

Vascular Endothelial Growth Factor Stimulates Rat Cholangiocyte Proliferation Via an Autocrine Mechanism

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Background & Aims: Vascular endothelial growth factor (VEGF) is secreted by several epithelia and modulates cellular functions by autocrine and paracrine mechanisms. The role of VEGF in cholangiocyte pathophysiology is unknown. We evaluated the role of VEGF in the regulation of cholangiocyte proliferation in rats that underwent bile duct ligation. **Methods:** The expression of VEGF-A and VEGF-C and their receptors in cholangiocytes from normal and BDL rats was evaluated. Normal or BDL rats were treated with recombinant-VEGF-A or recombinant-VEGF-C or anti-VEGF antibodies, and proliferation of cholangiocytes was evaluated in situ by morphometry and in vitro by proliferating cell nuclear antigen immunoblots and MTS assay. In vitro, normal rat cholangiocyte cultures were stimulated with r-VEGF-A or r-VEGF-C and proliferation and signal transduction were evaluated. **Results:** We found that (1) cholangiocytes express messenger RNA and protein for VEGF-A, VEGF-C, VEGF receptor 2 (VEGFR-2), and VEGF receptor 3 (VEGFR-3) and secrete VEGF; (2) secretion of VEGF and expression of VEGFR-2 and VEGFR-3 increases in BDL cholangiocytes; (3) blocking VEGF in vivo by anti-VEGF-A or anti-VEGF-C antibodies decreases cholangiocyte proliferation; (4) the in vivo administration of r-VEGF-A or r-VEGF-C induces cholangiocyte proliferation in normal rats; and (5) in vitro, VEGF-A increases normal rat cholangiocyte culture proliferation by activation of inositol 1,4,5-triphosphate/Ca²⁺/protein kinase C α and phosphorylation of Src/ERK1/2. **Conclusions:** Cholangiocytes secrete VEGF and express VEGFR-2 and VEGFR-3, all of which are amplified in BDL cholangiocytes. VEGF induces cholangiocyte proliferation by activation of inositol 1,4,5-triphosphate/[Ca²⁺]_i/protein kinase C α and phosphorylation of Src/ERK1/2. VEGF mediates the adaptive proliferative response of cholangiocytes to cholestasis.

Vascular endothelial growth factor (VEGF) is a family of related growth factors that includes VEGF-A, -B, -C, -D, and -E and placenta growth factor.¹⁻⁴ VEGF is a mitogen for vascular endothelial cells and regulates vascular pathophysiology, including vasodilatation, vascular permeability, migration, and survival of endothelial cells.²⁻⁴ The expression of VEGF and its receptors is not restricted to vascular endothelial cells because their expression has been detected in vascular smooth muscle cells, osteoblasts, regenerating myotubes, and hematopoietic stem cells.²⁻⁵ VEGF is secreted by several epithelia, where it modulates cell growth by autocrine and paracrine mechanisms.^{6,7} Two distinct tyrosine kinase receptors have been identified for VEGF (VEGFR), VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1), which share 44% amino acid homology.²⁻⁴ A third receptor, VEGFR-3 (Flt-4), has been identified.²⁻⁴ While VEGF-A interacts with VEGFR-2, VEGF-C interacts with VEGFR-3.²⁻⁴

The function of the intrahepatic biliary tree is linked with its vascular supply sustained by the peribiliary arterial plexus (PBP).⁸ Alterations of intrahepatic bile duct mass are associated with changes of the PBP architecture.^{8,9} Following bile duct ligation (BDL), the PBP

Abbreviations used in this paper: BDL, bile duct ligation; bp, base pairs; BSA, bovine serum albumin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GGT, γ -glutamyltransferase; IP₃, inositol 1,4,5-triphosphate; NRICC, normal rat intrahepatic cholangiocyte culture; PBP, peribiliary arterial plexus; PCNA, proliferating cell nuclear antigen; PKC, protein kinase C; r-VEGF, recombinant vascular endothelial growth factor; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

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undergoes marked proliferation, thus supporting the increased nutritional and functional demands from the proliferated bile ducts.⁸ However, the proliferation of the PBP occurs only after the hyperplasia of the intrahepatic biliary epithelium.⁸ This finding suggests a cross-talk mechanism between cholangiocytes and endothelial cells that mediates the adaptive changes of these cells during liver damage.¹⁰

The aims of this study were to (1) evaluate the expression of VEGF-A and VEGF-C and their receptors in normal and proliferating cholangiocytes after BDL and (2) determine the role and transduction mechanisms by which VEGF-A and VEGF-C regulate cholangiocyte proliferation.

Materials and Methods

Materials

Reagents were purchased from Sigma Chemical Co (St Louis, MO) unless otherwise indicated. The antibodies against proliferating cell nuclear antigen (PCNA), VEGF-A and VEGF-C, and the VEGF receptor subtypes VEGFR-1, VEGFR-2, and VEGFR-3 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The recombinant mouse VEGFs (r-VEGF-A and r-VEGF-C) were purchased from Leinco Technologies, Inc. (St Louis, MO). The inositol 1,4,5-trisphosphate (IP₃) [³H] kit, for the determination of intracellular IP₃ levels, was purchased from Amersham (Piscataway, NJ). The rabbit polyclonal antibody recognizing von Willebrand factor (Factor VIII-related antigen) was purchased from Chemicon International, Inc. (Temecula, CA). The antibodies for rabbit anti-phospho-Src (Tyr 139), mouse anti-total Src, mouse anti-p-JNK (which detects JNK1, JNK2, and JNK3 phosphorylated at Thr-183 and Tyr-185 of JNK1), rabbit anti-total JNK (which reacts with total JNK1, JNK2 p54, and JNK3), mouse anti-p-p38 (which detects Tyr-182 phosphorylated mitogen-activated protein kinase p38, Mxi2, and p38β), mouse anti-p38 (which detects total p38 and p38β), rabbit anti-ERK1 (which detects p44 and p42), rabbit anti-ERK2 (which detects p44 and p42), and goat anti-pERK (which detects phosphorylated p44 and p42) were purchased from Santa Cruz Biotechnology, Inc.

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-hour light/dark cycle and fed ad libitum rat chow. The studies were performed in (1) normal rats and 1-week BDL rats; (2) rats that (immediately after BDL) were treated with a daily intraperitoneal injection of nonimmune serum, anti-VEGF-A or anti-VEGF-C antibody, mouse anti-human VEGF antibody (which immunoneutralizes VEGF; Calbiochem Biochemicals, San Diego, CA),^{11,12} or combined anti-VEGF A + C antibody (10 ng/day) for 1 week; and (3) normal rats treated by intra-

peritoneally implanted Alzet osmotic minipumps (Durect Corp., Cupertino, CA) with 0.2% bovine serum albumin (BSA), r-VEGF-A, or r-VEGF-C (2.5 nmol · kg⁻¹ · h⁻¹) with 0.2% BSA for 1 week. BDL was performed as described.¹⁰ The dose (nanomolar range) of anti-VEGF-A, anti-VEGF-C, r-VEGF-A, or r-VEGF-C administered to rats was chosen according to the concentration (nanomolar range) of VEGF found in the serum of our animals (see following text). This dose was similar to that found in rats and humans.^{13,14} Before each procedure, animals were anesthetized with sodium pentobarbital (50 mg/kg body wt intraperitoneally). Study protocols were performed in compliance with institution guidelines.

Normal Rat Intrahepatic Cholangiocyte Cultures and Purified Cholangiocytes and Hepatocytes

Normal rat intrahepatic cholangiocyte cultures (NRICCs) were obtained as described by us.¹⁵ Hepatocytes were isolated by collagenase perfusion. Cholangiocytes (97%–100% pure by γ-glutamyltransferase [GGT] histochemistry)¹⁶ were obtained by immunoaffinity separation.^{17–20} Cell number and viability (>97%) were assessed by trypan blue exclusion.

