pared. Immunostaining was performed using primary antibodies against TβR-I (sc-398, rabbit polyclonal; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), TβR-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 TβR-I and TβR-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; TβR-I (1:50), TβR-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 μg/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-β1 and BMP7 levels of 66 samples obtained from 57 IPH patients were determined using enzyme-linked immunosorbent assay kits (Quantikine Human TGF-β1 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-β1 or BMP7, and incubated for 2 hours at room temperature. After washing, the plate was incubated with anti-TGF-β1 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 ± 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-β1 and BMP7 on Cellular Phenotype of HMVECs

RT-PCR analysis showed that HMVECs expressed receptors for TGF-β (TβR-I, TβR-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-β1 significantly reduced the expression of CD34 mRNA in HMVECs, and induced mRNA expression of S100A4,

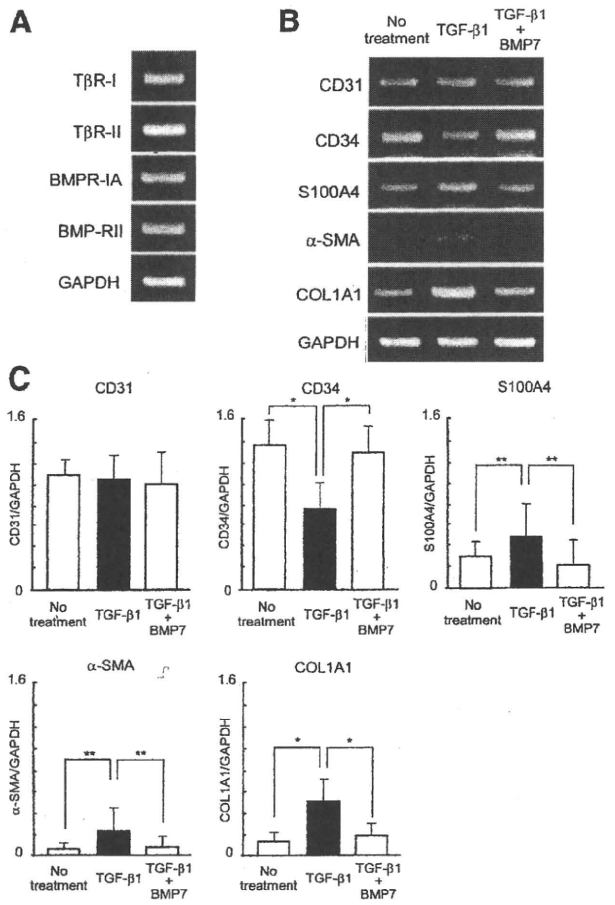

Figure 1. Effects of TGF-β1 and BMP7 on cellular phenotype of HMVECs at the mRNA level. HMVECs were treated with TGF-β1 (10 ng/ml) alone or in combination with BMP7 (100 ng/ml) for 5 days, and phenotypic changes were examined using RT-PCR as described in the *Materials and Methods*. HMVECs expressed receptors for TGF-β (TβR-I, TβR-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-β1 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-β1 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 ± 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-β1 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-β1 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-β1-induced COL1A1 expression in HMVEC in a dose-dependent fashion (Figure 2C).

Morphological and Phenotypic Alterations of HMVEC by TGF-β1 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-β1, the cellular morphology of HMVEC changed from epithelioid into spindle-shaped appearance (Figure 3). Immunocytochemistry showed that the spindle-shaped HMVEC following TGF-β1 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-β1 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 ± 6.3% to 52.7 ± 18.3% following the treatment. Again, the addition of BMP7 in the culture medium inhibited the morphological and phenotypic conversion of HMVECs by TGF-β1 (Figure 3). These results indicated that TGF-β1 could induce myofibroblastic features in HMVECs, and BMP7 antagonized the effects of TGF-β1.

Reduction of CD34 Expression in Portal Vein Endothelium of IPH Livers

Immunostaining of liver sections demonstrated that both TβR-I and TβR-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 ± 25.6%) (Figure 4D). In CVH/LC, the pSmad2-labeling

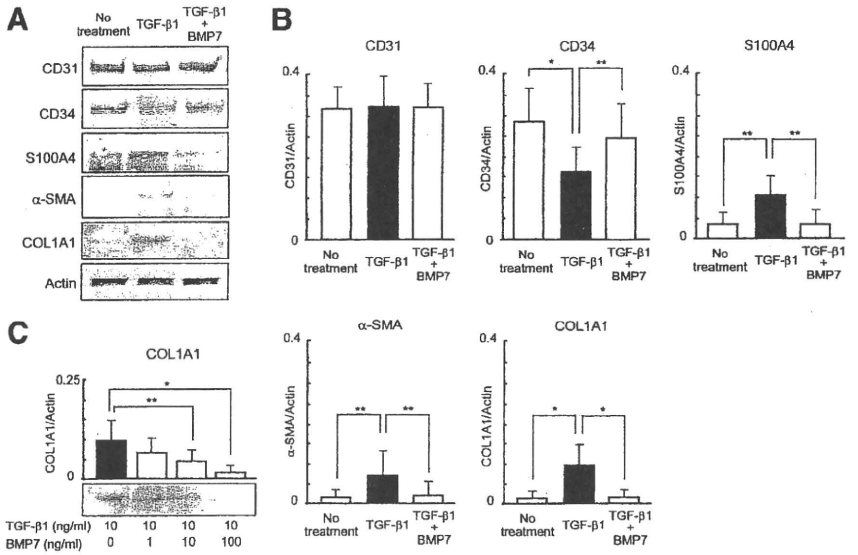

Figure 2. Effects of TGF-β1 and BMP7 on cellular phenotype of HMVECs at the protein level. HMVECs were treated with TGF-β1 (10 ng/ml) alone or in combination with BMP7 (100 ng/ml) for 5 days, and phenotypic changes were examined by Western blotting using protein extracts from HMVEC. Consistent with the 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 ± 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 ± 19.9% and 67.9 ± 26.9%, respectively, while those of biopsy livers were 69.2 ± 9.7% and 91.8 ± 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

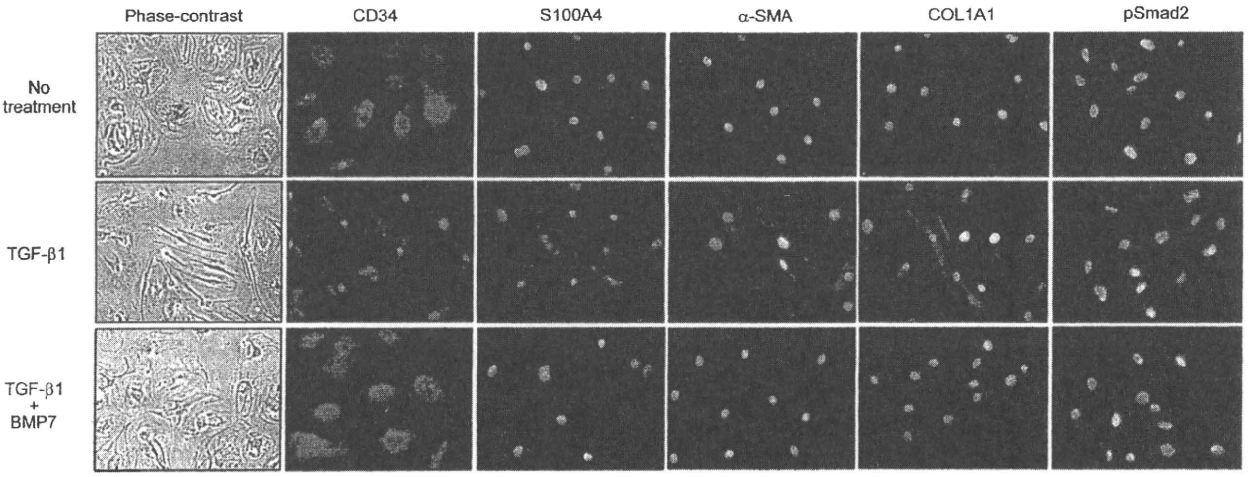

Figure 3. Morphological and phenotypic alterations of HMVECs by TGF-β1 and BMP7. HMVECs grew in a form of epithelioid, sheet-like appearance under the phase-contrast microscopy, and a 5-day treatment with TGF-β1 (10 ng/ml) changed the cellular morphology of HMVECs from epithelioid into spindle-shaped 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 ×1000.

