Gastrointestinal, Hepatobiliary and Pancreatic Pathology

Endothelial to Mesenchymal Transition via Transforming Growth Factor- β 1/Smad Activation Is Associated with Portal Venous Stenosis in Idiopathic Portal Hypertension

Azusa Kitao,^{*,†} Yasunori Sato,^{*}
Seiko Sawada-Kitamura,^{*} Kenichi Harada,^{*}
Motoko Sasaki,^{*} Hiroyasu Morikawa,[‡]
Susumu Shiomi,[§] Masao Honda,[¶] Osamu Matsui,[†]
and Yasuni Nakanuma^{*}

From the Departments of Human Pathology,^{*} Radiology,[†] and Gastroenterology,[¶] Kanazawa University Graduate School of Medicine, Kanazawa; and the Departments of Hepatology,[‡] and Nuclear Medicine,[§] Osaka City University Graduate School of Medicine, Osaka, Japan

Idiopathic portal hypertension (IPH) represents non-cirrhotic portal hypertension of unknown etiology, mainly due to stenosis of peripheral portal veins. This study was performed to clarify the mechanism of portal venous stenosis in IPH from the viewpoint of the contribution of the endothelial to mesenchymal transition of the portal vein endothelium via transforming growth factor- β 1 (TGF- β 1)/Smad activation. *In vitro* experiments using human dermal microvascular endothelial cells demonstrated that TGF- β 1 induced myofibroblastic features in human dermal microvascular endothelial cells, including spindle cell morphology, reduction of CD34 expression, and induction of S100A4, α -smooth muscle actin, and COL1A1 expression, as well as the increased nuclear expression of phospho-Smad2. Bone morphogenic protein-7 preserved the endothelial phenotype of human dermal microvascular endothelial cells. Immunohistochemical analysis showed that endothelial cells of the peripheral portal veins in IPH were characterized by the decreased expression of CD34 and the enhanced nuclear expression of phospho-Smad2; these results also confirmed the expression of S100A4 and COL1A1 in the portal vein endothelium. Serum TGF- β 1 levels in patients with IPH were significantly higher than those of healthy volunteers and patients with chronic viral hepatitis/liver cirrhosis, while an elevation of serum bone morphogenic protein-7 lev-

els was not observed. These results suggest that the endothelial to mesenchymal transition of the portal venous endothelium via TGF- β 1/Smad activation is associated with portal venous stenosis in IPH, and bone morphogenic protein-7 may therefore be a suitable therapeutic candidate for IPH. (*Am J Pathol* 2009, 175:616–626; DOI: 10.2353/ajpath.2009.081061)

Idiopathic portal hypertension (IPH) is a condition of non-cirrhotic presinusoidal portal hypertension of unknown etiology, primarily affecting adults.^{1–3} Clinical presentations of IPH include splenomegaly, pancytopenia, gastroesophageal varices, and subcapsular parenchymal atrophy of the liver.¹ Histologically, phleboscrosis and stenosis of peripheral portal veins associated with dense portal fibrosis are common and characteristic findings of IPH, accounting for portal hypertension due to presinusoidal block.^{4–6} Hepatic function tends to be well preserved even at an advanced disease stage, but hepatic failure can lead to fatal outcome in some patients.⁷ To date, a radical treatment is not available other than liver transplantation,^{8,9} because the mechanism of portal venous stenosis of IPH has not been clarified.

IPH in patients is reported to be complicated with collagen vascular diseases such as systemic sclerosis.^{10,11} Systemic sclerosis is a disease that causes excessive collagen production and deposition, vascular damage, and inflammation in multiple organs including skin, lung, and the gastrointestinal tracts.¹² Patients with

Supported by the Japanese Study Group of Intrahepatic Hemodynamics Alterations (Chairmen: Professor Makoto Hashizume, Kyushu University Graduate School of Medicine, Fukuoka; Professor Fuminori Moriyasu, Tokyo Medical School, Tokyo, Japan).

A.K. and Y.S. contributed equally to this work.

Accepted for publication May 5, 2009.

Address reprint requests to Yasuni Nakanuma, MD, PhD, Department of Human Pathology, Kanazawa University Graduate School of Medicine, 13-1 Takara-machi, Kanazawa 920-8640, Japan. E-mail: pbcpsc@kenroku.kanazawa-u.ac.jp.

the disease show an increased deposition of collagen types I and III in various organs, with type I being the most abundant.¹² Transforming growth factor- β (TGF- β) contributes greatly to the fibrotic processes, and elevation of circulating TGF- β level has been reported in patients with systemic sclerosis.^{13,14} Although excessive collagen deposition in systemic sclerosis appears to be mediated by complex networks of various factors, one possible mechanism of the cutaneous fibrogenesis is that dermal microvascular endothelial cells transform into myofibroblastic cells, thereby contributing to the collagen deposition in the dermis.^{15,16}

Recently, it has been described with increasing frequency that vascular endothelial cells have an ability to acquire matrix-producing myofibroblastic features, providing proof of principle for the process of endothelial to mesenchymal transition (EndMT).^{17–19} EndMT is a phenomenon reported to occur during embryonic cardiovascular development,^{20,21} and also occur under several pathological conditions including cardiac fibrosis and carcinoma-associated interstitial fibrosis.^{17,18} EndMT is a phenotypic conversion characterized by the down-regulation of vascular endothelial markers such as CD31 and von Willebrand factor, and the emergence of myofibroblastic markers such as S100A4/fibroblast-specific protein-1 and α -smooth muscle actin (α -SMA).^{16–18} TGF- β 1 acts as a potent inducer of EndMT both *in vitro* and *in vivo*.^{17,18,20}

TGF- β binds to TGF- β receptor type II (T β R-II), and it recruits the TGF- β receptor type I (T β R-I). T β R-I subsequently phosphorylates Smad2 and Smad3, which form hetero-oligomers with Smad4. They translocate from the cytoplasm to the nucleus, where they regulate transcription of target genes.²² Bone morphogenic protein-7 (BMP7) is a member of the TGF- β superfamily, and a promising TGF- β antagonist.^{23,24} BMP7 binds and activates BMP type II receptor that subsequently form complex with BMP receptor type IA. The receptors activated by BMP7 phosphorylate Smad1, 5 and 8, which counteract Smad2/3 phosphorylation by TGF- β . Indeed, BMP7 has been shown to inhibit EndMT in a mouse model of cardiac fibrosis.¹⁷

In most types of chronic liver disease, hepatic fibrosis is mediated by myofibroblasts, which generally originate from hepatic stellate cells. Portal fibroblasts and bone-marrow derived fibrocytes are other candidates of cell types of myofibroblast precursor in the liver.²⁵ Given the facts that systemic sclerosis is a clinical complication of IPH, and EndMT is one of the possible causative mechanism of excessive collagen deposition in systemic sclerosis, it is plausible that, in IPH, stenosis of peripheral portal veins with dense collagenous fibrosis around them is mediated by EndMT of the endothelial cells of portal vein, and the endothelial cell may be another contributor of the portal fibrosis.