Expression of VEGF Receptors and VEGF-A and VEGF-C

The expression of VEGF receptors (VEGFR-1, VEGFR-2, and VEGFR-3) and VEGF-A and VEGF-C was evaluated by (1) immunohistochemistry (VEGFR-1, VEGFR-2, VEGFR-3, VEGF-A, and VEGF-C) or immunofluorescence (VEGFR-1) in paraffin-embedded liver sections (n = 6 per group, 5 μm thick), (2) reverse-transcription polymerase chain reaction (AmpliTaq DNA polymerase; PE Applied Biosystems, Foster City, CA) in total RNA (1 μg) from cholangiocytes by the GenElute (Sigma-Aldrich, St. Louis, MO) total mammalian RNA kit, and (3) immunoblots of protein (10 μg) from whole cholangiocyte lysates and NRICCs.

Immunohistochemistry for VEGFR-1, VEGFR-2, VEGFR-3, VEGF-A, and VEGF-C was performed as follows. Following incubation with the selected antibody, liver sections were rinsed with 1× phosphate-buffered saline, incubated with a biotinylated secondary antibody (DAKO LSAB2, Milan, Italy), with DAKO ABC (Dako LSAB2), and developed with 3,3'-diaminobenzidine. Sections were analyzed in a coded fashion using an Olympus BX-51 light microscope (Tokyo, Japan) with a Videocam (Spot Insight; Diagnostic Instrument, Inc, Sterling Heights, MI) and processed with an Image Analysis System (IAS - Delta Sistemi, Rome, Italy). For immunofluorescence for VEGFR-1, following blocking of nonspecific binding of immunoglobulin G, BDL liver sections were incubated with a polyclonal antibody against VEGFR-1. Following washes, the specimens were incubated with a biotin-conjugated secondary antibody. Sections were incubated in a dark chamber with streptavidin CY3 (Jackson Europe Ltd; Soham, Cambridgeshire, UK) for 15 minutes. After washing, the sections were mounted with Vectorshield mounting medium with 4',6-diamidino-2-phenylindole (Vector Laboratories, Bur-

lingame, CA) and examined using a Reichert Jung Diastar fluorescence microscope (Hicksville, NY).

Specific oligonucleotide primers for VEGFR-2 were based on the rat VEGFR-2 sequence²¹ (sense 5'-GTGATTGCCATGT-TCTTCTGGC-3' and antisense 5'-TCAGACATGAGAGCTC-GATGCT-3', 337 base pairs [bp]). Specific oligonucleotide primers for rat VEGFR-3 were designed based on the human VEGFR-3 sequence²² (sense 5'-CACTCCCGCCATACGCCA-CATCAT-3' and antisense 5'-CTGCTCTCTATCTGCT-CAAACTCC-3', 450 bp). Specific oligonucleotide primers for VEGF-A were based on the rat VEGF-A sequence²³ (sense 5'-ACCTCCACCATGCCAAGT-3' and antisense 5'-TAGTTC-CCGAAACCCTGA-3', expected fragments bands: 434 bp [VEGF 120-amino acid isoform], 565 bp [VEGF 164-amino acid isoform], and 687 bp [VEGF 188-amino acid isoform]). Specific oligonucleotide primers for VEGF-C were based on the rat VEGF-C sequence²⁴ (sense 5'-CTGTGGACCCAACAAG-GAGT-3' and antisense 5'-CAGGCACATTTTCCAGGATT-3', 243 bp). The equality of the RNA used was assessed by reverse-transcription polymerase chain reaction for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (sense 5'-GTGACTTCAACAGCAACTCCCATTTC-3' and antisense 5'-GTTATGGGGTCTGGGATGGAATTGTG-3', 294 bp); primers were based on the rat GAPDH sequence.²⁵ Standard reverse-transcription polymerase chain reaction conditions were used with 1 μ g of total messenger RNA (mRNA) (35 step cycles: 30 seconds at 94°C, 30 seconds at 52°C, and 45 seconds at 72°C). The polymerase chain reaction samples for VEGFR-2, VEGFR-3, VEGF-A (434 bp [VEGF 120-amino acid isoform] and 565 bp [VEGF 164-amino acid isoform]), and VEGF-C were run on agarose gels, and the bands were excised and removed from the gel with the Qiaquick Gel Extraction Kit (Qiagen, Valencia, CA). We were not able to sequence the VEGF-A isoform (687 bp [VEGF 188-amino acid isoform]) due to the low yield of this message after gel elution. The purified fragments were sequenced by Davis Sequencing (Davis, CA).

The expression of VEGFR-2, VEGFR-3, VEGF-A, and VEGF-C was evaluated by immunoblots of protein (10 μ g) from whole cholangiocyte lysates from normal and BDL rats. The intensity of the enhanced chemiluminescence-visualized bands was determined by scanning video densitometry using the ChemiImager 4000 Low Light Imaging System (Alpha Innotech Corp, San Leandro, CA).

Measurement of Hepatocyte and Cholangiocyte VEGF Secretion and VEGF Serum Concentration

To determine VEGF secretion, primary cultures of hepatocytes or cholangiocytes (1×10^6) were incubated at 37°C for 0 or 6 hours. Then cells were centrifuged and the supernatant (100 μ L) was transferred to a tube and analyzed for VEGF concentration by enzyme-linked immunosorbent assay using commercially available kits (Peninsula Laboratories Inc, San Carlos, CA). Hepatocyte or cholangiocyte VEGF secretion (nanogram per 1×10^6 cells) was calculated as the difference between the amount of VEGF detected at 6 hours

and the amount detected at time 0. We measured by enzyme-linked immunosorbent assay kits (Peninsula Laboratories Inc) the changes in VEGF concentration in serum from normal and BDL rats.

Effect of Administration of Anti-VEGF-A or Anti-VEGF-C Antibody on Body Weight, Inflammation, Necrosis, Lobular Damage, Apoptosis, and Cholangiocyte Growth

To evaluate the effects of VEGF neutralization on cholangiocyte proliferation, we tested 2 antibodies (anti-VEGF-A and anti-VEGF-C) that bind to the functional domains of VEGF-A and VEGF-C, respectively (Santa Cruz data sheets and <http://www.ebi.uniprot.org>) and an additional antibody (anti-VEGF) reported to block all 3 isoforms of VEGF-A identified in cholangiocytes.^{11,12} To demonstrate that the mouse anti-human VEGF antibody^{11,12} interacts with rat bile ducts, we have performed immunohistochemistry with this antibody in normal and BDL liver sections.

We evaluated the degree of portal inflammation, necrosis, and lobular morphology (disarrangement of hepatocytes). Ten randomly selected portal areas and surrounding lobular areas were evaluated using light microscopy of H&E-stained sections (5 μ m thick, 3 sections evaluated per each group of animals, n = 2). For quantification of damage, the following categories were applied: 0, null; 1, limited to portal area; 2, intermediate; and 3, panlobular. Liver sections were examined in a coded fashion by light microscopy with an Olympus BX-40 (Tokyo, Japan) microscope equipped with a camera. Apoptosis was determined by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling analysis²⁶ (Wako Chemicals, Tokyo, Japan) in sections evaluated in a coded fashion with a microscope (U-PMTVC; Olympus Optical Co, Tokyo, Japan); 200 cells per slide were counted in 10 nonoverlapping fields.

Cholangiocyte proliferation was evaluated by measurement of (1) the number of PCNA- and CK-19-positive cholangiocytes and GGT-positive ducts in liver sections (5 μ m thick, 3 slides analyzed per animal)^{16,20,27} and (2) PCNA protein expression in protein (10 μ g) from whole cholangiocyte lysates. Proliferation of vascular endothelial cells was evaluated by immunohistochemistry for PCNA and Factor VIII-related antigen (a marker of endothelial cells)²⁸ in liver sections (5 μ m, 3 slides analyzed per animal) from normal, 1-week BDL, and 2-week BDL rats. Sections were counterstained with hematoxylin and examined with a microscope (Olympus BX 40; Olympus Optical Co). For PCNA, CK-19, VEGFR-1, VEGFR-2, and VEGFR-3 staining, more than 100 cholangiocytes were counted in a random, blinded fashion in 3 different fields for each group of animals. For GGT staining, more than 20 bile ducts were counted in 7 different fields for each group of animals.

