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HEPATOLOGY

Gene expression profiling in whole liver of bile duct ligated rats: VEGF-A expression is up-regulated in hepatocytes adjacent to the portal tracts

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Abstract

Background and Aim: It would be of clinical importance to clarify molecular mechanisms of cholangiocytes proliferation for the treatment of intractable cholestatic diseases. The aim of this study was to elucidate gene expression profiling in the whole liver of bile duct ligated (BDL) rats using microarray analysis. In addition, the localization and time course of up-regulated expression of vascular endothelial growth factor (VEGF) was investigated.

Methods: Male Sprague-Dawley rats were used. The whole liver was removed from BDL and sham-operated rats at day 2 after the procedure, and microarray analysis was performed using an array on which 3757 rat cDNA clones spotted. The up-regulation of VEGF expression was investigated by RT-PCR using livers at day 1, 2, 4 and 7, and immunoblotting and immunohistochemistry at day 2.

Results: Marked proliferation of bile ducts was observed in livers of BDL rats. By microarray analysis, 38 up-regulated and 17 down-regulated transcripts were detected in whole liver of the BDL rat. The expression of VEGF-A was significantly elevated in the BDL rats at day 2; the VEGF-A/GAPDH ratio was 4.030 ± 2.493 in BDL rats and 1.159 ± 0.125 in sham-operated rats ($P = 0.0330$). The up-regulation of VEGF-A expression was maximal at day 2. Immunoblotting also demonstrated up-regulated expression of VEGF-A at the protein level. Immunostaining of VEGF revealed that the expression was evident in hepatocytes adjacent to the portal tracts, and scarcely observed in hepatocytes at the centrilobular area or cholangiocytes.

Conclusion: Gene expression profiling in the whole liver of the BDL rats revealed 38 up-regulated and 17 down-regulated transcripts. In addition, the up-regulated expression of VEGF was mainly observed in hepatocytes surrounding to the portal tracts.

Introduction

Cholestasis is defined as the impairment of bile formation by several causes.[1,2] Clinically, cholestasis is classified into two categories depending on the level at which impairment of bile formation is located: hepatocellular cholestasis, and ductal cholestasis.[2] In chronic cholestatic liver diseases, including primary biliary cirrhosis and primary sclerosing cholangitis, the intrahepatic bile ducts and cholangiocytes lining the ducts are usually damaged due various known and unknown reasons, including autoimmune, metabolic, and genetic disorders, adverse effect of drugs, infections and malignancy, possibly leading to a ductopenia and biliary cirrhosis.[1] In contrast, the residual cholangiocytes are known to proliferate in response to the damage.[3] Thus, the course of the cholestatic diseases depends upon a balance between damage of the bile ducts and the compensatory proliferative mechanism of residual cholangiocytes.[4] Therefore, it would be of great clinical importance to stimulate the residual cholangiocytes for proliferation and to reconstruct the intrahepatic biliary tree in patients with intractable cholestatic diseases.

It has been well investigated as to which stimuli cholangiocytes tend to proliferate in various cholestatic models, such as bile duct ligated (BDL) and partial hepatectomized animals. These include cytokines (interleukins 1α and 6, tumor necrosis factor-α), growth factors (transforming growth factor [TGF]-α, hepatocyte growth factor, epidermal growth factor [EGF]),[5–9] hormones and peptides (estrogens, acetylcholine, parathyroid hormone-related peptide, growth hormone),[10–13] and bile salts (lithocholic acid, taurocholic

acid, taurolithocholic acid).[14,15] In this study, our primary aim was to elucidate gene expression profiling in the whole liver of the BDL rats, not in the biliary epithelium, using microarray analysis. We believe that the cholangiocytes' proliferative response is achieved not only in the biliary epithelium itself, but also in the close and complex interactions between cholangiocytes and other cells including hepatocytes and inflammatory cells, and thus it is crucial to focus on the whole liver. Gene expression profiling of the whole liver was previously performed using BDL mice.[16]

In addition to the aforementioned humeral factors that affect cholangiocyte proliferation, based on the importance of blood supply to proliferating bile ducts, it is suggested that vascular endothelial growth factor (VEGF) may be also involved in the proliferation of cholangiocytes.[17] Indeed it was reported that proliferation of the peribiliary vascular plexus was observed only around large proliferating ducts.[14] Moreover, in patients with primary biliary cirrhosis, a prototype of chronic cholestatic diseases in adults, marked expression of VEGF was immunohistochemically observed in sinusoidal endothelial cells in the liver, but not in controls.[18] In an experimental bile duct ischemia model, cholangiocytes showed up-regulated VEGF expression and proliferation after 48 h of arterial deprivation.[19] Additionally, VEGF-C as well as VEGF-A may play a role in the proliferative activities of cholangiocytes.[20] Therefore the second aim of this study was to measure expression of VEGF-A in BDL rats at mRNA as well as protein level.

