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From Cell Biology to Tissue Engineering

Cytoglobin-expressing cells in the splenic cords contribute to splenic fibrosis in cirrhotic patients

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Summary. Background and Aim. Among several noninvasive evaluation methods of portal hypertension (PH), the measurement of spleen stiffness is a reliable method for predicting esophageal variceal bleeding; however, the underlying mechanisms for increased stiffness remain unclear. We attempted to elucidate the pathological changes to the spleen and the underlying mechanisms in patients with PH.

Methods. Histological examination was performed using splenic tissues from 42 patients with PH who underwent laparoscopic splenectomy, and the results were compared with those from patients without PH.

Results. In addition to splenic sinus congestion, diffuse fibrosis was detected in the splenic cords in the red pulp of patients with PH. The degree of the fibrosis was well correlated with severity in thrombocytopenia and splenomegaly. Cells expressing α-smooth muscle actin dramatically increased in the splenic cord. Cytoglobin (Cygb) expression was detected in human splenic cords as reported in animal reticular cells, and fluorescent double immunostaining revealed that these cells expressed α-smooth muscle actin in patients with PH, suggesting transformation of Cygb-expressing cells to myofibroblastic cells. Expression levels of nicotinamide adenine dinucleotide phosphate oxidase (NOX) 2, nitrotyrosine, and transforming growth factor-β were markedly upregulated in the red pulp of patients with PH, implying a significant role of oxidative stress in the mechanism for splenic fibrosis.

Conclusion. Splenic fibrosis progresses along with advancement of PH. Cygb-expressing cells in the splenic cord possibly participate in this process through mechanisms including oxidative stress.

Key words: Cytoglobin, Oxidative stress, Portal hypertension, Splenomegaly, Splenic fibrosis

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Introduction

Effective antivirus treatment has shown that even advanced hepatic fibrosis can be ameliorated by relieving causative stress (Shiratori et al., 2000; Liaw et al., 2004; Chang et al., 2010). However, whether advanced portal hypertension (PH) and its complications could be reversible is still controversial. Variceal bleeding and thrombocytopenia are the major complications of advanced liver cirrhosis with PH (Garcia-Tsao, 2001). The risk of variceal bleeding can be estimated using the gastrointestinal endoscope, whereas several noninvasive methods to predict the risk have been recently proposed (Qi et al., 2018). Among them, the measurement of spleen stiffness has been reported to be one of the most reliable (Colecchia et al., 2012). In patients with cirrhosis, spleen stiffness evaluated by ultrasonographic elastography gradually increases along with the advancement of PH (Berzigotti, 2017). Congestion of splenic sinus is believed to be the major cause of splenomegaly, and it possibly contributes to increased spleen stiffness.

The appearance of splenic fibrosis in PH has been reported in early studies (Yamamoto, 1978; Terayama et al., 1994), and fibrosis was observed in the splenic cords of the red pulp. Such fibrosis possibly accelerates the

increase in spleen stiffness in patients with PH in combination with the congestion of splenic sinus. However, no systematic analysis of human splenic fibrosis exists so far, and its mechanism is totally obscure.

In the current study, we histologically investigated congestion of the splenic sinus and fibrosis within the splenic cords in patients with splenomegaly and thrombocytopenia who underwent laparoscopic splenectomy for the purpose of ameliorating thrombocytopenia. We found that splenic fibrosis appears within the splenic cords in patients with advanced splenomegaly and thrombocytopenia in conjunction with congestion of the splenic sinus and that Cygb-expressing cells, which have been reported to affect fibrogenesis in several organs (Nakatani et al., 2004), are possibly responsible for the fibrosis in the splenic cord through the mechanism, including oxidative stress.

Materials and methods

Patients

Forty-two patients with PH, who underwent laparoscopic splenectomy for the purpose of ameliorating thrombocytopenia between 2008 and 2011 at Hyogo College of Medicine, were analyzed in this study. Characteristics and clinical data from these patients are summarized in Table 1. Splenic specimens were also obtained from two patients with normal liver function. One patient underwent splenectomy along with gastrectomy against gastric cancers in relatively early stages and another underwent distal pancreatectomy with splenectomy for pancreatic cystic tumor with low-grade malignancy, and they were analyzed as splenic tissues in patients without PH.

Histological analysis of human splenic tissues

Histological analysis was performed as previously reported (Yada et al., 2015). Tissues were fixed in 10% buffered formalin and embedded in paraffin. Sections were stained with hematoxylin-eosin and examined by light microscopy. Sirius Red/Fast Green staining was performed to evaluate the expression of fibrillar collagens (Yada et al., 2015). Azan-Mallory staining was also performed to visualize collagen fibers as well as reticular fibers (Wu et al., 2019). To detect alpha-smooth muscle actin (α-SMA), a monoclonal anti-α-SMA antibody (Diagnostic Biosystems, Pleasanton, CA) was used. For the detection of endothelial cells of splenic sinus or penicillar arterioles in the splenic cords, a monoclonal anti-CD141 (Abcam, Cambridge, MA, USA) or an anti-CD34 (DAKO, Santa Clara, CA) was used, respectively (Steiniger et al., 2007). A rabbit polyclonal antibody against cytoglobin (Cygb) was used to visualize Cygb-expressing cells in the red pulp (Nakatani et al., 2004). Nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOXs) were detected using antibodies against NOX1 (Signalway Antibody, College Park, MD, USA), NOX2 (Santa Cruz Biotechnology Inc, Dallas, TX, USA), and NOX4 (Proteintech, Chicago, IL, USA). Similarly, anti-nitrotyrosine (Millipore Sigma, Burlington, MA, USA) or anti-transforming growth factor-β (TGF-β; Novus Biologicals, Littleton, CO, USA) antibody was used for immunostaining.

