Imatinib mesylate (STI-571) attenuates liver fibrosis development in rats

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Yoshiji, Hitoshi, Ryuichi Noguchi, Shigeki Kuriyama, Yasuhide Ikenaka, Junichi Yoshii, Koji Yanase, Tadashi Namisaki, Mitsuteru Kitade, Tsutomu Masaki, and Hiroshi Fukui. Imatinib mesylate (STI-571) attenuates liver fibrosis development in rats. Am J Physiol Gastrointest Liver Physiol 288: G907–G913, 2005. First published December 23, 2004; doi:10.1152/ajpgi.00420.2004.—It is widely recognized that activated hepatic stellate cells (HSC) play a pivotal role in development of liver fibrosis. A platelet-derived growth factor (PDGF) tyrosine kinase inhibitor, on development of experimental liver fibrosis. The rat model of pig serum-induced hepatic fibrosis was used to assess the effect of daily oral administration of STI-571 on the indexes of fibrosis. STI-571 markedly attenuated development of liver fibrosis and hepatic hydroxyproline and serum fibrosis markers. The number of α-smooth muscle actin-positive cells and mRNA expression of α2-(I)-procollagen, tissue inhibitor of metalloproteinases-1, and transforming growth factor-β were also significantly suppressed by STI-571. Our in vitro study showed that STI-571 markedly attenuated PDGF-BB-induced proliferation and migration and α-SMA and α2-(I)-procollagen mRNA of activated HSC in a dose-dependent manner. STI-571 also significantly attenuated PDGF-BB-induced phosphorylation of PDGFR-β, MEK1/2, and Akt in activated HSC. Because STI-571 is widely used in clinical practice, it may provide an effective new strategy for antifibrosis therapy.

LIVER FIBROSIS DEVELOPMENT is a characteristic feature of chronic liver diseases, regardless of their etiology, such as hepatitis virus B and hepatitis C (1, 10, 22). In human liver, fibrogenesis underlies development of hepatocellular carcinoma (HCC) in ≈90% of cases, and HCC is an ominous complication of cirrhosis in 30% of the patients (2). It has been reported that the risk of HCC increases along with the progression of hepatic fibrosis (21). Furthermore, the existence of fibrosis accelerates experimental hepatocarcinogenesis (28). Taken together, an effective therapeutic strategy for suppressing liver fibrosis should improve the overall prognosis of patients with chronic liver diseases.

Despite efforts to develop antifibrotic agents, no drugs have been approved as antifibrotic agents in humans (10). Because liver fibrosis develops with chronic liver disease, therapies should be well tolerated safely over decades, with good targeting to the liver and few adverse effects on the other organs. Several agents, such as interferon (IFN) and the herbal medicine Sho-saiko-to have been used for prevention of hepatic fibrosis. However, it has been reported that the antifibrotic effect of IFN alone can be observed only in virologically responding patients (18, 31) and that IFN therapy is not always convenient for all patients because it is an injection therapy. Also, Sho-saiko-to cannot be used for all patients with liver cirrhosis because of the risk of development of severe lethal interstitial pneumonia. Because there is no approved antifibrotic agent, an alternative approach may be to find a clinically used orally available compound that also shows antifibrotic activity until new drugs become widely available.

It is widely recognized that activated hepatic stellate cells (HSC) play a pivotal role in development of liver fibrosis (9, 24). The increased number of HSC during liver fibrogenesis reflects the activity of several growth factors and their cognate tyrosine kinase receptors (24). In particular, platelet-derived growth factor (PDGF)-BB is the most potent proliferating stimulus for HSC (10, 22). The effects of PDGF are mediated by dimeric transmembrane receptors composed of α and β-subunits with an intrinsic protein tyrosine kinase activity (12). It has been reported that the β-isof orm of the PDGF receptor (PDGFR-β), which binds PDGF-BB, is mainly up-regulated along with HSC activation in CCl4- and bile duct ligation-induced liver injuries (16, 17, 32). Recently, compounds capable of inhibiting PDGFR kinase have been developed. Imatinib mesylate (STI-571), also known as Gleevec is a protein tyrosine kinase inhibitor of the 2-phenylaminojirimidine class that was developed initially for its selective action against the Bcr-Abl fusion protein, which exists in nearly all patients with chronic myeloid leukemia (CML) (6). At concentrations required for Bcr-Abl tyrosine kinase inhibition, STI-571 also inhibits the activities of PDGF and c-Kit tyrosine kinase receptors (4, 5). Recent clinical studies demonstrated the safety and efficacy of STI-571 for treatment of CML and c-Kit-positive gastrointestinal stromal tumors (7, 15). STI-571 has also been shown to inhibit PDGFR signaling in vivo in several animal models of experimental tumors (13, 20, 27). Furthermore, it has been reported that treatment of CML with STI-571 resulted in a marked regression of bone marrow fibrosis in clinical practice (3).