Methods

Animals and experimental protocol

Male Sprague-Dawley rats (230–250 g) were purchased from Japan Laboratory Animals (Tokyo, Japan), and were anesthetized with an intraperitoneal injection of pentobarbital (5 mg/100 g body weight). The common bile duct was ligated at two sites and cut at the distal end. The abdomen was closed and the animals were allowed to recover. Sham-operated rats served as controls.

The animals were killed at day 2 for cDNA array analysis, when the maximal proliferation of cholangiocytes occurred after BDL,[21] and at days 1, 4, and 7 for time-course analysis of VEGF expression. BDL and sham-operated rats were killed under anesthesia with pentobarbital after overnight fasting. Liver samples of all rats were immediately stored in RNA*later* solution (Ambion, Austin, TX, USA), or fixed in phosphate-buffered formalin. The study protocols were in compliance with the institution's guidelines.

Analysis of gene expression profile in the liver using cDNA microarray

Total RNA was prepared from livers using RNeasy Mini Kit (Qiagen, Basel, Switzerland). cDNA was synthesized from 1 μg of total RNA using BD PowerScript Reverse Transcriptase (BD Clontech, Palo Alto, CA, USA). Thereafter Cy3 reactive fluorescent dye (Amersham Bioscience, Piscataway, NJ, USA) was coupled to cDNA, and hybridized to 3757 rat cDNA clones spotted onto the BD Atlas Glass 3.8 I Microarray (BD Clontech). The microarray slide was scanned for fluorescence emission using a Gene Pix Scanner (Axon Instruments, Foster City, CA, USA), and analyzed using Array Gauge (Fuji Photo Film, Tokyo, Japan).

Real-time PCR for quantification of VEGF-A mRNA

For quantification of VEGF mRNA, we performed real-time PCR using Light-Cycler system (Roche, Mannheim, Germany). It is well known that there are several isoforms in VEGF, namely VEGF-A, VEGF-B, VEGF-C and VEGF-D.[22] Of these, we focused on VEGF-A, a key regulator of blood vessel growth, and determined as an up-regulated gene in the BDL rat liver by cDNA array analysis in this study.

As a number of splicing variants of rat VEGF-A have been reported, we designed primers and probes for real-time PCR to amplify as many splicing variants as possible. Thus, the sequences of primers we designed were as follows; a forward primer, 5′-ATT GAG ACC CTG GTG GAC-3′ (M32167: 164–181), and a reverse primer, 5′-CCT ATG TGC TGG CTT TGG-3′ (M32167: 354–337), which amplify AF215725, AF215726, AF222779, AF261751, AY033503, AY033504, AY033506, AY033507, AY033508, AY702972, L20913, M32167, but fail to amplify AF062644, AF080594, AY033505. In addition, we designed two probes for real-time PCR, one (5′-TTC ATC ATT GCA GCA GCC CGC ACA C-3′) labeled with fluorescein, and the other (5′-GCA TTA GGG GCA CAC AGG ACG GCT T-3′; M32167 245-221) labeled with Light-Cycler Red640.

The PCR was performed according to the manufacturer's instruction. In brief, 20 μL of cDNA, made from 1 μg of total RNA using SuperScript III First Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) was diluted by 1:500, and 5 μL was used as a template. The final volume of the reaction was 20 μL, and final concentrations of $MgCl_2$, 5′ primer, 3′ primer, fluorescein probe, LC-Red probe were 3 μmol/L, 0.3 μmol/L, 0.3 μmol/L, 0.2 μmol/L and 0.4 μmol/L, respectively. The PCR reaction consisted of denaturing for 10 min at 95°C, followed by 45 cycles of denaturing for 10 s at 95°C, annealing for 15 s at 60°C, and extension for 7 s at 72°C. The display mode was F2/1. In addition, we amplified glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcript as an internal standard, using primers as follows: forward primer, 5′-GAA GGT GAA GGT CGG AGT-3′, reverse primer, 5′-GAA GAT GGT GAT GGG ATT TC-3′.

