Expression of Connective Tissue Growth Factor in the Human Liver with Idiopathic Portal Hypertension

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Idiopathic portal hypertension (IPH) is a disorder of unknown etiology, clinically associated with portal hypertension in the absence of cirrhosis. This study was designed to delineate the characteristics of IPH RNA expression in liver specimens from patients with IPH. Liver specimens from patients with IPH and patients without liver diseases underwent cDNA expression analysis and in situ hybridization studies. Connective tissue growth factor (CTGF) levels in serum were examined in 76 patients with IPH, 84 patients with hepatitis C virus infection (including those with cirrhosis), and 38 healthy volunteers. Among 588 genes sorted on macroarray, seven up-regulated genes, including CTGF, were detected. In situ hybridization studies showed that positive reactions for CTGF mRNA were most intense in the epithelial cells of proliferating bile ducts within portal tracts in patients with IPH. In the liver parenchyma, there was no appreciable staining of hepatocytes, sinusoidal endothelial cells, or hepatic stellate cells (HSCs), and there were few positive signals for CTGF mRNA in normal liver. The serum CTGF level in patients with IPH was significantly higher than the value in healthy volunteers. Six (8%) of the 76 patients with IPH had serum CTGF levels greater than 80 ng/mL, far exceeding the level of any patient with cirrhosis. In conclusion, overexpression of CTGF is one of the most important features of IPH.

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INTRODUCTION

Idiopathic portal hypertension (IPH), or Banti's syndrome, is relatively frequent in several Asian countries, including Japan and India, as compared with the prevalence in the United States and Europe (1–3). Because IPH is characterized by prominent portal fibrosis despite no fibrotic changes in the liver parenchyma, this disease also has been referred to as hepatoportal sclerosis (4) or noncirrhotic portal fibrosis (2). Portal hypertension without hepatic cirrhosis is considered to result from increased portal venous flow associated with splenomegaly and from increased portal vascular resistance (5). The principal pathological changes in IPH are moderate portal fibrosis, destruction of intrahepatic terminal portal radicles, and parenchymal atrophy of the liver secondary to portal malperfusion (6–8). IPH is not associated with hepatic injury, perisinusoidal fibrosis in the liver parenchyma, or pseudonodule formation. Accumulated evidence indicates that IPH is an entirely distinct disease from liver cirrhosis. Aetiopathogenically, IPH has been associated with chronic abdominal infection (9), abnormal T-cell activation by continuous antigen stimulation (10), immunological abnormalities initiated by HLA-DR antigen expression by the portal tract microvasculature (11,12), exposure to toxic substances or drugs (13), and blood coagulation abnormalities (14). However, the pathogenesis of IPH remains poorly understood.

Recently, differential expression analysis by cDNA array has shed light on new factors related to liver cancer (15) and chronic viral hepatitis (16). To our knowledge, IPH has not been studied previously by array analysis. In the present study, we first attempted to identify RNA expression patterns related to IPH by macroarray analysis. We detected several overexpressed genes in the liver with IPH. One of them was connective tissue growth factor (CTGF), a member of the CCN family that plays an important role in matricellular regulation related to internal and external cell signaling (17). CTGF is characterized by a modular architecture, containing domains homologous to insulin-like growth factor-binding proteins and the C-terminal domains of some types of

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collagens and mucins. CTGF is overexpressed in a number of fibrotic diseases, including systemic sclerosis (18), pulmonary fibrosis (19,20), pancreatic fibrosis (21), renal fibrosis (22), and liver fibrosis (23–27). To examine the role of CTGF in the development of portal fibrosis in the liver of patients with IPH, we performed in situ hybridization analysis. We also examined serum CTGF levels in patients with IPH, viral hepatitis, or cirrhosis to evaluate the relations between CTGF and the clinical features of these diseases.

MATERIALS AND METHODS

Patients

We examined i) 76 patients with IPH; ii) 24 patients with chronic hepatitis C and fibrosis, stage 1 (CHC-F1, no bridging); iii) 24 with chronic hepatitis C and fibrosis, stage 2 (CHC-F2, porto-portal bridging); iv) 24 with chronic hepatitis C and fibrosis, stage 3 (CHC-F3, porto-central bridging); v) 12 with chronic hepatitis C and fibrosis, stage 4 (CHC-F4, cirrhosis); and vi) 38 healthy volunteers. IPH was histopathologically diagnosed according to the criteria proposed by the Japanese Study Group of Intrahepatic Haemodynamic Alterations (12,28). Wedge liver biopsy specimens and peripheral blood samples were collected from each patient. All samples from patients with IPH were provided by the Japanese Study Group of Intrahepatic Haemodynamic Alterations.

