GASTROINTESTINAL, HEPATOBILIARY, AND PANCREATIC PATHOLOGY

Matricellular Protein Periostin Contributes to Hepatic Inflammation and Fibrosis

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Periostin actively contributes to tissue injury, fibrosis, atherosclerosis, and inflammatory diseases; however, its role in hepatic fibrosis is unclear. Herein, we revealed that periostin expression was significantly up-regulated in carbon tetrachloride- and bile duct ligation-induced mice with acute and chronic liver fibrosis. Deficiency in periostin abrogated the development of liver fibrosis in mice. Carbon tetrachloride treatment significantly increased α -smooth muscle actin, fibronectin, and collagen I levels in wild-type mice, which were unaffected in periostin-knockout mice. Periostin-deficient mice showed a significantly reduced area of collagen deposition and decreased levels of serum alanine aminotransferase and aspartate aminotransferase compared with wild-type mice after 2 weeks of carbon tetrachloride administration. Chemokine ligand 2, IL-6, IL-1 β , tumor necrosis factor- α , and tissue inhibitor of metalloproteinases 1 mRNA levels were significantly lower in periostin-deficient mice than in wild-type mice after carbon tetrachloride treatment. Periostin colocalized with hepatic stellate cell-derived collagen I and α -smooth muscle actin in mouse acute and chronic fibrotic liver tissues. Transforming growth factor (TGF)- β 1 markedly induced periostin expression in primary mouse hepatic stellate cells. Periostin-deficient mice showed significantly lower levels of TGF- β 1 and TGF- β 2 compared with wild-type mice after carbon tetrachloride treatment. High levels of periostin in patients with acute or chronic hepatitis correlated with TGF- β 1 and TGF- β 2 expression in serum from patients with hepatitis. Data indicate that periostin is a novel mediator of hepatic fibrosis development. (*Am J Pathol* 2015, 185: 786–797; <http://dx.doi.org/10.1016/j.ajpath.2014.11.002>)

Liver fibrosis is a common consequence of chronic liver damage, such as damage due to hepatotropic viruses (mainly hepatitis B and C viruses), toxins, excessive alcohol intake, and autoimmunity. Liver fibrosis is characterized by alteration of tissue architecture and deposition of collagen-rich extracellular matrix (ECM). Chronic inflammatory stimuli can cause hepatocyte death, and the apoptotic hepatocytes, in turn, stimulate the hepatic resident macrophages (ie, Kupffer cells) and the recruited inflammatory leukocytes to secrete proinflammatory cytokines and pro-fibrogenic factors, which activate collagen-producing hepatic stellate cells (HSCs). These immunological and tissue repair responses result in excessive collagen deposition and compromised liver function.^{1–3} HSCs are a major source of ECM proteins in the damaged liver. Under

physiological conditions, HSCs maintain a quiescent phenotype. After liver injury, the quiescent HSCs can be activated by several fibrogenic stimuli, including transforming growth factor β (TGF- β), platelet-derived growth factor, and inflammatory cytokines that are mainly secreted by Kupffer cells. The activated HSCs transdifferentiate into

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myofibroblasts and produce excessive ECM proteins, which result in excessive collagen deposition and subsequent liver fibrosis.^{1–3}

Matricellular proteins are a family of secreted ECM proteins that includes osteopontin (OPN), tenascins, cysteine-rich acidic secreted protein (SPARC), CCNs, thrombospondins, and periostin. These non-structural ECM proteins are important during embryonic development but are typically restricted to tissue remodeling and wound repair in the normal adults.^{4–6} Periostin (encoded by *Postn*) was originally identified in MC3T3-E1 osteoblast cells as a critical regulator of bone and tooth development and maintenance.^{7,8} Periostin is mainly expressed in collagen-rich fibrous connective tissues, including heart valves, tendons, corneas, the perichondrium, and the periodontal ligament, which are subjected to constant mechanical stresses during embryonic development or pathogenesis.^{9–11} Current evidence in animal models and patients demonstrates that periostin is also involved in the pathobiology of various inflammatory diseases, including tissue injury, fibrosis, arthritis, atherosclerosis, and other inflammatory diseases.^{12–16} Periostin is a critical mediator of the healing process of myocardial infarction,^{17,18} and plays a critical role in fibrosis of bronchial asthma and chronic allergic skin inflammation.^{13,19} Periostin-deficient mice show a reduction in pulmonary fibrosis.¹⁴ Interestingly, as a critical developmental factor and a secreted ECM protein, periostin is also overexpressed in various types of human cancers and contributes to tumorigenesis and metastasis.^{5,20–23} Collectively, these findings suggest that periostin actively contributes to tissue injury, inflammation, fibrosis, and tumor initiation and progression.^{15,24} Interestingly, a recent report reveals that periostin actively contributes to obesity-induced hepatosteatosis.²⁵ However, whether periostin is involved in liver fibrosis progression has not yet been studied.²⁶