To clarify this, the involvement of EndMT in portal venous stenosis of IPH was examined by the use of microvascular endothelial cells *in vitro*, and by means of histological analysis using liver tissue sections of IPH. Measurement of the serum TGF- β 1 and BMP7

levels was also performed using samples obtained from IPH patients.

Materials and Methods

This human study was performed with the approval of the ethics committee of Kanazawa University Graduate School of Medicine.

Cell Culture

Human dermal microvascular endothelial cells (HMVEC) were purchased from Cell Applications, Inc. (San Diego, CA), and were maintained with endothelial growth medium (CADMEC growth medium, Cell Applications, Inc.). HMVECs were then treated with either TGF- β 1 (10 ng/ml; R&D systems, Inc., Minneapolis, MN) alone or in combination with BMP7 (up to 100 ng/ml; R&D systems, Inc.) for 5 days, and phenotypic changes of HMVECs were examined as described below. Experiments were conducted with HMVECs at passages 2 to 4.

Reverse Transcription-PCR and Quantitative Real-time PCR

Reverse transcriptase (RT) PCR was performed using total RNA (1 μ g) extracted from HMVECs. Total RNA was extracted using an RNA extraction kit (RNeasy mini; Qiagen, Tokyo, Japan) and was used to synthesize cDNA with reverse transcriptase (ReverTra Ace; Toyobo Co., Osaka, Japan). The sequences of the primers and conditions for PCR used are shown in Table 1. The PCR products were subjected to 2% agarose gel electrophoresis and stained with ethidium bromide.

Quantitative real-time PCR was performed according to a standard protocol using the SYBR Green PCR Master Mix (Toyobo Co.) and ABI Prism 7700 Sequence Detection System (PE Applied Biosystems, Warrington, UK). Cycling conditions were incubation at 50°C for 2 minutes, 95°C for 10 minutes, and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Fold difference compared with glyceraldehyde-3-phosphate dehydrogenase expression was calculated.

Western Blot Analysis

Total proteins were extracted from HMVEC using T-PER protein extraction reagent (Pierce Chemical Co., Rockford, IL). First, 5 μ g of the protein was subjected to 10% SDS-polyacrylamide gel electrophoresis, and then electrophoretically transferred on to a nitrocellulose membrane. The membrane was incubated with primary antibodies against CD31 (1:200, JC70A, mouse monoclonal; DakoCytomation, Glostrup, Denmark), CD34 (1:250, QBEND10, mouse monoclonal; Immunotech, Marseilles, France), S100A4 (1:200, rabbit polyclonal; Abcam Inc., Cambridge, MA), α -SMA (1:200, 1A4, mouse monoclonal; DakoCytomation), pro-COL1A1 (1:200, goat polyclonal, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and actin (1:3000, AC-15, mouse monoclonal; Abcam

Table 1. Sequences of the Primers and PCR Conditions Used in this Study

Gene	Sequences	Annealing temperature (°C)	PCR cycles	Product size (bp)
<i>TβR-I</i>	5'-GGCCATTTACTGGAATGAG-3' 5'-GGCTTAGAAATGGCCAAAA-3'	55	40	386
<i>TβR-II</i>	5'-GTGGAGACACTTACAAAGCT-3' 5'-GAAACTTGGGCTAACTGAGA-3'	55	40	250
<i>BMP-RIA</i>	5'-GGTTTCATAGCGGCAGACAT-3' 5'-CTTTCCTTGGGTGCCATAAA-3'	55	40	198
<i>BMP-RII</i>	5'-GCTAAAATTTGGCAGCAAGC-3' 5'-CTTGGGCCCTATGTGTCCT-3'	55	40	224
<i>CD31</i>	5'-CCTGCTAAGTTAATGTTGGG-3' 5'-GAAAAAACACGCTCTAACC-3'	62	40	269
<i>CD34</i>	5'-CCAATCTGACCTGAAAAAGC-3' 5'-CCACCGTTTCCGTGAATA-3'	55	30	216
<i>S100A4</i>	5'-GGTGACAAGTTCAAGCTCAA-3' 5'-CTGGGAAGCCTTCAAAGAAT-3'	55	40	214
<i>α-SMA</i>	5'-GAAATGAACGTTTCCGCTGC-3' 5'-CAGACAGAGTATTGCGCTC-3'	62	40	268
<i>COL1A1</i>	5'-GTCTTCTGCAACATGGAGAC-3' 5'-CAGTGGTAGGTGATGTTCTG-3'	62	40	245
<i>GAPDH</i>	5'-GAGTCAACGATTGGTCTG-3' 5'-TTGATTTTGGAGGATCTC-3'	55	30	240

Inc.). The protein expression was detected using an EnVision+ system (DakoCytomation) and a HISTOFINE system (Nichirei, Tokyo, Japan). 3,3'-diaminobenzidine tetrahydrochloride was used as the chromogen. Semiquantitative analysis of the results was performed using NIH J image software (National institutes of Health, Bethesda, MD). The fold difference compared with actin expression was calculated.

Immunocytochemistry

HMVECs were fixed with 4% paraformaldehyde for 15 minutes, and permeabilized for 3 minutes with 0.1% Triton X-100. After blocking, the cells were incubated for 1 hour at room temperature with primary antibodies against CD34 (1:200, Immunotech), S100A4 (1:100, Abcam Inc.), α-SMA (DakoCytomation), pro-COL1A1 (1:100, goat polyclonal, Santa Cruz Biotechnology, Inc.), and phospho-Smad2 (pSmad2) (1:100, Ser465/467, rabbit polyclonal; Cell Signaling Technology, Inc., Danvers, MA). The protein expression was detected using the alkaline phosphatase-labeled polymer, the HISTOFINE system (Nichirei). Color development was performed using the Vector Red alkaline phosphatase substrate kit (Vector Laboratories, Burlingame, CA), and nuclei were stained with 4'-6'-diamidino-2-phenylindole. The signals were detected under immunofluorescence confocal microscopy.

