SPANDIDOS PUBLICATIONS

Amelioration of liver fibrogenesis by dual inhibition of PDGF and TGF-ß with a combination of imatinib mesylate and ACE inhibitor in rats

HITOSHI YOSHIJI[1], SHIGEKI KURIYAMA[2], RYUICHI NOGUCHI[1], YASUHIDE IKENAKA[1], JUNICHI YOSHII[1], KOJI YANASE[1], TADASHI NAMISAKI[1], MITSUTERU KITADE[1], MASAHARU YAMAZAKI[1], KIYOSHI ASADA[1], TAKEMI AKAHANE[1], TATSUHIRO TSUJIMOTO[1], MASAHITO UEMURA[1] and HIROSHI FUKUI[1]

[1]Third Department of Internal Medicine, Nara Medical University, Nara; [2]Third Department of Internal Medicine, School of Medicine, Kagawa University, Kagawa, Japan

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Abstract. Both platelet-derived growth factor (PDGF) and transforming growth factor-ß (TGF-ß) are known to be pivotal cytokines in liver fibrosis development. The aim of our current study was to elucidate the effects of dual inhibition of PDGF and TGF-ß by combination of the clinically used imatinib mesylate (STI-571) and perindopril (an ACE-inhibitor; ACE-I), respectively, on ongoing liver fibrosis development in rats. The effects of STI-571 and ACE-I at clinically comparable low doses were examined in a rat model of CCl4-induced liver fibrogenesis. Treatment with both STI-571 and ACE-I inhibited liver fibrogenesis and suppressed activation of hepatic stellate cells (HSCs). Administration of both agents exerted a more potent inhibitory effect than administration of either single agent. Our *in vitro* study demonstrated that STI-571 and ACE-I suppressed PDGF receptor (PDGFR) phosphorylation and TGF-ß expression in activated HSCs, respectively. Dual suppression of PDGF and TGF-ß with a combination of clinically comparable low doses of STI-571 and ACE-I exerted a significant inhibitory effect on ongoing liver fibrosis development. Since both agents are widely used in clinical practice, this combination therapy may provide a new strategy against liver fibrosis in the future.

Introduction

It is now widely recognized that activated hepatic stellate cells (HSCs) play a pivotal role in liver fibrosis development (1,2). The increased number of HSCs during liver fibrogenesis reflects the activity of several growth factors (1). Among the growth factors, transforming growth factor-ß (TGF-ß) and platelet-derived growth factor (PDGF) are key mediators in liver fibrogenesis (3,4). Liver fibrosis is characterized by the excessive deposition of extracellular matrix (ECM), which leads to a severe pathological disturbance in the liver. TGF-ß is an important cytokine in regulating the production of ECM. Strategies aimed at disrupting TGF-ß synthesis and the signaling cascade markedly suppressed liver fibrosis development. Animal and culture studies using soluble TGF-ß receptors or other means such as neutralizing monoclonal antibody and dominant negative receptor gene transfer have shown promising results (5-7). PDGF is the most potent proliferating stimulus for HSCs (4,8). It has been reported that the PDGF receptor (PDGFR) is up-regulated along with HSC activation in carbon tetrachloride (CCl4)- and bile duct ligation-induced liver injuries (9-11). Dominant-negative soluble PDGFR or anti-sense PDGF gene transfer significantly attenuated experimental liver fibrosis development (12,13). It had been believed that hepatic fibrosis was a passive and irreversible process due to the collapse of the hepatic parenchyma and its substitution with collagen-rich tissue. However, improved understanding of the mechanism underlying hepatic fibrosis revealed that even advanced liver fibrosis is potentially reversible (3,4). It has been reported that the risk of hepatocellular carcinoma (HCC) increases with the progression of hepatic fibrosis (14). Furthermore, the existence of fibrosis itself accelerates experimental hepatocarcinogenesis (15). Taken together, an effective therapeutic strategy for suppressing liver fibrosis should improve the overall prognosis of patients with chronic liver diseases. Although efforts are being made to develop new drugs against liver fibrosis, targeting TGF-ß and PDGF in particular, there are no approved anti-fibrotic agents to date. An alternative approach may be to find a clinically used orally available compound that also shows inhibitory effects on TGF-ß and PDGF until new drugs become widely available.