Immunofluorescence staining

Fluorescence immunohistochemistry was performed as previously reported (Yada et al., 2015). An anti-α-SMA monoclonal antibody (Diagnostic Biosystems) and an anti-CD141 monoclonal antibody (Abcam) were used to investigate the relationship between α-SMA-expressing cells in the splenic cords and endothelial cells of splenic sinus. Alexa Fluor 555 tyramide (Invitrogen, Carlsbad, CA, USA) was used to visualize the CD141-positive cells. Alexa Fluor 488-conjugated streptavidin (Invitrogen) was used to visualize the α-SMA-positive cells. In the same manner, fluorescence immunostaining was also performed using an anti-α-SMA monoclonal antibody (Diagnostic Biosystems), an anti-Cygb rabbit polyclonal antibody. Fluorescence immunostaining was observed using a confocal laser scanning microscope, LSM510 (Carl Zeiss, Jena, Germany).

Image analysis

Image analysis was used for semiquantitative assessment of each immunostaining. At a magnification of 200×, the ratio of the area positive for each immunostaining including CD34, Sirius Red, or α-SMA was defined in five fields randomly selected in each sample using free software available from the National Institutes of Health (Image J; http://rsb.info.nih.gov/ij). The mean value of the five fields was used as a representative data point of the sample. Similarly, the percentage of splenic sinus area bounded by the CD141-positive endothelial cells was also determined at a magnification of 400×.

Western blot analysis

Western blot analysis was performed as described

Table 1. Characteristics of patients with portal hypertension (n=42).

	Median (Min-Max)
Age, years; median (range)	60 (47-72)
Sex, M/F, n	20/22
Viral infection, HBV/HCV/NBNC/HBV+HCV, n	0/41/0/1
Platelet counts (×103/μL); median (range)	59.5 (21-111)
WBC counts (μL); median (range)	3130 (1470-6890)
Serum total bilirubin (mg/dL); median (range)	1.2 (0.4-4)
Serum albumin (mg/dL); median (range)	3.7 (2.7-4.8)
Prothrombin activity (%); median (range)	78.6 (47.2-114.7)
Splenic volume (mL); median (range)	428 (96-1508)

elsewhere (Uyama et al., 2012). Namely, splenic tissues were homogenized in sample buffer (62.5 mmol/l Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate [SDS], 1 mM NaF, 1 mM Na_3VO_4, and 10% glycerol) and boiled for 10 min. Samples were separated by SDS polyacrylamide gel electrophoresis (5-20% gradient gel; ATTO, Tokyo, Japan) and electroblotted onto polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). After blocking with Tris-buffered saline containing 5% skim milk and 0.1% Tween 20 overnight, the membrane was incubated with primary antibodies for 2 h. Primary antibodies for Cygb, NOX2, NOX4, nitrotyrosine, and TGF-β were the same as those used in immunohistochemistry. An anti-β-actin antibody (Merk, Darmstadt, Germany) was used to detect β-actin. After washing, the membrane was then incubated with a secondary antibody for 1 h. After washing, the antigens were detected with enhanced chemiluminescence (ECL) using an ECL substrate (Amersham, Roosdaal, Netherlands). Immunoreactive bands were captured using the LAS-3000 imaging system (Fujifilm, Tokyo, Japan).

Measurement of total collagen amounts in splenic tissues

The total collagen amount in splenic tissues was quantitatively determined by colorimetric methods using a commercially available kit (Total Collagen Assay Kit, QuickZyme Biosciences, Leiden, Netherlands).

Measurement of splenic volume

All patients underwent abdominal computed tomography before splenectomy. Preoperative splenic volume was analyzed using a three-dimensional image-processing software (Synapse Vincent: version 3, Fujifilm, Tokyo, Japan).

Statistical analysis

Statistical analyses were performed using Statview version 5.0.1 (SAS Institute Inc., Cary, NC, USA). Correlations among obtained data were analyzed using standard Pearson correlation analysis. Scheffé multiple comparison test was used to analyze the relation between the Sirius Red-positive fibrotic area (%) and the grade of decrease in platelet counts. A value of $P<0.05$ was considered to be statistically significant.

Results

Congestion of the splenic sinus in patients with PH

We first analyzed splenic sinus in the red pulp of patients with PH in comparison with that of patients without PH. An immunohistochemical study using an antibody against CD141, which recognizes endothelial cells of the splenic sinus (Steiniger et al., 2007), revealed that the width of the splenic cord was narrowed by the increased area of the splenic sinus in patients with PH (Fig. 1B,D). Using image-analyzing software, we semiquantitatively measured the percentage of the splenic sinus area, which was bounded by the CD141-positive cells, and compared it with the platelet counts in peripheral blood in each patient (Fig. 1F). A significant negative correlation was detected between these two parameters. Meanwhile, the area of CD34-positive penicillar arterioles per observed field did not markedly change in the patients with or without PH (Fig. 1A,C), and no significant correlation was detected between the percentage of CD34-positive area and the platelet counts in each patient (Fig. 1E).

Fibrosis in the splenic cord in patients with PH

We next analyzed splenic fibrosis in patients with PH, investigating the expression of fibrillar collagens in the spleen using Sirius Red staining. In low-magnified views (×40), thickening of the splenic capsules and trabeculae was detected in patients with PH compared with those in patients without PH (Fig. 1G,H). In higher magnified views, a significant amount of fibrillar collagens appeared in the red pulp in patients with PH (Fig. 1J), which was only slightly detected in the splenic cords in patients without PH (Fig. 1I). At much higher magnifications, network-patterned expression of fibrillar collagens was observed in the splenic cord, surrounded by congested splenic sinuses in patients with PH (Fig. 1L), while only a small amount of fibrillar collagens was detected in the splenic cords in patients without PH (Fig. 1K). Azan-Mallory staining, which can visualize collagen fibers as well as reticular fibers, revealed significant advancement of fibrosis in the splenic cords in patients with PH compared with that in patients without PH (Fig. 1M,N). In addition, total collagen content in splenic tissues, which was quantitatively measured, significantly increased in patients with PH compared with that in patients without PH (Fig. 1O).