Liver Specimens

A total of 44 liver specimens were used. Twenty-four specimens corresponded to IPH. Clinicopathological features of IPH cases were summarized in Table 2. Both liver biopsy and autopsy materials were included. The IPH livers of autopsy cases were collected as previously described, and all autopsy livers were obtained at an advanced disease stage corresponding to Stage III or Stage IV.^{1,7} Histology of the liver confirmed the diagnosis of IPH, and five cases corresponded to incomplete septal cirrhosis. Complication of systemic sclerosis was observed in three cases.

As controls, liver specimens obtained from patients with chronic viral hepatitis/liver cirrhosis (CVH/LC) (*n* = 10), and histologically normal livers (NL) (*n* = 10) were used. The causes of CVH/LC were viral infection of hepatitis B (*n* = 2) and hepatitis C (*n* = 8). NL specimens were obtained from patients undergoing a partial hepatectomy for the diseases other than hepatobiliary disorders such as metastatic colon cancer, and macroscopically and microscopically normal areas were used.

Immunohistochemistry

Liver specimens were fixed with neutral formalin, and 4-μm thick paraffin-embedded tissue sections were pre-

Table 2. Clinicopathological Features of IPH Cases Used for Histological Analysis

N	Age (years)	Sex (male:female)	Specimen (biopsy:autopsy)	Splenomegaly	Esophageal varices	Liver weight* (g)	Splenic weight* (g)	Liver fibrosis†	Systemic sclerosis
24	59 ± 20	7:17	6:18	22(92%)	17(77%)	779 ± 293	378 ± 176	5(21%)	3(13%)

*Liver and splenic weight were the value of autopsy cases.
†Liver fibrosis corresponded to incomplete septal cirrhosis.

pared. Immunostaining was performed using primary antibodies against T β R-I (sc-398, rabbit polyclonal; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), T β R-II (sc-220, rabbit polyclonal; Santa Cruz Biotechnology, Inc.), CD34 (Immunotech), and pSmad2 (Cell Signaling Technology, Inc.). After deparaffinization, antigen retrieval was performed by incubating with 1 mg/ml of trypsin for 10 minutes at 37°C for T β R-I and T β R-II staining, and by incubating with 20 mg/ml of proteinase K for 6 minutes at room temperature for pSmad2 staining. To block the activity of endogenous peroxidase, sections were immersed in 0.3% hydrogen peroxidase in methanol for 20 minutes at room temperature. After pretreatment with blocking serum (DakoCytomation), sections were incubated overnight at 4°C with individual primary antibodies; T β R-I (1:50), T β R-II (1:50), CD34 (1:200), and pSmad2 (1:100). Then sections were incubated with secondary antibodies conjugated to peroxidase-labeled polymer, using the EnVision+ system (DakoCytomation). Color development was performed using 3,3'-diaminobenzidine tetrahydrochloride and the sections were slightly counterstained with hematoxylin. Negative controls were done by substitution of the primary antibodies with nonimmunized serum, resulted in no signal detection.

Double Immunofluorescence Staining

Double immunofluorescence staining of CD34 and S100A4, CD34 and α -SMA, and CD34 and COL1A1 was performed for the liver sections. Deparaffinized sections were incubated 1 hour at room temperature with the anti-CD34 antibody (1:200, Immunotech). The sections were incubated with secondary antibodies conjugated to alkaline phosphatase-labeled polymer, the HISTOFINE system (Nichirei). Color development was performed using the Vector Red alkaline phosphatase substrate kit (Vector Laboratories, Burlingame, CA). Then, antigen retrieval was performed by incubating with 20 mg/ml of proteinase K for 6 minutes at room temperature for the staining of S100A4. After microwaving in 10 mmol/L citrate buffer pH 6.0 for 10 minutes, the sections were incubated overnight at 4°C with the anti-S100A4 antibody (1:100, Abcam Inc.), the anti- α -SMA antibody (1:200, DakoCytomation), and the anti-pro COL1A1 antibody (1:100, Santa Cruz Biotechnology, Inc.). Alexa Fluor 488 (10 μ g/ml; Molecular Probes, Eugene, OR) was used as a secondary antibody. Nuclei were stained with 4',6-diamidino-2-phenylindole, and the sections were observed under immunofluorescence confocal microscopy.

Histological Assessment

Semiquantitative analysis was performed for the sections stained with the anti-CD34 antibody. In each section, a total of 20 peripheral portal tracts were randomly selected. For liver biopsy specimens, all portal tracts in the specimen were evaluated, because they usually did not contain 20 portal tracts. As described later, the endothelial cells of peripheral portal vein of IPH frequently showed reduced immuno-expression of CD34. The CD34

signal intensity of the endothelial cells was compared between the portal vein and the escorting hepatic artery in the same portal tract. The CD34 signal intensity was regarded as being reduced when less CD34 expression was observed in more than 2/3 circumference of one portal vein. The percentage of the number of portal veins with reduced CD34 expression was calculated in each case, and was defined as CD34-reduction index. The histological assessment was performed by two independent investigators (A.K. and Y.S.).

For immunostained sections of pSmad2, a total of 100 nuclei of the endothelial cells of peripheral portal vein were randomly selected in each section. The percentage of the endothelial cells positive for pSmad2 was determined, and was defined as pSmad2-labeling index. For the determination of pSmad2 expression in HMVECs, a total of 100 nuclei of HMVECs immunostained with the anti-pSmad2 antibody were evaluated, and pSmad2-labeling index was calculated in the same manner.

Enzyme-Linked Immunosorbent Assay

The serum TGF- β 1 and BMP7 levels of 66 samples obtained from 57 IPH patients were determined using enzyme-linked immunosorbent assay kits (Quantikine Human TGF- β 1 Immunoassay and Quantikine Human BMP7 Immunoassay; R&D Systems, Inc.) according to the manufacturer's instructions. As controls, serum samples obtained from 16 healthy volunteers and 19 patients with CVH/LC (hepatitis B, $n = 9$; hepatitis C, $n = 10$) were used. Samples were added to a 96-well plate coated with a monoclonal antibody for TGF- β 1 or BMP7, and incubated for 2 hours at room temperature. After washing, the plate was incubated with anti-TGF- β 1 or anti-BMP7 antibody conjugated to horseradish peroxidase for 2 hours at room temperature. Color development was performed using a substrate solution for 30 minutes and the absorbance at 450 nm was measured.

Statistics

The data were expressed as the mean $\pm$ SD. Statistical significance was determined using the Mann-Whitney U -test and the Pearson correlation test. A P value less than 0.05 was accepted as the level of statistical significance.