Compounds capable of inhibiting PDGFR kinase have been developed. Imatinib mesylate (STI-571) is a protein-

Correspondence to: Dr Hitoshi Yoshiji, Third Department of Internal Medicine, Nara Medical University, Shijo-cho 840, Kashihara, Nara 634-8522, Japan
E-mail: yoshijih@naramed-u.ac.jp

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tyrosine kinase inhibitor of the 2-phenylamonioyrimideine class that was initially developed for its selective action against the Bcr-Abl fusion protein, which exists in nearly all patients with chronic myeloid leukemia (CML) (16). At concentrations required for Bcr-Abl tyrosine kinase inhibition, STI571 also inhibits the activities of PDGFR kinase receptors (17,18). We have previously reported a marked suppressive effect of STI-571 on pig-serum-induced liver fibrosis development associated with the inhibition of activated HSCs (19). To suppress TGF-ß, we employed an angiotensin-converting enzyme inhibitor (ACE-I) in the current study. It has been reported that angiotensin-II (AT-II) mediated modulation of TGF-ß ligand expression. AT-II increased TGF-ß gene expression in a dose-dependent manner in activated HSCs *in vitro* (20). We and others reported that suppression of AT-II by ACE-I markedly attenuated experimental liver fibrosis development and suppressed TGF-ß (3,20).

It has been reported that a combination therapy against liver fibrogenesis may exert a synergistic rather than additive effect (4). In the current study, to evaluate the feasibility of future clinical application, we examined the effects of the combination treatment of STI-571 and ACE-I at clinically comparable low doses on ongoing liver fibrosis development in rats to achieve dual inhibition of PDGF and TGF-ß, and attempted to investigate the possible mechanisms involved.

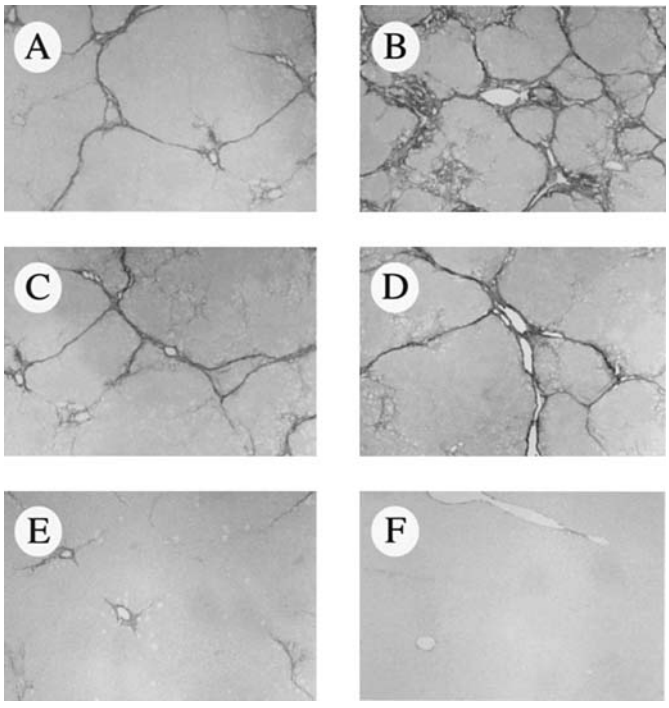

Figure 1. Microphotographs of liver sections from the CCl4-treated rats. (A and B) The control group after CCl4 treatment for 4 and 16 weeks, respectively. (C and D) The STI-571- and ACE-I-treated groups, respectively. (E) STI-571 and ACE-I combination-treated group. (F) Corn-oil-treated negative control group. The 4- and 16-week treatment with CCl4 resulted in moderate and marked liver fibrosis development, respectively. Single treatment with either STI-571 or ACE-I from week 4 suppressed ongoing liver fibrosis development, but the combination treatment with STI-571 and ACE-I exerted a much more potent anti-fibrotic effect. No fibrosis development was found in the corn oil-treated group. (S-R staining, original magnification of x40).

