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Suppression of fibrogenic gene expression and liver fibrosis using a synthetic prostacyclin agonist

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ABSTRACT

Chronic injury and inflammation in the liver are associated with the development of liver fibrosis. Expressions of transforming growth factor-β1 (TGF-β1) and hepatocyte growth factor (HGF) participate in the development and suppression, respectively, of liver fibrosis. Here, we investigated the effect of ONO-1301, a synthetic prostaglandin I_2/IP receptor agonist, on liver fibrosis and on changes in the hepatic expressions of genes that regulate the progression of fibrosis in mice. Liver fibrosis was caused by the repetitive administration of CCl_4 for 12 weeks, with ONO-1301 being administered during the last 4 weeks. The expressions of fibrogenic genes: TGF-β1, connective tissue growth factor, α-smooth muscle actin, type-I collagen, and type-III collagen were upregulated by chronic liver injury, which was associated with the expansion of myofibroblasts and the development of liver fibrosis. Treatment with ONO-1301 increased hepatic HGF mRNA expression, but decreased the expressions of TGF-β1, connective tissue growth factor, α-smooth muscle actin, and type-I and type-III collagen, which was associated with the suppression of myofibroblast expansion and liver fibrosis. Neutralizing antibody for HGF significantly attenuated the suppressive action of ONO-1301 on liver fibrosis and fibrogenic gene expressions. The therapeutic action of ONO-1301 on liver fibrosis may have occurred partly through HGF-mediated pathways.

Chronic injury and inflammation are associated with the onset of fibrotic change in tissues, particularly in structurally organized and complicated tissues composed of epithelial and mesenchymal cells, such as the liver, kidney, and lung. Liver fibrosis/cirrhosis is caused by long-term alcohol abuse or hepatitis viral infection. Advanced cirrhosis is generally irreversible and is often associated with variceal hemorrhage or the development of hepatocellular carcinoma. Hence, liver cirrhosis is a major cause of morbidity and mortality with liver diseases worldwide. Approaches that can promote the remodeling of the excess extracellular matrix that has been associated with the reorganization of the hepatic structure and function are critical for the establishment of a therapeutic base.

Growth factors play roles in the cellular and molecular pathways that lead to fibrogenic events. Among the growth factors, transforming growth factor-β1 (TGF-β1) is a key mediator of fibrogenesis (2, 11, 23). TGF-β1 transcriptionally activates the expression of connective tissue growth factor (CTGF)

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Abbreviations: α-SMA, α-smooth muscle actin; CREB, cAMP response element binding protein; CTGF, connective tissue growth factor; HGF, hepatocyte growth factor; PKA, protein kinase-A; TGF-β1, transforming growth factor-β1

and collagen via the Smad signaling pathways (4, 8, 31). TGF-β1 plays a principal role in the differentiation of fibroblasts and fibroblastic cells into myofibroblasts, which is the predominant cell type that is responsible for tissue fibrosis (23, 34). TGF-β1 enhances the expressions of tissue inhibitor of metalloproteinase-1 and plasminogen activator inhibitor-1, thereby promoting the accumulation of an extracellular matrix (23, 32). However, hepatocyte growth factor (HGF) inhibits the profibrotic action of TGF-β1 by stabilizing or inducing Smad transcriptional corepressors (5, 39). HGF facilitates either the expression or the activation of proteases involved in the proteolysis of an extracellular matrix, including matrix metalloproteinases, and urokinase-type plasminogen activators (9, 30). TGF-β1 and HGF have counteractvive effect on the proliferation of normal epithelial cells, and HGF facilitates the apoptosis of myofibroblasts (16). Previous studies using animal models of tissue fibrosis have provided evidence that HGF exerts anti-fibrotic actions on tissue fibrosis, including liver fibrosis/cirrhosis (20, 27, 35).

ONO-1301 was developed as a new type of prostaglandin I_2 (PGI_2)/IP receptor agonist lacking the typical prostanoid structures, including a 5-membered ring and allylic alcohol (12). Prostacyclin and its analogues are not stable *in vivo*, whereas ONO-1301 is chemically and biologically more stable than prostacyclin and its analogues because of the absence of prostanoid structures. Furthermore, the presence of a 3-pyridine radical in ONO-1301 confers inhibitory activity for thromboxane synthase, by which ONO-1301 escapes the desensitization of the action. Because we previously showed that PGI_2 analogues induce the gene expression of HGF (22), we speculated that ONO-1301 might enhance expression of the HGF gene and exert biological action through the induction of HGF. The onset of acute liver injury was strongly suppressed in a mouse model by the administration of ONO-1301, while the suppression of acute liver injury by ONO-1301 was reversed by the neutralization of endogenous HGF (36).