Our aim was to explore the role of periostin in liver fibrosis. Herein, by using murine experimental models of liver fibrosis, we demonstrate that periostin is up-regulated in carbon tetrachloride- and bile duct ligation (BDL)-induced acute and chronic liver fibrosis and that liver fibrosis is dramatically decreased in periostin-deficient mice. The level of periostin is significantly elevated in the serum of patients with acute or chronic hepatitis. We also demonstrate that TGF- β signaling is involved in periostin-related liver fibrosis. Our observations identify periostin as a potential diagnostic or therapeutic target for hepatic fibrosis.

Materials and Methods

Experimental Models of Liver Fibrosis

All experimental procedures involving animals were performed in accordance with animal protocols approved by the Institutional Animal Care and Use Committee of Xiamen University (Xiamen, China). Heterozygous

B6;129-*Postn*^{tm1Jmol/J} (*Postn*^{+/-}) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Wild-type and periostin-deficient mice were generated from crossing *Postn*^{+/-} mice with *Postn*^{+/-} mice. Littermate controls were used in all experiments. All mice were housed in a specific pathogen-free environment. In the carbon tetrachloride-induced acute fibrosis model, male mice were injected i.p. with carbon tetrachloride (diluted 1:5 in sterile mineral oil) or vehicle (mineral oil) at a dose of 2.5 mL/kg body weight twice per week for 2 weeks. In the carbon tetrachloride-induced chronic fibrosis model, male mice were injected i.p. with the same dose of carbon tetrachloride twice per week for 8 weeks. Mice were euthanized 72 hours after the final i.p. injection. To perform BDL, the common bile duct was ligated distally. We performed the sham operation similarly, except that the bile duct was not ligated. Mice were euthanized 1 or 3 weeks after BDL.

Cell Culture

The immortalized mouse macrophage cell line RAW264.7 was routinely cultured in Dulbecco's modified Eagle's medium (Gibco, Carlsbad, CA) with 10% fetal bovine serum and 1% penicillin-streptomycin at 37°C and saturated with 5% CO₂ in a humidified atmosphere.

IHC and Immunofluorescence

Immunohistochemistry (IHC) and immunofluorescence staining were performed as described.²⁷ The primary antibodies used included antibodies to periostin (AdipoGen, Liestal, Switzerland), fibronectin (BD Biosciences, Franklin Lakes, NJ), α -smooth muscle actin (α -SMA; Santa Cruz Biotechnology, Santa Cruz, CA), collagen I, β -actin, and glyceraldehyde-3-phosphate dehydrogenase (Millipore, Billerica, MA).

Immunoblotting Analysis

Total proteins were extracted from liver tissues. Immunoblotting analysis was performed as described.²⁷

Real-Time PCR

Total RNA was extracted from liver tissues or cells using TRIzol reagent, according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Reverse transcription was performed as previously described.²⁷ The expression levels of the mRNAs were determined by real-time PCR using SYBR Premix Ex Taq (Takara, Liaoning, China) and the pre-designed primers listed in Table 1.

H&E and Sirius Red Staining

Before staining, paraffin-embedded sections (5 μ m thick) were dewaxed and hydrated. For hematoxylin and eosin (H&E) staining, nuclei were stained with hematoxylin,

