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Research Paper

Smad3 Specific Inhibitor, Naringenin, Decreases the Expression of Extracellular Matrix Induced by TGF-β1 in Cultured Rat Hepatic Stellate Cells

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Purpose. During the process of liver fibrogenesis, transforming growth factor-β (TGF-β) plays an essential role in modulating extracellular matrix (ECM) gene expression, and a growing body of evidence suggests that this is a Smad3-dependent process in the activated hepatic stellate cells (HSCs). Naringenin showed a significantly protective effect on experimental rat liver fibrosis, in our efforts to elucidate its antifibrosis molecular mechanisms and to find a novel target based on Smad3 signaling for challenging fibrosis diseases.

Methods. In this study, reverse transcription-polymerase chain reaction and Western blot assays were used to investigate the inhibitory effect of naringenin on ECM formation induced by TGF-β1 in the HSC-T6 cells.

Results. Naringenin reduced not only the accumulation of ECM, including collagen Iα1 (Col Iα1), fibronectin (FN), and plasminogen activator inhibitor-1 (PAI-1), but also the production of Smad3 induced by TGF-β1 in both mRNA and protein levels in a dose-dependent manner. Moreover, naringenin selectively inhibited the transcription of Smad3, but not other Smads involved in TGF-β1 signaling pathways.

Conclusion. Our data demonstrate that naringenin can exert antifibrogenic effects by directly or indirectly down-regulating Smad3 protein expression and phosphorylation through TGF-β signaling.

KEY WORDS: extracellular matrix; liver fibrosis; naringenin; Smad3; transforming growth factor-1.

INTRODUCTION

Liver fibrosis is the common result of multiple forms of chronic liver disease, most commonly viral hepatitis, excess alcohol consumption, or chronic biliary obstruction. It is a wound-healing response gone awry (1), with the formation of a "hepatic scar". Histologically, fibrosis is marked by disruption of the hepatic architecture and deposition of excess abnormal extracellular matrix (ECM), with increased fibrillar collagens, proteoglycans, and fibronectin (2,3), in the result of both increased production and decreased degradation of matrix components (4).

The cells primarily responsible for extracellular matrix production in the fibrotic liver are hepatic stellate cells (HSCs). These perisinusoidal cells, formerly called lipocytes or Itocells, normally play an important role in maintaining liver homeostasis through growth factor secretion, retinoid storage and release, and production of extracellular matrix components essential for both the architectural integrity of the space of Disse and for phenotypic stability of the surrounding cells (5). In chronic liver disease, HSCs undergo transdifferentiation to fibrogenic myofibroblasts that produce much of the excess abnormal matrix of the hepatic scar.

Many mediators of HSC transdifferentiation *in vitro* have been identified, including growth factors, lymphocytes, apoptotic fragments of hepatocytes, oxidative stress, and matrix components (6,7). Growth factors that are particularly important include transforming growth factor β (TGF-β), which is essential for HSC transdifferentiation *in vivo*. TGF-β is unquestionably one of the most important mediators of liver fibrosis, although its specific effects on HSC, whether direct or indirect, have been frustratingly difficult to identify (8). There is extensive evidence demonstrating that TGF-β is both necessary and sufficient for the development of hepatic fibrosis in experimental animals. Mice null for TGF-β1 or its downstream signaling mediator Smads develop less fibrosis in response to carbon tetrachloride treatment than wild-type animals, and there are now multiple reports of anti-TGF-β agents, including soluble and dominant negative TGF-β receptors and inhibitory signaling molecules, that prevent or lead to resolution of fibrosis in experimental animals (9–15). TGF-β plays an essential role in modulating ECM gene expression, and a growing body of evidence suggests that this is a Smad3-dependent process. Using differential hybridization of a cDNA expression array containing 265 known

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ABBREVIATIONS: Col Iα1, collagen Iα1; DMEM, Dulbecco's Modified Eagle Medium; ECM, extracellular matrix; FCS, fetal calf serum; FN, fibronectin; HSCs, hepatic stellate cells; LC_{50}, 50% lethal concentration; PAI-1, plasminogen activator inhibitor 1; TBST, Tris-buffered saline/Tween 20; TGF-β1, transforming growth factor β1.

ECM-related genes, a number of collagen gene promoters were identified in human dermal fibroblasts that were induced by TGF-β1 and dependent upon Smad3 (16). In all cases, activation of the promoter by TGF-β was blocked by both dominant-negative Smad3 and inhibitory Smad7 expression vectors and promoter transactivation by TGF-β did not occur in Smad3 null mice.