In the present study, we examined the effect of STI-571 on development of liver fibrosis induced by administration of pig serum, especially in conjunction with HSC activation. We also examined the effect of STI-571 on the cell signal transduction pathway in activated HSC.

MATERIALS AND METHODS

Animals. Male Fischer 344 rats, 6 wk of age (Japan SLC, Hamamatsu, Shizuoka, Japan), were housed in stainless steel, mesh...
Fig. 1. Photomicrographs of liver sections from rats treated with pig serum (control, group 1: A), STI-571 (5 mg/kg, group 2: B), and PBS with (group 3: C) and without (group 4: D) STI-571. STI-571 significantly attenuated development of liver fibrosis (B).

No evidence of fibrosis was found in the PBS-treated group (C). Sirius red staining. Original magnification: ×40.

cages under control conditions of temperature (23 ± 3°C) and relative humidity (50 ± 20%), with 10–15 air changes per hour and light illumination for 12 h/day. After 1 wk of acclimatization, the rats were divided into four experimental groups, including one control group. The animals were allowed access to food and tap water ad libitum throughout the acclimatization and experimental periods.

Compounds and animal treatment. STI-571 (generously supplied by Novartis Pharma, Basel, Switzerland) was stored at room temperature and diluted in sterile water as necessary at the time of use. Pig serum was purchased from Cosmo Biologicals (Tokyo, Japan); the same lot of serum was used in all experiments. The rats were randomly divided into four groups (n = 10 in each group). Groups 1 and 2 received 0.5 ml of pig serum intraperitoneally twice weekly for 8 wk. Group 1 was fed the basal diet throughout the experiment and was designed as a positive control group. Group 2 was treated with STI-571 by gavage once a day at 5 mg/kg. Groups 3 and 4 were injected with PBS, instead of pig serum, and were fed the basal diet with and without STI-571, respectively. At the end of all experiments, the rats were killed under ether anesthesia. All animal procedures were performed according to approved protocols and in accordance with the recommendations for the proper care and use of laboratory animals and were approved by the animal care and use committee of Nara Medical University.

Histological and immunohistochemical examinations and hepatic hydroxyproline content. In all experimental groups, 5-μm-thick sections of formalin-fixed and paraffin-embedded livers were processed routinely for Sirius red staining for determination of liver fibrosis development. Immunohistochemical staining of α-smooth muscle actin (α-SMA) was performed as previously described (36, 37) using paraffin-embedded serial sections with a primary anti-α-SMA antibody (DAKO, Kyoto, Japan). Computer-assisted quantitative analyses of fibrosis development and the immunopositive cell area were carried out with an image-analyzing system (Fuji-BAS 2000, Fuji, Tokyo, Japan) in six ocular fields (×40 magnification) per specimen as described previously (35, 38). We did not count the α-SMA-positive vessels in the portal area, which were assumed to be hepatic arteries. We included only the α-SMA-positive cells in the sinusoidal lining for image analysis. Hepatic hydroxyproline content (μg/g wet liver) was determined as previously described with 200 μg of frozen samples (n = 10) (33, 35).

Evaluation of RNA expressions of α2-(I)-procollagen, tissue inhibitor of metalloproteinases-1, and transforming growth factor-β by real-time PCR. The mRNA expressions of α2-(I)-procollagen, tissue inhibitor of metalloproteinases-1 (TIMP-1), and transforming growth factor-β (TGF-β) were evaluated by real-time PCR as described previously (34, 36). The mRNA was extracted from the whole liver of the animals in each experimental group (n = 10). For cDNA synthesis, Taqman reverse transcription reagents were used as described in the manufacturer’s manual of the ABI Prism 7700 Sequence Detection System (PE Applied Biosystems, Foster City, CA), which was used for real-time PCR amplification following the Taqman Universal PCR Master Mix Protocol (PE Applied Biosystems). The primers for
the respective genes have been described elsewhere (36, 37). Relative 
quantification of gene expression was performed as described in the 
manual, with GAPDH used as an internal control. The threshold cycle 
and the standard curve method were used to calculate the relative 
amount of the target RNA as described by PE Applied Biosystems. 
The sequence was as follows: hold at 50°C for 2 min, 60°C for 30 
min, and 94°C for 5 min followed by 45 cycle repeats 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 ampli-
fied DNA.