Cholangiocyte proliferation was also evaluated by immunoblots for PCNA in protein (10 μ g) from whole cholangiocyte lysates. The intensity of the enhanced chemiluminescence-

visualized bands was determined by scanning video densitometry using the ChemiImager 4000 Low Light Imaging System.

Effect of Administration of r-VEGF-A or r-VEGF-C on Normal Cholangiocyte Proliferation

To show that VEGF stimulates cholangiocyte proliferation, normal rats were treated with 0.2% BSA, r-VEGF-A, or r-VEGF-C for 1 week. We measured cholangiocyte proliferation by evaluating the number of PCNA- and CK-19-positive cholangiocytes and GGT-positive ducts in liver sections (3 slides analyzed per animal, 5 μm thick).

r-VEGF-A and r-VEGF-C Stimulates NRICC Proliferation

In NRICCs, we evaluated the effect of r-VEGF-A and r-VEGF-C (100 nmol/L) on intracellular IP_3 and Ca^{2+} levels,²⁹ 2 second messengers activated by VEGF.^{30,31} For IP_3 measurements, NRICCs (5×10^6) were incubated for 1 hour at 37°C and stimulated for 10 minutes at 22°C with 0.2% BSA, r-VEGF-A, or r-VEGF-C (100 nmol/L with 0.2% BSA) in the absence or presence of PP2 (an Src inhibitor, 1 $\mu\text{mol/L}$ for 10 minutes).³² IP_3 levels were assessed by the IP_3 [^3H] kit (Amersham).²⁹

Before Ca^{2+} measurements, NRICCs were incubated for 1 hour at 37°C to regenerate the membrane receptors damaged by trypsin digestion. Calcium fluorescence measurements in NRICCs stimulated with 0.2% BSA, 100 nmol/L r-VEGF-A, or 100 nmol/L r-VEGF-C for 10 minutes were performed using fluo-3 AM (Molecular Probes, Eugene, OR) and a Fluoroskan Ascent FL (ThermoLabsystems, Helsinki, Finland) microplate reader equipped with 3 injectors.^{29,33} Ionomycin (10 $\mu\text{mol/L}$) was used at the end of each calcium determination to ensure cholangiocyte responsiveness.

To determine the effects of r-VEGF-A and r-VEGF-C on the phosphorylation of protein kinase C (PKC)- α , which regulates cholangiocyte function,^{18,34–36} NRICCs were cultured until 70% confluent¹⁵ and stimulated for 90 minutes¹⁸ with 0.2% BSA or r-VEGF-A or r-VEGF-C (100 nmol/L with 0.2% BSA). The expression of total and phosphorylated cholangiocyte PKC- α was evaluated by immunoblots.¹⁸ After evaluating the phosphorylated PKC- α expression, membranes were stripped to determine total PKC- α protein expression. Stripping buffer from Pierce Biotechnology, Inc. (Rockford, IL) was used according to the manufacturer's instructions.

NRICCs were cultured until 70% confluent¹⁵ and stimulated with (1) 0.2% BSA (basal) for 48 hours or (2) r-VEGF-A or r-VEGF-C (100 nmol/L with 0.2% BSA) for 48 hours in the absence or presence of 2-hour preincubation with BAPTA/AM, a [Ca^{2+}]_i chelator³⁷ (5 $\mu\text{mol/L}$), Gö6976, a PKC- α inhibitor³⁸ (1 $\mu\text{mol/L}$). Although some studies³⁹ refer to Gö6976 as an inhibitor of Ca^{2+} -dependent PKCs rather than PKC- α , other studies have shown^{40–43} that Gö6976 is a selective inhibitor of the PKC- α isozyme and PKC- β I and does not affect the kinase activity of the Ca^{2+} -independent PKC- δ , PKC- ϵ , and PKC- ζ isozymes even at micromolar

levels. Subsequently, we evaluated cholangiocyte proliferation by PCNA immunoblots. To show that r-VEGF-A and r-VEGF-C induce an actual increase in cholangiocyte cell number, NRICCs were cultured until 70% confluent,¹⁵ seeded into 96-well plates (10,000 cells/well), and subsequently stimulated with 0.2% BSA or r-VEGF-A or r-VEGF-C (100 nmol/L with 0.2% BSA) for 48 hours. We evaluated cell replication by a cell proliferation assay (MTS assay, CellTiter 96Aqueous; Promega Corp, Madison, WI). Changes in cell number were evaluated by measurement of absorbance (490 nm).

NRICCs were cultured until 70% confluent¹⁵ and stimulated with 0.2% BSA or r-VEGF-A or r-VEGF-C (100 nmol/L with 0.2% BSA) for 48 hours. Subsequently, we evaluated the changes in the phosphorylation of Src (at the Tyr 139 residue), mitogen-activated protein kinases p38 and p42/44 (ERK1/2), and JNK by immunoblots. The intensity of the enhanced chemiluminescence-visualized bands was determined by scanning video densitometry using the ChemiImager 4000 Low Light Imaging System.

Statistical Analysis

All data are expressed as mean \pm SEM. Differences between groups were analyzed by the Student unpaired *t* test when 2 groups were analyzed and analysis of variance when more than 2 groups were analyzed, followed by an appropriate post hoc test.

Results

Cholangiocytes Express VEGFR-2, VEGFR-3, VEGF-A, and VEGF-C and Secrete VEGF

Bile ducts from normal and BDL rats express VEGFR-2 and VEGFR-3 but not VEGFR-1 (Figure 1A). Immunohistochemistry shows that the expression of VEGFR-2 and VEGFR-3 was higher in bile ducts from BDL rats compared with bile ducts from normal rats (46.0% \pm 3.2% [BDL] vs 12.0% \pm 1.2% [normal] VEGFR-2-positive cholangiocytes [$P < .05$] and 49.0% \pm 2.9% [BDL] vs 25.0% \pm 1.9% [normal] VEGFR-3-positive cholangiocytes [$P < .05$]). Immunofluorescence for VEGFR-1 in BDL liver sections showed the absence of positivity for VEGFR-1 in cholangiocytes (Figure 1B); limited positivity for VEGFR-1 was observed only at the endothelial level (Figure 1B).

Cholangiocytes from normal and BDL rats express the message for VEGFR-2 and VEGFR-3 (Figure 1C). The polymerase chain reaction fragment for rat VEGFR-2 is 98% homologous to the Norway rat VEGFR-2 sequence⁴⁴ (Gene Bank NM 013062). The polymerase chain reaction fragment for rat VEGFR-3²² is 100% homologous to the rat VEGFR-3 sequence²² (Gene Bank NM 053652). The expression of GAPDH was similar in cholangiocytes from normal