Because of the pleiotropic biological actions of TGF-β1 mediated by multiple signaling pathways, therapies targeting TGF-β expression/activation or binding of TGF-β to its receptors may potentially induce a number of unwanted side effects. Agents that target specific signaling pathways downstream of the TGF-β receptors are more likely to have the desired effects while avoiding complications. Because Smad3 plays such a critical role in mediating the pathobiology of fibrotic disease, as demonstrated by the previously cited studies, inhibition of Smad3 signaling could be a prime target for intervention in fibrotic conditions.

Naringenin (4′,5,7-trihydroxyflavanone), a natural predominant flavanone, has a wide range of pharmacology properties, such as anticancer, antimutagenic, anti-inflammatory, and antiatherogenic activity (17–20). Naringenin was recently shown to reduce the accumulation of collagen fibers by reduced HSCs activity in dimethylnitrosamine-induced liver injury rats and to exhibit antifibrogenic effects (21). However, the information is not available on the molecular mechanisms for its antifibrogenic activity. One may assume that naringenin exerts its antifibrogenic effects through inhibition of Smad3 signaling.

In the present study, we attempt to elucidate the antifibrosis molecular mechanisms of naringenin and, furthermore, to find a new target based on Smad3 signaling for challenging fibrosis diseases. We demonstrate for the first time that naringenin selectively inhibits the expression of Smad3 mRNA and protein, but not Smad2, Smad4, and Smad7, which in turn significantly attenuates the TGF-β1-induced deposition of PAI-1, Col Iα1, and FN in cultured rat HSCs. These results suggest that naringenin exerts its antifibrogenic effects by directly or indirectly down-regulated Smad3 protein expression and phosphorylation through TGF-β1 signaling.

MATERIALS AND METHODS

Materials

Naringenin was purchased from Shanxi Huike Botanical Development Co. (China). SB-431542, synthesized and kindly provided by institute of Pharmacology Toxicology of the Academy of Military Medical Sciences, was stored as a stock solution in DMSO, which was used after diluting with medium for each assay. Trizol reagent and RevertAid™ M-MLV Reverse Transcriptase were obtained from Fermentas Life Science (Lithuan); *Taq* polymerase, dNTPs, and DNA marker were from TaKaRa Biotechnology Co. (Dalian, China). Primers for rat PAI-1, Smad3, Smad2, Smad7, Smad4, type I, type II, and Col Iα1, FN, and β-actin (as internal control) were designed by our group in accordance with gene sequence in GeneBank. Cytokine recombinant human TGF-β1 was purchased from R&D system (Minneapolis, MN, USA). Precision protein standards and protein molecular weight markers were obtained from Bio-Rad (Hercules, CA, USA). All antibodies, including primary and second antibodies, were provided by Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA); Phospho-Smad3 antibody (Ser433/435) was purchased from Cell Signaling Technology Inc. (USA). All other reagents were analytically pure.

Cell Culture

HSC-T6 cells, derived from immortalized and activated rat HSCs transfected by SV40, were kindly donated by Professor Friedman from Mount Sinai Medical Center and were maintained at 37°C in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal calf serum (FCS), 100 units/mL penicillin, and 100 μg/mL streptomycin under a 5% CO_2 atmosphere.

Cell Viability Assays

HSC-T6 cells were seeded overnight at 10,000 cells/well in 96-well plates in DMEM media, and the subconfluent cells in culture (75%) were washed twice with phosphate-buffered saline and serum-starved for 24 h with serum-free medium. To determine the optimal concentration of naringenin for use in these experiments, dose-dependent cytotoxicity was initially performed with different concentrations of naringenin added to culture for 48 h. Cell viability was determined by incubating each well with 10 μl of WST-1 for 4 h at 37°C. Metabolically active cells cleave WST-1 to water-soluble formazan, which is directly quantitated with an enzyme-linked immunosorbent assay plate reader (Bio-Rad 3550). Each experiment was performed at least twice, and treatment for cell line was done in triplicate.