Atlas cDNA Expression Arrays

A part of the wedge biopsy specimens was frozen, and poly A^+ RNA was extracted from the frozen tissue, using a Micro-Fast Track kit (Invitrogen, Carlsbad, CA, USA). To analyze differences in gene expression between livers with IPH and normal livers, Atlas cDNA expression macroarray membranes (Clonotech, Mountain View, CA, USA) were used. Each μg of poly A^+ RNA was treated with 5 μL DNase I at 37°C for 1 h. RNA was precipitated at –80°C for 30 min with a 0.1volume of 3 mol/L sodium acetate, pH 5.2, and a 2.5volume of 100% ethanol. The RNA was then converted into ^{32}P-labeled first strand cDNA with Superscript II reverse transcriptase. Unincorporated ^{32}P-labeled nucleotides were removed by Chroma Spin-200 column chromatography. After prehybridization, heat-denatured probes were hybridized to Atlas cDNA expression array membranes for 18 h at 68°C. The membranes were washed in 2X SSC/1% SDS for 60 min at room temperature, followed by 0.1X SSC/1.5% SDS at 68°C for 40 min. Autoradiography was then performed. Membranes were exposed to film, and signals were quantitated by a Fuji BAS2000 bio-imaging analyzer (Fuji Photo Film, Tokyo, Japan). The intensities of hybridization signals were measured in duplicate for each gene. Housekeeping genes such as GAPDH were hybridized with equivalent intensities on all filters. To select differentially expressed genes, maximum ratios of differences in expression were calculated for each gene.

Histology

Another part of the liver specimens underwent histological analysis. Samples were fixed in a periodate-lysine 3% paraformaldehyde (PLP) solution for 24 h at 4°C. Paraffin sections were mounted on silane-coated slides. Serial sections were stained with hematoxylin and eosin (H-E) and azan. For in situ hybridization, deparaffinized sections were treated with 0.2 M HCl for 15 min, followed by digestion with 1.5 mg/mL proteinase K for 15 min at 37°C. The sections were then post-fixed with 4% paraformaldehyde in PBS for 5 min and treated with 2 mg/mL glycine twice for 15 min each. After washing with PBS, the samples were soaked in 2X standard saline citrate buffer (SSC) with 50% formamide and subjected to hybridization. A 2100-bp fragment of CTGF cDNA was subcloned into the EcoRI site of Bluescript plasmid and used to prepare probes. The DNA was linearized by using Xba I to prepare the antisense strand and Xho I to prepare the sense strand. The probes were labeled with digoxigenin-11-UTP by using a DIG RNA-labeling kit (Boehringer Mannheim Biochemic, Mannheim, Germany). Aliquots of labeled RNA probes (1 mg/mL) in a mixture containing 50% formamide, 10% dextran sulfate, 1X Denhardt's solution, 100 g/mL tRNA, 5X SSC, 0.25% sodium dodecylsulfate, 1 mM ethylenediaminetetraacetic acid (EDTA), and 50 mM NaH2PO4 were placed on the slides under a coverslip. Hybridization was performed in a humidified chamber for 18 h at 45°C. The slides were washed in 2X SSC with 50% formamide at 50°C. Nonhybridized probes were digested in 2.5 mg/mL RNase A, 500 mM NaCl, 1 mM EDTA, and 10 mM Tris-HCl, pH 8.0, for 15 min at 37°C. They were then rinsed for 15 min in 2X SSC and in 0.2X SSC twice at 50°C. The digoxigenin-labeled probes were visualized with a DIG nucleic acid detection kit (Boehringer Mannheim Biochemica).