Immunoblotting of VEGF-A protein

The whole rat liver, immediately stored in RNA*later* solution, was homogenized, and protein concentration was measured using Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, USA) using the Bradford method. After heated at 95°C for 5 min, 10 μg of protein samples, mixed with 8 μL of water and 2 μL of ×10 Tris-SDS sample buffer (Daiichi-Kagaku, Tokyo, Japan) was loaded on an 10% e-PAGEL (Atto, Tokyo, Japan), and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at 20 mA using pageRun system (Atto). Thereafter, separated proteins were electrophoretically transferred onto ClearBlot Membrane-P (Atto).

After transfer, the membranes were incubated in blocking solution (PBS with 3% non-fat dry milk) at room temperature for 30 min, and then for 1 h with mouse monoclonal anti-VEGF antibody (C-1) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted at 1:50 with blocking solution. After washing four times with phosphate-buffered saline (PBS) with 0.05% Tween20, the

strips were incubated for an additional 1 h with horseradish peroxidase conjugated with goat-mouse IgG (1:2000, Zymed, San Francisco, CA, USA). The strips were washed as above and visualized with the ECL immunodetection system (Amersham, Buckinghamshire, UK).

Immunohistochemical staining of VEGF

We performed immunohistochemical staining of the liver using formalin-fixed paraffin-embedded tissue sections with the rapid microwave procedure.[23] In brief, deparaffinization and standard antigen retrieval by microwave irradiation were performed on paraffin sections. Then all sections were incubated with 5% bovine serum albumin (BSA; Sigma) for 5 min. Mouse monoclonal anti-VEGF antibody (C-1) (Santa Cruz Biotechnology) was diluted 200-fold in Tris-buffered saline (TBS) including 5% BSA and applied to the specimens, followed by incubation at 4°C for overnight. After washing with TBS for 5 min, peroxidase-conjugated immune polymer reagent for mouse monoclonal antibody (Envision-PO-plus for mouse, Dako, Glostrup, Denmark) was applied as secondary antibody and incubated for an hour. After washing with TBS, sections were developed with 3-3′ diaminobenzidine (DAB, Sigma), and counter-stained with hematoxylin.

Statistical analysis

Statistical analysis in the current study was performed using SAS software version 9.1 (SAS Institute, Cary, NC, USA). Comparison between unpaired collection of data, that is data from BDL and sham-operated rats, was performed using on-parametric test, Mann–Whitney's *U*-test. We regarded the difference as significant if the calculated *P*-value was less than 0.05.

Results

Morphological changes in the livers of the BDL rats

Liver histology of the livers from BDL and sham-operated rats are shown in Fig. 1. In sham-operated rats, livers appear almost normal, except for the findings of centrilobular ballooning and fatty change of hepatocytes (Fig. 1a), and these changes were also found in those of BDL rats (Fig. 1b). In contrast, we confirmed marked proliferation of bile ducts as the morphological changes specific to the livers of BDL rats. In addition, distinct infiltration of mononuclear cells and necrosis in the portal area was also observed in livers of BDL rats (Fig. 1b).

Gene expression profile in whole liver of the BDL rat

The expression profiles of each gene in BDL and sham-operated rats are shown in Fig. 2. As indicated, most of genes among the 3757 cDNA were equally expressed in both materials. However, based on the premise that more than three-fold difference in expression in whole liver between BDL and sham-operated rats was significant, 38 up-regulated (Table 1) and 17 down-regulated (Table 2) transcripts were identified.