Preparation of RNA and Protein Extracts

HSC-T6 cells were plated at a confluence of approximately 75% in the wells of 6-well dish and allowed to attach overnight. Cells were treated with 10 ng/mL TGF-β1 for 12 h before being treated with a variety of concentrations of naringenin or SB-431542 for 12 h to determine RNA levels, and for 24 h to analyze TGF-β-regulated protein expression. Treated cells were harvested, washed, and lysed in ice-cold buffer [250 mM Tris–HCl, pH 7.4, containing a mixture of protease inhibitors (100 μg/mL phenylmethylsulfonyl fluoride, 1 μg/mL E-64, 0.5 μg/mL leupeptin, and 1.7 μg/mL peptatin A)]. The debris was removed by centrifugation at 13,000 × *g* for 20 min at 4°C. Total protein amount in the extracts was measured using the Bio-Rad Bradford reagent. Total RNA was isolated from HSC-T6 cells as described by Chomczynski and Sacchi.

Reverse Transcription-Polymerase Chain Reaction Assay

After treatment, RNA was harvested with Trizol (Invitrogen, Carlsbad, CA, USA). For each reaction, 1 μg of total RNA was reverse-transcribed to cDNA using RT kit (MBI) for reverse transcription-polymerase chain reaction (RT-PCR) (Ambion) as recommended by the manufacturer. The resulting cDNA was subsequently subjected to 40 cycles of PCR. PCR products were quantitated to confirm that they were in linear range of amplification. PCR products were quantitated by Quantum RNA 18S internal standards (Ambio).

The primers used in this study include: rat TGF-β type I receptor (Geneback L26110), forward, 5′-GTCGCTGCCTG-CTTCTCATC-3′, reverse, 5′-CGGGGAATTAGGTC-GATTTC-3′ (product size 202 bp); rat TGF-β type II receptor (Geneback S67770), forward, 5′-CTGCACATCGTCCTG-TGGAC-3′, reverse, 5′-TTCTTCCTCCACACGGCCA C-3′ (product size 239 bp); rat Smad2 (Geneback AF056001), forward, 5′-ACAACAGGCCTTTACAGCTTC-3′, reverse, 5′-CTCTGTGGCTCAATTCCTGC-3′ (product size 350 bp); rat Smad3 (Geneback NM013095), forward, 5′-CTGGCTA-CCTGAGTGAAGATG-3′, reverse, 5′-TGTGAAGCGT-GGAATGTCTC-3′ (product size 211 bp); rat Smad4 (Geneback AB010954), forward, 5′-CCTCATGTGATC-TATGCCCG-3′, reverse, 5′-GTGGAAGCCACAGGAA-TGTT-3′ (product size 395 bp); rat Smad7 (Geneback AF042499), forward, 5′-CCCTGGGGGGCTTTCAGATT-3′, reverse, 5′-CGGACTTGATGAAGATGGGG-3′ (product size 308 bp); rat FN (Geneback X15906), forward, 5′-TCCAAT-GGTGCCTTGTGCCA-3′, reverse, 5′- GTTCCCAACA-CACGTGCAACC-3′ (product size 253 bp); rat PAI-1 (Geneback M24067), forward, 5′-GGCTTCATGCCC-CACTTCTTC-3′, reverse, 5′-TACTCGTGCCCATCCG-GAGT-3′ (product size 341 bp); rat Col Iα1, forward, 5′-ACTTTGCTTCCCAGATGTCC-3′, reverse, 5′-CCATC-CAAACCACTGAAACC-3′ (product size 349 bp); rat β-actin (Geneback BC063166), forward, 5′-TGGCGCTTTTGACT-CAGGAT-3′, reverse, 5′-AGCCCTGGCTGCCTCAAC-3′ (product size 452 bp). The conditions for RT-PCR were as follows: cDNA synthesis and predenaturation were at 42°C for 60 min, followed by 94°C for 10 min. PCR amplification sequence was performed for 40 cycles at 94°C for 45 s, 57°C for 45 s (except at 55°C for Smad7), and 72°C for 20 s. After amplification, each sample was applied to 1.2% agarose/ethidium bromide gel. The resolved PCR products were photographed under UV illumination.

Western Blot

For Western blot, treated cells were harvested by the addition of ice-cold lysis buffer as described above. Centrifuged lysates (30 μg) from each sample were analyzed by SDS-polyacrylamide gel electrophoresis and transferred to a PVDF membrane by semidry transfer. The membranes were blocked for 1 h at room temperature in Tris-buffered saline containing 0.1% Tween 20 and 3% BSA. Blots were probed overnight at 4°C with the following primary antibodies: anti-Smads, anti-phospho-Smad3, anti-PAI-1, anti-NF, anti-Col Iα1, and anti-β-actin antibodies. This was followed by incubation with the appropriate horseradish peroxidase-conjugated secondary antibody at a dilution of 1:1,000 for 1 h. Detection was achieved by enhanced chemiluminescence (Amersham Pharmacia Biotech) and exposed to film. Filters were quantitated by scanning densitometry using a Bio-Rad model 620Video Densitometer with a 1-D Analyst software package for Macintosh.