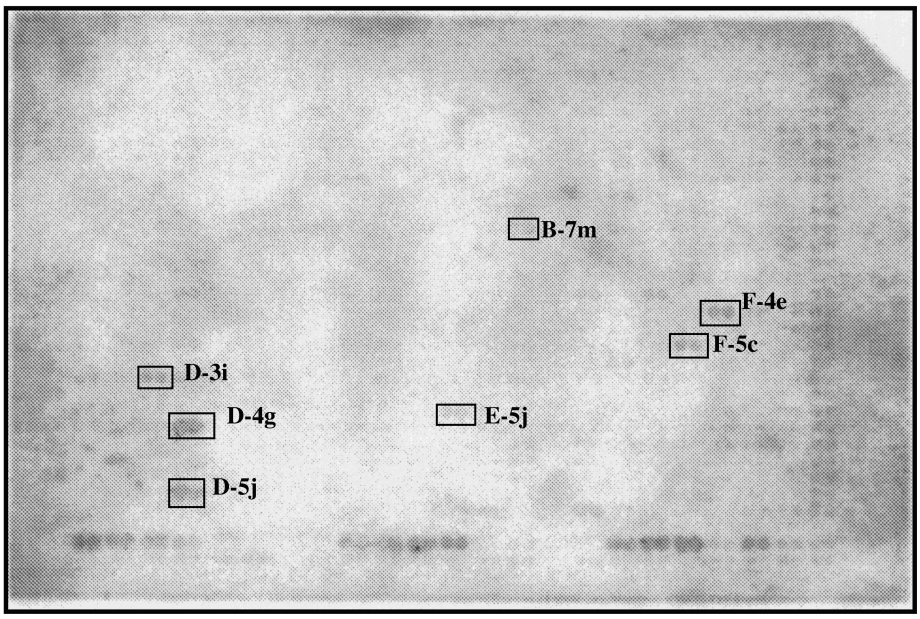
Enzyme-linked Immunosorbent Assay (ELISA)

To detect human CTGF, a sandwich ELISA method was employed with the use of two different anti-human CTGF mAbs, M295 and 8-86-2, both of which were provided by Japan Tobacco Inc. A 96-well ELISA plate (Corning Inc., Corning, NY, USA) was coated with 50 μL of anti-human CTGF mAb M295 at a concentration of 10 μg/mL in phosphate-buffered saline (PBS) for 1 h at room temperature. After washing, unbound sites were blocked by incubation with 200 μL of a blocking reagent (Block Ace; Dainippon Pharmaceutical Co., Osaka, Japan) for 2 h at room temperature. After washing three times with 0.1% Tween 20 in PBS, 50 μL of sample was added to the well and incubated for 1 h. After washing three times, 50 μL of 2 μg/mL biotinylated anti-human CTGF mAb 8-86-2 in 1% bovine serum albumin and PBS with 0.1% Tween 20 was added. The plates were incubated for 1 h at room temperature and subsequently washed three times. Then, 50 μL of a 1/1,000 dilution of streptavidin β-D-galactosidase (Gibco BRL, Gaithersburg, MD, USA) was added. After 1 h of incubation, the

plates were washed three times, and 50 μL of 1% 4 methyl-umbelliferyl-β-D-galactoside (Sigma Aldrich Japan Corp., Tokyo, Japan) was added. After 10 min of incubation, the fluorescence intensity of the well was determined at 460 nm (excitation, 355 nm) with a Wallac 1420ARVOsx fluorometer (GE Healthcare Bio-Science Corp., Piscataway, NJ, USA).

Statistical Analysis

The Japanese Study Group of Intrahepatic Haemodynamic Alterations established a database from case report forms collected by nationwide epidemiologic surveys in 1999 and 2003. We analyzed 29 patients with IPH who were followed up by these surveys. CTCF levels were classified as low or high on the basis of the median value of 20 ng/mL. Factors included in univariate analysis were as follows: age, sex, hepatic coma, ascites, splenomegaly on computed tomography or ultrasonography, liver fibrosis on histopathological examination, platelet count, albumin level, and indocyanine green retention rate at 15 min. All statistical analyses were performed with Statistical Analysis System (SAS), ver. 8.2.



B-7m	heat shock 70-kDa protein 1	E-5j	Leukosialin S
D-3i	CCAAT-binding transcriptional factor	F-4e	Connective tissue growth factor
D-4g	T-lymphocyte activated protein	F-5c	Placental growth factor
D-5j	Heregulin Alpha		

Figure 1. cDNA expression on macroarray analysis. One gene appeared as two spots on the membrane. Seven overexpressed genes were identified as shown above.

RESULTS

Expression of CTGF mRNA in Livers with IPH

To define differences in gene expression between livers with IPH and control livers, maximum ratios of differences in expression between these tissues were calculated for each gene. Among 588 genes sorted, seven up-regulated genes were identified: T-lymphocyte activated protein, Leukosialin S (both of which are related to T-cell activation), heat shock 70-kDa protein 1, placental growth factor, Heregulin alpha, CCAAT-binding transcriptional factor, and CTGF (Figure 1).