Results

Effects of TGF- β 1 and BMP7 on Cellular Phenotype of HMVECs

RT-PCR analysis showed that HMVECs expressed receptors for TGF- β (T β R-I, T β R-II) and BMP7 (BMP receptor type IA, BMP type II receptor) (Figure 1A). In this study, CD31 and CD34 were used as markers of vascular endothelial cells, and S100A4 and α -SMA as markers of myofibroblastic cells. Treatment of HMVECs with TGF- β 1 significantly reduced the expression of CD34 mRNA in HMVECs, and induced mRNA expression of S100A4,

Figure 1. Effects of TGF- β 1 and BMP7 on cellular phenotype of HMVECs at the mRNA level. HMVECs were treated with TGF- β 1 (10 ng/ml) alone or in combination with BMP7 (100 ng/ml) for 5 days, and phenotypic changes were examined using RT-PCR as described in the *Materials and Methods*. HMVECs expressed receptors for TGF- β (T β R-I, T β R-II) and BMP7 (BMP receptor type IA, BMP type II receptor) (A). RT-PCR (B) and real-time PCR (C) showed that treatment of HMVECs with TGF- β 1 reduced the expression of CD34 mRNA, and induced the mRNA expression of S100A4, α -SMA, and COL1A1. All of these changes of HMVECs following TGF- β 1 treatment were inhibited by the addition of BMP7 in the culture medium (B, C). The data represent three independent experiments (A, B), and the mean $\pm$ SD of six per group (C). * P < 0.01; ** P < 0.05.

α -SMA, and COL1A1, while mRNA expression of CD31 was unchanged (Figure 1, B and C). Western blot analysis showed that proteins of the molecules showed similar changes to those of mRNA following TGF- β 1 treatment, and semiquantitative analysis of the Western blotting confirmed this tendency (Figure 2, A and B). All of these phenotypic changes of HMVECs following TGF- β 1 treatment were blocked by the addition of BMP7 in the culture medium (Figures 1, B and C, and 2, A and B). In addition, BMP7 reduced TGF- β 1-induced COL1A1 expression in HMVEC in a dose-dependent fashion (Figure 2C).

Morphological and Phenotypic Alterations of HMVEC by TGF- β 1 and BMP7

In the endothelial growth medium, HMVEC grew in a form of epithelioid, sheet-like appearance under the phase-contrast microscopy. Following 5-day treatment with

TGF- β 1, the cellular morphology of HMVEC changed from epithelioid into spindle-shaped appearance (Figure 3). Immunocytochemistry showed that the spindle-shaped HMVEC following TGF- β 1 treatment exhibited reduced expression of CD34, and increased expression of S100A4, α -SMA, and COL1A1 (Figure 3), which were consistent with the results of RT-PCR and Western blot analysis. TGF- β 1 treatment increased the expression of pSmad2 in the nuclei of HMVECs (Figure 3), and the percentage of HMVECs positive for pSmad2 were significantly increased from $8.2 \pm 6.3\%$ to $52.7 \pm 18.3\%$ following the treatment. Again, the addition of BMP7 in the culture medium inhibited the morphological and phenotypic conversion of HMVECs by TGF- β 1 (Figure 3). These results indicated that TGF- β 1 could induce myofibroblastic features in HMVECs, and BMP7 antagonized the effects of TGF- β 1.

Reduction of CD34 Expression in Portal Vein Endothelium of IPH Livers

Immunostaining of liver sections demonstrated that both T β R-I and T β R-II were diffusely expressed in the liver including portal vein endothelium of IPH (Figure 4A), as well as NL and CVH/LC. Immunohistochemical expression of CD34 was observed in the endothelial cells of portal vein, hepatic artery, and hepatic vein in all liver specimens without exceptions. The signal intensity of CD34 immunostaining was almost equal among these vessels in NL (Figure 4B). In IPH, reduction of CD34 expression in the endothelial cells of peripheral portal vein was frequently observed when compared with those of the escorting hepatic artery in the same portal tract (Figure 4B). The reduction of CD34 expression was observed in peripheral portal veins of IPH regardless of the presence or absence of luminal narrowing. In CVH/LC, CD34 expression in the portal endothelial cells tended to be preserved in most of the cases (Figure 4B), but several cases showed reduction of CD34 expression. Semiquantitative analysis of the results of CD34 immunostaining was performed as described in the *Materials and Methods*. As shown in Figure 4C, the CD34-reduction index was significantly increased in IPH, when compared with that of NL and CVH/LC.

Enhanced Expression of pSmad2 in IPH Livers

Positive immunostaining for pSmad2 were rarely seen in sections of NL (Figure 4B). By contrast, many IPH cases showed diffuse and strong nuclear expression of pSmad2 throughout the liver, including portal vein endothelium, hepatocytes, and biliary epithelial cells (Figure 4B). Several cases of CVH/LC showed positive immunohistochemical signals of pSmad2 in the liver, but most of the cases lacked its expression (Figure 4B).

Semiquantitative analysis of the immunohistochemical results showed that the expression of pSmad2 was negligible in NL, whereas IPH livers showed a high incidence of pSmad2 expression in portal vein endothelium ($73.8 \pm 25.6\%$) (Figure 4D). In CVH/LC, the pSmad2-labeling

Figure 2. Effects of TGF-β1 and BMP7 on cellular phenotype of HMVECs at the protein level. HMVECs were treated with TGF-β1 (10 ng/ml) alone or in combination with BMP7 (100 ng/ml) for 5 days, and phenotypic changes were examined by Western blotting using protein extracts from HMVEC. Consistent with the RT-PCR results, TGF-β1 reduced the expression of CD34 protein, and induced the protein expression of S100A4, α-SMA and COL1A1, and the addition of BMP7 inhibited these phenotypic changes (A). Semiquantitative analysis of the Western blotting confirmed this tendency (B). BMP7 inhibited TGF-β1-induced COL1A1 protein expression in a dose-dependent manner (C). The data represent five independent experiments (A), and the mean ± SD of five per group (B, C). **P* < 0.01; ***P* < 0.05.

index was $12.4 \pm 4.0\%$, and there was a statistically significant difference between the index of IPH and CVH/LC groups. This study examined three cases of systemic sclerosis complicated with IPH. Interestingly, these cases of systemic sclerosis exhibited high value of the pSmad2-labeling index as well as the CD34-reduction index (Figure 4, C and D, data indicated by white circles). When the pSmad2-labeling index was plotted against the CD34-reduction index for all 44 cases, they showed a fine liner correlation (Figure 4E), suggesting a causal relationship between the enhanced expression of pSmad2 and the reduced expression of CD34 in portal vein endothelium.