RESULTS

Effect of Naringenin on HSC-T6 Cell Viability and Growth

To assess the potential toxicity of naringenin to HSC-T6 cells, the treated cells were examined by MTT assay. Exposure of the HSC-T6 cells to naringenin resulted in an LC_{50} (50% lethal concentration) value of 250 μM. The viability cure (Fig. 1) showed that HSC-T6 cells could be treated with naringenin at concentrations up to 50 μM for up to 48 h without impact on cell viability. Naringenin at concentrations ranging from 10 to 50 μM slightly enhanced cell proliferation. However, at 100 μM, naringenin seem toxic to cells.

Effect of Naringenin on TGF-β1-Induced Expression of PAI-1 mRNA and Protein in HSC-T6 Cells

Smad3 phosphorylation correlates closely with TGF-β1-induced PAI-1 expression (22). In the present study, we asked whether naringenin can inhibit TGF-β1-mediated biological effects in HSC-T6 cells. For this purpose, the cells were pretreated with 10 ng/mL TGF-β1 and then exposed to naringenin. TGF-β1 responsiveness in the cells was initially analyzed by monitoring the up-regulation of PAI-1 mRNA expression. Treatment of HSC-T6 cells with 10 ng/mL TGF-β1 for 12 h led to a significant increase in PAI-1 expression. However, it was significantly reduced in HSC-T6 cells treated with naringenin in a dose-dependent manner (Fig. 2a).

To study whether the above-mentioned changes in PAI-1 gene expression were also associated with change in levels of PAI-1 protein, we estimated, by Western blot, the ratio of the optical density of PAI-1 protein, based on the β-actin protein band under control conditions and after naringenin treatment with pretreatment of TGF-β1 in HSC-T6 cells (Fig. 2a and b). The results demonstrated that TGF-β1 treatment had a significant effect on the expression level of the 60-kDa band corresponding to PAI-1 in TGF-β1-stimulated cells. We observed that naringenin significantly suppressed the up-regulation of PAI-1 in TGF-β1-stimulated cells. These data demonstrate that naringenin inhibits TGF-β1-dependent up-regulation of PAI-1 expression at both mRNA and protein levels.

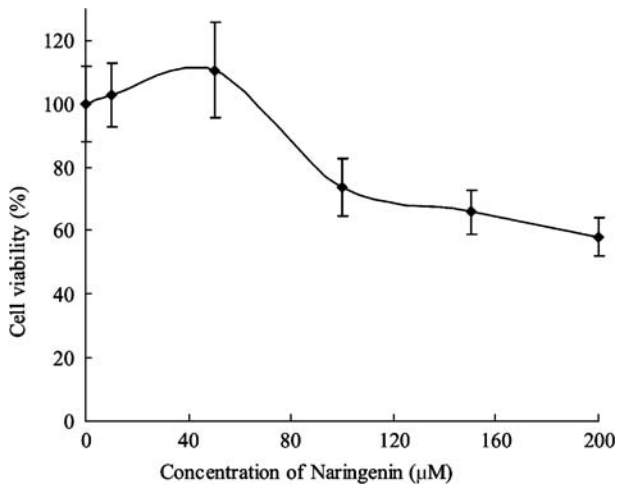

Fig. 1. Growth inhibition of naringenin to HSC-T6 cells. Cells treated with a variety of naringenin concentrations for 48 h; viability of the cells was examined by MTT. Each concentration was replicated three times. Data were expressed as mean values with the corresponding standard deviations. LC_{50} was performed using the 82798-LC_{50} program.