Materials and methods

Animals. Male Fisher 344 rats, aged 6 weeks, were purchased from Japan SLC Inc. (Hamamatsu, Shizuoka, Japan). They were housed in stainless-steel, mesh cages under controlled conditions of temperature (23±3˚C) and relative humidity (50±20%), with 10-15 air changes per hour and light illumination for 12 h a day. The animals were allowed access to food and tap water *ad libitum* throughout the acclimatization and experimental periods.

Animal treatment. STI-571 and perindopril, an ACE-I, were generously supplied by Novartis Pharma (Basel, Switzerland) and Daiichi Pharmaceutical Co. (Tokyo, Japan), respectively. The rats were divided into 4 groups (n=10 in each group). All experimental groups received CCl4 (2 ml/kg/BW dissolved in 150 μl of corn oil) twice a week to induce liver fibrosis. After 4 weeks of treatment with CCl4, administration of STI-571 and ACE-I was started. The doses of STI-571 and ACE-I were 5 and 2 mg/kg/day by daily gavage, respectively. The doses of these agents were almost comparable to those used in clinical practice (19,20). Rats that received only corn oil were examined as a negative control group. After 16 weeks of treatment with CCl4, all rats were sacrificed under anesthesia. All animal procedures were performed according to approved protocols, in accordance with the standard recommendations for the proper care and use of laboratory animals, and approved by the Animal Care and Use Committee of Nara Medical University.

Hepatic fibrosis indices. In all experimental groups, 5-μm-thick sections of formalin-fixed and paraffin-embedded livers were routinely processed with Azan-Mallory (A-M) staining to determine the liver fibrosis development. The immunohistochemical staining of α-smooth muscle actin (α-SMA) was performed as previously described using paraffin-embedded sections with a primary anti-α-SMA antibody (Dako, Kyoto, Japan) (22). Semiquantitative analyses of fibrosis development and the immunopositive cell area were carried out using the Fuji-BAS 2000 image analyzing system (Fuji, Tokyo, Japan) in six microscopic visual fields (original magnification, x40) per specimen from five rats. We did not count the α-SMA-positive vessels in the portal area, as they were assumed to be hepatic arteries, and only included the α-SMA-positive cells in the sinusoidal lining for image analysis. The hepatic hydroxyproline content was determined as previously described with 300 mg of frozen samples (22). The hydroxyproline content was expressed as μg/g wet liver. Alanine amino-transferase aspartate (ALT) and total bilirubin (T. Bil) were assessed using routine laboratory methods. The serum hyaluronic acid and procollagen III-N-peptide (P-III-P) were also measured as described previously (21).

RNA expression of α2-(I)-procollagen and TGF-ß by real-time PCR. The isolation and culture of activated HSC have been described previously (22). The expression of α2-(I)-procollagen and TGF-ß was evaluated by real-time poly-chain reaction (PCR) as described previously (23,24). For cDNA synthesis, Taqman reverse transcription reagents were used as described in the manufacturer's manual of the