In this study, liver specimens of IPH included both liver biopsy and autopsy materials. Because all autopsy livers of IPH were obtained at an advanced disease stage, the specimens of liver biopsy might reflect

pathological changes at an earlier disease stage of IPH, when compared with those of autopsy livers. In autopsy IPH livers, the CD34-reduction index and the pSmad2-labeling index were $68.3 \pm 19.9\%$ and $67.9 \pm 26.9\%$, respectively, while those of biopsy livers were $69.2 \pm 9.7\%$ and $91.8 \pm 6.7\%$, respectively. These results indicated that the occurrence of reduction of CD34 expression and induction of pSmad2 expression in the portal vein endothelium was not a phenomenon limited to the end stage of the disease, and might be closely associated with the disease progression.

Colocalization of CD34 and Mesenchymal Markers in Portal Vein Endothelium of IPH Livers

Co-expression of CD34 and S100A4 was observed in portal vein endothelium of IPH (Figure 5, arrow), but the

Figure 3. Morphological and phenotypic alterations of HMVECs by TGF-β1 and BMP7. HMVECs grew in a form of epithelioid, sheet-like appearance under the phase-contrast microscopy, and a 5-day treatment with TGF-β1 (10 ng/ml) changed the cellular morphology of HMVECs from epithelioid into spindle-shaped appearance. Immunostaining showed that the spindle-shaped HMVECs following TGF-β1 treatment showed reduced expression of CD34, and increased expression of S100A4, α-SMA, COL1A1, and pSmad2. Addition of BMP7 (100 ng/ml) in the culture medium inhibited the phenotypic changes of HMVECs by TGF-β1. The protein expression was visualized by a Vector Red reaction under immunofluorescence confocal microscopy, and nuclei were stained with 4',6-diamidino-2-phenylindole (blue). Original magnification $\times 1000$.

Figure 4. Reduction of CD34 expression and induction of pSmad2 expression in portal vein endothelium of IPH livers. Immunostaining of TGF- β receptors (T β R-I, T β R-II), CD34 and pSmad2 was performed for liver sections of normal liver (NL), chronic viral hepatitis/liver cirrhosis (CVH/LC) and idiopathic portal hypertension (IPH). T β R-I and T β R-II were diffusely expressed in the portal vein endothelium of IPH (**A**), as well as NL and CVH/LC. Endothelial cells of peripheral portal vein of IPH showed reduced expression of CD34, and increased expression of pSmad2 (**B**). The CD34-reduction index and the pSmad2-labeling index of portal vein endothelium were determined as described in the *Materials and Methods* section. Portal vein endothelium of IPH showed a significant reduction of CD34 expression (**C**) and a significant induction of pSmad2 (**D**) when compared with those of NL and CVH/LC. The data of white circles represent those from the cases of IPH complicated with systemic sclerosis (**C**, **D**). The CD34-reduction index and the pSmad2-labeling index showed a fine linear correlation (**E**). **Arrowheads** indicate nuclei of pSmad2-positive portal vein endothelium (**B**). HA, hepatic artery. PV, portal vein. White bars = 50 μ m; black bars = 20 μ m. * P < 0.01.

expression was limited in a small number of portal veins. Portal vein endothelium of NL and CVH/LC lacked double-positive signals of CD34 and S100A4 (Figure 5). Co-expression of CD34 and α -SMA was rarely seen in all experimental groups (data not shown). Double immunofluorescence staining of CD34 and COL1A1 showed that portal vein endothelium of IPH occasionally co-expressed CD34 and COL1A1 (Figure 5, arrowheads), and the portal veins showing double-positive signals irregularly distributed in an individual liver independently of the presence or absence of luminal narrowing. Portal vein endothelium of NL and CVH/LC typically lacked such double-positive signals (Figure 5).

Elevation of Circulating TGF- β 1 Level in IPH

The serum TGF- β 1 and BMP7 levels of 66 samples obtained from 57 IPH patients, 16 healthy volunteers, and 19 patients with CVH/LC were determined using an enzyme-linked immunosorbent assay. The serum TGF- β 1 level of healthy controls, CVH/LC, and IPH were 40.3 ± 17.6 ng/ml, 29.3 ± 10.8 ng/ml, and 53.0 ± 23.4 ng/ml, respectively, and the TGF- β 1 level in IPH patients was significantly higher than the value of the other two groups (Figure 6A). While the serum BMP7 level of healthy controls, CVH/LC, and IPH were 5.0 ± 2.4 pg/ml, 22.5 ± 12.4 pg/ml, and 6.6 ± 5.5 pg/ml, respectively, and the serum

Figure 5. Colocalization of CD34 and mesenchymal markers in portal vein endothelium of IPH livers. Double immunofluorescence staining of CD34 and S100A4 protein, and CD34 and COL1A1 was performed for liver sections of normal liver (NL), chronic viral hepatitis/liver cirrhosis (CVH/LC), and idiopathic portal hypertension (IPH). Figures shown were merged images in which the expression of CD34 was colored in red, and the expression of S100A4 and COL1A1 was colored in green. Nuclei were stained with 4',6-diamidino-2-phenylindole. Coexpression of CD34 and S100A4 was observed in portal vein endothelium of IPH (arrow), but the expression was limited in a small number of portal veins of IPH. Portal vein endothelium of NL and CVH/LC typically lacked double-positive signals of CD34 and S100A4. Portal vein endothelium of IPH occasionally showed colocalization of CD34 and COL1A1 (arrowheads). PV, portal vein. White bars = 30 μ m.

BMP7 level in patients with CVH/LC showed a significant increase compared with those of the other two groups (Figure 6B). When the serum TGF- β 1 level was plotted against the serum BMP7 level for all cases examined, a significant inverse correlation was observed between them (Figure 6C).

Discussion

In this study, TGF- β 1 induced phenotypic conversion of HMVECs into collagen-producing myofibroblast-like cells,

and BMP7 preserved the endothelial phenotype. *In vivo*, endothelial cells of peripheral portal vein of IPH were characterized by the decreased expression of CD34, and the enhanced expression of pSmad2 and COL1A1. Importantly, the serum TGF- β 1 level of IPH patients was significantly elevated when compared with the value of the healthy controls and CVH/LC. These results suggest that EndMT of the portal vein endothelium via TGF- β 1/Smad activation is closely associated with the pathogenesis of portal venous stenosis of IPH. Our hypothesis on the mechanism of portal venous stenosis of IPH is illustrated in Figure 7.