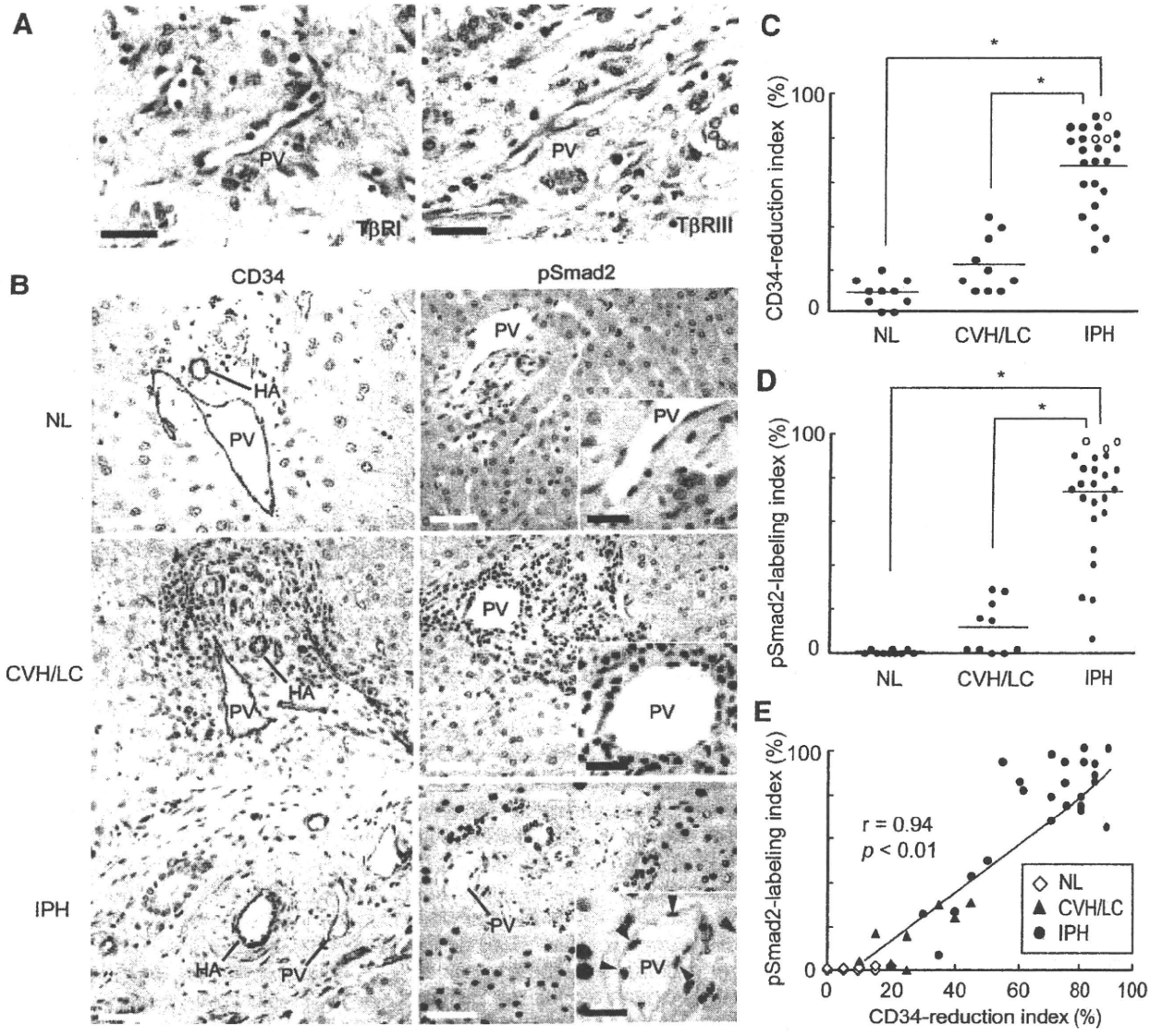

Figure 4. Reduction of CD34 expression and induction of pSmad2 expression in portal vein endothelium of IPH livers. Immunostaining of TGF-β receptors (TβR-I, TβR-II), CD34 and pSmad2 was performed for liver sections of normal liver (NL), chronic viral hepatitis/liver cirrhosis (CVH/LC) and idiopathic portal hypertension (IPH). TβR-I and TβR-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 liner correlation (**E**). **Arrowheads** indicate nuclei of pSmad2-positive portal vein endothelium (**B**). HA, hepatic artery. PV, portal vein. White bars = 50 μm; black bars = 20 μm. $^*P < 0.01$.

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-β1 Level in IPH

The serum TGF-β1 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-β1 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-β1 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

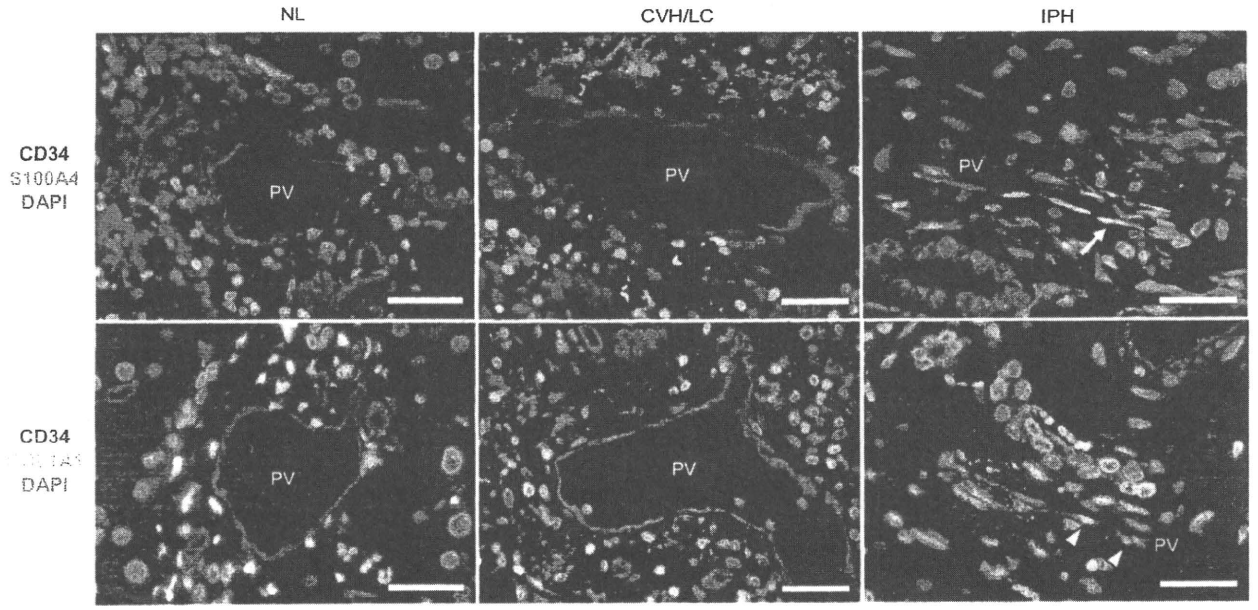

Figure 5. Colocalization of CD34 and mesenchymal markers in portal vein endothelium of IPH livers. Double immunofluorescence staining of CD34 and S100A4 protein, and CD34 and COL1A1 was performed for liver sections of normal liver (NL), chronic viral hepatitis/liver cirrhosis (CVH/LC), and idiopathic 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 μm.