In the present study, the therapeutic action of ONO-1301 on liver fibrosis and the possible involvement of HGF in its action were examined. The expression of fibrogenic growth factors TGF-β1 and CTGF along with the expansion of myofibroblasts and collagen in the liver was decreased by ONO-1301, and this was associated with the suppression of hepatic extracellular matrix accumulation. Neutralizing antibody for HGF significantly, though not entirely, attenuated the anti-fibrogenic action of ONO-1301. We suggest that ONO-1301 exerts therapeutic action on liver fibrosis partly through HGF-mediated pathways, which gives it considerable therapeutic value.

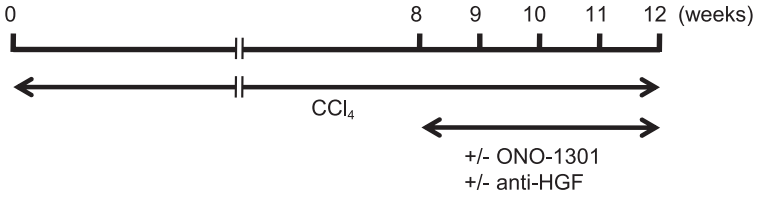
MATERIALS AND METHODS

Animal experiments and reagents. Eight- to nine-week-old female ICR mice (SLC, Shizuoka, Japan) were used. Liver fibrosis was caused by the repetitive administration of CCl_4 in mice (Fig. 1A). CCl_4 (25% in w/v) in olive oil was injected subcutaneously at 4.0 mL/kg body weight for 12 weeks. ONO-1301 (0.5% in w/v in carboxymethylcellulose) was perorally administered at a dose of 3.0 mg/kg body weight. Anti-rat/mouse HGF IgG in saline was intraperitoneally administered at 8.0 mg/kg body weight. Animal experiments were carried out according to the Guidelines for Experimental Animal Care issued by the Prime Minister's Office of Japan. The administration of reagents and the collection of tissues were carried out by Nihon Bioresearch, Inc. (Hashima, Gifu, Japan).

ONO-1301 was obtained from ONO Pharmaceutical Co., Ltd. (Osaka, Japan). Recombinant rat HGF was expressed in Chinese hamster ovary cells and purified from the culture supernatant, essentially as described elsewhere (36). The purity of rat HGF exceeded 98% as determined by SDS-polyacrylamide gel electrophoresis and protein staining with Coomassie brilliant blue. Female Japanese white rabbits (Japan SLC, Hamamatsu, Japan) weighing about 2 kg were immunized by subcutaneous injection of recombinant rat HGF (50 μg/rabbit) in complete Freund's adjuvant, and boosted once or twice at 2-week intervals by injecting rat HGF in incomplete Freund's adjuvant. The antiserum titer was monitored using an enzyme-linked immunosorbent assay. IgGs from anti-rat HGF serum were purified, using Protein A Sepharose Fast Flow (GE Healthcare, UK).

RNA preparation and quantitative RT-PCR. Total RNA was extracted using Sepasol®-RNA I Super (Nacalai Tesque, Kyoto, Japan). First-strand cDNAs were synthesized using SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, USA) with oligo$(dT)_{12\text{-}18}$ primers. The primer sequences for mRNA quantification are listed in Fig. 1B. A PRISM 7000 real-time PCR system (Applied Biosystems, Foster City, USA) and a Power SYBER Green PCR Master Mix (Applied Biosystems) were used for amplification and online detection. Experimental samples were matched to the standard curve gener-

A



B

Genes	Forward primers	Reverse primers
GAPDH	5'-aactttggcattgtggaagg-3'	5'-cacattgggggtaggaacac-3'
HGF	5'-tgttccatgtgggacaagaa-3'	5'-caacgggaaatagggcaata-3'
TGF-β1	5'-gcaacaacgccatctatgag-3'	5'-tccacatgttgctccacact-3'
CTGF	5'-agcagctgggagaactgtgt-3'	5'-gctgctttggaaggactcac-3'
α-SMA	5'-actactgccgagcgtgagat-3'	5'-cttctccagggaggaagagg-3'
Type-I collagen	5'-gaaacccgaggtatgcttga-3'	5'-gggtccctcgactcctacat-3'
Type-III collagen	5'-gcacagcagtccaacgtaga-3'	5'-tctccaaatgggatctctgg-3'

Fig. 1 The primer sequences used for quantitative reverse transcription-PCR (A), and experimental schedule for liver fibrosis caused by CCl_4 (B).

ated by amplifying serially diluted products using the same PCR protocol.