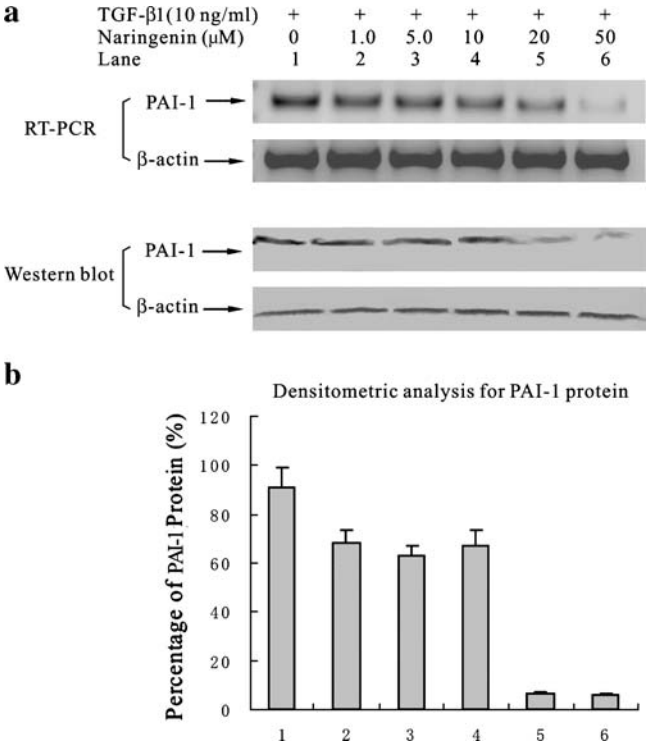

Fig. 2. Naringenin inhibits TGF-β1 induced PAI-1 gene expression in HSC-T6 cells. Cells were stimulated with 10 ng/mL TGF-β1 for 12 h before adding different naringenin concentrations ranging from 0 to 50 μM for 12 h incubation for RT-PCR analysis or 24 h incubation for Western blot. (a) The level of PAI-1 mRNA was determined using RT-PCR (upper panel) and the level of PAI-1 protein was measured by Western blot (lower panel). (b) Values of densitometric scan are means ± SD of three experiments. Results presented were obtained in triplicate and are representative of three independent experiments.

Naringenin Reduces TGF-β1-Induced Expression of Profibrotic Genes

To test whether naringenin regulates extracellular matrix production in HSC-T6 cells in the presence of TGF-β1, the stimulatory effect of TGF-β1 on profibrotic genes in the cells was first analyzed by determining the up-regulation of Col Iα1and FN mRNA expression. Treatment of HSC-T6 cells with 10 ng/mL TGF-β1 for 12 h led to a significant increase in Col Iα1and FN expression. TGF-β1 induced the expression of Col Iα1, and FN mRNA was significantly reduced in HSC-T6 cells treated with naringenin in a dose-dependent manner (Fig. 3a). To study whether changes in Col Iα1 and FN gene expression were also associated with changes in levels of Col Iα1 and FN proteins, we used Western blot to estimate the ratio of the optical density of Col Iα1 and FN proteins, based on the β-actin protein band under control conditions and after naringenin treatment with pretreatment of TGF-β1 in HSC-T6 cells (Fig. 3a and b). Both Col Iα1 and FN proteins decreased by almost 10-fold. Taken together, our results thus far demonstrate that naringenin is a potent inhibitor of TGF-β1 signaling as judged by its effect on TGF-β1-regulated synthetic events.

Naringenin Inhibits TGF-β1-Induced Expression of Smad3 and Smad3 Phosphorylation

Smad3 is the essential mediator of TGF-β signaling, because it directly activates genes encoding transcriptional regulators and signal transducers through Smad3/4 DNA-binding motif characteristic of immediate early target genes of TGF-β. In the present study, we examined via RT-PCR and Western blot whether naringenin can inhibit the TGF-β1-mediated expression of Smad3 and phosphorylation of Smad3 in HSC-T6 cells. As shown in Fig. 4a and b, Smad3-mRNA levels were markedly down-regulated in HSC-T6 cells treated with naringenin in a dose-dependent manner for 12 h, which in turn, resulted in a significant decrease in Smad3 protein levels in a dose-dependent manner for 24 h. In the TGF-β1 signaling pathway, phosphorylation of the cytoplasmic signaling molecules Smad3 by TβRII is the first step in signal transduction.