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-β1 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-β1 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-β1 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-β1/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.

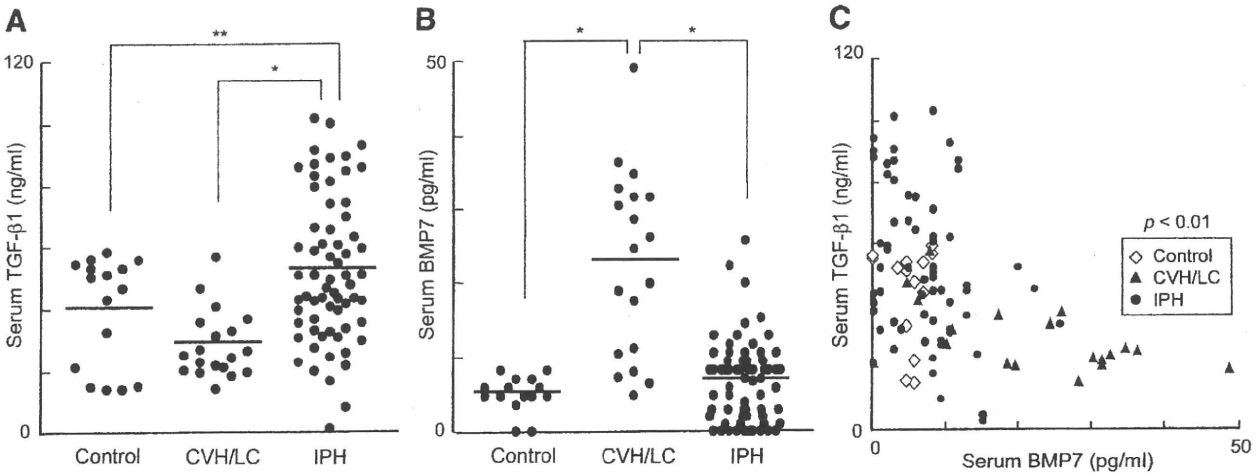

Figure 6. Elevation of circulating TGF-β1 levels in IPH. The serum TGF-β1 and BMP7 levels of 66 samples obtained from 57 patients with idiopathic portal hypertension (IPH), 16 healthy volunteers, and 19 patients with chronic viral hepatitis/liver cirrhosis (CVH/LC) were determined using an enzyme-linked immunosorbent assay. Serum obtained from IPH patients contained a significantly high level of TGF-β1 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-β1 level was plotted against the serum BMP7 level for all cases examined, a significant inverse correlation was observed between them (**C**). $^{*}P < 0.01$; $^{**}P < 0.05$.

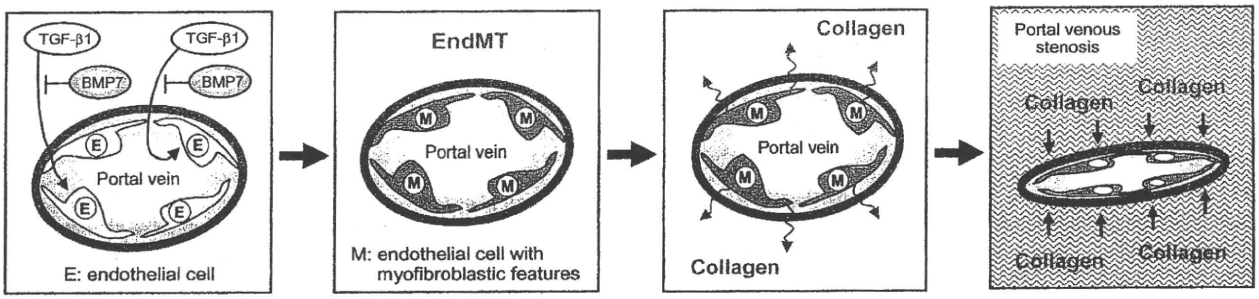

Figure 7. Proposed mechanism of the portal venous stenosis of IPH. TGF-β1 induces endothelial to mesenchymal transition (EndMT) of endothelial cells of peripheral portal vein of idiopathic portal hypertension (IPH). Endothelial cells acquire myofibroblastic features via Smad activation, and produce extracellular 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-β1.

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 fund 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-β1, 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-β1 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-β1/Smad activation is a possible mechanism of portal venous stenosis of IPH.

TGF-β1 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-β1, 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-β1 is different between portal vein endothelium and hepatic stellate cells of IPH; ie, portal vein endothelium of IPH may be hyperresponsive to TGF-β1 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 disproportionally 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-β1 level in IPH raises a question as to the cellular sources of TGF-β1. In the liver, hepatic stellate cells, macrophages, hepatocytes, and bile duct epithelial cells are candidates of cell types that produce TGF-β1.[29–31] The spleen is an organ that closely associates with the disease pathogenesis of IPH, and macrophages have been shown to produce TGF-β1 in

the spleen.[32,33] In cases of systemic sclerosis, myofibroblasts, fibroblasts, vascular endothelial cells, macrophages, lymphocytes, and platelets are potential sources of TGF-β1.[34–37] To determine cellular sources of TGF-β1 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-β1 level. In addition to its contribution to hepatic fibrosis, TGF-β1 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-β1 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-β1 and BMP7 levels in this study suggests a possibility that TGF-β1 and BMP7 may have an antagonistic effect on their expression to each other. Although the inverse correlation between serum TGF-β1 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-β1 level, and BMP7 can be a candidate of therapeutic agents for IPH.

In conclusion, the present study demonstrated an involvement of TGF-β1 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-β1. Our data indicate that TGF-β is a potential target of therapy of IPH, and BMP7 can be a possible therapeutic agent.

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第70回日本血液学会総会
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日本人の血栓性素因

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Key words : Venous thromboembolism, Thrombophilia, Protein S

はじめに

静脈血栓塞栓症（venous thromboembolism, VTE）は環境因子と遺伝因子からなる多因子疾患として知られている[1]。VTEの遺伝因子として，凝固制御因子であるアンチトロンビン，プロテインC，プロテインSの先天性欠損症が広く知られている[2,3]。また，白人種では，比較的頻度の高い第V因子Leiden変異[4]とプロトロンビンG20210A変異[5]がVTEの遺伝因子として知られている。日本人にはこれらの変異は見いだされておらず[6~8]，本研究で同定された日本人約55名に1人見られるプロテインS Lys196Glu変異が遺伝因子と考えられる[9,10]。VTEの環境因子として，手術，外傷，がん，脊椎損傷，長期臥床，経口避妊薬，妊娠，産褥期が知られている[11]。このように，VTEの発症に関する遺伝因子および環境因子が危険因子として明らかになっているにもかかわらず，米国ではVTEは依然として患者数が減っていないようである。こうしたことを背景として，2007年にBlood誌のPerspectiveにVTEが取り上げられ[12]，2008年3月号のArterioscler. Thromb. Vasc. Biol. 誌には，VTEに関する8つの総説が掲載された[13]。こういった背景には，VTEという疾患の重要性を米国で喚起し啓蒙する目的があるようである。

VTEの発症は加齢で増加する。米国ミネソタ州の1966年～1990年の統計によると，VTE発症者数は55歳から増加し，80歳では約100人年に1回の発症となり，80歳以上では45歳以下と比べて約1,000倍上昇するという[12]。フランスのブレスト地区の34.2万人を対象に，1998年度の1年間に行われたVTE発症の調査結果でも，VTE発症は加齢により大きく増加し，75歳以上の年間発症率は100人に1人に達していた[14]。超高齢化社会を迎えた本邦でも，VTE発症は診断法の普及とともに増加すると考えられ，VTE発症に関与する因子を，日本人を対象に調査することは，本疾患の予防と治療を考える上で重要であろう。