In vitro assays of activated HSC. HSC were isolated from the liver 
of the Fischer 344 rats as described previously (35), with a minor 
modification. The cells were plated at a density of $5 \times 10^5$ cells/ml on uncoated 60-mm plastic dishes. After 5 days in culture, HSC became myofibroblast-like, with reduced lipid vesicles and increased immunoreactive α-SMA; 7 days after plating, all the cells were well spread and became α-SMA-positive as described previously (30).

The effects of STI-571 (0.1–10 μM) on PDGF-BB (10 ng/ml)-induced in vitro proliferation and migration of activated HSC were examined as described previously (16, 35). Cell proliferation was quantified via conversion of tetrazolium 3-(4,5-diethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by cells cultured in 96-well plates ($n = 6$ per group). Cell migration activity was assessed by a Transwell chemofilter assay as described previously (16). Cells adhering to the undersurface of the membrane were counted in 10 random high-power fields ($\times 400$), and the mean count was determined from duplicate experiments. We also examined the effect of STI-571 on mRNA expression of PDGF-BB-induced α-SMA and α2-(I)-procollagen in activated HSC ($n = 6$ per group). Gene expression of activated HSC was also measured by real-time PCR, as described for measurement in the whole liver.

Fig. 3. Immunohistochemical analysis of α-smooth muscle actin (α-SMA). α-SMA-immunopositive cells were significantly suppressed in liver of an STI-571-treated rat (B) compared with control. Strong immunopositive staining was observed in the fibrotic septa (a). No α-SMA-positive cells could be found in the PBS-treated group (C and D). Original magnification: ×40 (A–D) and ×200 (a).

Fig. 4. Effect of STI-571 on α-SMA-positive cells and mRNA expression of α2-(I)-procollagen, tissue inhibitor of metalloproteinases-1 (TIMP-1), and transforming growth factor-β (TGF-β) in whole liver. A: number of α-SMA-positive cells was significantly reduced by STI-571 treatment compared with control ($P < 0.01$). STI-571 treatment also markedly suppressed mRNA expression of α2-(I)-procollagen (B), TIMP-1 (C), and TGF-β (D) in the liver compared with control ($P < 0.01$). Inhibitory effects of STI-571 on mRNA expression of α-SMA, α2-(I)-procollagen, TIMP-1, and TGF-β resulted in almost parallel reductions. Values are means ± SE ($n = 10$). *Statistically significant difference between groups ($P < 0.01$).
**Phosphorylation of PDGFR, MEK1/2, and Akt.** To examine the effect of STI-571 on the phosphorylation of PDGFR-β by PDGF-BB (10 ng/ml) in activated HSC, immunoprecipitation and Western blotting were performed as described previously. PDGF-BB-induced phosphorylation of MEK1/2 and Akt was measured by ELISA (FACE, Active Motif, Carlsbad, CA). It has been reported that the PDGF-BB-induced maximum phosphorylation of PDGFR, MEK1/2, and Akt was achieved at 1, 5, and 10 min after treatment, respectively, in activated HSC (29). The cell lysate for each experiment was prepared at the respective time after PDGF-BB stimulation with or without 0.1–10 μM STI-571. The liver lysates were immunoprecipitated with antiphosphotyrosine and then subjected to SDS-PAGE. Antityrosine (4G10) and anti-PDGFR-β were purchased from Upstate Biotechnology (Lake Placid, NY). Before Western blotting, we stained each membrane with Ponceau solution (Sigma, St. Louis, MO) to confirm that the same amounts of protein were immunoprecipitated (data not shown). The blots were developed using an amplified alkaline phosphatase immunoblot assay kit (Bio-Rad, Tokyo, Japan). Total and phosphorylated MEK1/2 and Akt were measured by ELISA according to the manufacturer’s instructions. Phosphorylation of MEK1/2 and Akt was achieved at 1, 5, and 10 min after treatment, respectively, in activated HSC (29). The cell lysate for each experiment was prepared at the respective time after PDGF-BB stimulation with or without 0.1–10 μM STI-571. The liver lysates were immunoprecipitated with antiphosphotyrosine and then subjected to SDS-PAGE. Antityrosine (4G10) and anti-PDGFR-β were purchased from Upstate Biotechnology (Lake Placid, NY). Before Western blotting, we stained each membrane with Ponceau solution (Sigma, St. Louis, MO) to confirm that the same amounts of protein were immunoprecipitated (data not shown). The blots were developed using an amplified alkaline phosphatase immunoblot assay kit (Bio-Rad, Tokyo, Japan).