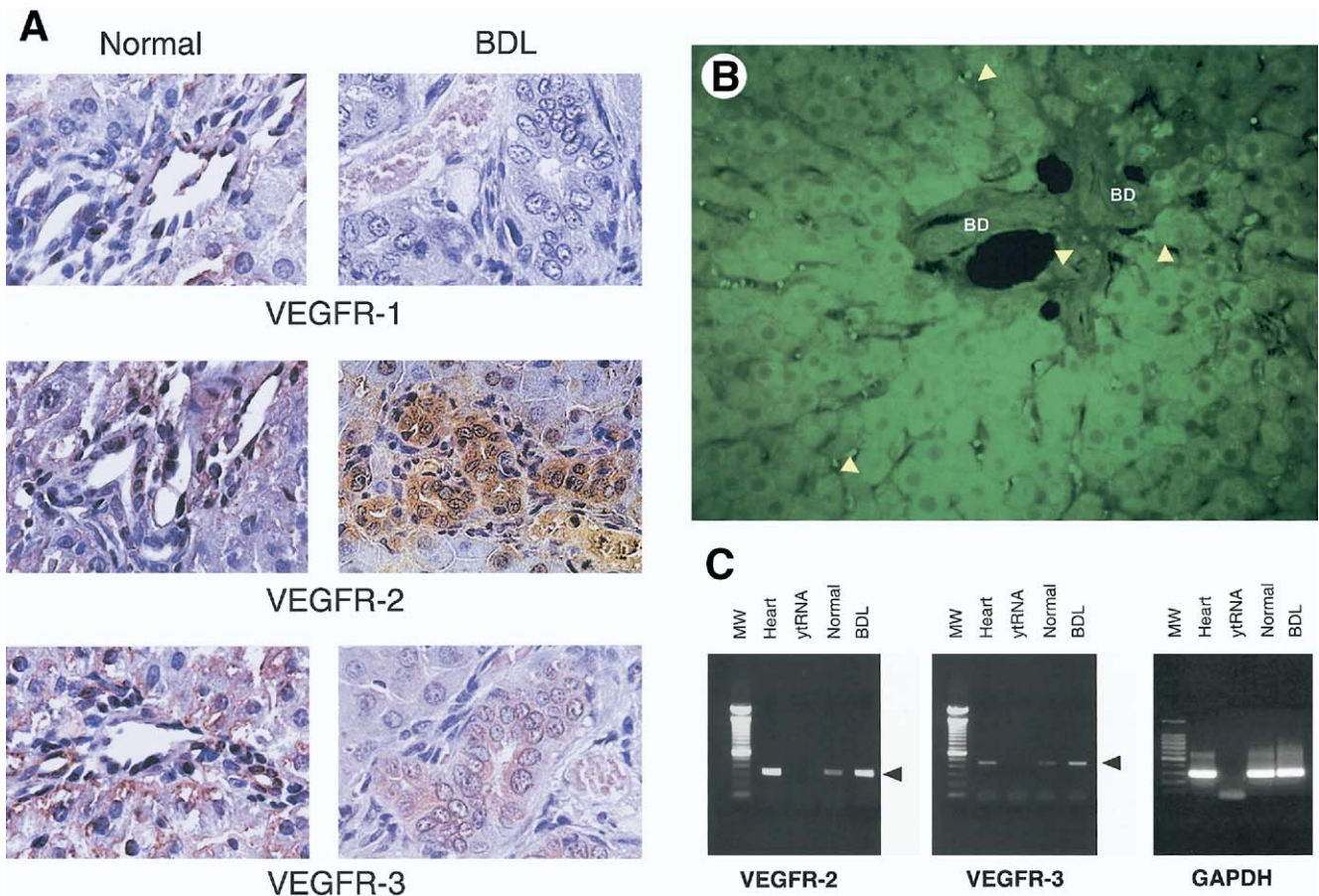


Figure 1. (A) Immunohistochemistry for VEGFR-1, VEGFR-2, and VEGFR-3 in liver sections from normal and BDL rats and (B) immunofluorescence for VEGFR-1 in liver sections from BDL rats. (A) Bile ducts from normal and BDL rats express VEGFR-2 and VEGFR-3 but not VEGFR-1 (original magnification 40 \times). (B) Immunofluorescence in liver sections from 1-week BDL rats shows the absence of positivity for VEGFR-1 in cholangiocytes. In liver sections, the vessels were negative for VEGFR-1; rare, limited, and mild positivity for VEGFR-1 was observed only at the endothelial level. BD, interlobular bile ducts. Arrowheads indicate Factor VIII-related antigen-positive endothelial cells (original magnification 40 \times). (C) Reverse-transcription polymerase chain reaction analysis showed that cholangiocytes from normal and BDL rats express the message for VEGFR-2 and VEGFR-3. For VEGFR-2 and VEGFR-3, total RNA from rat heart and yeast transfer RNA (yRNA) were the positive and negative controls, respectively. For GAPDH, total RNA from rat kidney and yeast transfer RNA were the positive and negative controls, respectively. VEGFR-2 mRNA, 337 bp; VEGFR-3 mRNA, 450 bp; GAPDH mRNA, 294 bp. Normal and BDL lanes are cholangiocytes.

and BDL rats (Figure 1C). By immunoblots, the protein for VEGFR-2 and VEGFR-3 was expressed by cholangiocytes from normal and BDL rats and NRICCs (not shown).

Immunohistochemistry shows that the expression of VEGF-A and VEGF-C was higher in bile ducts from BDL rats compared with bile ducts from normal rats (Figure 2A). Both normal and BDL cholangiocytes express the mRNA for VEGF-A (687, 565, and 434 bp) and VEGF-C (243 bp) (Figure 2B). Similar to previous studies,²³ 3 bands (VEGF 188–amino acid isoform, 687 bp; VEGF 164–amino acid isoform, 565 bp; and VEGF 120–amino acid isoform, 434 bp) originating from alternative splicing of mRNA for 3 different VEGF-A isoforms were present in normal and BDL cholangiocytes (Figure 2B). The expression of GAPDH was similar in cholangiocytes from normal

and BDL rats (Figure 2B). Analysis of the sequences indicated 99.8% homology for the sequence of rat VEGF-A (VEGF 164–amino acid isoform, 565 bp)²³ and 100% homology for the previously reported sequence of rat VEGF-A (VEGF 120–amino acid isoform, 434 bp)²³ (Gene Bank AY702972). Analysis of the sequences indicated 100% homology for the sequence of rat VEGF-C.²⁴ We found VEGF in the supernatant of primary cultures of normal cholangiocytes and hepatocytes (Figure 2C), indicating that cholangiocytes and hepatocytes secrete VEGF. Following BDL, the amount of VEGF secreted by cholangiocytes into the supernatant increased compared with the VEGF secreted by normal cholangiocytes (Figure 2C). VEGF secretion was decreased in BDL hepatocytes compared with normal hepatocytes (Figure 2C).

Administration of Anti-VEGF Antibodies Decreases Cholangiocyte Proliferation

No changes in body weight were observed between BDL rats treated for 1 week with nonimmune serum (180.57 ± 6.30 g), anti-VEGF-A (170.53 ± 3.88 g), or anti-VEGF-C (177.91 ± 2.97 g) antibodies. There were no significant differences in the degree of portal inflammation, necrosis, and lobular damage among BDL rats treated with nonimmune serum (1.2 ± 0.2 [portal inflammation], 1.4 ± 0.2 [necrosis], and 1.4

± 0.2 [lobular damage]), anti-VEGF-A (1.0 ± 0.01 [portal inflammation], 1.2 ± 0.2 [necrosis], and 1.0 ± 0.01 [lobular damage]), or anti-VEGF-C (1.2 ± 0.2 [portal inflammation], 1.4 ± 0.2 [necrosis], and 1.2 ± 0.2 [lobular damage]). The administration of anti-VEGF-A or anti-VEGF-C antibody to BDL rats significantly ($P < .05$) increased the number of apoptotic cholangiocytes per portal tracts compared with BDL rats treated with nonimmune serum (5.60 ± 0.51 [anti-VEGF-A antibody] and 4.80 ± 0.38 [anti-VEGF-C antibody] vs 2.40 ± 0.24 [nonimmune serum]). We have also tested an anti-VEGF antibody reported to immunoneutralize all 3 isoforms of VEGF-A^{11,12} and found that its administration in BDL rats induced cholangiocyte apoptosis (8.00 ± 0.84 apoptotic cholangiocytes per portal tract). When BDL rats were treated with anti-VEGF-A + C antibody, a further increase of cholangiocyte apoptosis was observed (12.2 ± 0.97 vs 2.40 ± 0.24 [BDL rats] apoptotic cholangiocytes per portal tract).