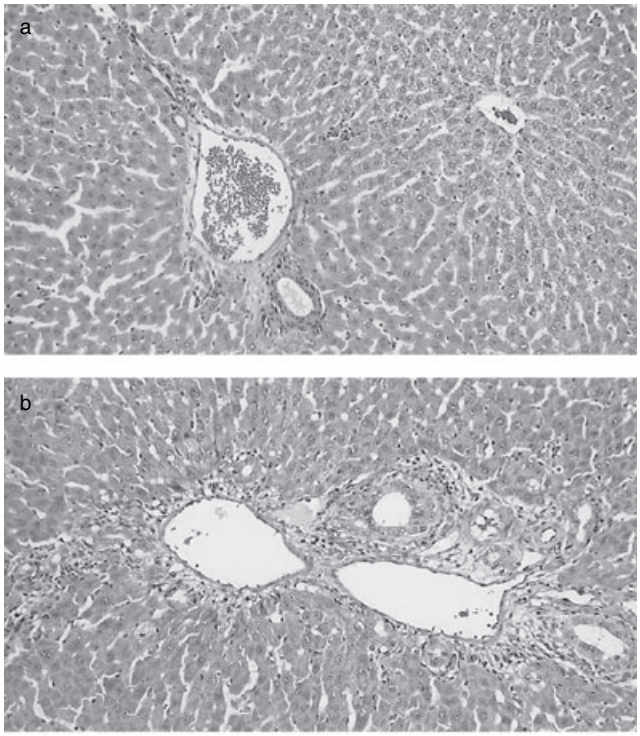

Figure 1 Morphological changes of the liver at day 2 after (a) sham operation and (b) bile duct ligation. In both, centrilobular ballooning and fatty change of hepatocytes were noted. Otherwise the liver of sham-operated rats appeared normal. In contrast, marked proliferation of bile ducts and distinct infiltration of mononuclear cells and necrosis at the portal area were remarkable in livers of BDL rats.

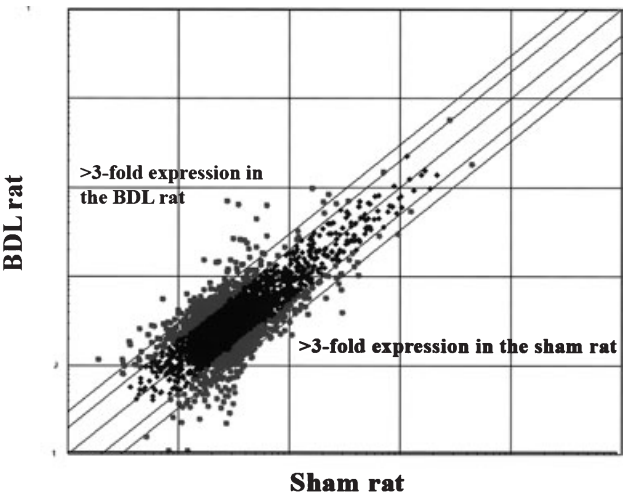

Figure 2 Expression profiles in whole liver of the bile duct ligation (BDL) and sham-operated rats of 3757 cDNA blotted onto cDNA array. Although most of genes among 3757 cDNA are equally expressed in both materials, 38 up-regulated and 17 down-regulated transcripts in whole liver of the BDL rat were identified, based on the premise that more than three-fold difference in expression was significant.

Table 1 Up-regulated (> threefold) genes in whole liver of the bile duct ligated rat at day 2

Ratio	Gene	GenBank no.
25.02	Lost on transformation 1	U72620
18.53	Cytoplasmic linker 2	AJ000485
10.04	Choline transporter	X66494
8.89	Tip associating protein	AF093139
8.72	Moesin	AF004811
8.58	Prostaglandin E synthase	AB041998
7.19	V-maf musculoaponeurotic fibrosarcoma (avian) oncogene family, protein G	AB026487
6.59	Lamin	X66870
6.42	Splicing factor, arginine/serine-rich 5 (SRp40, HRS)	AF020683
6.36	Carboxylesterase 1	X81395
6.00	Colony stimulating factor 1 receptor	X61479
5.74	Potassium voltage gated channel, shaker related subfamily, beta member 1	X70662
5.19	Somatostatin	M25890
4.80	Transforming growth factor-β type II receptor	L09653
4.58	Rabphilin-3A	U12571
4.40	Small zinc finger-like protein (TIM13)	AF144701
4.25	Aldolase A, fructose-bisphosphate	M12919
4.21	Unknown protein	AF092207
4.18	Retinoid X receptor gamma	AF016387
4.08	RAB6, member RAS oncogene family	AF148210
4.04	Linker for activation of T cells	AJ001184
3.69	Kreisler (mouse) maf-related leucine zipper homolog (b-maf)	U56241
3.69	Synuclein, gamma	X86789
3.65	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1	X52625
3.64	Urmodulin (Tamm-Horsfall protein)	M63510
3.60	Paired-like homeodomain transcription factor 3	AJ011005
3.60	Inositol triphosphate receptor type 2	X61677
3.47	Neurofilament protein, heavy polypeptide	AF031879
3.31	E-septin	AF170253
3.26	Stimulated by retinoic acid 14	AF009330
3.26	Double cortin and calcium/calmodulin-dependent protein kinase-like 1	U78857
3.25	Neurexophilin 3	AF042713
3.17	RANP-1	D50559
3.16	Vascular endothelial growth factor	M32167
3.14	Prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)	NM_017043
3.11	Tissue factor pathway inhibitor	D10926
3.11	Growth and transformation-dependent protein	M17412
3.08	Eukaryotic translation initiation factor 2B	Z48225

Up-regulation of VEGF-A expression in whole liver of the BDL rat

Vascular endothelial growth factor (M32167; 3.16, identical to VEGF-A) was found to be up-regulated in the BDL liver. Therefore, we focused on the differential expression of VEGF-A in the whole liver of the BDL rat.