Table 1 List of All Primers Used for Real-Time PCR

Gene	Forward primer	Reverse primer
<i>Postn</i>	5'-TAGCCCAATTAGGCTTGGCATCC-3'	5'-TAAGAAGGCGTTGGTCCATGCT-3'
<i>TGFB1</i>	5'-GTGTGGAGCAACATGTGGAACCTA-3'	5'-TTGGTTCAGCCACTGCCGTA-3'
<i>TGFB2</i>	5'-ATTGCCCTCCTACAGACTTGAG-3'	5'-CAGCACAGAAGTTGGCATTTGTA-3'
<i>ACTA2</i>	5'-AAGAGCATCCGACACTGCTGAC-3'	5'-AGCACAGCCTGAATAGCCACATAC-3'
<i>COL1IA1</i>	5'-GAAACCCGAGGTATGCTTGA-3'	5'-GACCAGGAGGACCAGGAAGT-3'
<i>COL3A1</i>	5'-GCACAGCAGTCCAACGTAGA-3'	5'-TCTCCAAATGGGATCTCTGG-3'
<i>TNF</i>	5'-AGGGTCTGGGCCATAGAACT-3'	5'-CCACCACGCTCTTCTGTCTAC-3'
<i>CCL2</i>	5'-ATTGGGATCATCTTGCTGGT-3'	5'-CCTGCTGTTTACAGTTGCC-3'
<i>IL6</i>	5'-CTCTGCAAGAGACTTCCATCCAGT-3'	5'-GAAGTAGGGAAGGCCGTGG-3'
<i>IL1B</i>	5'-TCTTTGAAGTTGACGGACCC-3'	5'-TGAGTGATACTGCCTGCCTG-3'
<i>TIMP1</i>	5'-AGGTGGTCTCGTTGATTCT-3'	5'-GTAAGGCCCTGTAGCTGTGCC-3'
<i>SPP1</i>	5'-ATTCTGGCAGCTCAGAGGAG-3'	5'-CTGTGGCGCAAGGAGATT-3'
<i>TNC</i>	5'-GCTACTGCCAGCATCTTTC-3'	5'-GAAGTCCCCTGAGGACTCTG-3'
<i>CTGF</i>	5'-ATCCACCAAAGTGAGAAG-3'	5'-TAATTTCCCTCCCGGTTAC-3'
<i>THBS1</i>	5'-CTTCACCTTTGCCACCTCTC-3'	5'-TGGTGAAGATGCTCTGGATG-3'
<i>SPARC</i>	5'-AGGGGAAGGAGGAGACAGAG-3'	5'-AAGAAGATCCAGGCCCTCAT-3'
<i>GAPDH</i>	5'-TGTGTCCGTCGTGGATCTGA-3'	5'-TTGCTGTTGAAGTCGCAGGAG-3'

followed by staining with eosin. For Sirius red staining, the dewaxed sections were dyed with Picrosirius Red solution (0.1% Direct Red 80 in saturated picric; Leagene, Beijing, China) for 1 hour, washed in acidified water, and dehydrated in three changes of 100% ethanol. The collagen deposition of Sirius red staining—positive area was quantified by Image Proplus version 6.0 (Media Cybernetics, Inc., Bethesda, MD).

Determination of Hepatic Hydroxyproline Content

The hepatic hydroxyproline content was determined in homogenized liver tissues. Briefly, liver samples were weighed and incubated in hydrochloric acid at 100°C for 5 hours. The acid hydrolysis was used to determine the content of hydroxyproline, according to the reagent kit's standard protocols (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The total hydroxyproline in collagen in whole liver specimens was quantified colorimetrically at 550 nm and expressed as per milligram of wet tissue weight.

Serum ALT and AST Assays

Ocular blood was collected and clotted at room temperature and centrifuged at $1000 \times g$ for 10 minutes, and the serum was collected. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) serum levels were determined by commercially available reagent kits (Biotech, Beijing, China), according to standard protocols.

Isolation of Primary HSCs

HSCs were isolated as described previously.^{28,29} Briefly, 2- to 3-month-old mice were anesthetized with phenobarbital sodium by i.p. injection and livers were perfused with Hanks' balanced salt solution (Sigma, St. Louis, MO), followed by Hanks' balanced salt solution containing collagenase II (Sigma) and Pronase (Roche, Basel, Switzerland). After

perfusions, the liver was digested in collagenase solution for 10 minutes in a 37°C incubator, then minced with tweezers and pipetted repeatedly, filtered with a 70- μ m filter, and centrifuged at $50 \times g$ at 4°C for 2 minutes twice. The supernatant was centrifuged at $250 \times g$ for 10 minutes at 4°C. HSCs were isolated by gradient centrifugation with Percoll (Sigma) and cultured in RPMI 1640 medium (Gibco) containing 10% fetal bovine serum and penicillin/streptomycin. After 24 hours, the medium was changed. The purity of the cultured HSCs was >90%, as determined by fluorescence of lipid droplets containing vitamin A.

Serum Periostin, TGF- β 1, and TGF- β 2 Assays

All clinical serum samples were collected under informed consent of the patients, and study protocols were in accordance with the ethical guidelines and were approved by the Institutional Medical Ethics Committee of Xiamen University. Human serum samples were obtained from 28 normal donors, 32 acute hepatitis patients, and 20 chronic hepatitis patients. Serum levels of periostin, TGF- β 1, and TGF- β 2 were quantified with enzyme-linked immunosorbent assay kits (Shanghai Yanhui Biotechnology, Shanghai, China).

Statistical Analysis

The results are presented as the means $\pm$ SEM for all experiments. Analysis was performed using one-way analysis of variance.