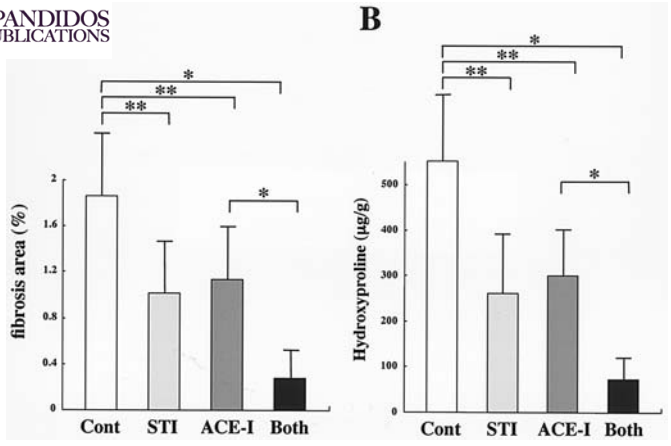

Figure 2. The effects of STI-571 and ACE-I on the fibrosis area (A) and hepatic hydroxyproline content (B) in the CCl4-treated liver. (A) The fibrosis area was evaluated by an image analyzer as described in Materials and methods. STI-571 and ACE-I significantly suppressed liver fibrosis development compared to the control group ($p<0.05$). The combination treatment with STI-571 and ACE-I revealed further inhibition compared to administration of STI-571 or ACE-I alone ($p<0.01$). (B) The inhibitory effects of STI-571 and ACE-I on hepatic hydroxyproline content were similar to those on the fibrosis areas. Cont, control group; STI-571 and ACE-I, STI-571- and ACE-I-treated group, respectively; both, STI-571 and ACE-I combination-treated group. The data represent the means ± SD (n=10). Statistically significant differences of $^{*}p<0.01$ and $^{**}p<0.05$ between the indicated groups.

ABI Prism 7700 Sequence Detection System (PE Applied Biosystems, Foster City, CA, USA), which was used for real-time PCR amplification following the Taqman Universal PCR Master Mix Protocol (PE Applied Biosystems). Relative quantification of gene expression was performed as described in the manufacturer's manual using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. The threshold cycle and standard curve method were used to calculate the relative amount of target RNA as described by PE Applied Biosystems. The following procedure was employed: 1 cycle at 50˚C for 2 min, 60˚C for 30 min, 94˚C for 5 min, 45 cycles at 94˚C for 1 min, 55˚C for 1 min, and 72˚C for 1 min. To prevent genomic DNA contamination, all RNA samples were subjected to DNase I digestion and checked by 40 cycles of PCR to confirm the absence of amplified DNA.

Phosphorylation of PDGFR. To examine the effect of STI571 and ACE-I on the phosphorylation of PDGFR in activated HSCs, immunoprecipitation (IP) and Western blotting (WB) were performed as described previously (24). To conduct IP, liver lysates were immunoprecipitated with anti-phosphotyrosine before conducting SDS-PAGE. Anti-tyrosine (4G10) and anti-PDGFR were purchased from Upstate Biotechnology (Lake Placid, NY, USA). Before WB, we stained each membrane with PONCEAU solution (Sigma, MI, USA) to confirm that equal amounts of protein were blotted (data not shown). The blot was developed using an amplified alkaline phosphatase immunoblot assay kit (Bio-Rad, Tokyo, Japan).

Statistical analysis. To assess the statistical significance of intergroup differences in the quantitative data, Bonferroni's multiple comparison test was performed after one-way analysis of variance (ANOVA). This was followed by Bartlett's test to determine the homology of variance.

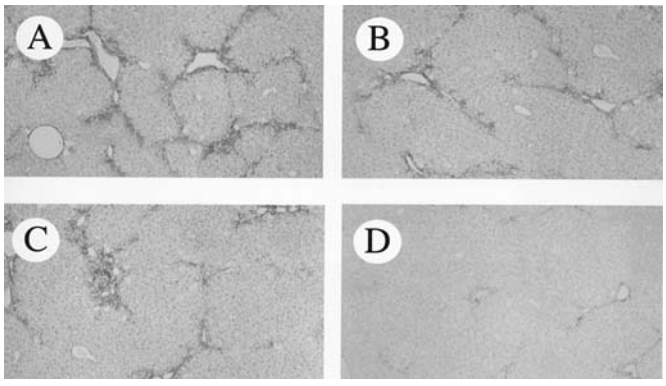

Figure 3. Immunohistochemical analysis of α-smooth muscle actin (α-SMA). The immunopositive cells of α-SMA were significantly reduced in number in the livers of the STI-571-treated (B), ACE-I-treated (C) and combination STI-571 and ACE-I-treated groups (D) compared to the control group (A). Original magnification, x40.