To confirm up-regulation of VEGF-A transcripts in other BDL rats, we killed five BDL and five sham-operated rats at day 2, and performed real-time PCR using total RNA derived from whole livers, in the same manner. We found that the expression of VEGF-A was significantly elevated in BDL rats compared to controls; the VEGF-A/GAPDH ratio was 4.030 ± 2.493 in BDL rats and 1.159 ± 0.125 in sham-operated rats ($P = 0.0330$; Fig. 3a).

In addition, to investigate the time course of up-regulated expression of VEGF-A, we killed animals at day 1, day 4 and day 10, and performed real-time PCR. We found that the up-regulation of VEGF-A expression was at a maximum at day 2, while the elevation was not clear at day 1, day 4 and day 7 (Fig. 3b).

Immunoblotting of VEGF-A

Next, we investigated whether VEGF-A expression is also up-regulated at the protein level. Immunoblotting using anti-VEGF antibody clearly demonstrated a 46-kDa band in each specimen (Fig. 4). In general, more distinct bands were observed in BDL rats compared to controls, suggesting that VEGF-A expression was apparently increased in BDL rats at the protein level as well. However, VEGF-A expression was also elevated in one sham-operated rat, and therefore statistical analysis with quantifying the bands using densitometry failed to show a significant difference between the two groups.

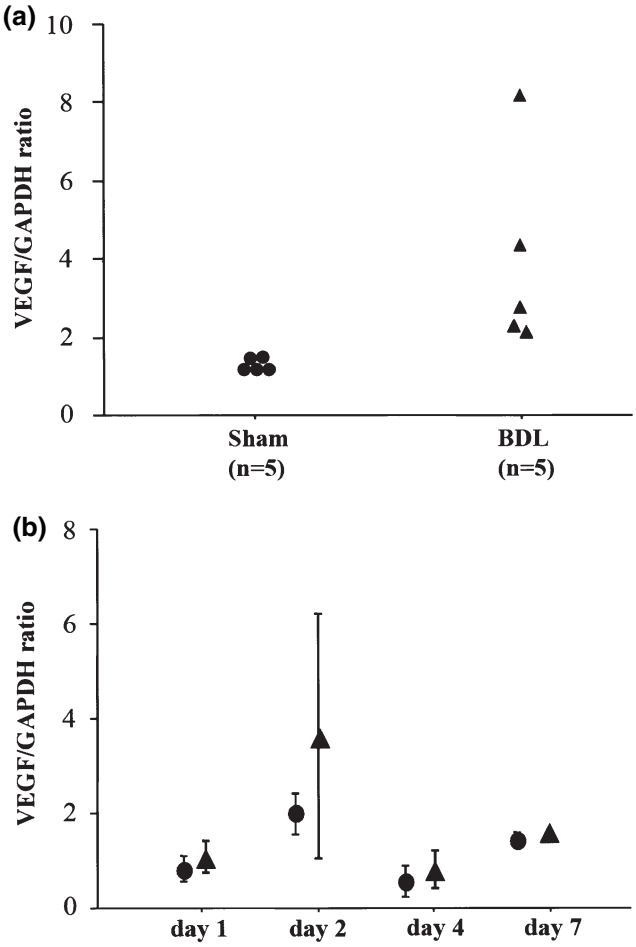

Figure 3 Expression of VEGF-A transcripts in the whole liver of (▼) bile duct ligation (BDL) and (●) sham-operated rats. Results are shown as the ratio of VEGF-A/GAPDH. (a) VEGF-A expression was significantly elevated in BDL rats compared to controls at day 2; the VEGF-A/GAPDH ratio was 4.030 ± 2.493 in BDL rats and 1.159 ± 0.125 in sham-operated rats ($P = 0.0330$). (b) In the time-course analysis, the up-regulation of VEGF-A expression was maximal at day 2, while the elevation was not clear at day 1, day 4 and day 7.

