Periostin cross-reacts with the renin-angiotensin system during liver fibrosis development

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Abstract. Periostin is a 90-kDa extracellular matrix protein, which is secreted primarily from fibroblasts and is expressed in the lungs, kidneys and heart valves. Angiotensin II (AT-II) serves pivotal roles in the pathogenesis of several diseases with accompanying fibrosis, including chronic liver diseases. AT-II induces periostin expression by regulating transforming growth factor-β1 (TGF-β1)/Smad signaling during cardiac fibrosis. The aim of the present study was to investigate the interaction between AT-II and periostin during liver fibrosis development. Fischer 344 rats were fed a choline-deficient L-amino-acid (CDAA)-defined diet for 12 weeks to simulate the development of steatohepatitis with liver fibrosis. Losartan, an AT-II type I receptor blocker, was administered to inhibit the effect of AT-II. The therapeutic effect of losartan on hepatic fibrosis development and on periostin expression was then evaluated. Several in vitro experiments were performed to examine the mechanisms underlying the interaction between AT-II and periostin in activated hepatic stellate cells (Ac-HSCs). Treatment with losartan suppressed the development of liver fibrosis induced by the CDAA diet, and reduced hepatic periostin expression. In addition, losartan treatment suppressed hepatic Ac-HSC expansion and hepatic TGF-β1 expression. In vitro analysis using LX2 HSC cells indicated that AT-II can augment TGF-β1 and collagen type I α1 mRNA expression via periostin expression, suggesting that the interaction between AT-II and periostin may serve a role in liver fibrosis development. In conclusion, blockade of AT-II-induced periostin may suppress the progression of liver fibrosis development.

Introduction

Liver fibrosis is a frequent and chronic clinical insult that induces wound-healing responses in the liver. It is characterized by the excessive deposition of extracellular matrix (ECM). Fibrosis is the predominant complication of chronic liver diseases that are caused by viral infection, autoimmune hepatitis, alcohol consumption, biliary obstruction and non-alcoholic fatty liver disease (1,2). The fibrotic process is characterized by the loss of parenchymal tissue and excessive accumulation of ECM, followed by activation of hepatic stellate cells (HSCs) (1,2).

The renin-angiotensin system (RAS) is a key mediator of arterial blood pressure and body fluid homeostasis; it serves an important role in the regulation of local hemodynamics in several organs (3-6), and in the progression of chronic liver diseases (3-6). In the RAS, angiotensin II (AT-II), which is an octapeptide produced by the enzymatic cleavage of angiotensin I by angiotensin I converting enzyme (ACE), serves a central role in the activation and proliferation of HSCs into activated HSCs (Ac-HSCs) (3). Previous studies have demonstrated that pharmacological inhibition of RAS effectively attenuates the development of liver fibrosis (3-7). For instance, inhibitors of the renin-angiotensin-aldosterone system, including ACE inhibitors (ACE-I), AT-II type I receptor blockers (ARBs), or selective aldosterone blockers (SABs), which are commonly used as antihypertensive agents, reportedly suppress the progression of hepatic fibrosis (4).

A previous study demonstrated that AT-II induced the expression of tissue inhibitor of metalloproteinase 1 (TIMP-1), which is a central molecule involved in fibrosis development (7). TIMP-1 was induced in Ac-HSCs, and this occurred via the AT-II type I receptor and phosphokinase C. In addition, a number of previous studies have indicated that the levels

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Abbreviations: AT-II, angiotensin II; TGF-β, transforming growth factor-β; CDAA, choline-deficient L-amino-acid; ARB, angiotensin II type I receptor blocker; Col1A1, collagen type I α1; ECM, extracellular matrix; HSC, hepatic stellate cells; ACE, angiotensin I converting enzyme; Ac-HSC, activated hepatic stellate cell; CSAA, choline-supplemented amino acid; ALT, alanine aminotransferase; RT-qPCR, reverse transcription quantitative polymerase chain reaction

Key words: periostin, angiotensin II, liver fibrosis, renin-angiotensin system, choline-supplemented amino acid
of periostin, a novel 90-kDa ECM protein, were reported to increase in a rat model of cardiac fibrosis development induced by continuous AT-II infusion, suggesting an interaction between AT-II and periostin (8,9). Periostin is secreted primarily from osteoblasts and fibroblasts and is expressed under normal conditions in the bone and to a lesser extent in the lungs, kidneys and heart valves of adult mammals (10). The effect of AT-II on periostin expression in hepatic fibrosis is currently unclear. Therefore, the aim of the present study was to explore the role of periostin in the development of liver fibrosis.