Results

Periostin Expression Is Up-Regulated in Mice with Acute or Chronic Liver Fibrosis

To determine whether periostin is involved in hepatic fibrosis, we used carbon tetrachloride to induce acute and

Figure 1 Periostin is up-regulated in mice with acute liver injury and chronic liver fibrosis. **A:** Immunofluorescence staining of periostin in carbon tetrachloride (CCl₄)—induced acute and chronic fibrotic liver in mice. **B:** Western blot analysis of liver periostin levels in the carbon tetrachloride—induced acute and chronic fibrotic mice and the control groups administered mineral oil. **C:** RT-qPCR analysis of liver periostin levels in the carbon tetrachloride—induced acute and chronic fibrotic mice and the control groups administered mineral oil. **D:** The serum periostin levels in the carbon tetrachloride—induced acute and chronic fibrotic mice and the control groups administered mineral oil as assessed by enzyme-linked immunosorbent assay. $n = 3$ (C and D). * $P < 0.05$. Scale bar = 50 μm (A). GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

chronic liver fibrosis in mice. After 2 weeks of carbon tetrachloride exposure, the expression of hepatic periostin was significantly increased, as determined by immunofluorescence assay (Figure 1A). Consistent with the data for acute fibrosis, the expression of hepatic periostin was also elevated in mice treated with carbon tetrachloride for 8 weeks compared with mice treated with mineral oil (Figure 1A). We further performed Western blot analysis and real-time quantitative RT-PCR (RT-qPCR) to analyze the liver periostin expression in the carbon tetrachloride—induced acute and chronic fibrotic mice and the control groups administered mineral oil. Compared with the expression levels in the control groups, hepatic periostin protein and mRNA levels were significantly up-regulated in the liver tissues of mice with acute and chronic liver fibrosis induced by carbon tetrachloride treatment (Figure 1, B and C). Moreover, the serum levels of periostin were also significantly increased in the carbon tetrachloride—induced acute and chronic fibrotic mice compared with those in mineral oil—treated control mice (Figure 1D). It has been recognized that the mice with carbon tetrachloride—induced acute liver fibrosis will spontaneously recover from acute liver fibrosis after stopping carbon tetrachloride injection for 1 month³⁰; thus, we further examined the level of periostin in the recovered livers. We observed that the expression of periostin was abolished in the recovered livers after stopping the injection of carbon tetrachloride for 1 month (Supplemental Figure S1A).

To confirm the relationship between high levels of periostin and liver injury and fibrosis, we further used mice undergoing BDL or a sham operation for 1 or 3 weeks, which represent early fibrogenesis and septal fibrosis, respectively. By using immunofluorescence, Western blot analysis, and RT-qPCR assays, we found that the protein and mRNA levels of periostin were also elevated in BDL-induced liver injury and fibrosis (Supplemental Figure S2, A–C). These findings suggest that periostin may play a critical role in carbon tetrachloride— and BDL-induced liver fibrosis.

Periostin Deficiency Ameliorates Liver Fibrosis in Mice

Periostin expression was significantly up-regulated in carbon tetrachloride— and BDL-induced acute and chronic mouse liver fibrosis but decreased in the recovered livers. Thus, we speculated that the deletion of periostin may reduce liver fibrosis. We further used the *Postn*^{-/-} mouse model treated with oil or carbon tetrachloride to determine the role of periostin in liver fibrosis. Periostin-knockout and wild-type mice were i.p. administered mineral oil or carbon tetrachloride for 2 or 8 weeks, and were sacrificed at 72 hours after final i.p. injection. It is well known that activated HSCs express α -SMA and secrete ECM proteins, such as collagen I, in response to liver injury. We therefore investigated the expression of α -SMA and collagen I in acute and chronic fibrotic livers in periostin-deficient mice by immunofluorescence analysis. We found that α -SMA and collagen

I were expressed in a low level in the livers of wild-type and periostin-deficient mice treated with mineral oil for 2 or 8 weeks (Supplemental Figure S3); however, α -SMA and collagen I levels were increased in wild-type mice after carbon tetrachloride treatment for 2 or 8 weeks, whereas these phenotypes were abolished in the livers of carbon tetrachloride-treated *Postn*^{-/-} mice (Figure 2). RT-qPCR analysis also revealed that carbon tetrachloride-treated *Postn*^{-/-} mice exhibited a decreased liver mRNA level of α -SMA and collagen I compared with wild-type counterparts with acute or chronic liver fibrosis (Figure 3, A and B). We also found that the mRNA level of collagen III α 1 was robustly reduced in periostin-deficient mice compared with wild-type mice with chronic fibrosis; however, we could not find a significant difference in wild-type and knockout mice treated with carbon tetrachloride for 2 weeks (Figure 3C). We further determined the reduced fibrosis in *Postn*^{-/-} mice by Sirius red staining and hepatic hydroxyproline quantification. After 2 or 8 weeks of carbon tetrachloride treatment, the area of collagen deposition (Figure 3, D and E) and the hydroxyproline content (Supplemental Figure S4) in *Postn*^{-/-} mice were markedly lower than in wild-type control mice. In addition, we detected the mRNA levels of several other fibrosis-related matricellular proteins, including OPN, CCN2, tenascin C, thrombospondin-1, and