本稿では，厚生労働省調査研究班で行った日本人のVTE発症に関する遺伝因子に関する研究を紹介する。

1. 研究対象集団

厚生労働省科学研究費補助金難治性疾患克服研究事業，血液凝固異常症に関する調査研究班（班長：池田康夫慶応大学教授）では「特発性血栓症サブグループ」を組織し，VTEの発症原因と発症メカニズムを明らかにし，VTEの治療と予防のための方策を検討するため，多施設共同でVTE患者の遺伝因子に関する研究を進めてきた。本サブグループは6施設（大阪大学：川崎富夫，京都府立医科大学：辻肇，名古屋大学：小嶋哲人，自治医科大学：坂田洋一，慶應義塾大学：村田満，国立循環器病センター：宮田敏行）で構成された。本サブグループでVTE患者約170名を登録し，一般住民を対象としてVTEの遺伝的背景を調査した。本研究の対照となる一般住民集団は，国立循環器病センター予防検診部が行っている都市部地域一般住民のコホート集団を用いた[15]。

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2. VTEの候補遺伝子解析

2-1. プロテインS遺伝子

2-1-1. 5個の候補遺伝子多型解析

候補遺伝子アプローチでは，機能との関連が明らかになっている次の5つの遺伝子変異を検討した[9]。ADAMTS13 Pro475Ser変異は，血栓性血小板減少性紫斑病（TTP）の原因遺伝子ADAMTS13に見られる変異である。本変異は国立循環器病センターが同定したミスセンス変異である。これによりプロテアーゼ活性は低下するが，TTPの発症をおこすまでには至らず，疾患との関連は明らかになっていない[16]。ADAMTS13活性を蛍光合成基質で測定すると，変異体は約30%の活性低下を示す[17]。プラスミノーゲンAla620Thr変異は，約30年前に自治医科大学により変異保有患者が報告された[18]。変異プラスミノーゲンは活性低下を示す[19~22]。プロテインS Lys196Glu変異は，名古屋大学および徳島大学・三重大学で同定された変異で，活性低下を示す[23~25]。第XII因子 −4C>T変異は九州大学で見いだされた変異である。変異により本来のATG配列より上流に新しいATGができるので，ここから翻訳が始まり本来のATGからの翻訳量が減少するため血中の第XII因子量が低下する[26]。Plasminogen activator inhibitor 1（PAI-1）4G/5Gはスウェーデンで同定された変異である。この変異はプロモーター領域にあり，転写因子の結合に影響を与え，PAI-1 mRNA量が変化する[27]。後2者は，白人種にも見られるが，前3者は白人種にはみられず，人種特異的な変異と思われる。

これら5多型をVTE患者群（161名）と一般住民群（3,651名）でタイピングし，多型頻度を比べた結果，プロテインS Lys196Glu変異の変異Gluアレル頻度が，一般住民群よりVTE群で有意に高く，変異Gluアレルが VTEの危険因子であることが判明した（オッズ比，5.6，95%信頼区域，2.90～9.46）（表1）[9]。他の4多型は，一般住民群とVTE群で頻度に差が見られず，VTEと関連を示さなかった。同じ時期に，九州大学濱崎直孝教授も日本人のVTEの遺伝因子の研究を発表し，プロテインS Lys196Glu変異が危険因子であることを報告した（オッズ比，3.7）[28]。このように，2つの独立した研究から本変異とVTEの関連が明らかになった。

私達の研究では，一般住民3,651人中にヘテロ接合体が66名，ホモ接合体はいなかったので，アレル頻度は0.9%と計算された。これは，以前名古屋大学が算出したアレル頻度0.8%[23]，今回九州大学が求めたアレル頻度0.8%[28]とよく一致した。これより，一般住民の約55人に1人がヘテロ接合体と算出された。この頻度から，約12,000人に1人がホモ接合体と計算された。日本人総人口を1億2,000万人とすると，約1万人がホモ接合体で

表1 VTE患者を対象とした5遺伝子多型の症例対照研究

		ADAMTS13 Pro475Ser	PLG Ala620Thr	PS Lys196Glu	FXII −4C>T	PAI-1 4G/5G
VTE群	Major homo	139	152	146	63	61
	Hetero	20	9	13	75	69
	Minor homo	1	0	2	23	30
	Total	160	161	161	161	160
	MAF	0.069	0.028	0.053	0.376	0.403
一般住民群	Major homo	3290	3501	3585	1513	1468
	Hetero	332	149	66	1651	1686
	Minor homo	17	0	0	486	497
	Total	3639	3650	3651	3650	3651
	MAF	0.05	0.02	0.009	0.359	0.367
	χ^2	2.179	0.987	75.464	0.372	3.402
	P	0.336	0.32	<0.0001	0.83	0.183
	Major allele	Pro	Ala	Lys	T	4G
	Minor allele	Ser	Thr	Glu	C	5G

MAF: minor allele frequency　　Kimura et al., Blood, 2006.

表2 プロテインS K196E変異

Characteristics	Description
遺伝子変異部位	cDNA, 586A>G[23] genomic DNA, 67951A>G[23]
アミノ酸変異	第2EGF様ドメイン内にあるLys196Glu（K196E）変異。成熟蛋白質のアミノ末端を1番とした場合は，Lys155Gluになる。
アレル頻度	日本人3,651人中では0.009[9] 日本人183人中では0.008[28] 日本人304人中では0.008[23]
VTEに対するオッズ比 (95%信頼区域)	5.58（3.11～10.01）[9] 3.74（1.06～13.2）[26]
血漿中のプロテインS活性	34人のヘテロ接合体では71.9±17.6%（平均±SD），正常人の平均値より16%低い活性を示す[30]
遺伝子変異の検出法	検出法1：変異プライマーを用いたRestriction fragment length polymorphism法[23] 次のプライマーを使ってPCRで434-bpを増幅し，*Hinf*I切断の違いを見る。E-alleleは404-bpと30-bpに分解される。 PCR primer 5'-CAATTTTAGAATTCCATGACATGAGA and mutagenic primer 5'-CCATCCTGCTCTTACCTTTACAATCTGACT. 検出法2：TaqMan法[9] PCR primers, 5'-ACCACTGTTCCTGTAAAAATGGTTT, 5'-TGTGTTTTAATTCTACCATCCTGCT. Probes, 5'-VIC-CAAATGAGAAAGATTGTAAAG-MGB (mutant E-allele)/ 5'-FAM-CAAATAAGAAAGATTGTAAAG-MGB (wild-type allele)
組み換えプロテインS Glu 196変異体を用いた機能解析	分子量：野生型と同一[24] 活性化プロテインCは結合しない[25]

あると推定された[10]。プロテインS Lys196Glu変異に関する情報を表2にまとめた。プロテインS Lys196Glu変異は，これまでのところ日本人にしか同定されていないが，韓国人や中国人にも本変異は存在するものと考えられる。

2-1-2. プロテインS Lys196Gluを保有するヘテロ接合体のプロテインS活性

プロテインS Lys196Glu変異は，1993年に名古屋大学と徳島大学・三重大学がそれぞれ独立にVTE患者に同定したミスセンス変異である。本変異はプロテインS分子の第2EGF様ドメイン内にある（表2）。本変異は変異を同定した地名に因んで，プロテインS Tokushima変異とも呼ばれる。血中に本変異を有する異常分子が存在するので，血中の抗原量の低下は見られない[23~25, 29]。本変異体は活性化プロテインC（APC）のコファクター活性を示さないことが発現実験で明らかとなっている[25]。