Appearance of α-SMA-positive cells in the splenic cord in patents with PH

We then examined the existence of fibroblast-like cells in the red pulp of the spleen, which are possibly responsible for the production of fibrillar collagens. In patients without PH, connective tissues such as splenic trabeculae and the walls of penicillar arterioles as well as a small number of cells in the splenic cords were positive for α-SMA in the red pulp (Fig. 2A,B). The expression of α-SMA markedly increased in the spleen, especially in the red pulp, in patients with PH (Fig. 2C,D). The expression of α-SMA in the red pulp was localized in the splenic cord (Fig. 2D,E). Semiquantitative image analysis revealed a significant increase in α-SMA-positive area in patients with PH (Fig. 2G). Fluorescent double immunostaining revealed that α-SMA-positive cells in the splenic cord exist

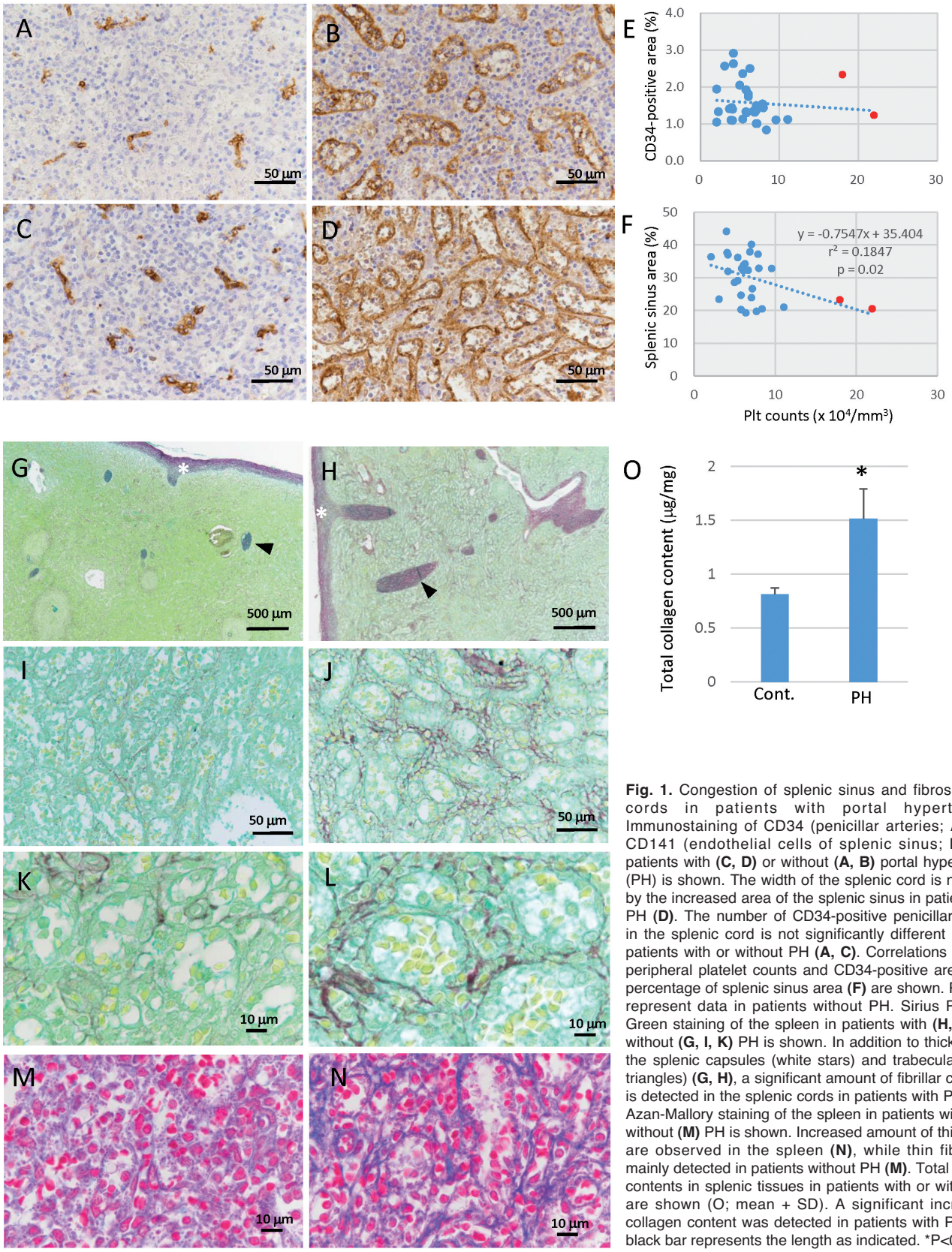

Fig. 1. Congestion of splenic sinus and fibrosis in the cords in patients with portal hypertension. Immunostaining of CD34 (penicillar arteries; **A**, **C**) or CD141 (endothelial cells of splenic sinus; **B**, **D**) in patients with **(C, D)** or without **(A, B)** portal hypertension (PH) is shown. The width of the splenic cord is narrowed by the increased area of the splenic sinus in patients with PH **(D)**. The number of CD34-positive penicillar arteries in the splenic cord is not significantly different between patients with or without PH **(A, C)**. Correlations between peripheral platelet counts and CD34-positive area **(E)** or percentage of splenic sinus area **(F)** are shown. Red dots represent data in patients without PH. Sirius Red-Fast Green staining of the spleen in patients with **(H, J, L)** or without **(G, I, K)** PH is shown. In addition to thickening of the splenic capsules (white stars) and trabeculae (black triangles) **(G, H)**, a significant amount of fibrillar collagens is detected in the splenic cords in patients with PH **(J, L)**. Azan-Mallory staining of the spleen in patients with **(N)** or without **(M)** PH is shown. Increased amount of thick fibers are observed in the spleen **(N)**, while thin fibers are mainly detected in patients without PH **(M)**. Total collagen contents in splenic tissues in patients with or without PH are shown (O; mean + SD). A significant increase in collagen content was detected in patients with PH. Each black bar represents the length as indicated. *P<0.05.

adjacent to the endothelial cells of splenic sinus and do not overlap (Fig. 2F).