In the current study it was observed that periostin expression, which was attenuated by ARB treatment, was significantly and exclusively increased in Ac-HSCs in the choline-deficient L-amino acid (CDAA)-defined diet-induced rat model of liver fibrosis. The in vitro experiments indicated that periostin augmented transforming growth factor (TGF) β1 and collagen type 1 α1 (Col1a1) mRNA expression in human Ac-HSCs, suggesting a potential role of an AT-II-periostin interaction in liver fibrosis development.

Materials and methods

Animals and reagents. A total of 22 male Fischer 344 rats (age, 6 weeks; weight, 100-120 g) were purchased from Japan SLC, Inc. (Hamamatsu, Japan). Rats were individually housed under the following regulated conditions: Controlled temperature (23.5±2°C) and relative humidity (50±10%), with 10-15 air changes/h and 12-h light/dark cycles. The losartan ARB was purchased from Merck Sharp and Dohme (Shanghai, China). AT-II and conventional chemical agents were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Human HSCs (LX2) were obtained from the Japanese Collection of Research Bioresources Cell Bank (JCRB, Osaka, Japan).

Animal treatment. The experimental period for all animal experiments was 12 weeks. Rats were randomly divided into the following 3 groups: G1 (n=6), the negative control group where rats received a choline-supplemented amino acid (CSAA) diet (Japan SLC, Inc., Shizuoka, Japan) and normal water; G2 (n=6), a liver fibrosis model group where rats received a CDAA-defined diet (Japan SLC, Inc.) and normal water; G3 (n=10), a liver fibrosis model group where rats received a CDAA-defined diet and were administered with 30 mg/kg/day losartan in their drinking water. The animals were allowed free access to food and water throughout the acclimation and experimental periods. All rats were sacrificed at the end of the experimental period, and blood was collected via cardiac puncture. Serum markers, including albumin, bilirubin and alanine aminotransferase (ALT) were measured at 15 min at 121°C. Endogenous peroxidase was blocked using 0.3% H2O2 for 15 min at room temperature, and then the sections were incubated overnight at 4°C with each primary antibody or a rabbit IgG isotypic control (Cat. No. ab27478; Abcam), incubated with MAX-PO Multi secondary antibody (cat. No. 424151; Nichirei Biosciences, Inc.) for 60 min at 37°C, and subsequently stained with 3,3’diaminobenzidine solution for 45 sec at 37°C. Sections were then counterstained with hematoxylin solution for 10 sec at room temperature. To quantify Sirius red, periostin and αSMA positive areas, 10 independent fields (magnification, x100) were semi-quantified using ImageJ software ver.1.45 (National Institutes of Health, Bethesda, MD, USA) and the mean ± standard deviation (SD) were calculated as described previously (10).

Cell culture and WST-1 assay. Human LX2 HSCs (3x10^3 cells/200 µl) were seeded into a 96-well plate and cultured in Dulbecco's Modified Eagle's Medium (Nacalai Tesque, Inc.) supplemented with 10% fetal bovine serum (Cat. No. FB-1365/500; Biosera, Kansas, USA) and 0.01% penicillin-streptomycin-L-glutamine solution in a 5% CO2 humidified atmosphere at 37°C. The growth medium was refreshed every other day. Cell viability was investigated using a WST-1 assay (cat. No. MK400; Promix WST-1 Cell Proliferation Assay System; Takara Bio, Inc., Otsu, Japan) in the presence of periostin and/or losartan, according to the manufacturer's protocol. Briefly, cells were incubated for 24 h before the addition of media containing periostin (0.1 or 1.0 µg/ml; Cat. No. C-60045; PromoCell GmbH, Heidelberg, Germany) or Losartan (10 µM; Cat. No. 12353-04; Nacalai Tesque, Inc.). Cells were incubated for a further 48 h and then analyzed using a WST-1 assay. WST positivity was assessed using a plate reader at 450 nm.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from liver tissue samples and LX2 cells using an RNeasy RNA extraction kit (Qiagen GmbH, Hilden, Germany). Total RNA (1 µg) was reverse transcribed to cDNA using a High Capacity RNA-to-cDNA kit (Cat. No. 4387406; Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the
Table I. Physical and serum parameters of rats in the three experimental groups.

| Parameter                  | G1       | G2       | G3       |
|----------------------------|----------|----------|----------|
| Number of rats             | 6        | 6        | 10       |
| Body weight (g)            | 325.1±8.5| 239±10.5b| 244±16.4b|
| Liver/body weight (%)      | 9.9±0.05 | 11±0.09a | 11.4±0.05a|
| Alb (g/dl)                 | 4.4±0.1a | 4.1±0.1  | 4.3±0.1a |
| Total bilirubin (mg/dl)    | 0.06±0.006| 0.13±0.01b| 0.12±0.03b|
| ALT (IU/l)                 | 57±10    | 245±50b  | 300±31b  |

Data are presented as the mean ± standard deviation. *P<0.05 and **P<0.01 vs. G1; †P<0.05 vs. G2, G1, choline-supplemented amino acid diet fed control rats; G2, CDAA-defined diet fed rats; G3, losartan-treated CDAA diet-fed rats; Alb, serum albumin; LDH, lactate dehydrogenase; ALT, alanine aminotransferase; CDAA, choline-deficient L-amino acid.