本変異のヘテロ接合体保有者の血中プロテインS活性を調べるため，一般住民1,862人の活性を測定し，遺伝

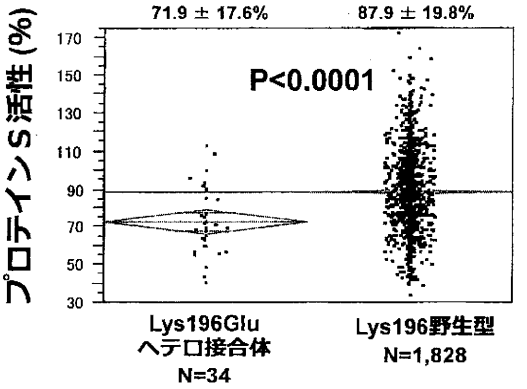

図1 プロテインS Lys196Glu 変異保有者の血中プロテインS活性[30]。一般住民1,862名の血中プロテインS活性を測定し，Lys196Glu 変異の遺伝型に分けて活性を比較した。ヘテロ接合体（34名）のプロテインS活性（71.9±17.6%，平均値±SD）は野生型（1,828名）のプロテインS活性（87.9±19.8%）より有意に低い（$p<0.0001$）。

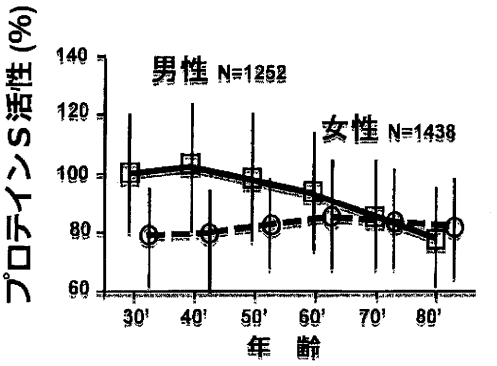

図2 プロテインS活性の性差と年齢差[10]。一般住民2,690名（男性1,252名，女性1,438名）のプロテインS活性を測定し，男女別に10歳毎に示した。男性は実線，女性は点線で示す。四角は男性の活性平均値，丸印は女性の活性平均値，SDは縦線で示した。

型との関連を調べた[30]。この研究では，34人がヘテロ接合体であり，ヘテロ接合体者のプロテインS活性は40%から110%まで広い範囲を示した（図1）。一方，野生型プロテインSをもつ1,828人の活性も40%から170%までの広い活性分布を示した。両群の活性値は，大きく重複しているので，血中のプロテインS活性だけではLys196Glu 変異を識別できないことを示唆した。しかし，遺伝型に分けて活性を比較すると，変異 Glu アレルのヘテロ接合体者は平均16%の活性低下を示すことが明らかとなった（プロテインS LysLys 型，1,828名：プロテインS活性 87.9±19.8%，平均±SD，プロテインS LysGlu 型，34名：プロテインS活性 71.9±17.6%，$P<0.0001$）[30]。

プロテインS Lys196Glu 変異はプロテインS活性を低下させることから，日本人に見いだされた血栓性素因と考えられる。このことから，本変異は他の血栓性疾患の素因になる可能性が考えられる。また，本変異は日本人だけでなく東アジア人に共通に見られる変異と考えられるので，韓国人や中国人を対象にした研究も進められるべきであろう[10]。本変異は白人種には見られない。白人種に見られる第V因子 Leiden 変異とプロトロンビン G20210A 変異は，保因者のハプロタイプ解析から，それぞれ21,340年前および23,720年前に生じた変異に由来する推定され，今日白人種に広く分布していることから，両変異の選択的な進化上の優位性（けがなどの出血性疾患に対する優位性）が考えられる[31]。同様の手法を用いると，プロテインS Lys196Glu 変異が生じた年代の推定も可能であろう。

図2に，一般住民2,690人を対象に測定したプロテインS活性を，性と年齢に分けて表示した[10]。プロテインS活性は，大きな性差が見られ，30歳代と40歳代の女性は同年代の男性より約20%活性が低い。男性は加齢によりプロテインS活性は低下するが，女性では加齢による変化は見られない。こういったプロテインS活性の変化により，活性値による欠損症の同定を難しくしている。また，プロテインS活性は多くの後天性因子の影響を受ける事が知られている。特に，妊娠時のプロテインS活性は，欠損症に匹敵する程度にまで低下することが知られており，注意が必要である[32,33]。

プロテインSの抗凝固活性は，APC 依存性と非依存性がある。APC 依存性抗凝固活性は，APC による活性型第V因子および活性型第VIII因子の分解を促進する。最近，APC 非依存性のプロテインSの機能として，Tissue factor pathway inhibitor（TFPI）の活性型第X因子阻害の促進活性が報告された[34]。このプロテインS活性は，組織因子経路の凝固活性を抑制することにより抗凝固として働き，大変注目される。プロテインS Lys196Glu 変異体の APC 非依存性の機能はまだ調べられていない。

プロテインSは血中では補体制御因子である C4BP と複合体を形成する。この親和性はとても強い。複合体のプロテインSは抗凝固能を持たず，遊離型だけが抗凝固能を示す。C4BP はα鎖だけで構成される分子とβ鎖を含む分子がある。プロテインSはβ鎖にのみ結合する。α鎖は炎症で増加するがβ鎖は増加しない。したがって，炎症時でも遊離プロテインS量は大きな変動はなく，プロテインSによる抗凝固能も大きく変化するものではないといわれている[35]。プロテインS活性を測定する際，

C4BPとの複合体が解離しない条件で活性測定する必要がある。このため，37℃での測定は避けるように報告されている[36]。

プロテインS欠損症は，欧米人に比べ，日本人に多く見られるという報告が相次いでいる[37~39]。既に述べたように，一般住民を対象に測定した結果では，プロテインS活性は幅広い値を示すため，欠損症を定義する客観的な明確な値がない。こういった，測定法の限界を勘案してもなお，プロテインS欠損症は日本人に多いようである。

2-1-3. プロテインS欠損症患者における *PROS1* 遺伝子欠損

プロテインS欠損症とみなされる程度にまで活性が低下した患者のプロテインS遺伝子の解析を行っても，その半数にしか原因変異は同定されず，残りの症例には変異を同定できないことが大きな問題になっている[40~42]。これは，重症血友病患者の遺伝子解析を思い起こさせる。この場合，第Ⅷ因子遺伝子のAlu配列特異的逆位（inversion）で説明された[43]。しかし，プロテインS遺伝子近傍にはAlu配列は存在しない。そこで，プロテインS遺伝子の大きな欠失を想定し，VTE患者163名のプロテインS遺伝子欠損の解析をmultiplex ligation-dependent probe amplification法で行った[44]。その結果，プロテインS活性21%を示す1名の患者の1アレルに遺伝子全域の欠失を認めた。163名中に1名に欠失を認めたので，高頻度に認められることにはならないが，プロテインS活性が50%以下で，かつミスセンス変異が同定されない患者とすると6名に絞られ，そのうちの1名が遺伝子欠失を保有していたことになる。プロテインSの遺伝子欠失はVTE患者に広く認められるものではないが，活性低下症例には存在することが明らかとなった。今後，プロテインS活性が低下しており，かつミスセンス変異が同定されない患者を対象に，プロテインS遺伝子の欠失を探索する必要があるかもしれない。

2-2. アンチトロンビン，プロテインC，プロテインSの遺伝子変異

アンチトロンビン，プロテインC，プロテインSの遺伝子変異は，VTEの遺伝的背景として知られているが，どれくらいの頻度でどういった変異が存在するのかについての研究はなかった。そこで，サブグループで収集したVTE患者173名を対象に3つの遺伝子の蛋白質コード全領域のDNAシークエンスを行った[45]。その結果を表3に示す。VTE患者54名にミスセンス変異などのアミノ酸変化を伴う遺伝子異常を同定した。プロテインS遺伝子欠損患者1名を加えると，総計55名，約32%の患者に，39種のアミノ酸の変化を伴う変異が同定された。