Immunoprecipitation and Western blot. Tissues were homogenized in lysis buffer consisting of 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mM EDTA, 100 mM NaF, 2 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 1 μg/mL aprotinin, and 2% Triton X-100, and were then incubated on ice for 30 min. After centrifugation, the supernatant was incubated with antibodies and precipitated by protein G-Sepharose at 4°C overnight. Immunoprecipitates were separated by 6% SDS-PAGE and electroblotted onto a polyvinylidene difluoride membrane (Bio-Rad). The membrane was incubated with 5% BSA in phosphate-buffered saline (PBS) at 4°C overnight, blotted with anti-α-SMA antibody (DAKO, Glostrup, Denmark) for 2 h, and subsequently labeled with horseradish peroxidase-conjugated antibody against mouse immunoglobulin for 1 h. The resultant signals were detected using ECL Plus immunoblotting detection reagents (Amersham Biosciences).

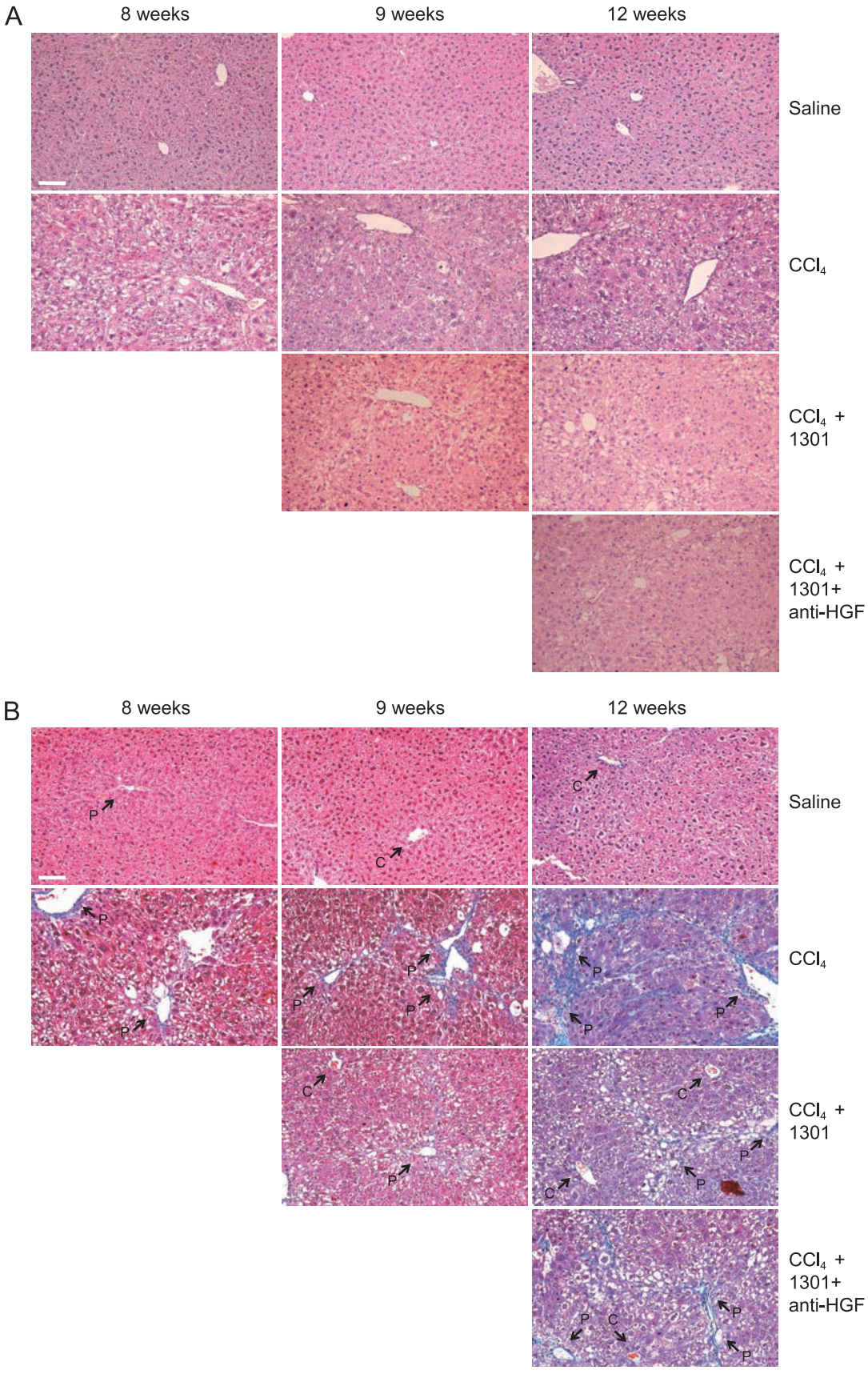
Histological analysis. For histopathology, tissues were fixed in 3.7% neutralized formaldehyde, embedded in paraffin, and tissue sections were analyzed by hematoxylin-eosin staining. To evaluate fibrotic changes, tissue sections were subjected to Masson-Trichrome and Sirius red staining. The fibrotic area was quantified in tissue sections subjected to Sirius red staining, using computer-aided image analysis with Lumina Vision (Mitani Corporation, Fukui, Japan). At least 3 fields at 100-fold magnification were captured and assessed in all the samples. For immunohistochemical detection of α-smooth muscle actin (α-SMA), the tissue sections were incubated with anti-α-SMA antibody (DAKO) for 1 h, and subsequently with horseradish peroxidase-conjugated antibody against mouse immunoglobulin (DAKO) for 1 h. The sections were visualized in chromogenic substrate solution containing 3,3'-diaminobenzidine hydrochloride and 0.01% hydrogen peroxide.