Progression of splenic fibrosis along with thrombocytopenia and splenomegaly

To investigate whether splenic fibrosis progresses along with advancement in thrombocytopenia, we compared the platelet counts and the percentages of fibrillar collagen or α-SMA-positive area per observed fields (×200) using image-analyzing software. Between the splenic fibrosis and platelet counts, a good negative correlation was detected ($P<0.01$; Fig. 3A, left). When the patients were divided into the three groups according to platelet count (less than $50\times10^3/\mu L$, between $50\times10^3/\mu L$ and $100\times10^3/\mu L$, and greater than $100\times10^3/\mu L$), significant differences in the degree of fibrosis were detected between each group (Fig. 3A, right). Similarly, a significant negative correlation was also detected between the percentages of α-SMA-positive area and platelet counts between the two parameters ($P<0.05$; Fig. 3B, left). A significant difference in the expression of α-SMA was also detected between the patients with platelet counts less than $50\times10^3/\mu L$ and the other two groups (Fig. 3B, right). Moreover, we compared the degree of splenic fibrosis with the preoperative splenic volume, which was calculated using three-dimensional image-processing software, and a strong positive correlation was detected between the two parameters ($P<0.001$; Fig. 4C).

Cygb-expressing cells participate in fibrosis in the splenic cord

We then investigated the characteristics of α-SMA-positive cells in the splenic cords. Cygb, the fourth globin, has been reported to be expressed in reticular cells in normal rat spleen (Nakatani et al., 2004). In the red pulp of patients without PH, Cygb-expressing cells existed in the splenic cords, and the shape of the cells