SPARC, by RT-qPCR assay. We found that OPN mRNA was obviously decreased in periostin-deficient mice compared with wild-type mice after carbon tetrachloride treatment for 2 or 8 weeks. However, the mRNA expression of CCN2, tenascin C, thrombospondin-1, and SPARC was not altered significantly in periostin-deficient mice compared with wild-type mice after carbon tetrachloride treatment for 2 or 8 weeks (Supplemental Figure S5).

Furthermore, *Postn*^{-/-} mice showed a significant reduced serum level of ALT (Figure 4A) and AST (Figure 4B) compared with wild-type mice after 2 weeks of carbon tetrachloride administration. We also observed a decreased signal of F4/80 IHC staining in *Postn*^{-/-} mouse liver, indicating that macrophage infiltration and fibrosis development in *Postn*^{-/-} mice was significantly attenuated compared with wild-type mice after 2 weeks of carbon tetrachloride administration (Figure 4C). In addition, the injured livers of wild-type mice had significantly increased mRNA expression for inflammatory and fibrotic genes, including those coding for chemokine ligand 2, IL-6, IL-1 β , and tumor necrosis factor- α , compared with expression in the mineral oil-treated controls. However, chemokine ligand 2, IL-6, IL-1 β , and tumor necrosis factor- α mRNA levels were significantly lower in *Postn*^{-/-} mice than in wild-type mice after carbon tetrachloride treatment for 2 weeks (Figure 4, D–G).

Figure 2 A deficiency in periostin reduces mouse liver fibrosis. Immunofluorescence analysis of periostin, α smooth muscle actin (α -SMA), and collagen I in wild-type and *Postn*^{-/-} mice after carbon tetrachloride (CCl₄) treatment for 2 or 8 weeks. Scale bar = 50 μ m.

Figure 3 Periostin-deficient mice develop less liver injury and fibrosis than wild-type mice after carbon tetrachloride (CCl₄) treatment. RT-qPCR analysis of hepatic mRNA levels of α smooth muscle actin (α -SMA; **A**), collagen I α 1 (**B**), and collagen III α 1 (**C**) in wild-type (black bars) and *Postn*^{-/-} (white bars) mice after carbon tetrachloride treatment for 2 or 8 weeks. Collagen deposition was analyzed by Sirius red staining (**E**) and quantification (**D**) in wild-type and *Postn*^{-/-} mice after carbon tetrachloride treatment for 2 or 8 weeks. *n* = 3 to 7 (**C**); *n* = 4 (**D** and **E**). **P* < 0.05, ***P* < 0.01, and ****P* < 0.001. Scale bar = 100 μ m (**E**).

Tissue inhibitor of metalloproteinase 1 plays a critical role in matrix remodeling, and its expression is significantly up-regulated during fibrosis and cirrhosis. *Postn*^{-/-} mice exhibited a significantly lower tissue inhibitor of metalloproteinase 1 mRNA level than wild-type mice after carbon tetrachloride treatment for 2 weeks (Figure 4H). Thus, these data suggest that a deficiency in periostin abrogates the development of liver fibrosis in mice.

Periostin Can Be Secreted by the Activated HSCs

It is widely accepted that liver fibrosis-related ECM proteins, such as fibronectin and collagen I, are mainly secreted by HSCs in response to liver injury. α -SMA is another well-known

hallmark of the activated HSCs. In the recovered mice, concomitant with the decreased expression of hepatic periostin, the hepatic levels of fibronectin, collagen I, and α -SMA also declined after stopping carbon tetrachloride injection for 1 month (Supplemental Figure S1, B–D). Moreover, periostin colocalized with fibronectin, collagen I, and α -SMA in fibrotic mouse livers after 2 weeks of carbon tetrachloride administration (Figure 5A). We also observed that periostin overlapped mostly with HSC-derived collagen I and α -SMA in mouse chronic fibrotic liver tissues (Figure 5B). Thus, these data suggest that periostin may be produced by activated HSCs in the progression of carbon tetrachloride-induced liver fibrosis.