Statistical analysis. Quantitative data are presented as the mean values ± SE. We used a Student's *t*-test to determine the statistical significance. $P < 0.05$ was considered significant.

RESULTS

Suppression of fibrous tissue expansion

Liver fibrosis was caused by the repetitive administration of CCl_4 in mice (Fig. 1A). Compared to the age-matched normal mice given saline alone, a significant increase in accumulation of collagen fibers was seen at 8 weeks after the initiation of CCl_4 administration, as seen in blue in Masson-Trichrome staining or red in Sirius red staining (Fig. 2B, C). Masson-Trichrome and Sirius red staining have been used to visualize collagen fibers and determine the


A
8 weeks
9 weeks
12 weeks
Saline
CCl4
CCl4 + 1301
CCl4 + 1301+ anti-HGF
B
8 weeks
9 weeks
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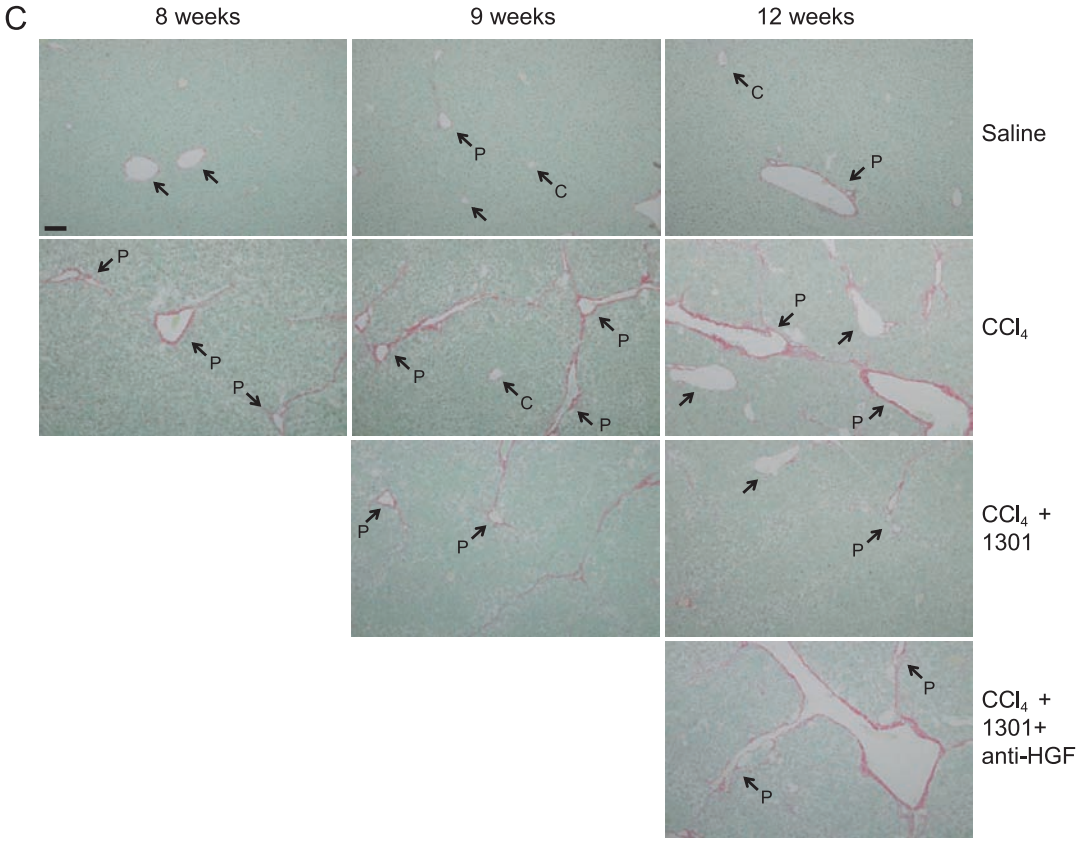
Saline
CCl4
CCl4 + 1301
CCl4 + 1301+ anti-HGF
P
C