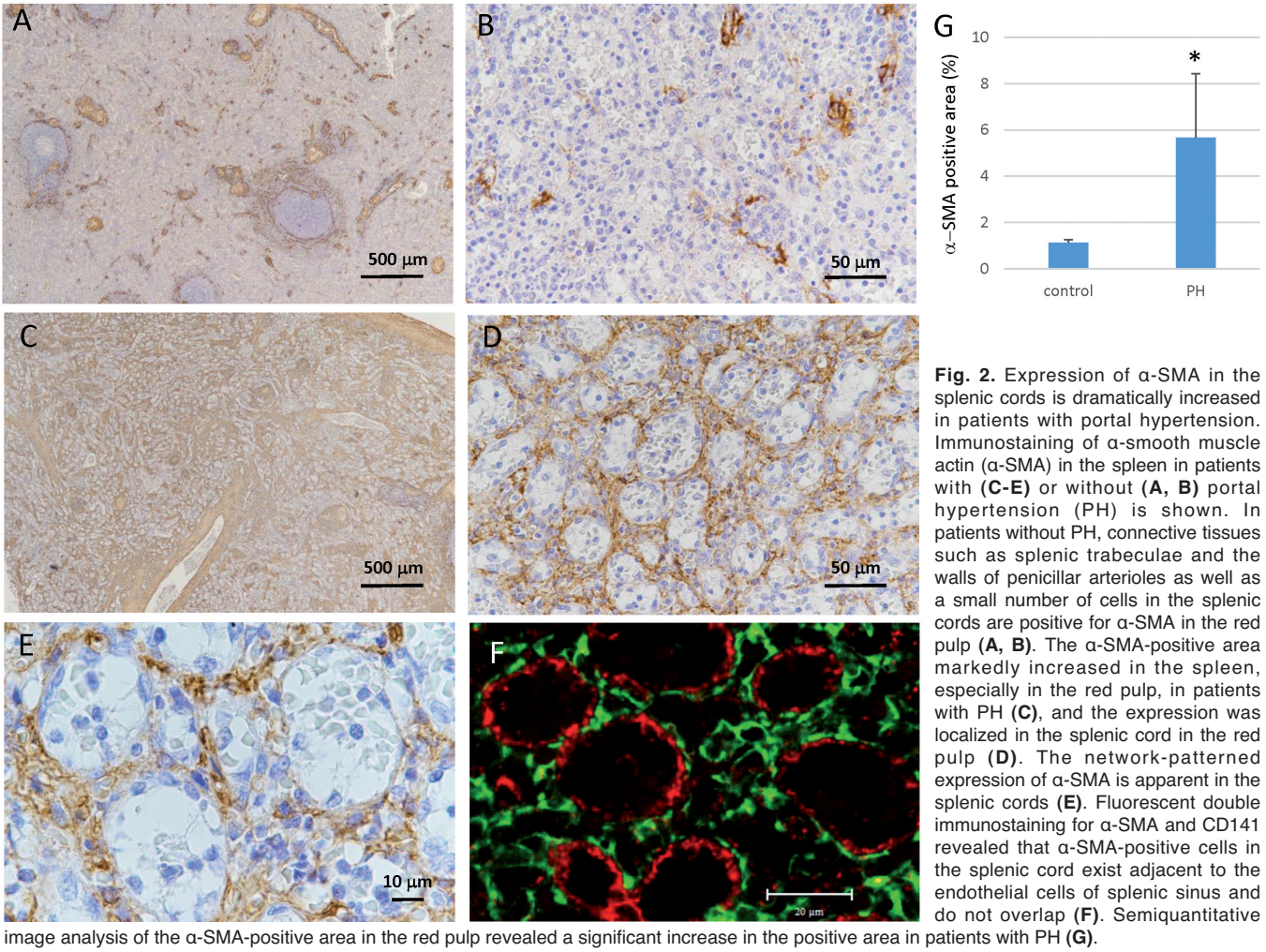

Fig. 2. Expression of α-SMA in the splenic cords is dramatically increased in patients with portal hypertension. Immunostaining of α-smooth muscle actin (α-SMA) in the spleen in patients with **(C-E)** or without **(A, B)** portal hypertension (PH) is shown. In patients without PH, connective tissues such as splenic trabeculae and the walls of penicillar arterioles as well as a small number of cells in the splenic cords are positive for α-SMA in the red pulp **(A, B)**. The α-SMA-positive area markedly increased in the spleen, especially in the red pulp, in patients with PH **(C)**, and the expression was localized in the splenic cord in the red pulp **(D)**. The network-patterned expression of α-SMA is apparent in the splenic cords **(E)**. Fluorescent double immunostaining for α-SMA and CD141 revealed that α-SMA-positive cells in the splenic cord exist adjacent to the endothelial cells of splenic sinus and do not overlap **(F)**. Semiquantitative image analysis of the α-SMA-positive area in the red pulp revealed a significant increase in the positive area in patients with PH **(G)**.

was small spindlelike or starlike possessing fine processes, which is similar to those cell shapes reported in rat spleen (Fig. 4A). In the red pulp of patients with PH, the shape of Cygb-expressing cells changed, elongating their cytoplasm to surround the splenic sinus (Fig. 4B). Fluorescent double immunostaining for Cygb and α-SMA revealed that many of the Cygb-expressing cells overlapped with α-SMA-positive cells in the splenic cords of patients with PH (Fig. 4D). Western blot analysis for Cygb using homogenates of splenic tissues revealed that the protein expression of Cygb in the splenic tissues markedly increased in patients with PH (Fig. 4E).

Expression of NOXs, nitrotyrosine, and TGF-β in the red pulp

We examined the expression of NOXs, which are responsible for the production of reactive oxygen species in phagocytic and nonphagocytic cells (De Minicis and Brenner, 2007). We analyzed the expression of NOX1, NOX2, and NOX4 in human spleen. The expression of NOX1 could not be detected in the human spleen (data not shown), whereas the expression of NOX2 and NOX4 was detected in the red pulp (Fig. 5 A-D). In patients with PH, NOX2 expression markedly increased mostly in the monocytes or macrophages (Fig. 5B), and NOX4 expression seemed to slightly increase mainly in the endothelial cells of splenic sinus (Fig. 5D). The expression of nitrotyrosine, an oxidative stress marker, was immunohistochemically analyzed in the red pulp. Nitrotyrosine expression dramatically increased in cells in splenic cords as well as in the endothelial cells of the splenic sinus in patients with PH (Fig. 5E,F). We also immunohistochemically investigated the expression of TGF-β, one of the most important cytokines in the progression of fibrosis, in the red pulp. TGF-β expression markedly increased in the red pulp in patients with PH compared with that in patients without PH (Fig. 5G,H). In high-power fields, the increased expression of TGF-β was detected in the mononuclear cells or macrophages as well as in the endothelial cells of splenic sinus in patients with PH (Fig. 5H, inset). Western blot analysis for NOXs, nitrotyrosine, and TGF-β revealed