To further determine whether activated HSCs produce periostin, we isolated primary HSCs from mice with oil

Figure 4 Liver injury and inflammation are decreased in the absence of periostin. Black bars indicate wild-type mice; white bars, *Postn*^{-/-}. **A** and **B**: *Postn*^{-/-} mice show significantly reduced serum levels of alanine aminotransferase (ALT; **A**) and aspartate aminotransferase (AST; **B**) compared with levels in wild-type mice after 2 weeks of carbon tetrachloride (CCl₄) administration. **C**: Immunohistochemical staining of F4/80-positive macrophages in wild-type and *Postn*^{-/-} mice after carbon tetrachloride administration for 2 weeks. **D–H**: RT-qPCR analysis of hepatic mRNA levels of chemokine ligand (CCL) 2 (**D**), IL-6 (**E**), IL-1β (**F**), tumor necrosis factor-α (TNF-α; **G**), and tissue inhibitor of metalloproteinase 1 (TIMP-1; **H**) in wild-type and *Postn*^{-/-} mice after carbon tetrachloride treatment for 2 weeks. *n* = 3 (**A**, **B**, and **D–H**). **P* < 0.05, ***P* < 0.01. Scale bar = 100 μm (**C**).

treatment, which were confirmed by phase-contrast microscopy and α-SMA immunofluorescence staining (Supplemental Figure S6). TGF-β1 is a critical profibrotic cytokine that activates HSCs during the development of hepatic fibrosis, so we assessed whether TGF-β stimulates periostin expression in HSCs. Primary mouse HSCs did not express periostin; however, the expression of periostin was dramatically increased in primary HSCs after 10 ng/mL TGF-β1 stimulation (Supplemental Figure S7). These results suggest that periostin can be secreted by the activated HSCs.

TGF-β Is Involved in Periostin-Related Mouse Liver Fibrosis

Because TGF-β1 significantly induces the expression of periostin in primary mouse HSCs, we further determined whether periostin could regulate the level of TGF-β in liver fibrosis. We found that the mRNA level of TGF-β1 and TGF-β2 was significantly increased in the liver tissues of mice with acute or chronic liver fibrosis compared with the mineral oil-treated control groups. However, periostin-deficient mice showed a significantly lower liver level of TGF-β1 and TGF-β2 mRNA than the wild-type mice after carbon tetrachloride treatment for 2 (Figure 6A) or 8 (Figure 6B) weeks. The serum levels of TGF-β1 and TGF-β2 in periostin-deficient mice were also significantly lower than those in the wild-type mice after carbon tetrachloride treatment for 2 weeks (Figure 6C). It has been reported that liver-resident and bone marrow-derived macrophages are the major

source of TGF-β in the fibrotic liver. To further evaluate whether periostin could induce TGF-β in macrophages in liver fibrosis, we detected the mRNA levels of TGF-β1 and TGF-β2 in the mouse macrophage cell line RAW264.7 after stimulation with human recombinant periostin protein. We found that recombinant periostin treatment strongly up-regulated the mRNA levels of TGF-β1 and TGF-β2 in RAW264.7 cells (Figure 6D). Therefore, these results indicate that TGF-β is involved in periostin-related mouse liver fibrosis development.

High Levels of Periostin Are Correlated with TGF-β1 and TGF-β2 Expression in Serum from Patients with Hepatitis

To further define the role of periostin in the development of human hepatitis and to determine the correlation between periostin and TGF-β, we detected the levels of periostin and TGF-β in the serum of patients with acute or chronic hepatitis. A high level of periostin was found in serum from patients with acute or chronic hepatitis compared with the serum from healthy controls without hepatitis (Figure 7A). Interestingly, the serum levels of TGF-β1 and TGF-β2 in acute and chronic hepatitis significantly increased compared with the serum levels in healthy controls without hepatitis. Moreover, there was a positive correlation between the levels of serum periostin and TGF-β1 in patients with acute or chronic hepatitis (Figure 7B). Another correlation was found between the levels of serum periostin and TGF-β2 in

Figure 5 Periostin colocalizes with collagen I, fibronectin, and α smooth muscle actin (α -SMA) in fibrotic mouse liver tissues. **A:** Immunofluorescence staining of periostin, collagen I, fibronectin, and α -SMA in acute fibrotic liver sections from carbon tetrachloride (CCl_4)-treated wild-type mice. **B:** Immunofluorescence staining of periostin, collagen I, and α -SMA in chronic fibrotic liver sections from carbon tetrachloride-treated wild-type mice. Scale bar = 50 μm (**A** and **B**).

patients with acute or chronic hepatitis (Figure 7C). In summary, these observations indicate that periostin expression is associated with TGF- β 1 and TGF- β 2 levels in human hepatic inflammation and fibrosis.