Administration of anti-VEGF-A, anti-VEGF-C, or the anti-VEGF antibody (known to immunoneutralize VEGF)^{11,12} to BDL rats induced a similar decrease in the number of PCNA- and CK-19-positive cholangiocytes and GGT-positive ducts compared with BDL rats treated with nonimmune serum (Table 1). The mouse anti-human VEGF antibody (which immunoneutralizes VEGF)^{11,12} was tested by immunohistochemistry to stain positively (not shown) bile ducts from normal and BDL rat liver sections. When BDL rats were treated simultaneously with anti-VEGF-A + C antibodies, there was a greater decrease in the

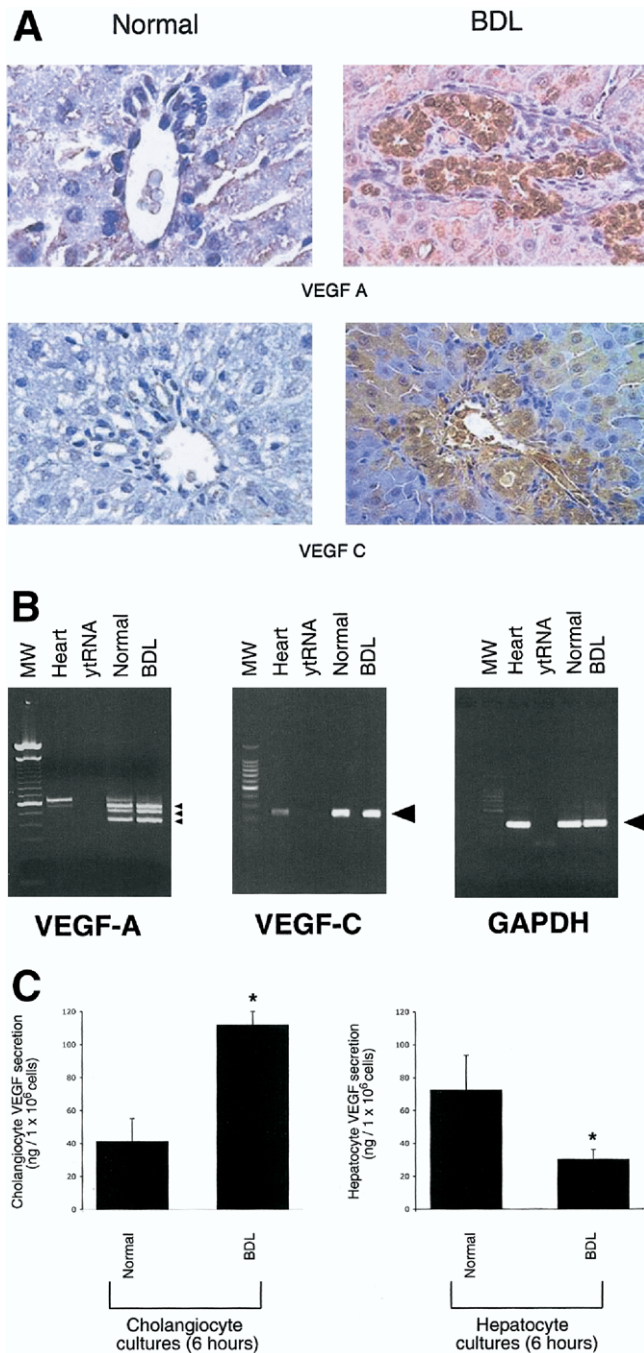


Figure 2. (A) Expression of VEGF-A and VEGF-C by immunohistochemistry in liver sections from normal and BDL rats. The expression of VEGF-A and VEGF-C was higher in bile ducts from BDL rats compared with bile ducts from normal rats (original magnification 25 \times). (B) Normal and BDL cholangiocytes express the mRNA for VEGF-A and VEGF-C. Three bands (687, 565, and 434 bp) originating from alternative splicing of mRNA for 3 different VEGF-A isoforms were present in normal and BDL cholangiocytes. For VEGF-A and VEGF-C, total RNA from rat heart and yeast transfer RNA (ytRNA) were the positive and negative controls, respectively. For GAPDH, total RNA from rat kidney and ytRNA were the positive and negative controls, respectively. Cholangiocytes express the mRNA for VEGF-A (687, 565, and 434 bp) and VEGF-C (243 bp); GAPDH mRNA, 294 bp. Normal and BDL lanes are cholangiocytes. (C) Measurement of VEGF secretion (nanogram per 1×10^6 cells) into the supernatant of primary cultures (6 hours) of cholangiocytes and hepatocytes from normal and BDL rats. We found VEGF in the supernatant of primary cultures of normal cholangiocytes and hepatocytes. Following BDL, the amount of VEGF secreted by cholangiocytes into the supernatant increased compared with the amount of VEGF secreted by normal cholangiocytes. VEGF secretion significantly decreased in BDL hepatocytes compared with normal hepatocytes. Data are mean \pm SEM of 8 (cholangiocytes) and 16 (hepatocytes) cell preparations. * $P < .05$ vs VEGF secretion of normal cholangiocytes or hepatocytes.

Table 1. Effect of Administration of Nonimmune Serum or Anti-VEGF Antibodies on the Proliferation of Cholangiocytes of BDL Rats

Treatment	PCNA-positive cholangiocytes (n = 5)	CK-19-positive cholangiocytes (n = 6)	Bile duct volume (%) (n = 11)
BDL + nonimmune serum	39.0 ± 1.9	133.8 ± 5.7	7.0 ± 0.6
BDL + anti-VEGF-A antibody	17.0 ± 1.0 ^a	82.7 ± 1.0 ^a	1.2 ± 0.2 ^a
BDL + anti-VEGF-C antibody	17.0 ± 1.3 ^a	77.0 ± 3.3 ^a	1.9 ± 0.3 ^a
BDL + anti-VEGF antibody	11.8 ± 1.1 ^a	71.3 ± 2.3 ^a	1.7 ± 0.2 ^a
BDL + anti-VEGF-A + C antibodies	6.4 ± 0.9 ^b	54.5 ± 3.7 ^b	0.4 ± 0.06 ^b

NOTE. Cholangiocyte proliferation was evaluated by measurement of the number of PCNA- and CK-19-positive cholangiocytes and GGT-positive ducts in liver sections. Administration of anti-VEGF-A, anti-VEGF-C, or the neutralizing anti-VEGF antibody to BDL rats decreased the number of PCNA- and CK-19-positive cholangiocytes and the number of GGT-positive ducts compared with BDL rats treated with nonimmune serum. When BDL rats were treated simultaneously with both anti-VEGF-A + C antibodies, there was a greater decrease in the number of PCNA- and CK-19-positive cholangiocytes and GGT-positive ducts compared with all the other groups.

^a*P* < .05 vs its corresponding value of BDL rats treated with nonimmune serum.

^b*P* < .05 vs the value of BDL rats treated with nonimmune serum or anti-VEGF antibodies.

number of PCNA- and CK-19-positive cholangiocytes and GGT-positive ducts compared with all the other groups (Table 1). Similarly, there was a significant (*P* < .05) decrease in PCNA protein expression (expressed as ratio to β-actin × 100 arbitrary units) in cholangiocytes from BDL rats treated with anti-VEGF-A (40.4 ± 5.1 arbitrary units, n = 8) or anti-VEGF-C (44.3 ± 6.8 arbitrary units, n = 8) compared with cholangiocytes from BDL rats treated with nonimmune serum (65.3 ± 6.5 arbitrary units, n = 8).

To show that following BDL, the proliferation of the PBP only occurs after that of cholangiocytes, we measured the number of PCNA-positive vascular endothelial cells and cholangiocytes in liver sections from rats with BDL for 1 or 2 weeks (Figure 3A). After BDL for 1 week, PCNA was expressed only by proliferating cholangiocytes (and in rare hepatocytes), whereas endothelial cells were negative for PCNA (Figure 3A). In contrast, after BDL for 2 weeks, both cholangiocytes and endothelial cells became positive for PCNA (Figure 3A). This was confirmed by the immunohistochemical expression of Factor VIII-related antigen (a marker of endothelial cells)²⁸ in liver sections from normal, 1-week BDL, and 2-week BDL rats (Figure 3B). The number of Factor

VIII-related antigen-positive cells increased, in comparison with normal rats, only after 2-week BDL but was unchanged after 1-week BDL, thus confirming that the proliferation of PBP occurs after that of the biliary epithelium.

Effect of Administration of Anti-VEGF-A or Anti-VEGF-C Antibody on Hepatocyte and Cholangiocyte VEGF Secretion and VEGF Serum Concentration

The secretion of VEGF from cholangiocytes (but not hepatocytes) from BDL rats treated with anti-VEGF-A or anti-VEGF-C antibody decreased compared with cholangiocytes from BDL rats treated with nonimmune serum (Table 2). In normal rats, we found a VEGF serum concentration (226.12 ± 5.6 ng/mL; n = 4) that was similar to that previously described in rats and humans.^{13,14} Following BDL, there was an increase in serum VEGF concentration (291.04 ± 10.04 [BDL] vs 226.12 ± 5.6 ng/mL [normal]; *P* < .05; n = 4).