Localization of CTGF mRNA Expression in Livers with IPH

H-E staining revealed enlarged portal tracts, containing proliferating bile ducts (Figure 2A). However, no necrotic or fibrotic changes were found in the parenchyma. On azan staining, collagen fibers, stained blue, were found only in portal tracts, and collagen bundles were negligible in the liver parenchyma (Figure 2B). On in situ hybridization, few positive signals for CTGF mRNA were seen in liver specimens from healthy volunteers (data not shown). Positive signals were most intense in epithelial cells of proliferating bile ducts within the portal tracts (Figure 2C). Signals were found in both the nuclei and cytoplasm. There were large bile ducts (Figure 2D), small ducts with an obvious lumen, and small ducts with an obscure lumen, the latter of which were situated at the periphery of the portal tracts and adjoined hepatocytes (Figure 2E). Endothelial cells of portal veins (Figure 2F) and capillaries (Figure 2F, inset) in the portal tracts were also positive. Abundant connective tissue was adjacent to the bile ducts and blood vessels. Spindle-shaped cells, possibly myofibroblasts, in the portal tract were embedded in this connective tissue and stained positively for CTGF mRNA (Figure 2D–G). In contrast, hepatocytes, sinusoidal endothelial cells, and HSCs in the liver parenchyma were not appreciably stained (Figure 2H).

Serum Levels of CTGF in Patients with IPH

The serum CTGF level was 19.1 ± 12.5 ng/mL in healthy volunteers (Figure 2). In patients with CHC-F1, F2, F3, and F4, serum CTGF levels were 36.1 ± 11.7 ng/mL, 31.7 ± 7.5 ng/mL, 32.1 ± 6.2 ng/mL, and 34.0 ± 9.8 ng/mL, respectively. Serum CTGF levels did not differ significantly among the different stages of fibrosis, but the levels in the patients were all significantly higher than the control value. In patients with IPH, the serum CTGF level was 28.0 ± 32.8 ng/mL, significantly higher than