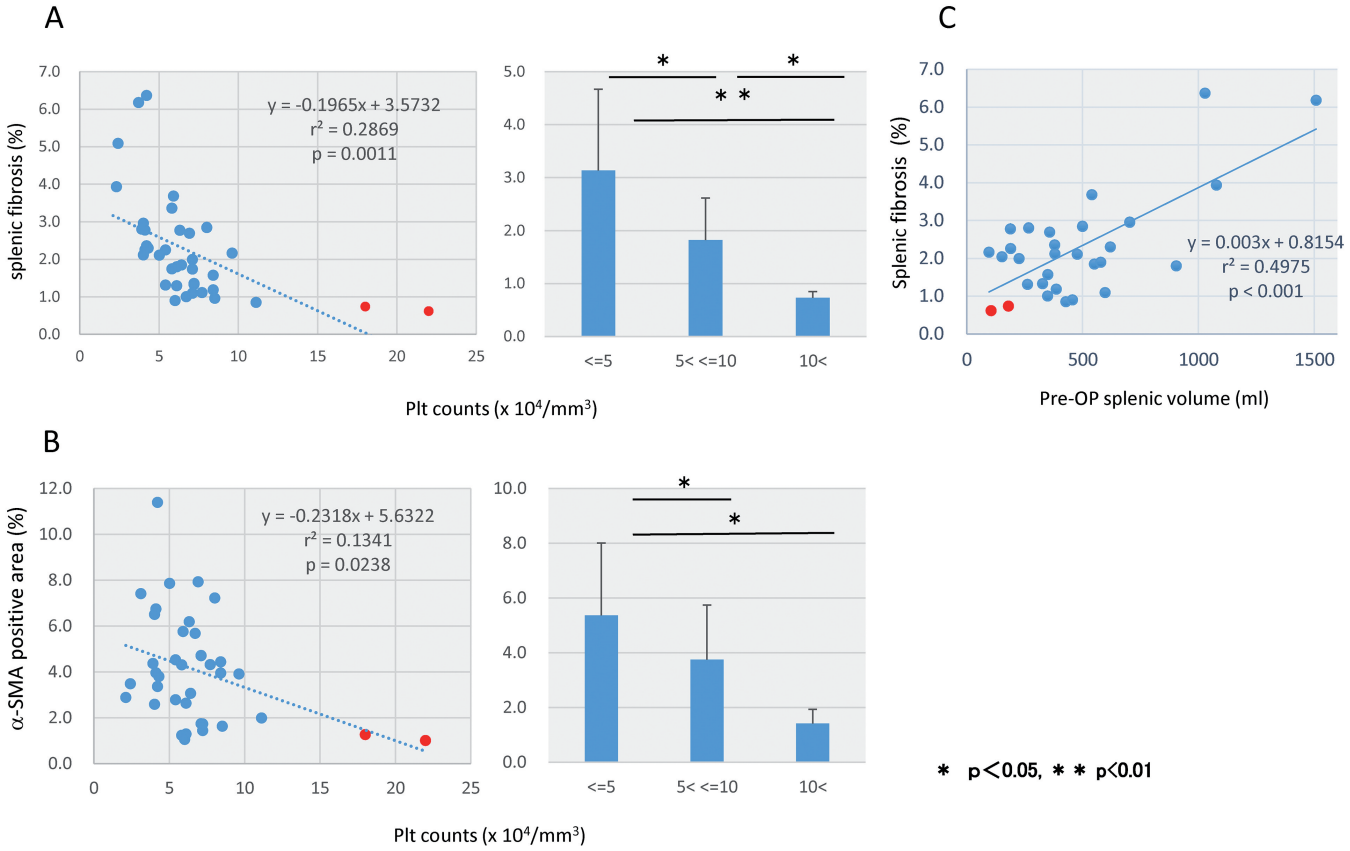

Fig. 3. Relationship between splenic fibrosis and thrombocytopenia or splenomegaly. Relationship between the peripheral platelet counts and the percentage of Sirius Red-positive area (%) **(A)** or the percentage of α-SMA-positive area (%) **(B)** is shown. Relationship between the peripheral platelet counts and the preoperative splenic volume is also shown **(C)**. These data indicate that splenic fibrosis progresses along with advancement in thrombocytopenia and splenomegaly. Red dots represent data in patients without portal hypertension. *P<0.05, **P<0.01.

that the protein expression of NOX2, nitrotyrosine, and TGF-β markedly increased in the splenic tissues in patients with PH compared with that in control patients (Fig. 5I). No remarkable change was detected in NOX4 expression.

Discussion

Congestion of splenic sinus and fibrosis in splenic cords in patients with PH

Evaluation of spleen stiffness using ultrasound is becoming a reliable noninvasive predictive tool for variceal bleeding in patients with PH (Berzigotti, 2017). However, the precise mechanism for the increase in splenic stiffness is still uncertain. Congestion of the splenic vein and splenic sinuses due to the increased pressure of the portal system is possibly a main cause of increased stiffness. Thus, we analyzed splenic congestion in the red pulp in patients with PH, in comparison with that in patients without PH. Narrowing of the splenic cords due to the dilated splenic sinus was detected in patients with PH as expected. A significant negative correlation was observed between splenic sinus