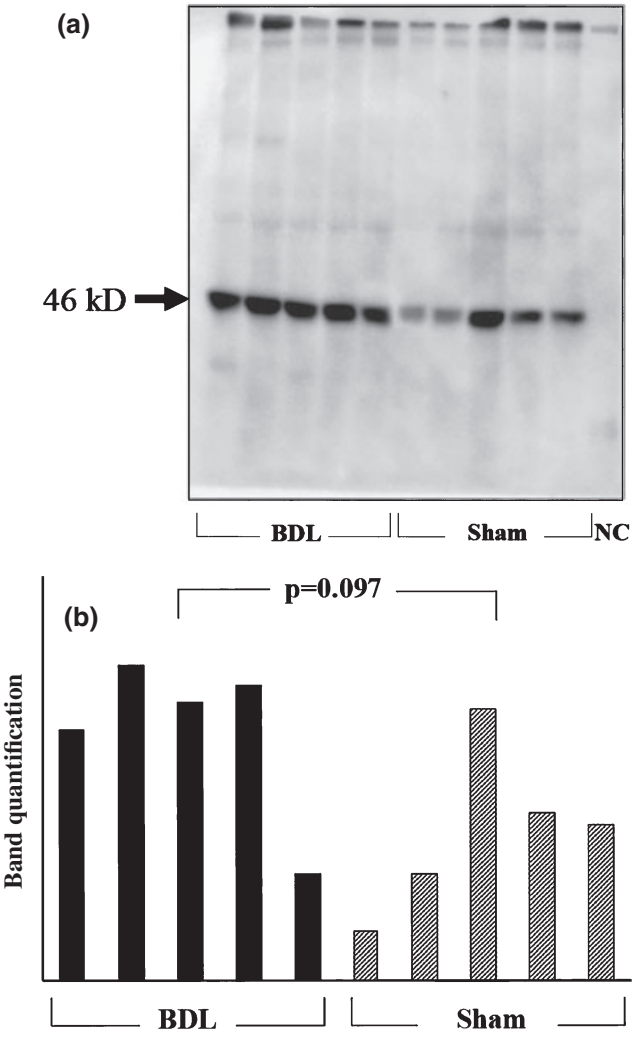

Figure 4 Immunoblotting using anti-VEGF antibody in the whole liver of bile duct ligation (BDL) and sham-operated rats. (a) Immunoblotting. A 46-kDa band (arrow) was demonstrated in each specimen. (b) Densitometry. Although more distinct bands were observed in BDL rats compared to sham-operated rats, statistical analysis with quantifying the bands failed to show a significant difference between two groups ($P = 0.0974$).

Immunohistochemical study of VEGF-A

Finally, we performed immunohistochemical analysis to locate the expression of VEGF-A in the liver of the BDL rats compared to sham-operated rats (Fig. 5). In the liver of BDL rats, VEGF-A expression was noted mainly in the hepatocytes. Of interest, the expression was clearly evident in hepatocytes adjacent to the portal tract, and scarcely observed in the centrilobular area (Fig. 5a). We confirmed that VEGF-A staining was mostly restricted to the hepatocytes adjacent to the portal tracts (Fig. 5b). In hepatocytes close to the portal tract, the cytoplasm was strongly stained with anti VEGF-A antibody, while weak expression was seen in biliary epithelial cells (Fig. 5c). In contrast, VEGF-A expression was barely observed in the liver of the sham-operated rats (Fig. 5d).

Discussion

In this study, we performed gene expression profiling of the whole liver at 2 days after the BDL procedure. A similar study, gene expression profiling using murine whole liver after BDL, was performed by Campbell *et al*.[16] They took advantage of Affymetrix gene chips to establish the gene expression profile, and identified 92 genes that were differentially expressed after BDL. In the current study, although the number of transcripts blotted onto the microarray we used was limited to 3757, at day 2 after BDL 55 differentially expressed genes were determined. Among them, we

Table 2 Down-regulated (> threefold) genes in whole liver of the bile duct ligated rat at day 2