Expression of periostin in the liver. The periostin protein levels in the liver tissues from rats in each experimental group were measured using an enzyme-linked immunosorbent assay kit (Cat. No. SEH339Ra; Cloud-Clone Corp., Katy, TX, USA) according to the manufacturer’s protocol. Protein was extracted from frozen liver using T-PER Tissue Protein Extraction Regent with HALT™ protease inhibitor cocktail (both from Thermo Fisher Scientific, Inc.). The protein concentration in each sample was measured at 280 nm using a NanoDrop™ Lite (Thermo Fisher Scientific, Inc.) and 100 µg protein was used for the assessment of periostin protein levels.

Statistical analysis. Results were presented as the mean ± SD. To assess the statistical significance of inter-group differences, one-way analysis of variance was performed followed by Bonferroni’s multiple comparison tests using software EZR version 3.1.2 (13). Bartlett’s test was subsequently performed to determine the homology of variance. P<0.05 was considered to indicate a statistically significant difference.

Results

Clinical parameters. Information regarding the number of rats in each group, their final body weights and relative liver weights in all experimental groups is provided in Table I. CDAA-fed rats in groups G2 and G3 exhibited significantly higher serum ALT levels, lower body weights and higher liver/body weight rates when compared with rats in the G1 group. This is consistent with the results described previously (11). Losartan-treated G3 rats did not exhibit significantly different clinical parameters from the rats in the G2 group (Table I).

Losartan reduces liver fibrosis development. The effects of clinically comparable doses of losartan on the development of liver fibrosis were investigated (3). The CDAA diet induced extensive fibrosis and severe steatohepatitis (Fig. 1). Treatment of rats in the G3 group with losartan resulted in significant attenuation of liver fibrosis when compared with the losartan-untreated G2 group (P<0.01); however the level of steatosis was unaffected (Fig. 1). No liver fibrosis development was observed in the CSAA diet-fed control rats in the G1 group (Fig. 1). Immunohistochemical analysis of αSMA was performed to confirm the inhibitory effect of losartan on HSC activation during the development of liver fibrosis. The number of Ac-HSCs, which express αSMA (1,2), were significantly reduced in the livers of the losartan-treated rats in the G3 group when compared with untreated rats in the G2 group (P<0.01; Fig. 2).
Immunohistochemistry was used to examine the levels of periostin in the experimental models, in order to investigate the association between liver fibrosis development and periostin. The CDAA diet significantly induced periostin expression along the fibrotic septa when compared with the CSAA diet (Fig. 3). Compared with rats in the G2 group, those in the G3 group exhibited a significant reduction in periostin expression (P<0.05; Fig. 3). No periostin expression was observed in rats from the G1 group.

RT-qPCR analysis indicated an inhibitory effect of losartan on the expansion of Ac-HSCs. This inhibitory effect was demonstrated by a decrease in the mRNA expression levels of Tgfb1 and Col1a1, which are representative markers of Ac-HSC expansion and directly correlate with liver fibrosis development (3) (Fig. 4). In addition, it was revealed that hepatic periostin RNA and protein levels were significantly increased in rats from the G2 group when compared with the G1 group (Fig. 5). By contrast, rats in the G3 group exhibited a significant reduction in periostin RNA and protein levels when compared with rats in the G2 group (Fig. 5). These results indicated that periostin expression may be induced during liver fibrosis, and this effect is inhibited by treatment with the losartan ARB.

In vitro analysis of the effects of periostin on Ac-HSCs. To further examine the possible involvement of periostin in liver fibrosis, in vitro assays were performed using the human LX2 Ac-HSC line. Periostin mRNA expression was confirmed in LX2 cells (data not shown). The mRNA expression levels of periostin in LX2 cells was significantly enhanced following treatment with 1 μM AT-II; however, this effect was significantly attenuated by supplementation with losartan (Fig. 6). This suggests that the RAS signaling pathway may positively regulate periostin expression. The direct effect of periostin on the viability of HSCs was then investigated. Exposure to
1 µg/ml periostin significantly induced the viability of LX2 cells (Fig. 6). Notably, this was not inhibited by treatment with losartan (Fig. 7). In addition, periostin significantly promoted the expression of TGF-β1 and Col1a1 in LX2 cells when compared with controls; however, treatment with losartan demonstrated no significant effect on the expression levels of these genes when compared with periostin-only treated cells (Fig. 8). These results suggest that AT-II may positively regulate periostin expression in HSCs, and periostin may serve an important autocrine role in the viability and activation of HSCs.