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

**RESULTS**

**Histological findings and fibrosis markers.** Sirius red staining showed that 8 wk of treatment with pig serum (group 4) resulted in development of marked liver fibrosis (Fig. 1A). In contrast, treatment with STI-571 (group 2) significantly attenuated fibrosis development (Fig. 1B) compared with group 1. No evidence of fibrosis development was found in the PBS-treated group with (group 3) or without (group 4) STI-571 (Fig. 1C and D, respectively). Densitometric analysis showed that the fibrosis areas were markedly suppressed in the STI-571-treated rats (P < 0.01; Fig. 2A). Liver hydroxyproline content was also significantly decreased in the STI-571-treated rats (Fig. 2B). There were no significant differences among the groups in serum levels of alanine transaminase and total bilirubin, body weight, liver weight, and health status (data not shown). Moreover, there were no decreases in the number of leukocytes and platelets in the STI-571-treated animals, indicating that STI-571 did not induce bone marrow suppression at the dose administered.

**Effect of STI-571 on activated HSC.** The number of α-SMA-immunopositive activated HSC significantly increased in the pig serum-injected group (group 1) by 10.2 ± 0.3% in comparison with the PBS group (group 2; Fig. 3A). High-power magnification revealed strong immunopositive-staining cells in the fibrotic septa (Fig. 3Aa). Treatment with STI-571 drastically reduced these positive cells in the liver (group 2; Fig. 3B). No marked increase in α-SMA-positive cells could be found in the PBS-treated group (groups 3 and 4; Fig. 3, C and D, respectively). The computer-assisted quantitative analysis showed significantly fewer α-SMA-positive cells in the STI-571 treated-group than in the control group (P < 0.01; Fig. 4A). We also performed real-time PCR analysis to elucidate the effect of STI-571 on mRNA expression of α2-(I)-procollagen, TIMP-1, and TGF-β in the whole liver in each experimental group. STI-571 treatment also markedly suppressed mRNA expression of α2-(I)-procollagen, TIMP-1, and TGF-β in the
liver compared with the pig-serum treated control group ($P < 0.01$; Fig. 4B).

**Effect of STI-571 on cultured HSC.** To elucidate the possible mechanisms, we examined the effect of STI-571 on cultured activated HSC from several aspects. First, we examined the effect of STI-571 on mRNA expression of $\alpha$-SMA and $\alpha_\text{2}(-I)$-procollagen by real-time PCR. STI-571 suppressed PDGF-BB-induced $\alpha$-SMA and $\alpha_\text{2}(-I)$-procollagen mRNA expression of activated HSC in a dose-dependent manner (Fig. 5, A and B, respectively). STI-571 also attenuated PDGF-BB-induced proliferation of activated HSC in a dose-dependent manner (Fig. 6A). In addition to the MTT assay, we performed a $^3$H incorporation study to evaluate in vitro proliferation of HSC and obtained similar results (data not shown). We also examined the effect of STI-571 on migration through the filter in response to the PDGF-BB gradient. Similar to its effect on proliferation, STI-571 significantly suppressed the PDGF-BB-induced cell migration in a dose-dependent manner (Fig. 6B).

**Phosphorylation of PDGFR$\beta$, MEK1/2, and Akt in activated HSC.** We next examined the effect of STI-571 on the signaling pathway in activated HSC. STI-571 attenuated the PDGF-BB-induced phosphorylation of PDGFR$\beta$ in a dose-dependent manner, whereas the protein level was not altered (Fig. 7). Phosphorylation of MEK1/2 and Akt was also elucidated. MEK1/2 phosphorylation was markedly upregulated by PDGF-BB, and STI-571 treatment suppressed the phosphorylation in a dose-dependent manner. To confirm the specificity of this protein kinase activity, we used a specific MEK1/2 inhibitor, U-0126. As illustrated in Fig. 8, U-0126 inhibited the PDGF-BB-induced MEK1/2 phosphorylation, suggesting that the effect of STI-571 was specific for MEK1/2. We also examined Akt phosphorylation in activated HSC. STI-571 significantly inhibited PDGF-BB-induced Akt phosphorylation in a dose-dependent manner (Fig. 9). Similar to the effect of U-0126 on MEK1/2, LY-294002, a specific inhibitor of Akt, inhibited the PDGF-BB-induced Akt phosphorylation. A parallel reduction was found in phosphorylation of PDGFR$\beta$, MEK1/2, and Akt by treatment with STI-571.