Ratio	Gene	GenBank no.
0.33	Calcitonin receptor	L14617
0.33	Malate dehydrogenase mitochondrial	X04240
0.32	Cytochrome P450, subfamily IIC (mephenytoin 4-hydroxylase)	J02657
0.32	Carcinoembryonic antigen-related cell adhesion molecule	M92848
0.31	Neural membrane protein 35	AF044201
0.30	Uteroglobin (Clara cell secretory protein)	J05536
0.30	Carbonic anhydrase 3	M22413
0.30	Adrenergic, beta 2-, receptor, surface	J03024
0.29	Calcitonin	V01228
0.27	Testosterone 6-beta-hydroxylase, cytochrome P450/6-beta-A (CYP3A2)	M13646
0.27	Outer mitochondrial membrane receptor rTOM20	U21871
0.26	Y box protein 1	M69138
0.25	Urinary plasminogen activator receptor 2	AF007789
0.24	Ras guanine nucleotide-releasing factor 1 (Rasgrf1)	AF044908
0.24	Metallothionein	J00750
0.21	Calmodulin-sensitive plasma membrane $Ca2^{+}$-transporting ATPase (PMCA3)	J05087
0.13	Betaine-homocysteine methyltransferase	AF038870

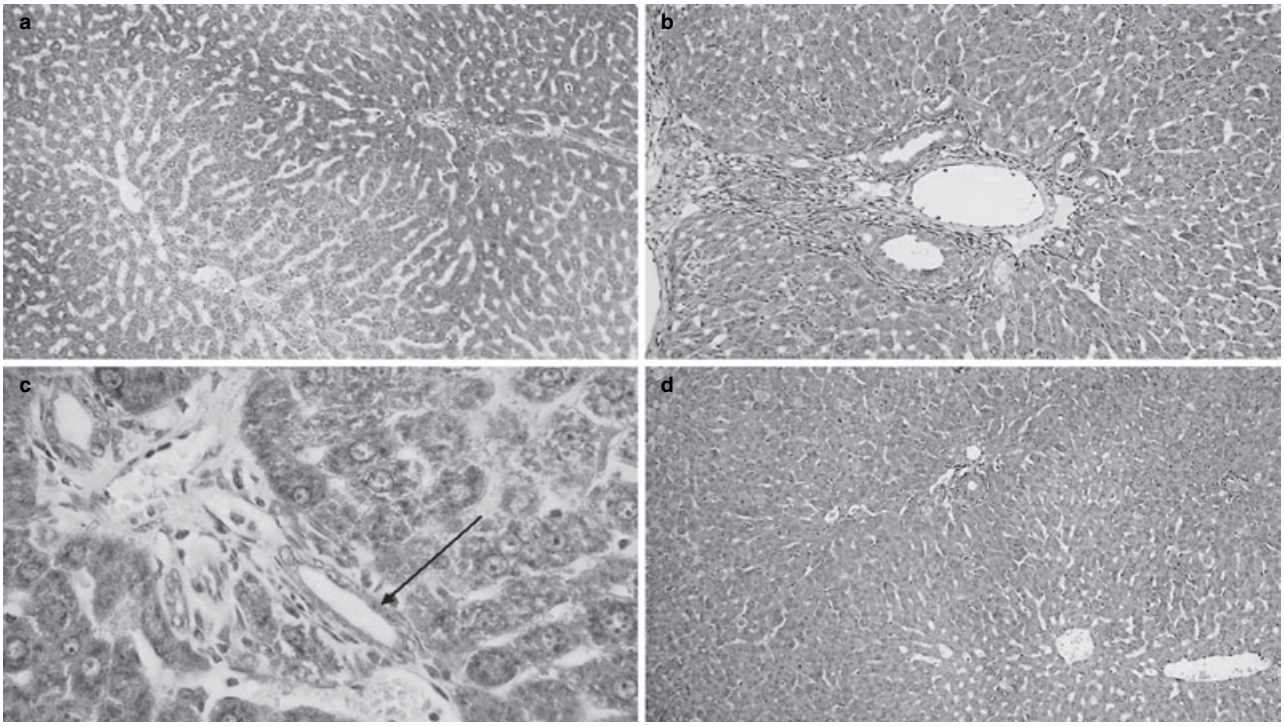

Figure 5 Immunohistochemical studies to locate the expression of VEGF-A in the liver. (a) VEGF-A expression was mainly demonstrated in hepatocytes, especially adjacent to the portal tract, and scarcely observed in the centrilobular area in the liver of BDL rats. (b) VEGF-A staining was mostly restricted to the hepatocytes surrounding to the portal tracts. (c) At the high-power magnification, the cytoplasm of hepatocytes close to the portal tract was strongly stained with anti VEGF-A antibody, while weak expression was seen in biliary epithelial cells. (d) In contrast, VEGF-A expression was barely observed in the liver of the sham-operated rats.

found several proteins and humeral factors that might be involved in cholangiocytes proliferation were overexpressed. For instance, choline transporter (X66494), originally cloned from rat spinal cord, is a part of the choline uptake system in the cholinergic nerve terminal and plays a role in synthesis of acetylcholine.[24] Therefore, the up-regulation of choline transporter may be related to the increased synthesis of acetylcholine in the liver, resulting in cholangiocytes proliferation.