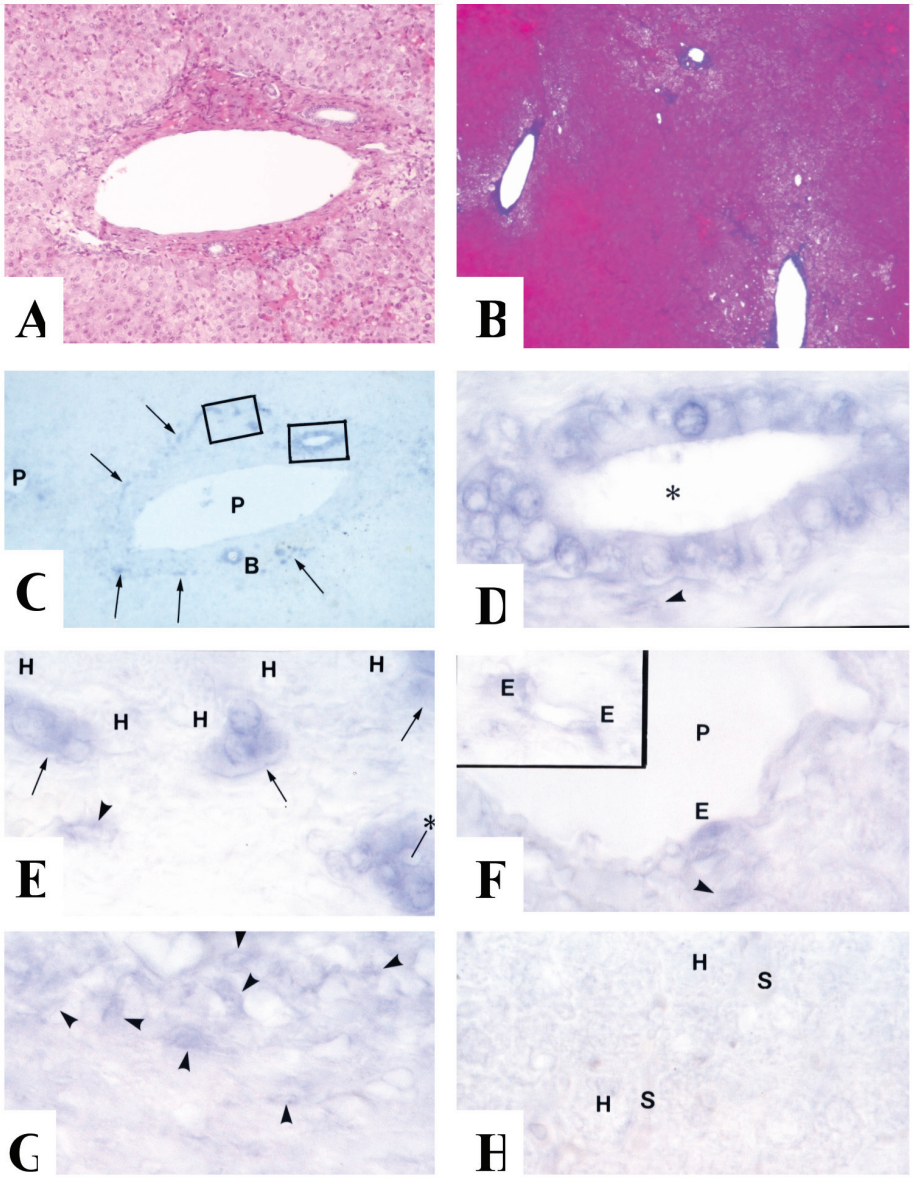


Figure 2. Histological examination of liver with IPH. (A) An enlarged portal tract containing proliferating bile ducts. Hematoxylin and eosin stain, x100. (B) Collagen fibers are seen in the fibrous portal tract. Azan stain, x40. (C-H) In situ hybridization shows positive signals for connective tissue growth factor (CTGF) mRNA in the epithelial cells of bile ducts (arrow), endothelial cells of portal tracts, and myofibroblasts (arrowhead) (C, x100 and D-H, x400). P, portal tract; B, bile duct; H, hepatocyte; S, sinusoid; E, endothelial cell; *, lumen.

the control value. In contrast to the patients with CHC-F1-4, some patients with IPH had markedly elevated serum CTGF levels. Serum levels of CTGF exceeded the control value + 2SD in 14 patients with IPH (Figure 3).

Univariate Analysis in Patients with IPH

No variable was significantly related to CTGF level (Table 1).

DISCUSSION

The first part of our study demonstrated that CTGF mRNA was overexpressed in the liver of patients with IPH. CTGF is well known to function as one of the downstream effectors of transforming growth factor-β (TGF-β), a potent fibrogenic cytokine (29). CTGF also mediates TGF-β-induced collagen synthesis (30) and induces angiogenesis (31), both of which have important roles in wound healing. In biliary atresia (25,26) and liver cirrhosis (23,24,29) accompanied by fibrosis, CTGF is highly expressed in liver tissue. Previous in situ hybridization studies have shown that myofibroblast-like cells, fibroblasts, and activated HSCs express CTGF in chronic active hepatitis (21,23) and contribute to fibrosis in the liver (24,25). Vascular endothelial cells in fibrous septa are also positive for CTGF mRNA (27). Furthermore, some ductular epithelial cells stain moderately for CTGF mRNA. In our patients with IPH, CTGF mRNA was strongly expressed in most proliferating ductular epithelial cells within the portal tracts and moderately expressed in vascular endothelial cells and portal myofibroblasts. Tsuneyama et al. also found that CTGF protein was strongly expressed in periductal mononuclear cells (32). Ductular proliferation is classified into two types: typical and atypical. The former results from proliferation of preexisting ductules, displaying an obvious lumen, while the latter results from ductular metaplasia of hepatocytes, showing an obscure lumen (33). In our study, expression of CTGF mRNA was associated with both types of ductular proliferation. In contrast, CTGF mRNA expression was not detected in the sinusoidal endothelial cells or HSCs in IPH. The distribution of CTGF mRNA signals was consistent with the localization of hepatic fibrosis. Namely, although fibrogenesis is induced in the liver parenchyma by hepatocellular injury in liver cirrhosis, it is confined to the portal tracts in IPH, with no appreciable damage or fibrosis of the parenchyma.

We did not investigate the six other genes detected on macroarray analysis. It is important to examine the roles of these factors not reported previously to

be related to IPH. The mechanism underlying the up-regulation of CTGF in IPH is also an important topic for future research.

Our study also showed that serum CTGF levels were elevated in patients with IPH. Serum CTGF levels have been reported to correlate with the progression of hepatic fibrosis (24). Recently, Gressner et al reported that the CTGF concentration reflected active fibrogenesis in chronic viral hepatitis and decreased in patients with hepatic cirrhosis (34). On the other hand, we found no significant differences in serum CTGF levels among the different stages of fibrosis in chronic hepatitis C. In the 29 patients for whom clinical data were available from the Japanese Study Group of Intrahepatic Haemodynamic Alterations, the serum CTGF level was not significantly related to any clinical characteristic; however, a trend toward significance was obtained for hepatic coma ($P = 0.09$). In the present study, we found no clinical features that were related to a high level of serum CTGF. Six (8%) of the 76 patients with IPH had serum CTGF levels higher than 80 ng/mL, far exceeding the highest level among the patient who had chronic hepatitis with fibrosis. Larger studies of patients with IPH are required to delineate the clinical characteristics of this disease and to define the implications of highly elevated serum levels of CTGF. Our results suggest that marked elevations of CTGF in serum are one of the unique features of IPH. Thus, CTGF might be a clinically useful marker for IPH.