Discussion

Fibrosis is the formation of excess fibrous connective tissues, which can be defined as an out-of-control wound-healing process.³¹ Hepatic fibrosis occurs as a wound-healing response after acute or chronic liver inflammation that

involves the progressive excess production and deposition of ECM components. During the inflammatory cascade in liver injury, resident macrophages and HSCs, together with recruited infiltrating leukocytes and bone marrow-derived cells, contribute to remodeling the ECM composition and tissue architecture. Fibrosis is characterized by the aberrant deposition of ECM proteins, such as collagens, fibronectins, and laminins. Collagen fibrillar formation is a critical step in fibrogenesis. As a non-structural ECM protein, periostin can regulate fibrillogenesis by changing the biophysical and biochemical properties of the ECM.^{10,12} Periostin binds to collagen I to promote fibrillogenesis

Figure 6 Periostin-deficient mice (black bars, A–C) show a significantly lower level of liver and serum transforming growth factor (TGF)-β1 and TGF-β2 than the wild-type mice (white bars, A–C) after carbon tetrachloride (CCl₄) treatment for 2 weeks. **A:** RT-qPCR analysis of TGF-β1 and TGF-β2 mRNA levels in the liver of wild-type and periostin-deficient mice after carbon tetrachloride treatment for 2 weeks. **B:** RT-qPCR analysis of TGF-β1 and TGF-β2 mRNA levels in the liver of wild-type and periostin-deficient mice after carbon tetrachloride treatment for 8 weeks. **C:** Enzyme-linked immunosorbent assay analysis of the serum levels of TGF-β1 and TGF-β2 in the wild-type and periostin-deficient mice after carbon tetrachloride treatment for 2 weeks. **D:** Periostin promotes the expression of TGF-β1 and TGF-β2 in the mouse macrophage cell line RAW264.7. RT-qPCR analysis of the mRNA levels of TGF-β1 and TGF-β2 in RAW264.7 cells after stimulation with 150 ng/mL human recombinant periostin protein for the indicated times. *n* = 3 (A–C). **P* < 0.05, ***P* < 0.01.

and also enhances collagen formation in the chronic phase of allergic skin inflammation.^{10,13} Periostin-null mice exhibit aberrant collagen I fibrillogenesis.⁹ Current data reveal that periostin can directly interact with collagen I, fibronectin, and Notch1 via its EMI domain and interact with tenascin C and bone morphogenetic protein 1 via its FAS I domains. Periostin can recruit bone morphogenetic protein 1 onto the fibronectin matrix to promote lysyl oxidase activity for collagen cross-linkage.^{12,32,33} These studies highlight the importance of periostin in ECM homeostasis and remodeling.^{6,15} Herein, our data showed that periostin not only increased concomitantly with up-regulated collagen I and fibronectin in mouse hepatic fibrosis and decreased together with down-regulated collagen I and fibronectin in the recovered livers but also colocalized with collagen I and fibronectin in hepatic fibrosis. Therefore, overexpressed periostin, collagen I, and fibronectin in acute and chronic liver inflammation accelerate fibrillogenesis, which ultimately leads to hepatic fibrosis.

Liver injury triggers inflammation and excessive production of ECM and is usually accompanied by tissue repair, but continuous liver injury may lead to liver fibrosis. Current evidence demonstrates that periostin undergoes a transient up-regulation in response to stresses or insults in the targeted tissue and the tissue may fail to repair appropriately if periostin is not adequate; however, sustained overexpression of periostin makes the tissue repair out of control.¹⁵ We detected the serum levels of ALT and AST in wild-type and periostin-deficient mice 48 hours after one injection of carbon tetrachloride and found that periostin-deficient mice exhibited a significantly increased serum level of ALT and AST compared with wild-type mice (Supplemental Figure S8). Meanwhile, our work showed that periostin-deficient mice had a reduction in serum ALT and AST activity compared with wild-type mice after 2 weeks of treatment with carbon tetrachloride. These data suggest that periostin may protect the liver from drug injury during early period and that periostin deficiency will ameliorate liver fibrosis during advanced development. Therefore, a timely expression and adequate periostin will facilitate tissue repair in response to liver injury insult in the early period, but a continuous high level of periostin or too much periostin will contribute to acute and chronic fibrosis in liver tissues.