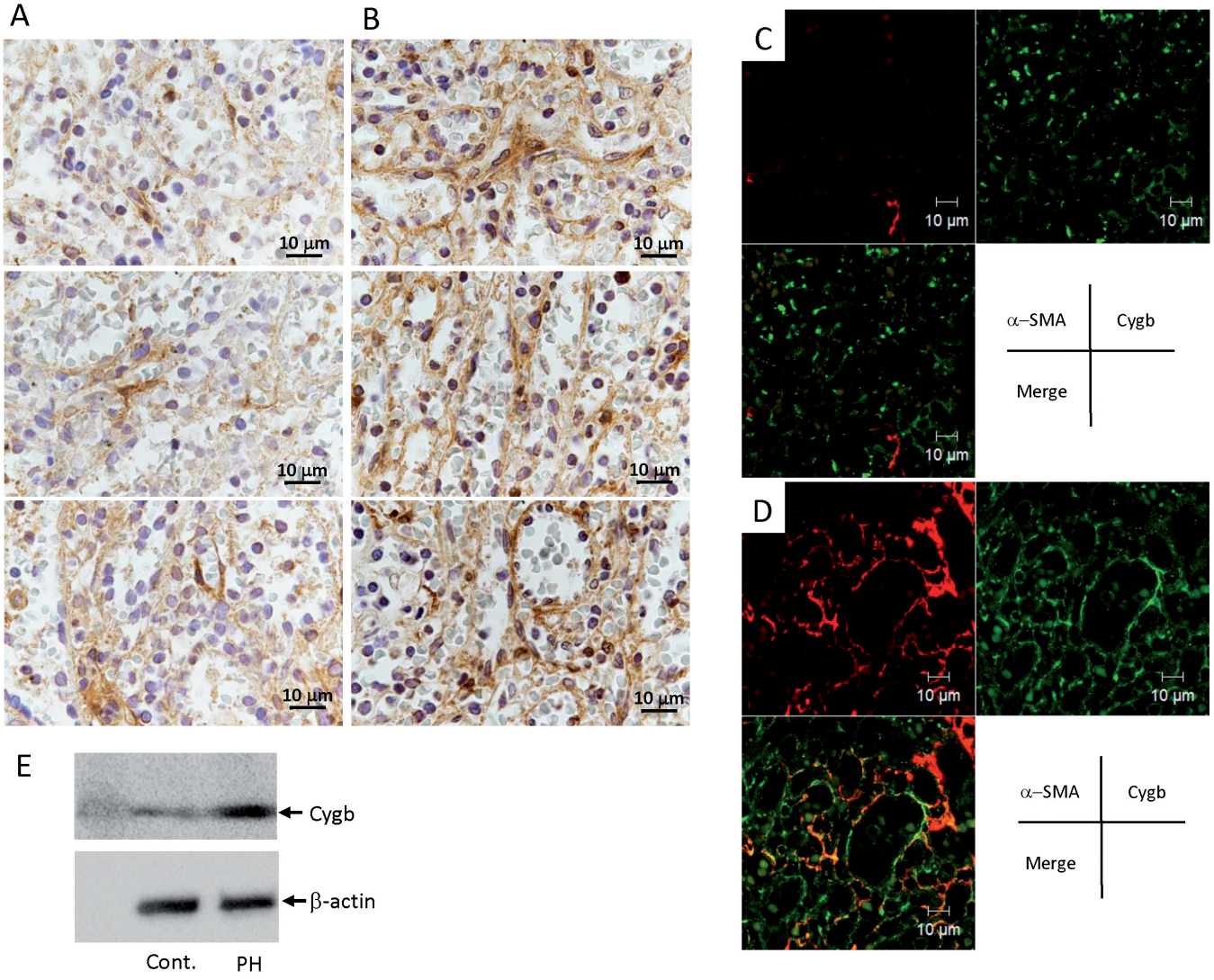

Fig. 4. Cytoglobin-positive cells express α-SMA in the splenic cord in patients with portal hypertension. Immunostaining of cytoglobin (Cygb) in the red pulp of the spleen in patients with **(B)** or without **(A)** portal hypertension (PH) is shown; three representative pictures in each group are shown. In the red pulp without PH, small spindle-like or starlike cells express Cygb in the splenic cord **(A)**. In patients with PH, Cygb-expressing cells elongate their cytoplasm, surrounding the splenic sinus **(B)**. Fluorescent double immunostaining for α-SMA (red) and Cygb (green) revealed that most of the Cygb-positive cells in the red pulp with PH express α-SMA **(D)**, whereas almost no Cygb-positive cell expresses α-SMA in patients without PH **(C)**. Representative Western blot for Cygb using splenic homogenate is shown **(E)**. A marked increase in Cygb expression is detected in patients with PH **(E)**.

dilatation and platelet counts in peripheral blood, which correlates well with the degree of PH (Peck-Radosavljevic, 2017).

Splenic fibrosis has been reported in PH in early studies (Yamamoto, 1978; Terayama et al., 1994), but no systematic analysis of splenic fibrosis exists thus far.