Figure 6. Elevation of circulating TGF- β 1 levels in IPH. The serum TGF- β 1 and BMP7 levels of 66 samples obtained from 57 patients with idiopathic portal hypertension (IPH), 16 healthy volunteers, and 19 patients with chronic viral hepatitis/liver cirrhosis (CVH/LC) were determined using an enzyme-linked immunosorbent assay. Serum obtained from IPH patients contained a significantly high level of TGF- β 1 when compared with that of healthy controls and CVH/LC (A), while the serum BMP7 level in patients with CVH/LC showed a significant increase compared with those of the other two groups (B). When the serum TGF- β 1 level was plotted against the serum BMP7 level for all cases examined, a significant inverse correlation was observed between them (C). * P < 0.01; ** P < 0.05.

Figure 7. Proposed mechanism of the portal venous stenosis of IPH. TGF- β 1 induces endothelial to mesenchymal transition (EndMT) of endothelial cells of peripheral portal vein of idiopathic portal hypertension (IPH). Endothelial cells acquire myofibroblastic features via Smad activation, and produce extracellular matrix molecules including collagen. Collagen depositions in peripheral portal tracts compress the portal veins, resulting in portal venous stenosis and presinusoidal portal hypertension. BMP7 may act as an inhibitor of EndMT by antagonizing the effects of TGF- β 1.

Dense portal fibrosis with luminal narrowing of peripheral portal veins is a common histological hallmark of IPH.¹ Parenchymal fibrosis, such as pericellular fibrosis and slender fibrous septa from the portal tracts, leading to incomplete septal cirrhosis, is also observed in several cases of IPH.¹ Because α -SMA-positive activated hepatic stellate cells are focally found in perisinusoidal area of IPH livers, parenchymal fibrosis may be explainable by the contribution of myofibroblasts.^{1,5} However, α -SMA-positive myofibroblast-like cells are rarely seen in the peripheral portal tracts of IPH,^{5,26} suggesting that other matrix-producing cells may exist in the portal tracts.

Since EndMT has been implicated in dermal fibrosis in patients with systemic sclerosis, a possible clinical manifestation of IPH,^{10,15,16} we focused on EndMT of the portal vein endothelium as a mechanism of portal venous stenosis of IPH. *In vitro*, it has been reported that vascular endothelial cells acquire myofibroblastic features, such as an increase in S100A4/ fibroblast-specific protein-1, α -SMA and type I collagen expression in response to TGF- β 1, which is accompanied by the reduced expression of vascular endothelial markers, CD31 and von Willebrand factor.^{17,18} In fact, this study confirmed that TGF- β 1 reduced the expression of CD34, and induced S100A4, α -SMA, and COL1A1 expression in HMVEC, which were accompanied by the increased nuclear labeling of pSmad2. In addition, portal vein endothelium of IPH showed the reduced CD34 expression and the increased nuclear expression of pSmad2. Although a question remains whether HMVEC and portal vein endothelium share the same endothelial phenotype, our data indicate that EndMT via TGF- β 1/Smad activation is a possible mechanism of portal venous stenosis of IPH.

TGF- β 1 is known to activate hepatic stellate cells, which in turn acquire myofibroblastic features and produce extracellular matrix proteins. This transition is a central process in LC due to various causes. However, no cirrhosis occurs in IPH despite the facts that activation of hepatic stellate cells occurs in focal areas of IPH livers, and incomplete septal cirrhosis is regarded as a late manifestation of IPH.^{1,5} In systemic sclerosis, dermal fibroblasts shows hyperresponsiveness to TGF- β 1, and the deficient expression of Smad7, an inhibitory Smad, may be responsible for the TGF- β hyperresponsiveness.²⁷ In addition, Smad6, another inhibitory Smad, is not expressed equally among cell types consisting the

liver.²⁸ From these results, it is suggested that the responsiveness to TGF- β 1 is different between portal vein endothelium and hepatic stellate cells of IPH; ie, portal vein endothelium of IPH may be hyperresponsive to TGF- β 1 when compared with that of hepatic stellate cells, which may be due to the abnormalities of the expression of inhibitory Smads, accounting for the fact that no cirrhosis occurs in IPH.

In this study, only a small fraction of portal endothelial cells of IPH exhibited transformed features in pathological examinations. Similarly, previous studies have shown the percentage of fibroblast-specific protein-1/CD31 double-positive cells remained in 3% of total cells in a mouse model of cardiac fibrosis, although nuclear staining of pSmad2/3 was present in 30% of endothelial cells.¹⁷ These results indicate that several but not all of the endothelial cells with positive nuclear expression of pSmad2 or pSmad2/3 undergo phenotypic changes into myofibroblast-like cells, which may lead to the uneven distribution of stenotic portal tracts in the liver of IPH. However, a precise mechanism remains to be studied. In addition, the induction of nuclear pSmad2 expression and the reduction of CD34 expression were observed even in peripheral portal veins without luminal narrowing of IPH. We speculate that these portal veins will gradually show luminal narrowing along with the collagen deposition around the portal veins, and these changes may slowly and disproportionately progress in the IPH liver.

Colocalization of CD34 and α -SMA was rarely seen in the portal vein endothelium of IPH. These observations were consistent with the results of previous studies regarding EndMT, showing that the occurrence of colocalization of CD31 and α -SMA in the vascular endothelium was a rare event *in vivo*, when compared with the frequency of the occurrence of colocalization of CD31 and S100A4/ fibroblast-specific protein-1.¹⁸ Therefore, vascular endothelial cells may be able to acquire the features of myofibroblasts *in vitro*, but they do not necessarily differentiate into myofibroblasts themselves *in vivo*.

The elevated serum TGF- β 1 level in IPH raises a question as to the cellular sources of TGF- β 1. In the liver, hepatic stellate cells, macrophages, hepatocytes, and bile duct epithelial cells are candidates of cell types that produce TGF- β 1.^{29–31} The spleen is an organ that closely associates with the disease pathogenesis of IPH, and macrophages have been shown to produce TGF- β 1 in

the spleen.^{32,33} In cases of systemic sclerosis, myofibroblasts, fibroblasts, vascular endothelial cells, macrophages, lymphocytes, and platelets are potential sources of TGF- β 1.^{34–37} To determine cellular sources of TGF- β 1 in IPH, further study is necessary. Also, the cellular sources of BMP7 are of interest, especially in patients with CVH/LC.