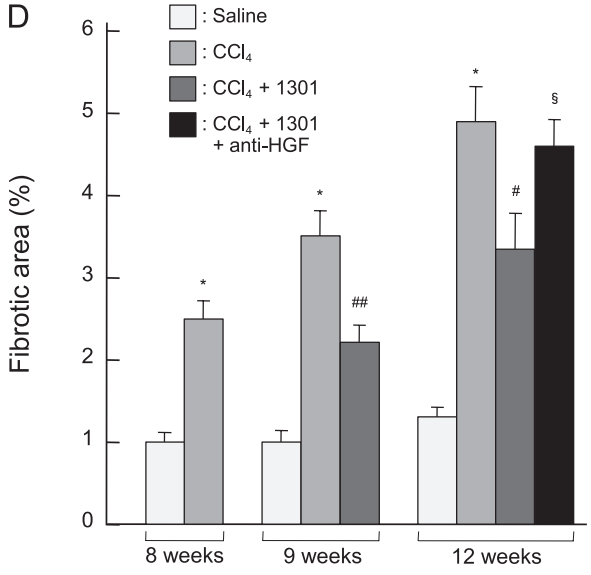


Fig. 2 Histological suppression of liver fibrosis by ONO-1301 administration. (**A**–**C**) Histological change of liver tissues. Liver tissue sections were analyzed by hematoxylin-eosin staining (**A**), Masson-Trichrome staining (**B**), and Sirius red staining (**C**). Bars represent 100 μm. Typical pericentral (C) and periportal (P) regions are indicated by allows in B and C. (**D**) Change in fibrotic areas. Fibrotic areas were determined by the image analysis in liver tissue sections subjected to Sirius red staining. 1301, ONO-1301. Each value represents the mean ± SE (n = 10 in each group). $^{*}P < 0.01$ vs. normal (vehicle) group; $^{\#}P < 0.05$ vs. CCl_4 group; $^{\#\#}P < 0.01$ vs. CCl_4 group; $^{\S}P < 0.05$ vs. CCl_4 + 1301 group.

quantity of the fibrotic area in the liver (7, 14). Because expression of type-I, type-III, and type-IV collagen genes was up-regulated in CCl_4-induced liver fibrosis (28), fibrous regions may be composed of mainly type-I collagen and other types of collagens. The image analysis in tissue sections stained with Sirius red indicated that fibrous tissue was increased 2.5-fold at 8 weeks, compared with saline-administered normal mice (Fig. 2D). The accumulation of collagen fibers further progressed thereafter by continuing CCl_4 administration, until 12 weeks after the initiation of CCl_4 administration. Therefore, mice were divided into experimental groups at 8 weeks and administered either CCl_4 + vehicles, CCl_4 + ONO-1301, or CCl_4 + ONO-1301 + anti-HGF IgG.

In control mice given CCl_4 alone, the accumulation of collagen fibers was obvious in the pericentral and periportal regions and in fibrous septa spread in the liver at 12 weeks (Fig. 2B). The administration of ONO-1301 clearly suppressed the accumulation of fibrous regions at 9 and 12 weeks (Fig. 2B, C). Image analysis indicated that ONO-1301 significantly suppressed the increase in the fibrotic area to 48.1 and 56.4% at 9 and 12 weeks, respectively, when the values in the vehicle-treated control and the CCl_4-treated mice were assumed to be the base line and 100%, respectively. When mice were administered neutralizing anti-HGF IgG in addition to CCl_4 and ONO-1301, the suppressive effect of ONO-1301 on the expansion of fibrous regions was significantly abrogated by the neutralization of the endogenous HGF (Fig. 2B–D). These results indicated that ONO-1301 suppressed the progression of fibrotic change in the liver that was caused by repetitive injury and that the suppressive action of ONO-1301 was at least partly mediated by HGF.

Suppression of myofibroblast expansion

In the liver, hepatic myofibroblasts play a major role in the biosynthesis and deposition of extracellular matrix components such as type-I collagen during fibrotic changes, and hepatic myofibroblasts are mainly derived from hepatic stellate cells and portal fibroblasts following their activation and differentiation (10). Because myofibroblasts are characterized by their expression of α-SMA, we analyzed the expansion of myofibroblasts in liver tissue by immunostaining and Western blot for α-SMA (Fig. 3).

In normal mice livers administered saline alone, the expression of α-SMA was very low with only a few sparsely distributed α-SMA-positive cells. Twelve weeks after treatment with CCl_4 alone, many α-SMA-positive cells appeared and were distributed particularly in the pericentral and periportal regions, and several α-SMA-positive cells expanded radially from the periportal regions. The expansion of α-SMA-positive cells in mice treated with CCl_4 + anti-HGF IgG was somewhat more obvious than what was seen in mice treated only with CCl_4. The administration of ONO-1301 into CCl_4-treated mice clearly suppressed the expansion of α-SMA-positive cells in the liver. When mice were administered anti-HGF IgG in addition to CCl_4 and ONO-1301, the suppressive effect of ONO-1301 on the expansion of α-SMA-positive cells disappeared because of the neutralization of HGF.