Among the differentially expressed genes, we paid particular attention to VEGF-A, as several investigators have recently

reported the potential importance of VEGF in cholangiocytes proliferation and cholestatic diseases.[17,19,20] We found up-regulation of VEGF-A expression in the liver of BDL rats. Molecular regulation of VEGF gene expression has been well investigated.[25,26] VEGF mRNA expression is enhanced by exposure to hypoxia in a variety of pathological conditions.[27] The response to hypoxic circumstances is mediated by the hypoxia-inducible transcription factor (HIF-1), which binds to hypoxia response elements (HRE), located in the promoter/regulatory regions of the VEGF gene.[26] Other inducers of VEGF expression include growth factors (EGF, TGF-α, TGF-β, keratinocyte growth factor, insuline-like growth factor-1, fibroblast growth factor, and platelet-derived growth factor), hormones (thyroid stimulating hormone, adrenocorticotropic hormone, gonadotropins and sex steroids), and specific oncogenic events (mutations of ras, p53, and Wnt-signaling pathway).[25]

In the BDL rats, the up-regulated expression of VEGF-A appears to be mainly located in hepatocytes adjacent to the portal tract (Fig. 5). In general, this area, regarded as Rappaport zone 1, is also contiguous to the entry system and is well supplied with oxygen and nutrient component, compared to zone 3.[28] Moreover, in the BDL rats hepatic arteries were kept intact, and thus oxygen supply to hepatocytes should be maintained even after BDL. Therefore, it is unlikely that low oxygen pressure to the hepatocytes resulted in up-regulated expression of VEGF. Instead, following BDL we detected significant necrosis in this area as well (Fig. 1b). As it was demonstrated that up-regulation of VEGF expression is enhanced in ischemic tumor cells close to hypoxic necrotic foci,[29] it was assumed that following the BDL necrosis in the portal tract occurred, which in turn resulted in the production of humeral factors contributing to up-regulated expression of VEGF-A mRNA in the surrounding hepatocytes.

Another interesting finding in this study is that overexpression of VEGF-A was mainly achieved in hepatocytes, primarily limited to those surrounding to the portal tracts. In the rat with arterial deprivation of the liver, it was bile duct cells that demonstrated the up-regulated expression of VEGF. *In vitro*, VEGF expression under hypoxic circumstances was confirmed in cholangiocytes by immunofluorescence as well as by RT-PCR.[19] In humans, immunohistochemical studies revealed VEGF expression in sinusoidal endothelial cells, not in bile ducts, in the livers of patients with primary biliary cirrhosis.[18] In contrast, in the common BDL model, which was similar to ours, Rosmorduc *et al*. described VEGF expression in more than 95% of hepatocytes at 2 weeks after the procedure.[30] In the current study, however, we demonstrated that up-regulated expression of VEGF-A was limited in hepatocytes surrounding portal tracts by immunohistochemical staining, and was very scarce in the centrilobular area (Fig. 5a). The disparity between these studies is probably due to the difference in oxygen supply to hepatocytes. In the current study, we performed detection of VEGF at day 2 following BDL, and probably oxygen supply was still maintained to hepatocytes as discussed above. Therefore humeral factors produced from the portal tract might play a crucial role in stimulation to adjacent hepatocytes. In contrast, in the study by Rosmorduc *et al.* VEGF detection was found at 2 weeks after BDL, when hypoxic circumstances prevailed, resulting in hypoxia-induced VEGF expression in >95% of hepatocytes.[30] In the current study, the up-regulated expression of VEGF mRNA was transient, almost limited to day 2. This finding suggests that humeral factors that induce VEGF expression might be secreted in a very short period, only at day 2. Although we failed to follow the time course of VEGF mRNA expression after day 7, it would be likely that up-regulation of VEGF was restored around 2 weeks after BDL, as demonstrated by Rosmorduc *et al.*, probably due to hypoxic circumstances in liver.

In conclusion, we performed gene expression profiling in the whole liver of BDL rats, even though the number of genes was limited. Moreover, we demonstrated that the up-regulated expression of VEGF was mainly observed in hepatocytes surrounding to the portal tracts. These results will contribute to the further molecular characterization of the proliferation of bile ducts in cholestatic livers.

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