In IPH livers, hepatocytes, as well as portal vein endothelium, showed diffuse and strong immuno-expression of pSmad2, which probably reflected the elevation of the serum TGF- β 1 level. In addition to its contribution to hepatic fibrosis, TGF- β 1 has an effect of growth inhibition or apoptosis induction on hepatocytes.^{28,38} Therefore, hepatic parenchymal atrophy frequently seen in IPH patients at an advanced disease stage may associate with the growth inhibitory effects of TGF- β 1 on hepatocytes, as well as the circulatory disturbance of the liver.

TGF- β induces connective tissue growth factor (CTGF) in various systems, and hepatic stellate cells are the major cellular sources of CTGF in the liver during liver fibrogenesis.³⁹ In patients with IPH, the serum CTGF level is significantly elevated than the value in healthy volunteers, and overexpression of CTGF seems to be one of the most important features of IPH.⁴⁰ The major cellular sources of CTGF in the liver of IPH has been shown to be periductal mononuclear cells, but hepatic stellate cells of IPH livers lack CTGF expression.^{5,40} In addition to CTGF, our data indicate that TGF- β is another novel factor involved in the pathogenesis of IPH.

From the view point of therapeutic interventions, it is important to note that BMP7 had striking effects on the preservation of endothelial phenotype. The inverse correlation between serum TGF- β 1 and BMP7 levels in this study suggests a possibility that TGF- β 1 and BMP7 may have an antagonistic effect on their expression to each other. Although the inverse correlation between serum TGF- β 1 and BMP7 has not been recognized in the previous literature and the mechanism remains unknown, there is a possibility that the administration of BMP7 in patients with IPH may improve the elevation of serum TGF- β 1 level, and BMP7 can be a candidate of therapeutic agents for IPH.

In conclusion, the present study demonstrated an involvement of TGF- β 1 in the pathogenesis of IPH. Although the mechanism of deposition of extracellular matrix proteins other than collagen such as elastin in the peripheral portal tracts is not fully understood,⁴¹ one plausible mechanism of the portal venous stenosis of IPH is due to excessive collagen deposition via EndMT of portal vein endothelium in response to TGF- β 1. Our data indicate that TGF- β is a potential target of therapy of IPH, and BMP7 can be a possible therapeutic agent.

References

1. Nakanuma Y, Tsuneyama K, Ohbu M, Katayanagi K: Pathology and pathogenesis of idiopathic portal hypertension with an emphasis of the liver: *Pathol Res Pract* 2001, 197:65–76
2. Dhiman RK, Chawla Y, Vasishta RK, Kakkar N, Dilawari JB, Trehan MS, Puri P, Mitra SK, Suri S: Non-cirrhotic portal fibrosis (idiopathic portal hypertension): experience with 151 patients and review of the literature. *J Gastroenterol Hepatol* 2002, 17:6–16
3. Okudaira M, Ohbu M, Okuda K: Idiopathic portal hypertension and its pathology: *Semin Liver Dis* 1996, 22:59–72
4. Tsuneyama K, Ohba K, Zen Y, Sato Y, Niwa H, Minato H, Nakanuma Y: A comparative histological and morphometric study of vascular changes in idiopathic portal hypertension and alcoholic fibrosis/cirrhosis. *Histopathology* 2003, 43:55–61
5. Tsuneyama K, Kouda W, Nakanuma Y: Portal and parenchymal alterations of the liver in idiopathic portal hypertension: a histological and immunohistochemical study. *Pathol Res Pract* 2002, 198: 597–603
6. Nakanuma Y, Hosono M, Sasaki M, Terada T, Katayanagi K, Nonomura A, Kurumaya H, Harada A, Obata H: Histopathology of the liver in non-cirrhotic portal hypertension of unknown aetiology. *Histopathology* 1996, 28:195–204
7. Sawada S, Sato Y, Aoyama H, Harada K, Nakanuma Y: Pathological study of idiopathic portal hypertension with an emphasis on cause of death based on records of Annuals of Pathological Autopsy Cases in Japan. *J Gastroenterol Hepatol* 2007, 22:204–209
8. Dumortier J, Bizollon T, Scoazec JY, Chevallier M, Bancel B, Berger F, Ducer C, Claudel-Bonvoisin S, Paliard P, Boillot O, Trepo C: Orthotopic liver transplantation for idiopathic portal hypertension: indications and outcome. *Scand J Gastroenterol* 2001, 36:417–422
9. Krasinskas AM, Egtesad B, Kamath PS, Demetris AJ, Abraham SC: Liver transplantation for severe intrahepatic noncirrhotic portal hypertension. *Liver Transpl* 2005, 11:627–634
10. Tsuneyama K, Harada K, Katayanagi K, Watanabe K, Kurumaya H, Minato H, Nakanuma Y: Overlap of idiopathic portal hypertension and scleroderma: report of two autopsy cases and a review of literature. *J Gastroenterol Hepatol* 2002, 17:217–223
11. Saito K, Nakanuma Y, Takegoshi K, Ohta G, Obata Y, Okuda K, Kameda H: Non-specific immunological abnormalities and association of autoimmune diseases in idiopathic portal hypertension. A study by questionnaire. *Hepatogastroenterology* 1993, 40:163–166
12. Charles C, Clements P, Furst DE: Systemic sclerosis: hypothesis-driven treatment strategies. *Lancet* 2006, 367:1683–1691
13. Verrecchia F, Mauviel A, Farge D: Transforming growth factor-beta signaling through the Smad proteins: role in systemic sclerosis. *Autoimmun Rev* 2006, 5:563–569
14. Snowden N, Coupes B, Herrick K, Illingworth K, Jayson MV, Brenchley PEC: Plasma TGF beta in systemic sclerosis: a cross-sectional study. *Ann Rheum Dis* 1994, 53:763–767
15. Karasek MA: Does transformation of microvascular endothelial cells into myofibroblasts play a key role in the etiology and pathology of fibrotic disease? *Med Hypotheses* 2007, 68:650–655
16. Chaudhuri V, Zhou L, Karasek M: Inflammatory cytokines induce the transformation of human dermal microvascular endothelial cells into myofibroblasts: a potential role in skin fibrogenesis. *J Cutan Pathol* 2007, 34:146–153
17. Zeisberg EM, Tarnavski O, Zeisberg M, Dorfman AL, McMullen JR, Gustafsson E, Chandraker A, Yuan X, Pu WT, Roberts AB, Neilson EG, Sayegh MH, Izumo S, Kalluri R: Endothelial-to-mesenchymal transition contributes to cardiac fibrosis. *Nat Med* 2007, 13:952–961
18. Zeisberg EM, Potenta S, Xie L, Zeisberg M, Kalluri R: Discovery of endothelial to mesenchymal transition as a source for carcinoma-associated fibroblasts. *Cancer Res* 2007, 67:10123–10128
19. Arciniegas E, Frid MG, Douglas IS, Stenmark KR: Perspectives on endothelial-to-mesenchymal transition: potential contribution to vascular remodeling in chronic pulmonary hypertension. *Am J Physiol Lung Cell Mol Physiol* 2007, 293:1–8
20. Liebner S, Cattellino A, Gallini R, Rudini N, Iurlaro M, Piccolo S, Dejana E: Beta-catenin is required for endothelial-mesenchymal transformation during heart cushion development in the mouse. *J Cell Biol* 2004, 166:359–367
21. Frid MG, Kale VA, Stenmark KR: Mature vascular endothelium can give rise to smooth muscle cells via endothelial-mesenchymal transition: in vitro analysis. *Circ Res* 2002, 90:1189–1196
22. Inagaki Y, Okazaki I: Emerging insights into transforming growth factor beta Smad signal in hepatic fibrogenesis. *Gut* 2007, 56: 284–292
23. Kinoshita K, Imuro Y, Otagawa K, Saita S, Inagaki Y, Nakajima Y, Kawada N, Fujimoto J, Friedman SL, Ikeda K: Adenovirus-mediated