Western blot analysis for α-SMA indicated that α-SMA expression in the livers of the control saline-treated mice was mostly undetectable, while it was strongly increased at 8 weeks after CCl_4 treatment (Fig. 3B). At 12 weeks, treatment with ONO-1301 for 4 weeks clearly suppressed the hepatic expression of α-SMA. However, the suppressive action of ONO-1301 on α-SMA expression was inhibited by treatment with anti-HGF IgG. Thus, ONO-1301 suppressed the expansion of α-SMA-positive myofibroblasts in CCl_4-treated mice, and the suppressive action of ONO-1301 on myofibroblast expansion was at least partly attributable to HGF.

Change in fibrogenic gene expression

When HGF mRNA levels in the liver were analyzed by quantitative RT-PCR, CCl_4 treatment did not change the HGF mRNA levels (Fig. 4), perhaps due to the increased expression of hepatic TGF-β1 (see below). ONO-1301 administration increased HGF mRNA expression 2.5-fold, compared with the level in control vehicle-treated mice (Fig. 4). We then analyzed the hepatic gene expression that is involved in the signaling and accumulation of extracellular matrix proteins (Fig. 4). TGF-β1 and CTGF play a key role in the progression and pathogenesis of liver fibrosis (2, 11, 23). At 12 weeks after the initiation of CCl_4 injection, the hepatic gene expressions of TGF-β1 and CTGF were increased 3.8- and 3.1-fold in mice, respectively, compared with saline-injected normal mice. Consistent with the up-regulation of TGF-β1 and CTGF expression, the gene expression of α-SMA, and type-I and type-III collagen were strongly induced after CCl_4-treatment. ONO-1301 significantly suppressed the gene expressions of TGF-β1, CTGF, α-SMA, type-I collagen, and type-III collagen. Neutralization of HGF largely reversed the suppressive effect of ONO-1301 on the expression of these genes.