表3 173名のVTE患者に見られたアミノ酸配列が変化する変異を有する患者数

遺伝子名	患者数
プロテインS	24*
プロテインC	12
アンチトロンビン	14
プロテインS+プロテインC	5
合計	55

*1名のプロテインS遺伝子欠失患者を含む

表4 2つの遺伝子に変異を有する5名の患者

プロテインS	プロテインC	VTE発症年齢	家族歴
Lys196Glu	Lys193del	57	情報なし
Lys196Glu	Arg221Trp	40	なし
Lys196Glu	Arg27Trp	39	あり
Lys196Glu	Val339Met	25	あり
Lys196Glu	Val339Met	55	なし

なかでも，プロテインS Lys196Gluは前述のように最も多くの患者に見られ，次いでプロテインCのLys193del変異とVal339Met変異が4名ずつの患者に同定された。このことから，これら3つの変異は日本人のVTEの危険因子として重要であることが明らかとなった。Val339Met変異保有者のプロテインCアミド活性は1名（77%）を除き，低い活性を示したが，Lys193del変異保有者はいずれもアミド活性は90%以上を示した。九州大学も本変異保有者を同定しているが，その抗凝固活性は低値を示していた[28]。このことから，Lys193del変異はプロテインCの抗凝固活性を低下させるがアミド活性には影響しないものと考えられた。プロテインCアミド活性は，その簡便性と優れた定量性から，血栓性素因のスクリーニングとして広く使われているが，Lys193del変異を持つ血漿は，アミド活性が低下しないことを理解する必要があろう。ちなみに，Lys196残基はプロテインC軽鎖のC末端から6残基目に位置する。

VTE患者のうち，5名はプロテインSとプロテインCの2つの遺伝子に変異を保有していた（表4）。この5名の患者はいずれもプロテインS Lys196Glu変異を保有していた。このことから，プロテインS Lys196Glu変異は他の変異と重複することによりVTE発症のリスクを上げると考えられた。

VTE患者55名にアミノ酸変化を伴う遺伝子変異を同定した（表3）ので，次にこれら遺伝子変異保有者の

VTE 発症年齢を変異非保有者と比較した。その結果，変異保有者の VTE は非保有者より有意に若年で発症することが判明し，遺伝子変異の VTE 発症への寄与が明らかになった（変異保有者 55 名，発症年齢 44.7±16.5 歳，変異非保有者：発症年齢 118 名，52.6±16.1 歳，$P=0.0031$）[45]。サブグループで収集した VTE 患者は，初発例や再発例，家族発症を有する例，がん患者など，種々の背景因子を持つ。VTE 発症と遺伝因子の関連をより明確にするためには，VTE 患者を連続例として収集する必要があると考える。

2-3. トロンボモジュリン遺伝子

トロンボモジュリン（TM）は，血管内皮細胞に恒常的に発現しており，凝固反応で生じたトロンビンに結合し，トロンビンがプロテイン C を活性化する際のコファクターとして働く。このことから，TM の活性低下は VTE に繋がる可能性がある。そこで，TM 遺伝子を解析した。VTE 患者 118 名の TM 遺伝子（TM 遺伝子は一つのエクソンでコードされている）をシークエンスし，変異を収集した。そのうち，頻度の高い変異を対照集団でタイピングし，多型頻度を比較することにより VTE との関連を検討した。また，可溶性 TM を測定し，遺伝子多型と可溶性 TM 量との関連を解析した。その結果，Ala455Val 変異を含むハプロタイプが，男性・女性共に可溶性 TM 量と関連を示し（$P<0.05$），男性で VTE に関連を示した（$P=0.02$，95％信頼区域　1.14～6.67）[46]。本多型と可溶性 TM 量との関連は，米国の研究でも見られたが，VTE との関連は他の研究では確認されていないので，慎重に検討する必要がある。

2-4. 組織因子経路インヒビター（TFPI）遺伝子

TFPI は組織因子経路の凝固反応を抑制するので，本因子の活性低下は VTE の素因となる可能性がある。TFPI は 3 つのクニッツ阻害ドメインからなる α 型と，2 つのクニッツ阻害ドメインをもつ β 型（alternative splice 型）がある。175 名の VTE 患者の TFPI 遺伝子の全エクソンをシークエンスし，変異を収集した結果，β 型だけをコードするエクソンにミスセンス変異 Asn221Ser を同定した。この Asn221 は glycosylphosphatidylinositol で修飾を受けると考えられる残基である。即ち，TFPIβ はこの Asn221 を介して glycosylphosphatidylinositol 化され，発現細胞である血管内皮細胞の表面に係留すると考えられる。そこで，本変異と血中の total TFPI 量および free TFPI 量との関連を調べた。total TFPI 測定法は TFPIβ も測定可能であるが，free TFPI 測定法は第 3 クニッツドメインを認識する抗体を用いるため，TFPIβ は測定されない。実際，total TFPI 量では，変異 Ser アレルを持つヒトは野生型のヒトより高値を示した。一方，free TFPI 量は遺伝型による差は認められなかった。この結果は，TFPIβ は Asn221 を介して細胞上に係留することを支持した，次に，本多型と VTE の関連を調べたが，有意な関連を見いださなかった。このことから，TFPI 遺伝子のミスセンス変異 Asn221Ser は，血中 TFPI 量に関連するものの，VTE 発症には影響しないと考えた[47]。しかし，変異 Ser221 を有する TFPIβ は血管内皮細胞上に係留できないので，その内皮細胞の抗凝固活性は低下すると考えられる。ある種の病態下では，この内皮細胞上の抗凝固活性が血栓の抑制に重要であり，そういった場合は本多型保有者は血栓傾向を示すかもしれない。

3. 白人種と日本人の VTE の遺伝的背景の相違

白人の VTE の遺伝的背景として，第 V 因子 Leiden 変異（Arg506Gln 変異）とプロトロンビン遺伝子の 3'非翻訳領域に 20210G>A 変異が知られている（表 5）[4, 5]。第 V 因子 Leiden 変異保有者は APC 添加の凝固時間の延長が見られない（APC 抵抗性と呼ばれる）。ヨーロッパ北部の一般住民の 10～15％および VTE 患者の 20～40％に見られる変異であるが，日本人を含む東アジア人には見られない[6, 7]。また，白人種はプロトロンビン遺伝子の 3'非翻訳領域に 20210G>A 変異をもつ。変異 A アレル保有者は血中プロトロンビン量が高く，VTE のリスクとなる。本変異は白人一般住民の約 2％および VTE 患者の 6

表 5　VTE の遺伝的素因

	比較的よくみられる変異	まれな変異
白人	第 V 因子 Leiden 変異 プロトロンビン 20210G>A 変異	プロテイン S プロテイン C アンチトロンビン
日本人	プロテイン S Lys196Glu 変異	プロテイン S プロテイン C アンチトロンビン

～8%に見られるが，日本人には同定されていない[8]。プロテインS，プロテインC，アンチトロンビンに見られる稀な変異は，白人・日本人を問わず，あらゆる人種に見られる。一方，日本人を対象にした研究から，VTEの遺伝的背景としてプロテインS Lys196Glu変異が明らかとなった[9, 28]（表5）。最近のヒトゲノム多様性の研究から，人種の違いによる遺伝子多型の頻度の違いが広く知られるようになってきた。

おわりに

私達はVTEの遺伝因子を明らかにするため，候補遺伝子のVTEへの関連を検討した。VTEへの関連は，症例対照研究で行ったが，この時，それぞれの血中因子の活性（もしくは抗原量）を中間形質として用いて，遺伝子多型の影響を検討した。中間形質である血中因子の活性に影響を与える遺伝子多型は幾つか同定できたものの，それらの多くはVTEとは関連を示さず，唯一プロテインS Lys196Glu変異がVTEと強い関連を示す結果となった。VTEの遺伝因子を検討する研究では，症例対照研究で関連を示し，かつ中間形質にも関連を示す遺伝因子を同定することが重要であろう。本稿で紹介したように，血栓症の遺伝因子には，人種による違いが明確に見られる。こうした遺伝因子の違いにより，VTEの頻度の人種による違いが一部説明できるようになった。今後，研究で明らかになった血栓症の遺伝因子をVTEの予防と治療にどのように使うのかが，重要になると思われる。

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A three-decade experience of radical open endvenectomy with pericardial patch graft for correction of Budd-Chiari syndrome

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Background: We previously reported the value of our operative procedure for Budd-Chiari syndrome (BCS) that comprised reconstruction of the occluded or severely stenosed inferior vena cava (IVC) using an autologous pericardium patch and reopening as many occluded hepatic veins as possible. Here, we present the long-term durability and efficacy of the autologous pericardium patch for reconstruction of the IVC in BCS.