TGF-β is a major factor accelerating the development of hepatic fibrosis by promoting the transdifferentiation and migration of HSCs. Activated HSCs can promote hepatic fibrosis progression through autocrine and paracrine loops of TGF-β–stimulated collagen production.³⁴ Periostin is a TGF-β–responsive gene product, which is increased in rat carotid arteries after injury and stimulation with TGF-β.³⁵ Periostin also can regulate TGF-β signaling in various diseases. Periostin-null mice exhibit markedly decreased phosphorylated Smad2 and Smad3 in endocardial cushions/leaflets at E14 compared with wild-type controls.³⁶ IL-13–induced periostin in asthma up-regulates the expression of TGF-β and

Figure 7 Periostin expression is associated with transforming growth factor (TGF)-β1 and TGF-β2 levels in human hepatic fibrosis. **A:** Enzyme-linked immunosorbent assay analysis of serum periostin, TGF-β1, and TGF-β2 levels in patients with acute or chronic hepatitis and healthy controls without hepatitis. **B:** Correlation analysis of the levels of serum periostin and TGF-β1 in patients with acute or chronic hepatitis. **C:** Correlation analysis of the levels of serum periostin and TGF-β2 in patients with acute or chronic hepatitis. *** $P < 0.001$.

collagen I. Periostin secreted by epithelial cells also cross-links with collagen I to alter the biomechanical properties of the airway.³⁷ Periostin deletion reduces muscular dystrophy and fibrosis by regulating TGF-β signaling.³⁸ Periostin accelerates pathological fibrosis in both bleomycin-induced murine skin sclerosis and human scleroderma by promoting myofibroblast differentiation and collagen synthesis via the regulation of TGF-β1 activity.³⁹ Interestingly, infiltrating breast cancer stem cells can educate lung fibroblasts by secreting factors such as TGF-β, which induces periostin expression in fibroblasts to generate a metastatic niche to support lung metastasis.²¹ Periostin and TGF-β are highly expressed in sprouting neovasculature niches to activate dormant cancer cells to undergo micrometastatic outgrowth.⁴⁰ These observations suggest that both periostin and TGF-β actively contribute to remodeling local tissue microenvironment in tissue injury, inflammation, fibrosis, and tumor metastasis. During liver fibrosis, quiescent HSCs can be activated into collagen I- and α-SMA-positive myofibroblasts after TGF-β stimulation. Herein, lower mRNA and protein levels of TGF-β1 and TGF-β2 were found in the livers of carbon tetrachloride-treated periostin-deficient mice compared with their wild-type counterparts. Moreover, high levels of periostin were correlated with TGF-β1 and TGF-β2 expression in serum from patients with acute or chronic hepatitis. Because TGF-β is a major profibrogenic cytokine and a central regulator of liver fibrosis, the capacity of periostin to influence TGF-β pathways suggests that HSC-derived periostin might function, at least in part, to regulate TGF-β activity in liver fibrosis. Conversely, TGF-β can induce a high level of periostin in the isolated primary HSCs, indicating that there may be a reciprocal regulatory mechanism between TGF-β and periostin in liver fibrosis. Taken together, our data demonstrate that persistent liver injury and inflammatory stimuli result in hepatic resident and recruited

macrophages to secrete profibrogenic cytokines, such as TGF-β; this cytokine activates HSCs to produce excessive ECM proteins, such as collagen I, fibronectin, and periostin. Among these ECM proteins, periostin, in turn, might induce macrophages to produce TGF-β, thereby generating a vicious circle in the development of liver fibrosis. Furthermore, periostin, as a remodeling matricellular protein, might directly bind with collagen I and fibronectin to promote fibrillogenesis, which partly accounts for acute and chronic liver fibrosis. In addition, the activated HSC-derived periostin also might serve as one of chemotactic factors to recruit macrophages into the damaging liver. Further studies are required to address these issues and the underlying molecular mechanisms.

It is of interest that several matricellular proteins, such as OPN,^{41–43} tenascin C,⁴⁴ and SPARC,^{45–47} are also involved in hepatic fibrosis. This study, thus, reinforces the notion that there may be functional similarities between matricellular proteins in remodeling the tissue microenvironment in hepatic fibrosis and other inflammatory diseases. An excessive or uncontrolled function of these matricellular proteins may contribute to aberrant remodeling and homeostasis of tissue microenvironment in various inflammatory diseases. In addition, OPN,⁴⁸ tenascin C,⁴⁹ periostin,^{21,50} and other matricellular proteins have been reported to promote tumor metastasis by modulating metastatic niches. These matricellular proteins are often associated with the need for ECM remodeling during embryonic development and are also highly expressed at sites of injury or inflammation or tumor sites within an adult organism. Therefore, a systemic evaluation of how these matricellular proteins regulate tissue inflammatory and tumor microenvironment will help in the development of therapeutic strategies to treat hepatic fibrosis and other inflammatory diseases.

Acknowledgments

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Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2014.11.002>.

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