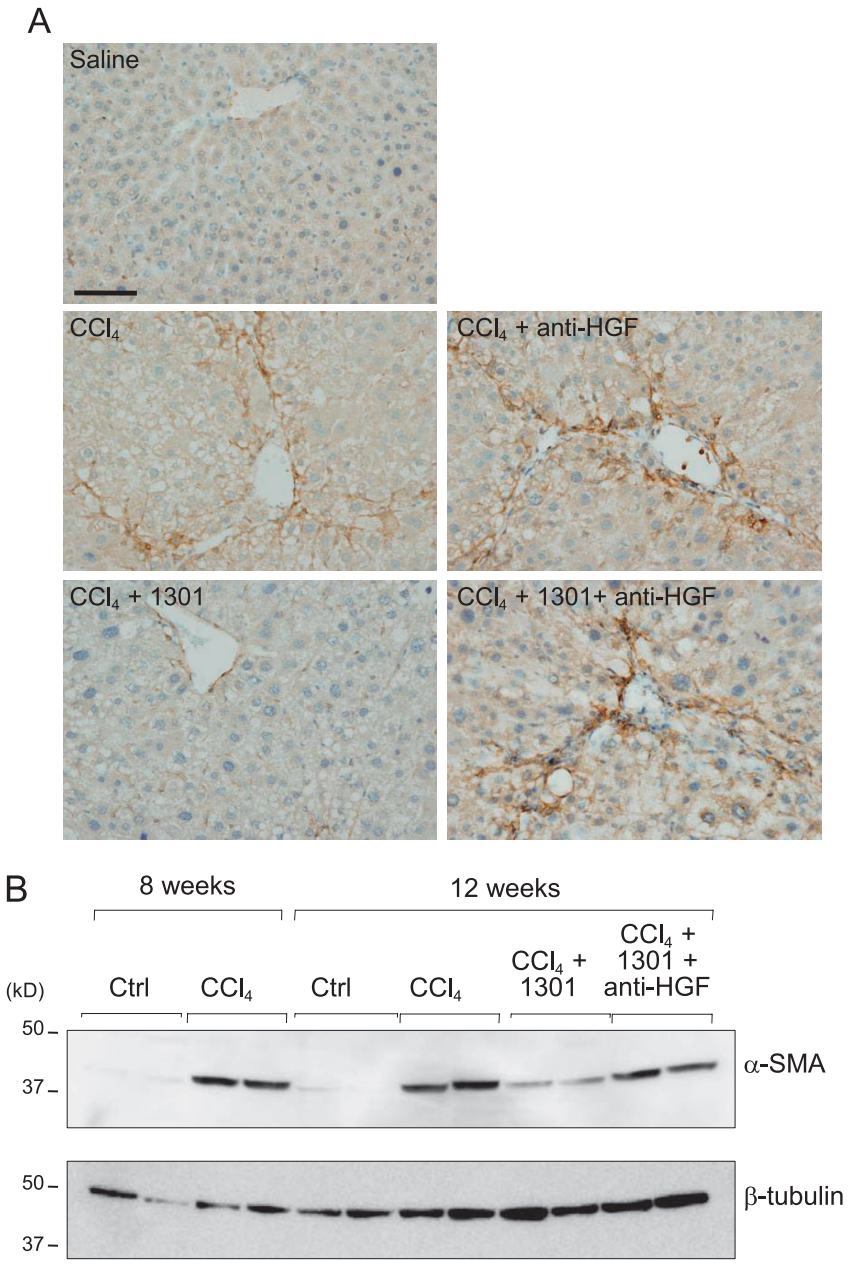


Fig. 3 Decreased expression of α-SMA in liver tissues by ONO-1301 administration. (**A**) Accumulation and distribution of α-SMA-positive myofibroblasts and/or myofibroblast-like cells. A bar represents 100 μm. (**B**) Change in hepatic expression of α-SMA. Expression of α-SMA protein was analyzed by Western blot using liver tissue extracts.

DISCUSSION

The gene expression of HGF is transcriptionally regulated by different types of extracellular signaling molecules, including interleukin-1, chemokines, growth factors, and prostaglandins (PGE_1/E_2 and PGI_2) (6, 15, 22, 26, 33). Prostaglandin receptors are G-protein-coupled receptors that evoke different effectors and signaling pathways. The IP receptor for PGI_2 activates adenylate cyclase upon ligand binding, thereby increasing the intracellular cAMP level. cAMP activates protein kinase-A (PKA), and the PKA-induced Ser133 phosphorylation in the cAMP response element binding protein (CREB) plays a major regulatory role in the transcriptional activation of target genes (24). The binding motif of CREB is located in the promoter region of HGF genes (18). We previously found that ONO-1301