Methods: We retrospectively analyzed a series of 53 consecutive patients (mean age, 48.4 ± 12.8 years; range, 24-76 years; 34 men) who underwent surgical treatment for BCS at our institution from 1979 to 2008. Patency of the IVC and hepatic veins was examined by venography at discharge. Patients attended an outpatient clinic every 1 or 2 months for follow-up. The reconstructed IVC was evaluated by enhanced computed tomography every 1 or 2 years.

Results: Two in-hospital (operative mortality, 3.7%) and 15 late deaths occurred. During a mean follow-up of 7.6 ± 6.5 years (range, 0.08-24.1 years), the reconstructed IVC became totally obstructed in three patients, of whom two underwent reoperation, and severely stenosed in two patients, who required percutaneous transvenous balloon venoplasty (PTV). The 5- and 10-year patency rates without reoperation or PTV for the reconstructed IVC were 90.5% and 84.3%, respectively. The cumulative 5- and 10-year survival rates were 89.8% and 70.7%, respectively.

Conclusion: The autologous pericardium patch is effective and durable for reconstructing a diseased IVC in BCS. (J Vasc Surg 2009;50:590-3.)

Budd-Chiari syndrome (BCS) is defined as chronic, progressive, and congestive liver dysfunction resulting in liver cirrhosis and hepatic failure due to hepatic inferior vena cava (IVC) obstruction concomitant with ostial obstruction of the hepatic vein. We previously described our corrective procedure for BCS that consists of reconstruction of the occluded IVC using autologous pericardium patch and reopening as many occluded hepatic veins as possible.[1-3]

Malignant involvement of the IVC or superior vena cava (SVC) is sometimes surgically treated using autologous pericardium, which is considered good material for patch reconstruction after partial resection of the IVC or SVC.[4-7] We previously demonstrated that autologous pericardium is more effective than bovine or horse pericardium and expanded polytetrafluoroethylene (ePTFE) for reconstruction of the IVC in dogs.[8] However, the long-term durability and efficacy of this autologous pericardium after patch reconstruction of the IVC in patients with BCS is unclear. To clarify the effectiveness and long-term durability of the autologous pericardium for patch reconstruction of the IVC, we retrospectively reviewed patients with BCS who had undergone our surgical corrective procedure.

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Competition of interest: none.

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PATIENTS AND METHODS

We enrolled 53 consecutive patients (34 men) who had undergone this corrective procedure for BCS at Ryukyu University Hospital between December 1979 and January 2008. The patients were a mean age of 48.4 ± 12.8 years (range, 24-76 years). Major symptoms or signs were lower extremity edema in 11, abdominal distension in 10, hepatic encephalopathy in 6, hepatomegaly in 5, superficial collateral veins on the abdomen in 5, varicose vein or pigmentation of the leg in 5, gastrointestinal bleeding in 4, and abdominal pain in 2. All patients had ascites at the operation. Three patients had associated Behçet's disease, 6 had a protein C deficiency, 5 had protein S deficiency, and 1 had antiphospholipid antibody syndrome. Esophageal varices were confirmed in 46 patients (87%), of which six (11.3%) had a history of hepatic encephalopathy.

The mean pressure gradient between the right atrium (RA) and IVC was 12.0 ± 3.4 mm Hg (range, 8-21 mm Hg). The 15-minute indocyanine green clearance test (ICG 15') was 30.4% ± 16.4% (range, 5.3%-68%). Preoperative venography showed that the obstructed or stenosed lesion of the IVC was 3.0 ± 2.6 cm (range, 0-12 cm) in length. Three patients had undergone previous catheter intervention to the IVC stenosis. Our surgical indication for BCS is an IVC obstruction or severe stenosis resulting in elevation of the pressure gradient between RA and IVC, with or without a hepatic vein with ostial obstruction, which is thought to be able to be reopened by our procedure, simultaneously.

The patients were placed in the left lateral position under unilateral ventilation, and the lateral site of the

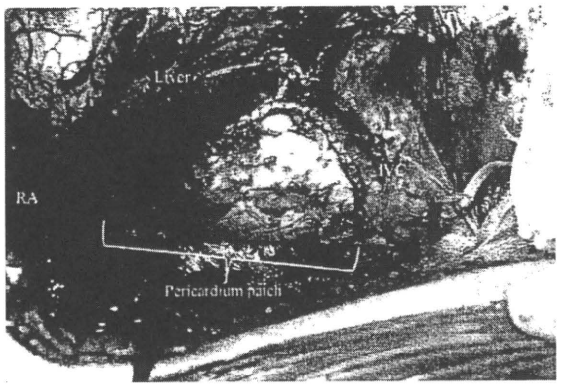

Fig 1. Reconstructed retrohepatic inferior vena cava *(IVC)* with fresh autologous pericardium patch. *RA,* right atrium.

hepatic IVC and the right atrium were exposed by dividing the diaphragm and right sixth intercostals by a thoracotomy. Harvested fresh autologous pericardium was immersed in saline and used to reconstruct the occluded or severely stenosed IVC (Fig 1). We concomitantly reopened as many occluded hepatic veins as possible under a partial cardiopulmonary bypass (CPB) through the right femoral artery and vein cannulation. A thickened caval wall that occluded the ostia of the hepatic veins was partially resected with some liver parenchyma to reopen and dilate the hepatic venous outflow. The concept of lateral patch augmentation and resection of liver parenchyma is to widen and deepen the occluded or severely stenosed hepatic IVC and normalize hepatic circulation from the portal vein to the RA through the hepatic veins.

All patients received continuous intravenous heparin sulfate at a dose of 100 U/kg/day within 24 to 48 hours postoperatively, followed by lifelong warfarin therapy at doses required to maintain the international normalized ratio between 1.5 and 2.5.

All surviving patients underwent venography and the ICG 15' test at discharge and were followed up every 1 or 2 months as outpatients. The reconstructed IVC was evaluated by enhanced computed tomography (CT) scanning every 1 or 2 years. During the follow-up period, 129 CT images of the surviving patients, with an average of 4.3 images per patient (range, 1-14 images) were reviewed. When restenosis or occlusion of the hepatic IVC was indicated on scans, the patients underwent venography. The last imaging study that confirmed graft patency served as the end point for graft patency calculations.

Statistical analysis was performed with Statview 5.0 software (SAS Institute, Cary, NC). All data were expressed as mean ± SD. The survival rate and the patency rate without reoperation and transvenous balloon venoplasty (PTV) for the reconstructed IVC were estimated using the Kaplan-Meier product limit method. For comparison between two groups, significance was determined by the t test. A value of $P < .01$ was considered significant.

RESULTS

The duration was 298.5 ± 83.4 minutes (range, 180-567 minutes) for the procedure and 30.5 ± 15.5 minutes (range, 13-71 minutes) for CPB. The harvested pericardium was 72 (range, 40-120) × 34 (range, 24-86) mm. Two patients died in the hospital at postoperative day 15 for an operative mortality of 3.7%. One patient died of severe liver failure and the other died of ventricular fibrillation from cardiac amyloidosis. The postoperative hospital length of stay was 49 ± 22 days (range, 15-124 days). Complications developed in 11 patients, consisting of pleural effusion in 5, postoperative bleeding in 2, wound infection in 2, renal dysfunction in 1, and acute hepatitis in 1. Symptoms were acceptably reduced in all survivors, with no evidence of hepatic failure.