Results

Effects of STI-571 and ACE-I on hepatic indices. Histological examination revealed that 4-week and 16-week treatment with CCl4 resulted in a moderate and a marked liver fibrosis development, respectively (Fig. 1A and 1B, respectively). Single treatment with either STI-571 or ACE-I from week 4 to 16 suppressed the ongoing liver fibrosis development, although the attenuation was not drastic ($p<0.05$) (Fig. 1C and 1D, respectively). The combination treatment with STI-571 and ACE-I exerted a more potent anti-fibrotic effect than administration of either single agent (Fig. 1E). No fibrosis development was found in the corn-oil-treated control group (Fig. 1F). Densitometric analysis showed that the fibrosis areas almost corresponded to the histological findings (Fig. 2A). Although both STI-571 and ACE-I suppressed liver fibrosis development compared to the control group ($p<0.05$), the combination treatment with both agents induced further inhibition in comparison to administration of either single agent alone ($p<0.01$). The hepatic hydroxyproline content showed results similar to those of the fibrosis areas (Fig. 2B). The serum fibrosis markers (i.e. hyaluronic acid and P-III-P) were also significantly suppressed by treatment with STI-571 and ACE-I, whereas the serum ALT and T. Bil levels did not change, suggesting that the inhibitory effects of STI-571 and ACE-I were not due to the non-specific cytoprotective effect. The body and liver weights also did not show any significant differences among the groups (data not shown).

Effects of STI-571 and ACE-I on HSC activation in the liver. Immunohistochemical analysis of α-SMA was carried out to examine the effects of STI-571 and ACE-I on HSC activation. The activated HSCs, which expressed α-SMA, were drastically reduced in number after treatment with STI-571 and ACE-I (Fig. 3A-C). Similar to the anti-fibrotic effect, combination treatment with both agents induced further inhibition of the α-SMA-positive cells compared

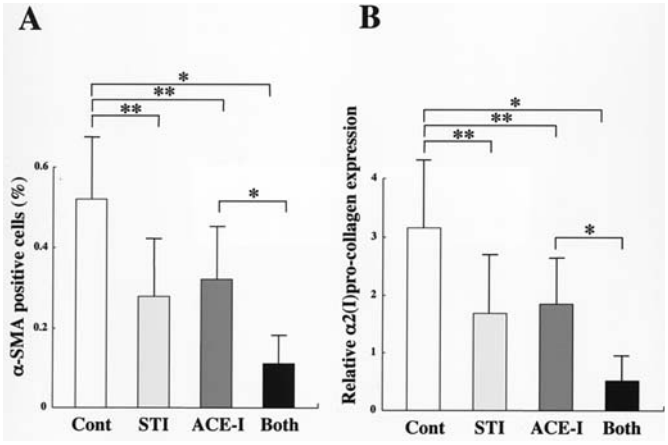

Figure 4. Densitometric analysis of the α-SMA-positive cells (A) and α-(I)-procollagen mRNA expression (B) in the CCl4-treated liver. The α-SMA-positive activated HSCs and α-(I)-procollagen mRNA were significantly reduced by STI-571 and ACE-I treatment. The inhibitory effects of STI-571 and ACE-I on α-SMA and α2-(I)-procollagen expression exerted almost parallel reductions. Cont, control group; STI-571 and ACE-I, STI-571- and ACE-I-treated group, respectively; both, STI-571 and ACE-I combination-treated group. The data represent the means ± SD (n=10). Statistically significant differences of $^{*}p<0.01$ and $^{**}p<0.05$ between the indicated groups.