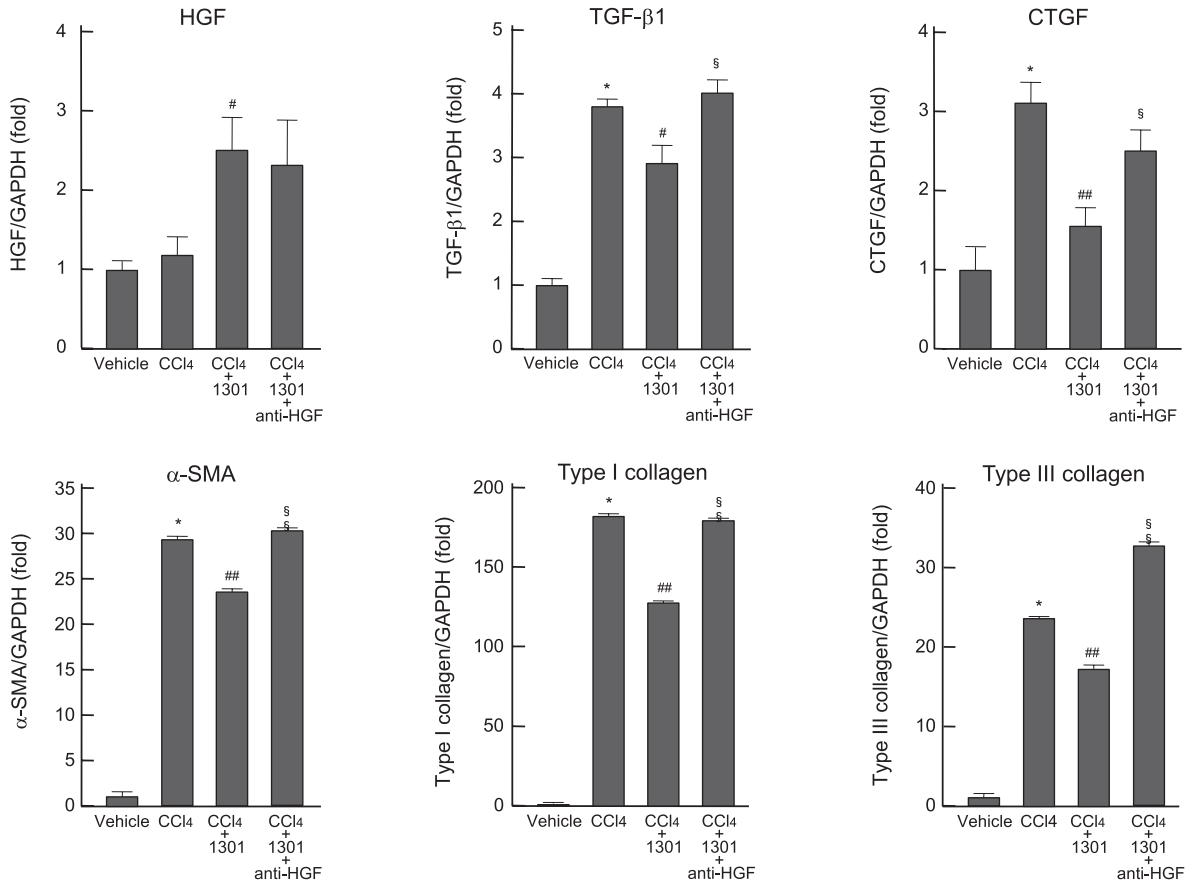


Fig. 4 Changes in the mRNA expression of HGF, TGF-β1, CTGF, α-SMA, type-I collagen, and type III collagen. RNA was prepared from liver tissues and each mRNA level was analyzed by quantitative RT-PCR. 1301, ONO-1301. Each value represents the mean ± SE (n = 4 in each group). $^{*}P < 0.01$ vs. normal (vehicle) group; $^{\#}P < 0.05$ vs. CCl_4 group; $^{\#\#}P < 0.01$ vs. CCl_4 group; $^{\S}P < 0.05$ vs. CCl_4 + 1301 group; $^{\S\S}P < 0.01$ vs. CCl_4 + 1301 group.

strongly enhanced the expression of HGF and induced CREB-Ser133 phosphorylation, and that the biological activity of ONO-1301 that enhanced HGF expression was cancelled by a selective inhibition of PKA (36). Thus, ONO-1301 up-regulates the gene expression of HGF via an IP receptor-mediated signaling pathway.

Recent studies have shown that ONO-1301 enhances the expression of HGF in different tissues and suppresses interstitial fibrosis in the kidney, fibrotic changes in myocardium caused by ischemia-reperfusion, and collagen deposition in bronchial tissue (13, 28, 37). It is important to note that the suppressive effect of ONO-1301 was partly reversed by the neutralization of endogenous HGF. We obtained evidence that ONO-1301 suppressed the progression of liver fibrosis caused by chronic liver injury and that selective neutralization of HGF significantly abrogated the suppressive effect of ONO-1301 on liver fibrosis, although not entirely. Collectively, ONO-1301 enhanced the expression of HGF and exerted an anti-fibrotic action on different tissues, and the anti-fibrotic action of ONO-1301 may have been at least partly mediated by HGF. These results suggest the importance of HGF in the anti-fibrotic action of ONO-1301 and that a common HGF-mediated mechanism underlies the anti-fibrotic action of ONO-1301.

HGF exerts biological and physiological activities through the Met receptor tyrosine kinase (3, 27), and the anti-fibrotic action of the HGF-Met pathway has been demonstrated in different models for different tissues. In the liver, the selective loss of Met receptor in hepatocytes has accelerated the development of liver fibrosis in response to chronic hepatic injury by CCl_4 (19). By contrast, the administration of HGF and the expression of the HGF gene suppressed the development of liver fibrosis/cirrhosis (20, 27, 35). HGF treatment accelerated the resolution of fibrosis in experimental animal models, in-

cluding renal and lung fibrosis (16, 25, 27, 38). It seems that the anti-fibrotic actions of HGF are explained by the characteristics of the HGF-Met pathway. Among the different types of growth factors, HGF is unique for the following points: 1) HGF preferentially targets the cells of an epithelial origin rather than the cells of a mesenchymal origin; 2) HGF facilitates cell death in myofibroblasts (16); and 3) HGF strongly enhances the expression and activity of different types of extracellular proteases involved in the proteolysis of extracellular matrix proteins (3, 9, 30). Furthermore, it must be emphasized that HGF and TGF-β1 counterbalance one another in their signaling and expressions. HGF suppresses the expression of TGF-β1 (35), while TGF-β1 suppresses the expression of HGF (1, 18, 21). HGF stabilizes or induces Smad transcriptional corepressors, thereby inhibiting the biological action of TGF-β1 (5, 38). HGF and TGF-β1 counteract miR-29 expression, thereby exhibiting an opposite effect on collagen synthesis in hepatic stellate cells (17). Thus, HGF and TGF-β1 counterbalance one another in their signaling and expressions, and HGF exerts biological activities that are involved in the suppression and improvement of fibrosis.

Because progressive fibrosis in organs and tissues caused by chronic inflammation, infection, and/or injury, which includes liver fibrosis/cirrhosis, is a major cause of suffering and death, the investigative approaches for the therapeutic intervention of fibrotic disorders are definitely important. For the treatment of chronic diseases, small molecules for therapeutic use have some manufacturing advantages over recombinant protein drugs: expense, chemical stability, and drug delivery. The slow-release material of ONO-1301 polymerized with poly (D, L-lactic-co-glycolic acid) exerts a therapeutic effect on renal and cardiac pathology (13, 29). Therapeutic approaches using slow-release ONO-1301 for the treatment of liver fibrosis/cirrhosis are worthy of further preclinical study.

Acknowledgements

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