All survivors underwent venography at discharge, which revealed the absence of occlusion and severe stenosis in the reconstructed IVC. The mean pressure gradient between the RA and IVC was significantly decreased from 12.0 ± 3.4 (range, 8-21) to 4.0 ± 3.0 (range, 0.0-12.0) mm Hg ($P < .001$). The ICG 15' was also significantly decreased from 30.4% ± 16.4% (range, 5.3%-68%) to 19.7% ± 14.6% (range, 5.5%-56.6%; $P < .001$). The serum bilirubin and platelet count were significantly recovered from 1.62 ± 0.7 (range 0.6-3.4) to 1.19 ± 0.67 (range, 0.3-4.0) mg/dL ($P < .001$) and from 11.5 ± 7.5 (range, 2.0-48.4) × $10^4/mm^3$ to 16.3 ± 7.8 (range, 3.6-35.0) × $10^4/mm^3$ ($P < .001$). The esophageal varices disappeared in 11 of 46 patients (23.9%).

No graft infections developed during the mean follow-up period of 7.6 ± 6.5 years (range, 0.08-24.1 years). Total obstruction of the reconstructed IVC occurred in three patients at 5, 6, and 7 years, respectively, after the initial surgery. Two of three patients with total obstruction, whose symptoms of lower extremity edema and esophageal varices deteriorated despite medical treatment, underwent a repeat procedure. One patient underwent repeated IVC reconstruction using a ring-reinforced ePTFE graft, and another underwent a Senning-type RA-liver anastomosis. Four presented with severe stenosis of the reconstructed IVC that was treated using PTV. The 5- and 10-year patency rates without reoperation or PTV for the reconstructed IVC were 90.5% and 84.3%, respectively (Fig 2). The remaining patients did well with considerable improvement of symptoms that were documented before surgery and no progression of the liver dysfunction during the follow-up period. Hepatocellular carcinoma developed in 13 patients (24.5%), and they underwent partial hepatic resection 5.3 ± 5.4 years (range, 0-17.6 years) after the BCS surgery, including a one-stage operation in three patients.

There were 15 late deaths caused by hepatocellular carcinoma in 2 patients, pneumonia in 2, respiratory failure in 1, arrhythmia in 1, suicide in 1, liver failure in 1, and an unknown cause in 7. The cumulative 5- and 10- year survival rates were 89.8% and 70.7%, respectively (Fig 3).

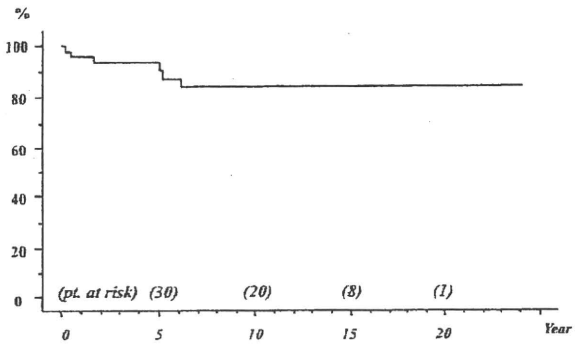

Fig 2. Cumulative patency rates without reoperation or percutaneous transvenous balloon venoplasty. Patency rates are 90.5% at 5 years and 84.3% at 10 years.

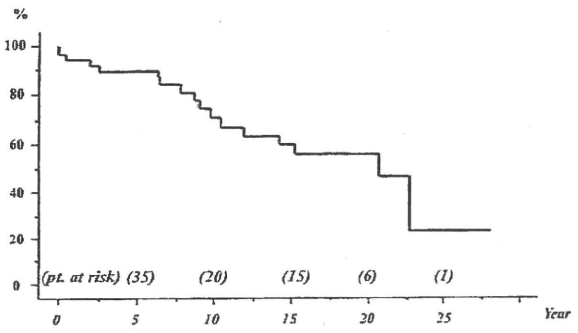

Fig 3. Cumulative survival rates were 89.8% at 5 years and 70.7% at 10 years.

DISCUSSION

These results suggest that autologous pericardium can serve as an effective and durable patch for IVC reconstruction in BCS. Because of its geometric and functional advantages, antibacterial and anticoagulant potential, long-term durability without calcification, and its cost, the autologous pericardium is considered an excellent material for reconstructive heart surgery[9-15] and for treating tumors with vascular involvement and thrombosis.[4-7] It has been applied in many reconstructive procedures such as right ventricular outflow tract repair of tetralogy of Fallot, aortic arch reconstruction in hypoplastic left heart syndrome, heart valve repair, and aortic root repair in aortic valve endocarditis.[9-15]

Our previous immunohistologic findings[8] also support the use of autologous pericardium for IVC reconstruction in terms of the quality of the neointima of grafts after patch reconstruction of the IVC in dogs. Moreover, the pericardium can be easily harvested through a thoracoabdominal incision approached through the retrohepatic IVC and RA in patients with BCS.

The autologous pericardium has some limitations, however. The size of the pericardium available for harvest using our approach might be insufficient for IVC repair in patients who have already undergone cardiac surgery or our procedure for BCS, so an alternative material is required for such patients. The ePTFE graft that has mostly been used for IVC replacement or patch reconstruction in patients with tumors or thrombus involving the IVC is considered a good material for treating patients with BCS from the viewpoints of size-matched IVC, resistance to compression, and high patency rates, regardless of anticoagulant therapy.[16-19]

One of two patients with an obstructed IVC required repeated patch augmentation with an external supported ePTFE graft 6 years after the first operation; however, severe edema of both feet developed 16 years after the repeat operation. Venography revealed severe stenosis of the proximal site of the graft, and the pressure gradient across this stenosis site had increased from 0 (at the first operation) to 8 mm Hg. PTV did not resolve his symptoms or reduce the pressure gradient due to sclerotic change at the stenosis site of previous ePTFE graft. Three other patients with severe stenosis of the autologous pericardium underwent successful PTV. These findings suggested that the anastomotic site of the ePTFE graft might undergo long-term sclerotic changes.

In the setting of anticoagulation therapy, inherited and acquired hypercoagulable states along with a variety of other causes can be identified in about 75% of patients with BCS, indicating that several etiologic factors are involved in 25% of these patients.[20] Therefore, our patients were administered indefinite postoperative warfarin therapy. In fact, six (11%) of our patients with BCS had inherited and acquired hypercoagulable states. Moreover, the two in-hospital deaths occurred in patients who had hypercoagulable states and presented fulminant liver failure before surgery.

Transjugular intrahepatic portosystemic shunt (TIPS) is thought to be feasible and effective in patients with fulminant or acute BCS, and is also useful as a bridge to liver transplantation. The long-term success of the TIPS is limited, however, particularly in patients with liver cirrhosis.[21,22] In our series, 32 patients (60.3%) had histologic liver cirrhosis, and their cumulative survival rates were 93.3% at 5 years and 78.2 % at 10 years. So, TIPS should be considered as a bridge to definitive surgery in case of fulminant or acute liver failure of BCS with liver cirrhosis.

CONCLUSIONS

Fresh autologous pericardium is an effective and durable patch for reconstruction of the IVC in BCS. This technique has a good long-term outcome. We consider that long-term anticoagulant therapy with warfarin is necessary for such hypercoagulable states.

AUTHOR CONTRIBUTIONS

Conception and design: HI, YM, TN, KA, SY, YK
Analysis and interpretation: YM, TN, KA, SY, YK
Data collection: HI, YM, TN, KA, SY, YK, KK
Writing the article: HI
Critical revision of the article: SY, YK, KK

Final approval of the article: HI, YM, TN, KA, SY, YK, KK
Statistical analysis: HI
Obtained funding: YK
Overall responsibility: HI

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