**DISCUSSION**

Over many years, there have been numerous attempts to develop medications that inhibit the progress of liver fibrosis. It is recognized that HSC play a central role on the basis of their ability to undergo activation during development of liver fibrosis and that the PDGF-PDGFR interaction plays a pivotal role in activated HSC bioactivities (10, 32). Accordingly, neutralization of PDGF activity, by ligand antagonists or receptor blockade, would be a potentially useful approach for antifibrotic therapy.

STI-571 was initially shown to inhibit Bcr-Abl-positive CML cells through selective inhibition of the Abl tyrosine kinase.
kinase (8). STI-571 is also a highly selective inhibitor of the tyrosine kinase activities of c-Kit and PDGFR (4, 5). In the present study, we found that STI-571 inhibited rat liver fibrosis development along with suppression of α-SMA-positive cells. α-SMA is one of the well-known makers of activated HSC (22). We observed that expression of TIMP-1 and TGF-β was also suppressed by STI-571. TIMP-1 has been shown to be increased in liver fibrosis development in murine experimental models and human samples, and it plays an important role in liver fibrogenesis by modulating extracellular matrix remodeling (14). Furthermore, TIMP-1 has been shown to exert an antiapoptotic activity in activated HSC (37). TGF-β is also the main fibrogenic cytokine with a well-established role in patients with cirrhosis and in animal-induced liver fibrosis (9). It has been reported that the main source of these molecules during liver fibrosis development was activated HSC. We found that the inhibitory effect of STI-571 on mRNA expression of α-SMA, α2-(I)-procollagen, TIMP-1, and TGF-β and fibrosis area exerted almost parallel reductions, indicating that suppression of activated HSC contributed to the antifibrotic effect of STI-571. We also confirmed the direct inhibitory effect of STI-571 on cultured activated HSC. STI-571 significantly suppressed PDGF-BB-induced mRNA expression of α-SMA and α2-(I)-procollagen mRNA. STI-571 also markedly inhibited PDGF-BB-induced proliferation and migration of activated HSC.

STI-571 displays no specificity for the α- or β-subunit of PDGFR (27). It has been reported that PDGFR-β is mainly upregulated after CCl4-induced liver injury (26, 32). Similar to the CCl4 model, we observed that PDGFR-β expression was mainly upregulated during development of liver fibrosis induced by pig serum (data not shown). It has been reported that PDGFR-β is dimerized and autophosphorylated on the tyrosine residues by PDGF-BB (25). This phosphorylation is followed by sequential activation of Ras, Raf-1, and MEK1/2. Another molecule that is required and recruited by PDGFR by sequential activation of Ras, Raf-1, and MEK1/2, and Akt, suggesting that the antifibrotic and migration associated with inhibition of phosphorylation of STI-571 significantly suppressed activated HSC proliferation and migration associated with inhibition of phosphorylation of PDGFR-β, MEK1/2, and Akt, suggesting that the antifibrotic effect of STI-571 is mainly mediated through suppression of the PDGFR-β-signaling cascade in activated HSC. In addition to PDGFR, STI-571 also inhibits the tyrosine kinase activity of c-Kit (27). However, it has been reported that expression of c-Kit was not detected in HSC but was localized only on the bile duct epithelial cells, making it unlikely that HSC proliferation is mediated by c-Kit (23).

It has been reported that daily intraperitoneal injection of STI-571 caused a marked decrease of proliferation in isolated HSC in an experimental model of rats with 48 h of acute bile duct ligation liver injury (17). In the present study, we performed an 8-wk chronic experiment with a different fibrosis model. Furthermore, STI-571 was administered by intraperitoneal injection in the previous study; in our present study, it was administered orally, which mimics clinical practice. Thus we believe that our study will be more relevant for future clinical application. The pig serum model induces liver fibrosis without severe inflammation (36). Because we would like to mainly focus on the effect of STI-571 on activated HSC without any effect by inflammation-induced cytokines, we employed this model in the present study. We also found a similar antifibrotic effect of STI-571 in the CCl4-induced liver fibrosis experimental model, suggesting that the antifibrotic effect of STI-571 is not a phenomenon specific to the experimental model (Y. Ikenaka, personal communication).

In conclusion, we found that treatment with the PDGFR tyrosine kinase inhibitor STI-571 markedly attenuated development of liver fibrosis via suppression of activated HSC. Because STI-571 is widely used in clinical practice, this drug may provide an effective new strategy for antifibrosis therapy.

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