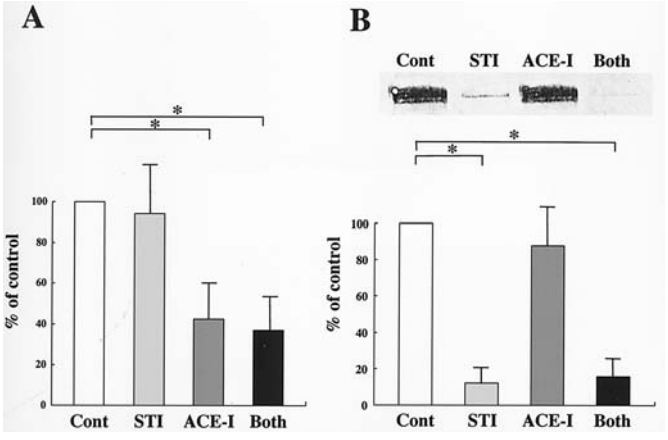

Figure 5. The effects of ACE-I and STI-571 on TGF-ß mRNA expression (A) and phosphorylation of PDGFR (B) in activated HSCs, respectively. (A) At a dose of 1 μM, ACE-I significantly inhibited TGF-ß expression, whereas STI-571 did not. (B) STI-571 (1 μM) attenuated PDGF-induced phosphorylation of PDGFR, whereas ACE-I did not. Densitometric analysis showed that the relative intensity of phosphorylated PDGFR was 12±8%, 88±27%, and 16±9%, in the STI-571-, ACE-I-, and combination-treated groups, respectively, compared with the control group. The data represent the means ± SD (n=6). *Statistically significant differences between the indicated groups ($p<0.01$).

with administration of either single agent alone (Fig. 3D). Computer-assisted semiquantitative analysis showed that α-SMA-positive cells in the STI-571- and ACE-I-treated groups were significantly reduced in comparison to the control group ($p<0.05$) (Fig. 4A). We also performed real-time PCR analysis to elucidate the effect of these agents on the α2-(I)-pro-collagen mRNA expression. Similar to their effects on α-SMA expression, STI-571 and ACE-I also markedly suppressed mRNA expression of α2-(I)-pro-collagen in the liver compared to the control group ($p<0.05$), and the combination treatment exerted further inhibitory effects (Fig. 4B). Noteworthy was the finding that the inhibitory effects of STI-571 and ACE-I on α-SMA, α2-(I)-pro-collagen mRNA expression and those on the fibrosis area exerted almost parallel reductions.

TGF-ß expression and PDGFR phosphorylation in activated HSCs. We examined whether TGF-ß mRNA expression and PDGFR phosphorylation were suppressed by treatment with ACE-I and STI-571, respectively. As shown in Fig. 5A, ACE-I suppressed the mRNA expression of TGF-ß compared to the control group, whereas STI-571 did not alter TGF-ß expression in activated HSCs. The inhibitory effect of the combination treatment was of similar magnitude to that of ACE-I alone. We next examined PDGF-BB-induced PDGFR phosphorylation in activated HSCs. In contrast to TGF-ß expression, STI-571 markedly attenuated PDGFR phosphorylation in activated HSCs. ACE-I did not affect phosphorylation, and the combination treatment also exerted suppression of a similar magnitude to that of STI-571 (Fig. 5B).

Discussion

In the current study, we revealed that CCl4-induced ongoing liver fibrosis development was significantly suppressed by combination treatment with STI-571 and ACE-I along with suppression of PDGFR phosphorylation and TGF-ß expression, respectively. We previously reported that simultaneous administration of pig serum and either STI-571 or ACE-I significantly inhibited liver fibrosis development (19,20). The pig serum model is known to induce liver fibrosis without severe inflammation (20). In the current study, we used the CCl4-induced liver fibrosis rat model, which is known to be associated with severe inflammation and necrosis. We also examined the effects of STI-571 and ACE-I on an ongoing liver fibrosis model in this study. Considering the possible future clinical application, examining the effect of test agents on the ongoing liver fibrosis model is much more relevant to the clinical situation than the simultaneous administration of a fibrotic stimulator and test agent. We therefore administered STI-571 and ACE-I after confirming the stage of established liver fibrosis.