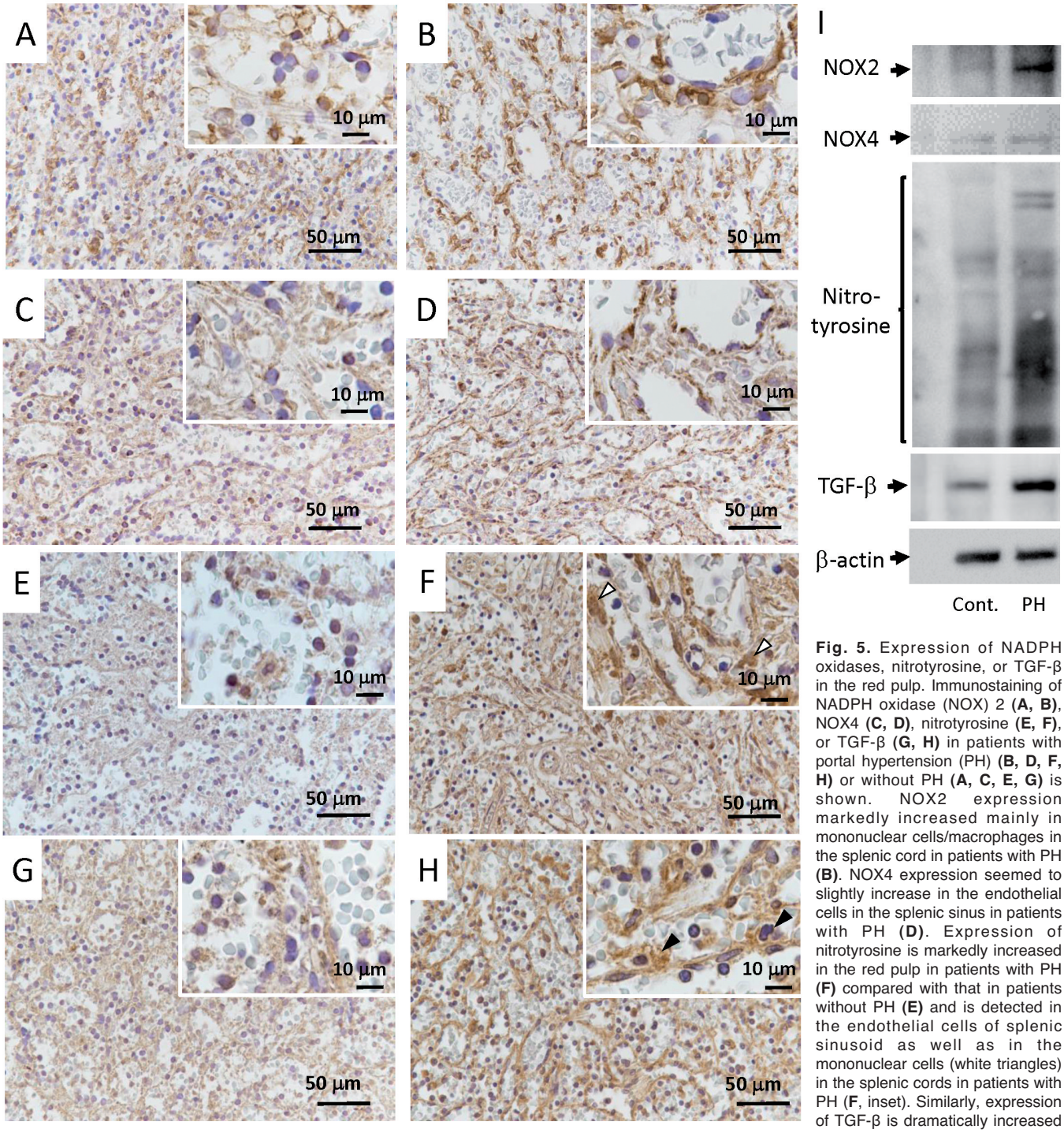

Fig. 5. Expression of NADPH oxidases, nitrotyrosine, or TGF-β in the red pulp. Immunostaining of NADPH oxidase (NOX) 2 **(A, B)**, NOX4 **(C, D)**, nitrotyrosine **(E, F)**, or TGF-β **(G, H)** in patients with portal hypertension (PH) **(B, D, F, H)** or without PH **(A, C, E, G)** is shown. NOX2 expression markedly increased mainly in mononuclear cells/macrophages in the splenic cord in patients with PH **(B)**. NOX4 expression seemed to slightly increase in the endothelial cells in the splenic sinus in patients with PH **(D)**. Expression of nitrotyrosine is markedly increased in the red pulp in patients with PH **(F)** compared with that in patients without PH **(E)** and is detected in the endothelial cells of splenic sinusoid as well as in the mononuclear cells (white triangles) in the splenic cords in patients with PH (**F**, inset). Similarly, expression of TGF-β is dramatically increased in the red pulp with PH **(H)** compared with that in patients without PH **(G)** and is detected in the endothelial cells in the splenic sinus as well as in the mononuclear cells (black triangles) in patients with PH (**H**, inset). Representative Western blot for NOX2, NOX4, nitrotyrosine, and TGF-β in patients with or without PH is shown **(I)**. Remarkable increase in protein expression was detected in NOX2, nitrotyrosine, and TGF-β.

Thus, we next analyzed fibrosis in the spleen and detected remarkable progression of splenic fibrosis in patients with PH. In addition to thickening of the splenic capsules and the splenic trabeculae, deposition of a significant amount of fibrillar collagens in the splenic cords in the red pulp was observed in patients with PH. We focused on the fibrosis in the splenic cords, because this fibrosis existed ubiquitously in the spleen and is possibly responsible for the increased stiffness detected by ultrasonography. We compared the degree of fibrosis, which was semiquantitatively analyzed using image-analyzing software, and the platelet counts in peripheral blood. As a result, a strong negative correlation was detected between the two parameters, suggesting that fibrosis in the splenic cords progresses along with the advancement of PH.

From these results, it was indicated that congestion of splenic sinus as well as splenic fibrosis are possibly responsible for the increased spleen stiffness detected in patients with PH.

Cygb-expressing cells in the splenic cords are possibly responsible for splenic fibrosis

Fibroblast-like cells expressing α-SMA contribute to the production of extracellular matrix in many organs (Carthy, 2018). We detected an increased α-SMA-positive area in the splenic cords, and the shape of the α-SMA-expressing cells was a network pattern, reminiscent of reticular cells that reside in this space. We hypothesized that the reticular cells may transform to a fibroblastic phenotype and produce fibrillar collagens in this space.

Cygb, the fourth globin discovered by Kawada et al. in hepatic stellate cells (Kawada et al., 2001), is expressed in several splanchnic organs including the spleen (Nakatani et al., 2004), and it plays significant roles in organ fibrogenesis (Thuy le et al., 2016). In normal rat spleen, the expression of Cygb is dominant in the red pulp and localized to the reticular cells in the splenic cords (Nakatani et al., 2004). Thus, we investigated the expression of Cygb in our human samples to examine our aforementioned hypothesis. As reported in rat spleen, expression of Cygb was detected in small spindle-shaped cells, most likely reticular cells, in the splenic cord in patients without PH. The Cygb-expressing cells transformed to myofibroblast-like cells expressing α-SMA in the splenic cords in patients with PH.

This is the first report that Cygb-expressing cells in the splenic cord contribute to the fibrogenic process in human spleen. This phenomenon is similar to that reported in human hepatic stellate cells in response to fibrogenic stimuli (Motoyama et al., 2014).

Oxidative stress and splenic fibrosis

The presence of Cygb in the pericytes of many organs serves an important function in maintaining homeostasis of the antioxidant systems (Thuy le et al., 2016). Thus, we speculated that oxidative stress may play significant roles in the mechanism of splenic fibrosis observed in this study. Indeed, it has been well documented that oxidative stress contributes to fibrosis in many organs (Richter and Kietzmann, 2016), and NOXs participate in this process (Richter et al., 2015). In the red pulp in patients with PH, expression of nitrotyrosine, an oxidative stress marker, was markedly increased, and expression of NOX2 was also upregulated. The NOX2 expression markedly increased in mononuclear cells or macrophages in the splenic cords, while NOX4 expression seemed to slightly increase in the endothelial cells of splenic sinus, suggesting more important roles of NOX2 in splenic fibrosis. The expression of TGF-β, one of the most important cytokines in fibrogenesis, was also increased in the red pulp of patients with PH. The expression was markedly upregulated in the endothelial cells of splenic sinus as well as in mononuclear cells or macrophages in the splenic cords. The relationship between TGF-β expression and NOXs activation has been well documented in other organs (Bataller et al., 2003; Cucoranu et al., 2005; Hecker et al., 2009).

From these results, it is strongly speculated that the increase in oxidative stress introduced by the upregulated NOX system, mainly NOX2 in human spleen, possibly contributes to the transformation of Cygb-expressing cells to α-SMA-expressing myofibroblastic cells in the red pulp.

Conclusion

It was demonstrated in this study that splenic fibrosis progresses along with advancement of PH and that this fibrosis in addition to the congestion of splenic sinus possibly contribute to increased spleen stiffness detected by ultrasound. Cygb-expressing cells in the splenic cord possibly participate in this process through mechanisms including oxidative stress, most likely produced by mononuclear cells or macrophages in the splenic cords.

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Compliance with Ethical Requirements. (1) Conflict of Interest (Coi) statements: Yuji Iimuro, Akito Yada, Toshihiro Okada, Ikuo Nakamura, Kazuhiro Suzumura, Jinyang Xu, Makoto Sudo, Shuhei Nishiguchi, Norifumi Kawada, Etsuro Hatano, and Jiro Fujimoto declare that they have no conflict of interest. (2) Informed Consent in Studies with Human Subjects: All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (the Ethics Committee of Hyogo University Graduate School of Medicine; approval number 201611-071) and with the Helsinki Declaration of 1975, as revised in 2008 (5). Informed consent was obtained from all patients included in this study.

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