VEGF Stimulation of Normal Cholangiocyte Proliferation Is Associated With Activation of IP₃/Ca²⁺/PKC-α and Phosphorylation of Src and ERK1/2

Administration of r-VEGF-A or r-VEGF-C to normal rats increased cholangiocyte proliferation evaluated by the enhanced number of PCNA- and CK-19-positive cholangiocytes and GGT-positive ducts in liver sections compared with normal rats treated with saline (Table 3).

Consistent with the concept that VEGF stimulates the proliferation of normal cholangiocytes by activation of the IP₃/Ca²⁺/PKC pathway, r-VEGF-A and r-VEGF-C increased cholangiocyte IP₃ (Table 4) and [Ca²⁺]_i (Table 5) levels in NRICCs compared with NRICCs treated with BSA. VEGF-A- and VEGF-C-induced increases in IP₃ levels were not blocked by the Src inhibitor PP2 (Table 4), a finding that shows that Src is downstream to IP₃/Ca²⁺.

In NRICCs, both r-VEGF-A and r-VEGF-C increased the phosphorylation of PKC-α (Table 6), r-VEGF-A and r-VEGF-C increased PCNA protein expression of NRICCs (Table 7), r-VEGF-A- and r-VEGF-C-induced increases in PCNA protein expression of NRICCs were blocked by BAPTA/AM and Gö6976 (Table 7). Both r-VEGF-A and r-VEGF-C increased absorbance (an index of changes in cell number) of NRICCs compared with basal values (Table 6). Furthermore, both r-VEGF-A and r-VEGF-C increased the phosphorylation of the Tyr139 residue (which positively regulates Src activity)⁴⁵ and ERK1/2 of NRICCs in NRICCs (Table 6). Neither r-VEGF-A nor r-VEGF-C altered the phosphorylation of p38 or JNK (not shown).

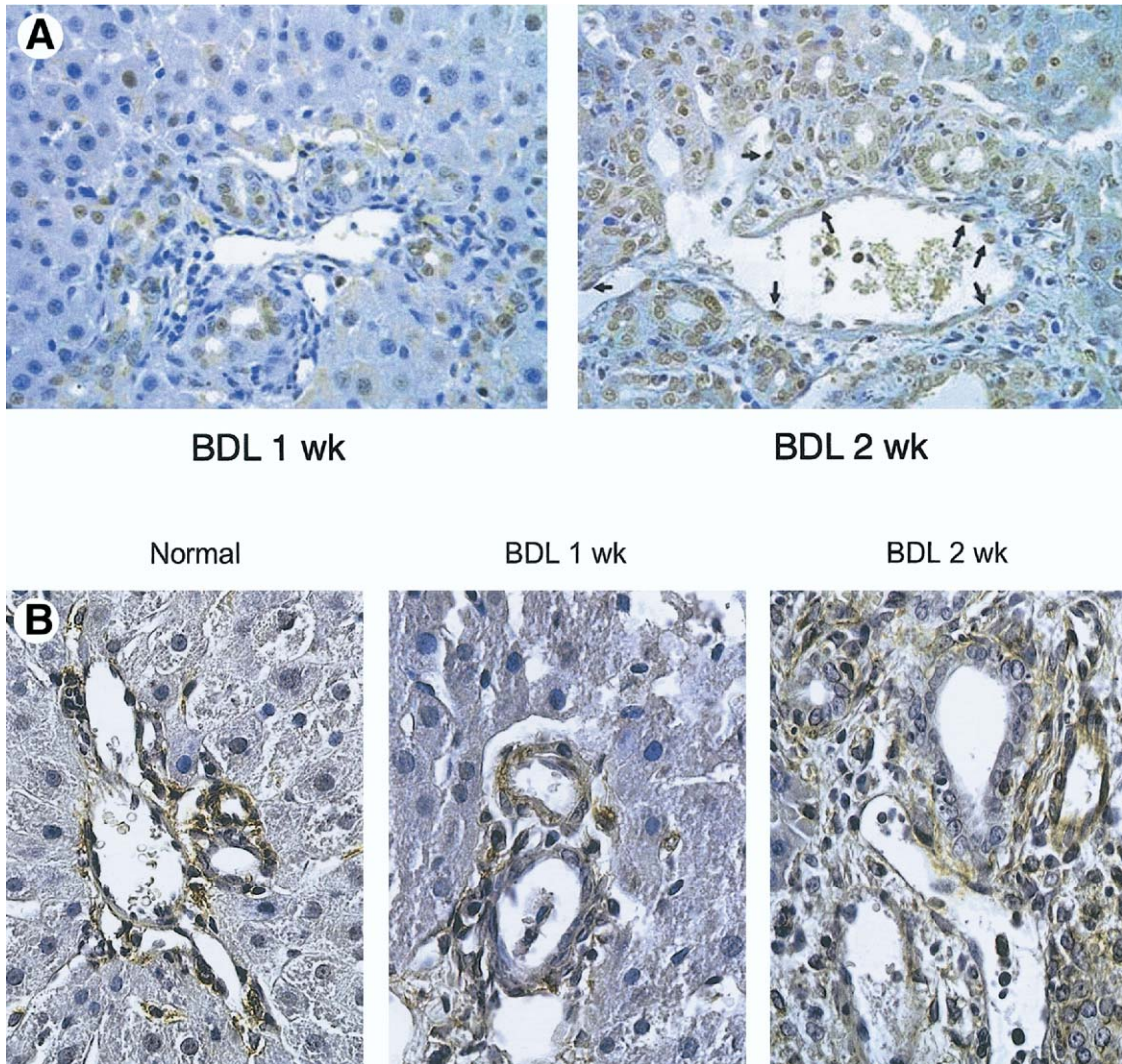


Figure 3. Immunohistochemistry for (A) PCNA in liver sections from 1- and 2-week BDL rats and (B) Factor VIII-related antigen in liver sections from normal, 1-week, and 2-week BDL rats. (A) In 1-week BDL rats, PCNA was expressed only by proliferating cholangiocytes (and in rare hepatocytes), whereas endothelial cells were negative for PCNA. After 2 weeks of BDL, both proliferating cholangiocytes and endothelial cells (*arrows*) became positive for PCNA. The data demonstrate that bile ducts *in vivo* proliferate first, then the arterial network (original magnification 40 \times). (B) The number of Factor VIII-related antigen-positive cells increased, in comparison with normal rats, only after 2-week BDL but was unchanged after 1-week BDL, thus confirming that the proliferation of PBP occurs after that of the biliary epithelium (original magnification 40 \times).

Discussion

Cholangiocyte proliferation is a typical hallmark of cholangiopathies, where it counteracts the loss/damage of intrahepatic bile ducts to compensate for the structural and functional derangement of the intrahepatic biliary tree.⁴⁶ Proliferating cholangiocytes display enhanced secretory activity,^{10,20} which is important for maintaining a normal ductal secretion despite the loss of damaged bile ducts.⁴⁶ Cholangiocyte proliferation is modulated by a complex system of growth factors, neuropeptides, and hormones.⁴⁷ Most of this information has been achieved in the BDL rat model, which is characterized by cholangiocyte proliferation^{10,17,18} followed by an adaptive proliferation of the PBP.⁸ Similar to previous findings,⁸ our study shows that

the proliferation of PBP occurs after that of the intrahepatic biliary epithelium. In support of this concept, we show that in 1-week BDL rats, PCNA is expressed only by proliferating cholangiocytes (and in rare hepatocytes), whereas endothelial cells are negative for PCNA. After 2 weeks of BDL, both cholangiocytes and endothelial cells become positive for PCNA. To determine that following BDL the growth of the PBP is due to the enlargement of bile duct mass, we evaluated the immunohistochemical expression of Factor VIII-related antigen in liver sections from normal and BDL rats. The finding that Factor VIII-related antigen is positive only in endothelial cells, and that the number of the marked cells increases only after 2-week BDL (but not after 1 week) where it correlated with the increased number