Since it is now widely recognized that many factors are involved in the development of liver fibrogenesis, the combination therapy of agents with different mechanisms of action would be better than a single agent treatment. Dual inhibition of PDGF and TGF-ß should be one of the most powerful combinations in molecular targeting therapy against liver fibrogenesis since it is widely accepted that both molecules play pivotal roles in liver fibrosis development. To examine the feasibility of their future clinical application, we employed clinically used agents, but not developing modalities such as gene therapy, to attenuate the biological activities of PDGF and TGF-ß. The main action of STI-571 is to inhibit PDGF, which is the strongest stimulator of proliferation of activated HSCs. It has been reported that STI-571 markedly suppressed PDGFR phosphorylation, and this effect has been shown to attenuate the proliferation of activated HSCs (19). Although AT-II also stimulates the proliferation of activated HSCs, its magnitude was not as strong as that of PDGF. However, AT-II plays an important role in the biology of

HSCs from different aspects, e.g. TGF-ß and stress production (25). AT-II is a potent inducer of TGF-ß synthesis through Smad2 in cultured HSCs *in vitro* (26,27). AT-II induces HSC proliferation and up-regulates TGF-ß expression via the AT1-R pathway (20). The increase in hepatic concentration of TGF-ß induced by bile duct ligation was attenuated in AT1-R knockout mice (28). In humans, it has been shown that the combination of AT-II and TGF-ß genotypic polymorphism is associated with advanced hepatic fibrosis (29). In the current study, we found that ACE-I but not STI-571 significantly suppressed TGF-ß expression in activated HSCs. Therefore, the different biological activities of STI-571 and ACE-I on activated HSCs could contribute to the combination inhibitory effects of STI-571 and ACE-I on liver fibrogenesis.

Extensive remodeling of ECM has been shown to play a pivotal role during the development of liver fibrosis (8). Alteration of the balance between matrix metalloproteinases (MMPs) and their inhibitors, tissue inhibitor of metalloproteinase (TIMP), has been shown to play a key role in maintaining the balance between ECM deposition and degradation (30). TIMP-1 expression was significantly up-regulated during liver fibrosis development, and TIMP-1 exerted a strong stimulatory effect on liver fibrogenesis (21,31). It has been reported that ACE-I suppressed TIMP-1 expression in activated HSCs (32). Furthermore, both STI-571 and ACE-I significantly inhibited TIMP-1 expression during experimental liver fibrosis development (19,20). The effects of STI-571 and ACE-I also at least in part contributed to the combined suppressive effect on liver fibrogenesis.

Emerging evidence from previous studies has shown that angiogenesis plays a pivotal role in many physiological and pathological processes, such as tumor growth, arthritis, psoriasis, and diabetic retinopathy (33,34). Although previously conducted studies to determine the molecular process associated with fibrosis and angiogenesis were performed independently, some studies have revealed that both biological phenomena emerged synergistically (35). We previously reported that neovascularization was significantly increased during the development of liver fibrosis, and suppression of angiogenic signaling markedly attenuated liver fibrogenesis (24). AT-II is now recognized as a multifunctional protein, and has been reported to exert potent pro-angiogenic activity (36). We also previously reported that ACE-I possessed strong anti-angiogenic activity at clinically comparable low doses (37). PDGF is also known to possess angiogenic activity, and STI-571 exerted marked anti-angiogenic activity both *in vitro* and *in vivo* (38,39). Endothelial cells (ECs) and pericytes are co-dependent, and their interaction is crucial for vascular maturation, remodeling, and maintenance (40). AT-II mainly acts on ECs, whereas PDGF acts on the pericytes. Similar to the different biological actions on activated HSCs, the anti-angiogenic activities of ACE-I and STI-571 from different aspects would contribute to the combined inhibitory effect on liver fibrosis development. Further studies to elucidate the exact mechanisms and respective roles of STI-571 and ACE-I should be conducted in the future.

In summary, we found in our present study that the combination treatment of PDGFR kinase inhibitor, STI-571, and ACE-I inhibitor, perindopril, significantly attenuated liver fibrosis development and suppressed the bioactivities of PDGF and TGF-ß. Noteworthy was that the inhibitory effects of STI-571 and ACE-I against ongoing liver fibrogenesis were achieved at clinically comparable low doses. Both agents are widely used in clinical practice, and the safety of long-term administration of both agents has been proven. The dual inhibition of PDGF and TGF-ß by combination treatment with STI-571 and ACE-I may provide a new strategy in anti-liver fibrosis therapy.

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