- expression of BMP-7 suppresses the development of liver fibrosis in rats. *Gut* 2007, 56:706–714
24. Sugimoto H, Yang C, LeBleu VS, Soubasakos MA, Giraldo M, Zeisberg M, Kalluri R: BMP-7 functions as a novel hormone to facilitate liver regeneration. *FASEB J* 2007, 21:256–264
25. Wynn TA: Cellular and molecular mechanisms of fibrosis. *J Pathol* 2008, 214:199–210
26. Sato Y, Harada K, Ozaki S, Furubo S, Kizawa K, Sanzen T, Yasoshima M, Ikeda H, Sasaki M: Cholangiocytes with mesenchymal features contribute to progressive hepatic fibrosis of the polycystic kidney rat. *Am J Pathol* 2007, 171:1859–1871
27. Dong C, Zhu S, Wang T, Yoon W, Li Z, Alvarez RJ, Dijke PT, White B, Wingley FM, Goldschmidt-Clermont PJ: Deficient Smad expression: A putative molecular defect in scleroderma. *Proc Natl Acad Sci USA* 2002, 99:3908–3913
28. Nguyen LN, Furuya MH, Wofraim LA, Nguyen AP, Houdren MS, Campbell JS, Knight B, Yeoh GC, Fausto N, Parks WT: Transforming growth factor-beta differentially regulates oval cell and hepatocyte proliferation. *Hepatology* 2007, 45:31–41
29. Nanji AA, Tahan SR, Golding M, Khwaja S, Rahemtulla A, Laijani EN: Role of transforming growth factor-beta1 in inhibiting endothelial cell proliferation in experimental alcoholic liver disease. *Am J Pathol* 1996, 148:739–747
30. Okuno M, Moriaki H, Imai S, Muto Y, Kawada N, Suzuki Y, Kojima S: Retinoids exacerbate rat liver fibrosis by inducing the activation of latent TGF-beta in liver stellate cells. *Hepatology* 1997, 26:913–921
31. Ramm GA, Nair VG, Bridle KR, Shepherd RW, Crawford DH: Contribution of hepatic parenchymal and nonparenchymal cells to hepatic fibrogenesis in biliary atresia. *Am J Pathol* 1998, 153:527–535
32. Sato Y, Sawada S, Kozaka K, Harada K, Sasaki M, Matsui O, Nakanuma Y: Significance of enhanced expression of nitric oxide synthases in splenic sinus lining cells in altered portal hemodynamics of idiopathic portal hypertension. *Dig Dis Sci* 2007, 52:1987–1994
33. Akahoshi T, Hashizume M, Tanoue K, Shimabukuro R, Gotoh N, Konishi K, Tsutsumi N, Sugimachi K: Role of the spleen in liver fibrosis in rats may be mediated by transforming growth factor beta-1. *J Gastroenterol Hepatol* 2002, 17:59–65
34. Needleman BW, Choi J, Burrows-Menzu A, Fontana JA: Secretion and binding of transforming growth factor-b by scleroderma and normal dermal fibroblasts. *Arthritis Rheum* 1990, 33:650–653
35. Gabrielli A, Di Loreto C, Taborro R, Candela M, Sambo P, Nitti C, Danieli MG, DeLustro F, Dasch JR, Danieli G: Immunohistochemical localization of intracellular and extracellular associated TGF-b in the skin of patients with systemic sclerosis (scleroderma) and primary Raynaud's phenomenon. *Clin Immunol Immunopathol* 1993, 68:340–349
36. Hasegawa M, Sato S, Takehara K: Augmented production of transforming growth factor-beta by cultured peripheral blood mononuclear cells from patients with systemic sclerosis. *Arch Dermatol Res* 2004, 296:89–93
37. Assoian RK, Komoriya A, Meyers CA, Miller DM, Sporn MB: Transforming growth factor-beta in human platelets. Identification of a major storage site, purification, and characterization. *J Biol Chem* 1983, 258:7155–7160
38. Oberhammer F, Bursch W, Pazefall W, Breit P, Erber E, Stadler M, Schulte-Hermann R: Effect of transforming growth factor beta on cell death of cultured rat hepatocytes. *Cancer Res* 1991, 51:2478–2485
39. Paradis V, Dargere D, Vidaud M, De Gouvello AC, Huet S, Martinez V, Gauthier JM, Ba N, Sobesky R, Ratzu V, Bedossa P: Expression of connective tissue growth factor in experimental rat and human liver fibrosis. *Hepatology* 1999, 30:968–976
40. Morikawa H, Tamori A, Nishiguchi S, Enomoto M, Habu D, Kawada N, Siomi S: Expression of connective tissue growth factor in the human liver with idiopathic portal hypertension. *Mol Med* 2007, 13:240–245
41. Sato Y, Sawada S, Nakanuma Y: Fibulin-5 is involved in phlebosclerosis of major portal vein branches associated with elastic fiber deposition in idiopathic portal hypertension. *Hepatol Res* 2008, 38:166–173