Table 2. Measurement of VEGF Concentration in the Supernatant of Primary Cultures of Hepatocytes and Cholangiocytes From Rats That (Immediately After BDL) Were Treated by a Daily Intraperitoneal Injection of Nonimmune Serum, Anti-VEGF-A Antibody, or Anti-VEGF-C Antibody for 1 Week

Treatment	Cholangiocyte VEGF secretion (ng/1 × 10 ⁶ cells)	Hepatocyte VEGF secretion (ng/1 × 10 ⁶ cells)
BDL + nonimmune serum	142.54 ± 4.6	31.74 ± 7.2
BDL + anti-VEGF-A antibody	73.29 ± 10.0 ^a	37.58 ± 8.8 ^b
BDL + anti-VEGF-C antibody	71.71 ± 9.6 ^a	32.70 ± 9.5 ^b

NOTE. Administration of anti-VEGF-A or anti-VEGF-C antibody reduced cholangiocyte (but not hepatocyte) VEGF secretion compared with cholangiocytes from BDL rats treated with nonimmune serum. Data are mean ± SEM of 8 (cholangiocytes) and 16 (hepatocytes) experiments.

^a*P* < .05 vs VEGF secretion of cholangiocytes from BDL rats treated with nonimmune serum.

^bNot significant vs secretion of hepatocytes from BDL rats treated with nonimmune serum.

of the proliferated ducts, demonstrates that the expansion of the PBP follows the increases of bile duct mass.

The proliferating bile ducts are characterized by markedly enhanced cholangiocyte secretory and proliferative activities.^{10,17,18,46} 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.^{8,10,17,18,46} Because the proliferation of the PBP follows the increase in the growth of bile ducts,⁸ it is reasonable to suppose that proliferating cholangiocytes modulate the adaptive response of the vascular bed.⁸

Table 3. Effect of Long-term Administration of r-VEGF-A, r-VEGF-C, or Saline on the Proliferation of Cholangiocytes of Normal Rats

Treatment	PCNA-positive cholangiocytes (n = 8)	CK-19-positive cholangiocytes (n = 8)	Bile duct volume (%) (n = 8)
Normal + saline	0.5 ± 0.2	19.4 ± 3.4	0.4 ± 0.08
Normal + r-VEGF-A	4.7 ± 0.5 ^a	32.2 ± 5.4 ^a	2.4 ± 0.2 ^a
Normal + r-VEGF-C	5.7 ± 0.7 ^a	37.7 ± 7.0 ^a	2.0 ± 0.1 ^a

NOTE. Cholangiocyte proliferation was evaluated by measurement of the number of PCNA- and CK-19-positive cholangiocytes and GGT-positive ducts in liver sections from the selected groups of animals. Administration of r-VEGF-A or r-VEGF-C increased the number of PCNA- and CK-19-positive cholangiocytes and GGT-positive ducts compared with normal rats treated with saline for 1 week. Data are mean ± SE.

^a*P* < .05 vs its corresponding value of normal rats treated with saline.

Table 4. Effect of r-VEGF-A and r-VEGF-C on IP₃ Levels of NRICCs

Treatment	IP ₃ levels (pmol/1 × 10 ⁶ cells)
Basal	0.18 ± 0.02
r-VEGF-A	0.32 ± 0.03 ^a
PP2 + r-VEGF-A	0.34 ± 0.01 ^a
r-VEGF-C	0.31 ± 0.08 ^a
PP2 + r-VEGF-C	0.29 ± 0.02 ^a

NOTE. Measurement of IP₃ levels of NRICCs stimulated at room temperature for 10 minutes with 0.2% BSA or r-VEGF-A or r-VEGF-C (100 nmol/L with 0.2% BSA) in the absence or presence of preincubation with PP2 (1 μmol/L for 10 minutes). Both r-VEGF-A and r-VEGF-C increased cholangiocyte IP₃ levels in NRICCs compared with NRICCs treated with 0.2% BSA. VEGF-A- and VEGF-C-induced increases in IP₃ levels were not blocked by the Src inhibitor PP2. Data are mean ± SEM of 7 experiments.

^a*P* < 0.05 vs its corresponding value of NRICCs treated with 0.2% BSA.

With this background, we evaluated the role of VEGF in modulating cholangiocyte pathophysiology in BDL rats. Our study has clinical implications, because in human cholangiopathies alterations of the PBP have been described to be associated with bile duct damage and to condition the development and progression of the disease.^{48,49} On the other hand, studies suggest that the activation of the VEGF system represents a protective mechanism during experimental liver damage, suggesting that regulation of VEGF hepatic synthesis or VEGF receptors could represent a potential therapeutic strategy for the management of liver injury.⁵⁰

We showed that (1) VEGFR-2 and VEGFR-3 are present in normal cholangiocytes and (2) the expression of VEGFR-2 and VEGFR-3 is increased in cholangiocytes following BDL. VEGF-A and VEGF-C protein were expressed in normal cholangiocytes, and their expression increased after BDL. Furthermore, VEGF is secreted by cholangiocytes and hepatocytes, because in the medium of short-term cultures of normal cholangiocytes and hepatocytes we found significant amounts of

Table 5. Effect of r-VEGF-A and r-VEGF-C on [Ca²⁺]_i Levels of NRICCs

Treatment	[Ca ²⁺] _i levels (nmol/L)
Basal	197.59 ± 1.85 (n = 5)
r-VEGF-A	301.10 ± 9.87 ^a (n = 5)
r-VEGF-C	294.06 ± 10.06 ^a (n = 5)

NOTE. Calcium fluorescence measurements in NRICCs stimulated with 0.2% BSA or 100 nmol/L r-VEGF-A or r-VEGF-C for 10 minutes were performed using fluo-3AM (Molecular Probes) and a Fluoroskan Ascent FL (ThermoLabsystems) microplate reader equipped with 3 injectors. Both r-VEGF-A and r-VEGF-C increased cholangiocyte [Ca²⁺]_i levels in NRICCs compared with NRICCs treated with 0.2% BSA. Data are mean ± SEM.

^a*P* < .05 vs its corresponding basal value.

Table 6. Effect of r-VEGF-A and r-VEGF-C on the Growth of NRICCs and Phosphorylation of PKC- α , Src139, and ERK1/2 in NRICCs

Treatment	Cell proliferation (MTS, absorbance at 490 nm) (n = 8)	pPKC- α to tPKC- α ratio $\times 100$ (n = 6)	pSrc139 to tSrc ratio $\times 100$ (n = 7)	pERK1 to tERK1 ratio $\times 100$ (n = 10)	pERK2 to tERK2 ratio $\times 100$ (n = 10)
Basal (0.2% BSA)	0.14 \pm 0.01	37.1 \pm 5.3	121.1 \pm 13.8	165.7 \pm 18.7	171.7 \pm 20.6
r-VEGF-A (100 nmol/L)	0.21 \pm 0.01 ^a	51.3 \pm 5.4 ^a	164.3 \pm 16.5 ^a	216.1 \pm 22.0 ^a	243.6 \pm 22.3 ^a
r-VEGF-C (100 nmol/L)	0.22 \pm 0.03 ^a	55.1 \pm 5.7 ^a	172.7 \pm 18.9 ^a	212.5 \pm 21.1 ^a	233.2 \pm 19.6 ^a

NOTE. r-VEGF-A and r-VEGF-C increased (1) proliferation of NRICCs evaluated by MTS assay (increased absorbance at 490 nm) compared with its basal value and (2) phosphorylation of PKC- α , Src139, and ERK1/2 of NRICCs.

^aP < .05 vs its corresponding value of NRICCs treated with 0.2% BSA.

