 38), impaired ductal secretion is associated with conditions causing reduction of cholangiocyte proliferation (1, 25, 36, 39). In agreement with this study, HAL-induced impairment of proliferation is associated with decreased basal and secretin-induced ductal secretion, with all these effects being prevented by VEGF.

After liver transplantation, ischemic lesions of bile ducts may occur presumably by surgical lesions of the hepatic artery (13, 15, 47). In cholangiopathies, mainly primary sclerosing...
cholangitis, lesion of the hepatic artery and its branches play a causal role in bile duct damage and in the related ductal cholestasis (13). Our study gives the pathophysiological basis for these cholestatic conditions since reduction of the blood supply through the hepatic artery impairs the proliferative and the repairing capacities of damaged ducts. The beneficial effects of VEGF may provide the experimental background for using this growth factor in the management of cholangiopathies.

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