**Discussion**

Periostin, also known as osteoblast-specific factor 2, is a recently characterized matricellular protein that binds to ECM components, such as type I collagen and fibronectin, and has been implicated in collagen fibrosis (14). This protein transmits signals from the ECM to cellular receptors to regulate a number of cellular functions, including cell adhesion, proliferation, migration and tissue angiogenesis (8).

The potential involvement of AT-II-induced periostin in fibrosis development was first demonstrated in the heart (15). Cardiac fibroblasts, the predominant secretory cells that produce ECM, are important targets of AT-II and are key mediators of cardiac fibrosis (16). Continuous AT-II infusion in a rat cardiac fibrosis model demonstrated extensive interstitial fibrosis and the abundant accumulation of periostin within the interstitial space, as determined by immunofluorescence analysis (9). In cultured adult rat cardiac fibroblasts, AT-II induced the expression of periostin mRNA and protein via the Ras/p38-mitogen activated protein kinase/cyclic adenosine monophosphate responsive element binding protein 1 and extracellular signal-related kinase 1/2-TGF-β1 signaling pathways (9,17). Taken together, these results suggest the involvement of an AT-II/periostin signaling pathway in cardiac fibrosis.

A recent report demonstrated an upregulation of periostin in cirrhotic liver tissues and activated HSCs, which suggested that periostin may be a potential biomarker for hepatic fibrosis (18). In addition, previous studies have demonstrated a...
possible involvement of AT-II in HSC-mediated liver fibrosis development (3-6,19). A previous report demonstrated that AT-II directly stimulated HSC proliferation in vitro, and RAS inhibitors, including ACE-I, ARB and SAB, attenuated hepatic fibrosis and suppressed the proliferation of Ac-HSCs (3-6,19). These medications are commonly used antihypertensive agents that reportedly suppress the progression of hepatic fibrosis. Bataller et al (20) demonstrated that activated but not quiescent HSCs produce AT-II, which stimulate HSC proliferation in an autocrine manner. However, the association between RAS and peristin in liver fibrosis development has not yet been elucidated. The current study demonstrated that inhibition of peristin with losartan markedly inhibited the development of liver fibrosis, as evidenced by a reduced number of Ac-HSCs in the liver. These results suggested a possible interaction between the RAS and peristin in liver fibrosis development.

In the current study, in vitro analysis of LX2 cells demonstrated that AT-II was able to induce peristin expression in Ac-HSCs, and this may serve a central role in liver fibrosis. In addition, these results suggested that Ac-HSCs may be a source of peristin. In addition, peristin treatment improved viability and induced the expression of profibrotic genes TGF-β1 and Collα1 in Ac-HSCs, which supports the hypothesis that peristin directly affects Ac-HSCs by augmenting liver fibrosis. Notably, this direct effect of peristin was not reversed by inhibition of the RAS with losartan. The authors hypothesize that the RAS may partially induce peristin expression in Ac-HSCs in an autocrine manner. Therefore, antifibrotic treatments that target peristin directly may provide an additional anti-fibrotic effect on ARB treatment. Notably, peristin has recently been investigated as a novel target for anti-fibrotic therapy in the liver (21,22).

Previous reports have demonstrated that loss of peristin in mice reduced the development of experimental liver fibrosis induced by a methionine-choline-deficient diet or treatment with carbon tetrachloride (21,22). In addition, small interfering RNA-induced loss of peristin reduced the proliferation and expression of Collα1 and αSMA mRNA in HSCs following treatment with TGF-β (18). These observations indicate the positive involvement of peristin on HSC activation and proliferation, which serves a central role in liver fibrosis. In addition, a successful anti-fibrotic treatment targeting a receptor of peristin (αv integrin), has been reported to be a potential alternative to anti-peristin treatment (23). Further examinations are required to discover novel anti-fibrotic treatment modalities targeting peristin.

In conclusion, the present study demonstrated that AT-II-induced peristin serves an important role in liver fibrosis development. Hepatic peristin expression was significantly attenuated in parallel with hepatic fibrosis development by a pharmacological blockade of the AT-II signal. These experiments indicated that AT-II can augment the activity of HSCs by the expression of TGF-β1 and collagen type I α1 via peristin expression, which may be crucial for liver fibrosis. The results of the present study suggest that peristin may function as a novel regulator of HSC activation, and targeting peristin may therefore present a useful strategy for the treatment of liver fibrosis. Further studies, which may employ a genetically altered peristin in a rodent liver fibrosis model, would allow further investigation into the role of peristin in the development of liver fibrosis.

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