VEGF. Following BDL, VEGF secretion increased in the medium of proliferating cholangiocytes (but not hepatocytes) isolated from BDL rats. Therefore, these findings indicate that the VEGF system (ligands and receptors) is activated and amplified in proliferating cholangiocytes after BDL. The VEGF concentration we found in the rat serum (nanomolar range) or in the supernatant of primary cultures of purified normal rat cholangiocytes (41.15 \pm 14.04 ng/mL) was much higher than that reported by Nichols et al⁵¹ in cyst fluid (average, 0.849 ng/mL) of patients with autosomal dominant polycystic kidney disease or in bile (0.27 ng/mL) of control subjects. However, VEGF secretion from the apical side of cholangiocytes into bile or cyst fluid likely represents only a portion of total secreted VEGF and this, other than species differences (human vs rats), could explain the divergence with respect to the study by Nichols et al. Furthermore, the serum levels of VEGF found in our study in normal rats were similar to that previously reported in rat and human serum.^{13,14}

Table 7. In Vitro Effect of r-VEGF-A or r-VEGF-C on PCNA Protein Expression of NRICCs

Treatment	PCNA to β -actin ratio $\times 100$
Basal	94.4 \pm 4.4
r-VEGF-A	121.6 \pm 14.4 ^a
BAPTA/AM + r-VEGF-A	97.9 \pm 7.0
Gö6976 + r-VEGF-A	96.1 \pm 5.9
r-VEGF-C	118.9 \pm 13.3 ^a
BAPTA/AM + r-VEGF-C	103.9 \pm 5.9
Gö6976 + r-VEGF-C	105.7 \pm 5.1 ^a

NOTE. In vitro effect of r-VEGF-A or r-VEGF-C on PCNA protein expression of NRICCs treated in vitro with 0.2% BSA (basal) or r-VEGF-A or r-VEGF-C (100 nm) in the absence or presence of BAPTA/AM (5 μ mol/L), and Gö6976 (1 μ mol/L). Both r-VEGF-A and r-VEGF-C increased PCNA protein expression of NRICCs. r-VEGF-A- or r-VEGF-C-induced increases in PCNA protein expression were blocked by BAPTA/AM, and Gö6976. Data are mean \pm SEM of 11 experiments.

^aP < .05 vs its corresponding value of NRICCs treated with 0.2% BSA.

To evaluate whether VEGF and related receptors regulate cholangiocyte proliferation, we injected BDL rats (immediately after BDL) for 1 week with different antibodies against VEGF. Proliferation of cholangiocytes was inhibited by the in vivo administration of different anti-VEGF antibodies, a finding that supports the concept that VEGF, secreted by cholangiocytes, modulates (through an autocrine mechanism) intrahepatic ductal mass. We tested 2 antibodies (anti-VEGF-A and anti-VEGF-C) that bind the functional domains of VEGF-A and VEGF-C, respectively (as evaluated in Santa Cruz data sheets and <http://www.ebi.uniprot.org>) and an additional antibody (anti-VEGF) reported to block all 3 isoforms of VEGF-A identified in cholangiocytes.^{11,12} In addition, when anti-VEGF-A and anti-VEGF-C antibodies were administered together, the inhibitory effects on cholangiocyte proliferation were higher than that obtained by each individual antibody. Because VEGF-A and VEGF-C activate similar signaling pathways, it should be expected that VEGF-A would be able to compensate in case of VEGF-C blockade and vice versa. In contrast, we found that the inhibition of either VEGF-A or VEGF-C induces the same effects. This could suggest a cross-reaction of the anti-VEGF-A and anti-VEGF-C antibodies, but this is only partial given the additional inhibitory effect of the 2 antibodies administered together in BDL rats.

The fact that, similar to other studies,^{50,52} hepatocytes secrete VEGF raises the possibility that cholangiocyte growth may be also regulated by a paracrine mechanism through hepatocyte VEGF secretion. However, because (1) hepatocyte VEGF secretion decreases following BDL and (2) hepatocyte VEGF secretion does not change following the administration of anti-VEGF antibodies, we propose that regulation of BDL cholangiocyte growth occurs mainly by an autocrine mechanism (by secretion of cholangiocyte VEGF secretion), although a paracrine mechanism (by hepatocyte secretion) cannot be ruled out completely by this study. Our finding that VEGF con-

centration decreases in the serum of BDL rats treated with anti-VEGF-A or anti-VEGF-C antibody compared with the serum of BDL rats treated with nonimmune serum suggests that the block of extracellular VEGF leads to impaired cholangiocyte proliferation, which is then followed by a decreased VEGF secretion. This should also explain why cholangiocytes isolated from BDL rats treated with anti-VEGF antibodies display a lower VEGF secretion in the supernatant even in the absence of anti-VEGF antibodies in the medium.

Furthermore, we injected normal rats with r-VEGF-A or r-VEGF-C and found an increase in cholangiocyte proliferation. To directly evaluate the role and mechanisms of action by which VEGF modulates cholangiocyte proliferation, we incubated NRICCs¹⁵ (which, similar to freshly isolated cholangiocytes, express VEGFR-2 and VEGFR-3) with r-VEGF-A or r-VEGF-C and showed the ability of VEGF to induce in vitro cholangiocyte proliferation. Next, to elucidate the signaling transduction pathways involved, we performed in vitro studies in NRICCs showing that VEGF increases intracellular IP₃ and Ca²⁺ levels and phosphorylation of PKC- α . VEGF receptors belong to the family of membrane-bound tyrosine kinase receptors, and therefore their intracellular signaling is mediated by tyrosine phosphorylation of several intracellular and membrane-bound proteins.⁵³ However, in many studies, it has been shown that VEGF also induces activation of PLC- γ ,^{54,55} leading to generation of diacylglycerol and IP₃ and subsequent PKC activation by an increase in intracellular Ca²⁺. This is particularly important in the modulation of endothelial cell proliferation because PKC blockers inhibit VEGF-induced endothelial cell proliferation.⁵⁶ In NRICCs, we observed that VEGF increased intracellular IP₃ and Ca²⁺ levels and enhanced activation of the Ca²⁺-dependent PKC- α and Src. In our previous reports,^{29,34,36,57,58} we showed that the calcium dynamics of cholangiocytes are in general slower and not characterized by a calcium spike.^{34,36,57} The method that we currently use results in calcium measurements that are an average signal of 400,000 cells rather than typical single-cell measurements. This approach gives us similar data^{29,58} to that obtained with measurements, which were made in single cells loaded with Fluo-3AM.^{34,36,57} Because the measurements are taken in a large number of cells, the cellular response to VEGF-A or VEGF-C is not synchronized in the studies. Thus, any peak(s) present will be muted and spread out over time, which is a factor contributing to the slow drift observed. The slow ionomycin response is again a factor of an average signal of many cells.

We also found that ERK1/2 (but not p38 and JNK) phosphorylation was enhanced in isolated cholangiocytes

incubated with VEGF. This latter result suggests a key role of the mitogen-activated protein kinase isoform, ERK1/2, in the activation of the proliferative machinery by VEGF, similar to other agents inducing cholangiocyte proliferation, such as estrogens and insulin-like growth factor I.^{32,59} On the other hand, in many different cell types, synergistic effects of VEGF, estrogens, and insulin-like growth factor I on modulation of cell proliferation have been described with convergence of the different signaling pathways at the level of the ERK system.^{60,61} We found that VEGF increased Tyr139 phosphorylation, which positively regulates Src activity⁴⁵ in NRICCs. Activation of Src has often been described in endothelial cells stimulated to proliferate by VEGF.⁴ Src is an adapter protein linked with G-protein-coupled receptors, and its phosphorylation has been considered an early step in VEGF signaling that determines the downstream activation of PLC- γ and therefore IP₃ formation and Ca²⁺ mobilization.⁶² On the other hand, Src activation could represent an early step in the activation of the RAS/RAF/MEK/ERK cascade. Our findings indicate that, in cholangiocytes, activation of Src is a downstream event occurring after IP₃ and Ca²⁺ mobilization because the specific Src inhibitor, PP2, did not influence VEGF-induced increases in IP₃ levels in NRICCs.

In conclusion, our study indicates that VEGF, likely by an autocrine mechanism, plays a major role in modulating cholangiocyte proliferation in response to cholestasis. The study suggests that administration of VEGF analogues or neutralizing anti-VEGF antibodies may represent an important strategy for the regulation of cholangiocyte proliferation/loss during the course of cholangiopathies.

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