In conclusion, macroarray analysis showed that seven genes, including CTGF, were overexpressed in IPH. CTGF was strongly expressed by proliferating ductular epithelial cells in fibrous portal areas and was not appreciably expressed in the liver parenchyma, suggesting that CTGF might contribute to periductal fibrosis in IPH. As compared with patients who have CHC accompanied by fibrosis or cirrhosis, some patients with IPH showed markedly higher serum levels of CTGF. Our findings suggest that overexpression of CTGF is one of most important pathological features of IPH.

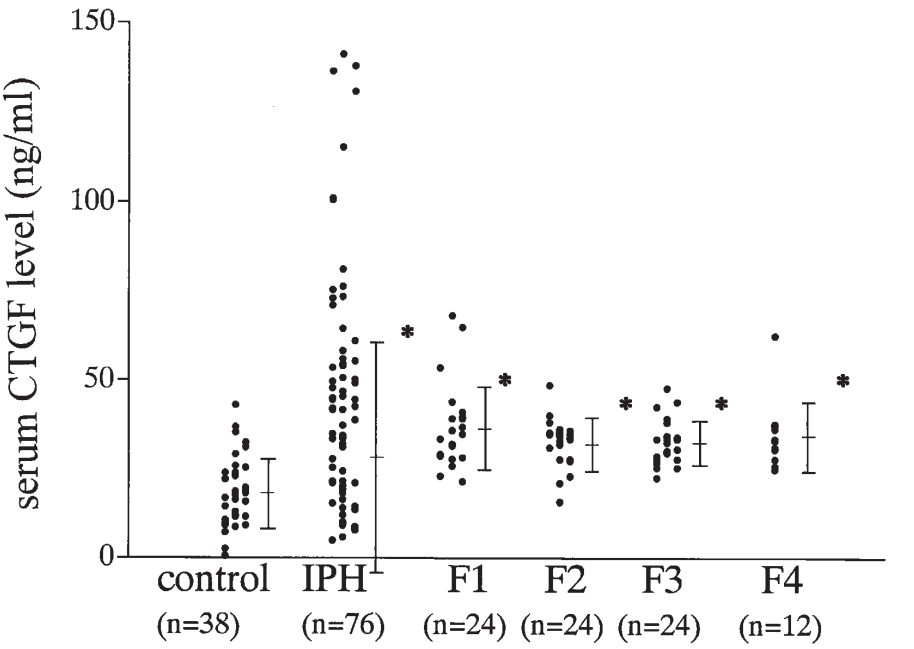


Figure 3. Serum levels of CTGF in patients with IPH, patients with HCV, and healthy volunteers (control). Patients with HCV were divided in four groups according to the stage of hepatic fibrosis: F1, periportal fibrous expansion; F2, porto-portal septa; F3, porto-central septa; and F4, cirrhosis. * means significantly higher than the control value.

ETHICAL CONSIDERATIONS

All study participants provided informed consent and this study protocol complied with the ethical guidelines of the Declaration of Helsinki (1975) and was approved by the Ethics Committee of Osaka City University Graduate School of Medical (approval number 368).

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Table 1. Results of univariate analysis in patients with IPH

Parameter	CTGF value (ng/mL)		
	< 21	> 21	*P*
Age (years)	58 ± 20	64 ± 11	0.605
Sex (male/female)	4/11	5/9	0.303
Hepatic coma	0 (0/15)	2(2/9)	0.092
Splenomegaly	14 (14/15)	11 (11/11)	0.392
Liver fibrosis	5 (5/9)	5 (5/9)	1.000
Platelet count (< $5.0 \times 10^3/mm^3$)	4 (4/15)	3 (3/14)	0.742
Albumin (< 3.0 g/dl)	1 (1/15)	0 (0/14)	0.325
ICG_R15^a (> 30%)	3 (3/11)	3 (3/11)	1.000

[a]ICG_R15, indocyanine green retention rate at 15 min.

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