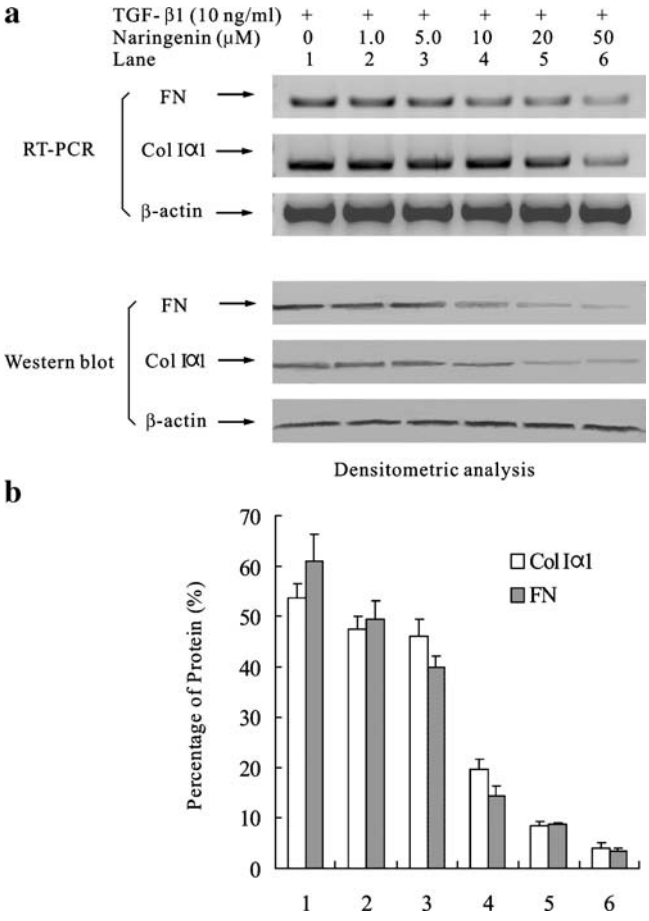

Fig. 3. Naringenin inhibits TGF-β1-dependent Col Iα1 and FN genes expression in HSC-T6 cells. Cells were treated with 10 ng/mL TGF-β1 for 12 h before adding different naringenin concentrations ranging from 0 to 50 μM for 12 h incubation for RT-PCR analysis or 24 h incubation for Western blot. (a) Levels of Col Iα1 and FN mRNA were determined using RT-PCR (upper panel) and levels of Col Iα1 and FN protein were measured by Western blot (lower panel). (b) Values of densitometric scan are means ± SD of three experiments. Experiments were performed in triplicate and data presented are representative of three independent experiments.

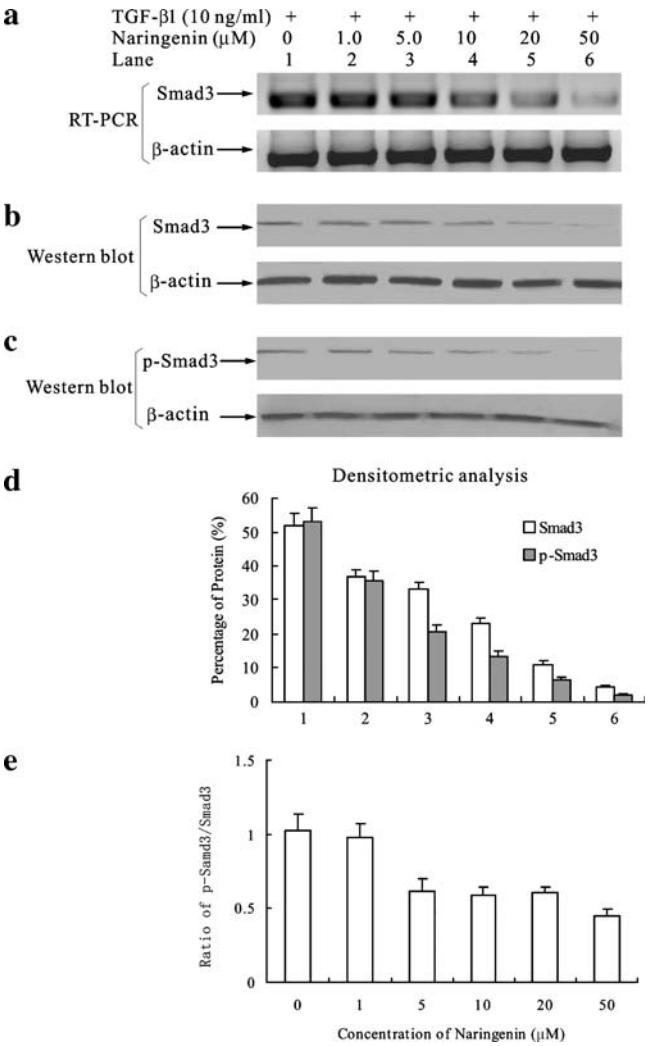

Fig. 4. The effect of naringenin on the expression of Smad3 and the phosphorylation of Smad3. Cells were treated with 10 ng/mL TGF-β1 for 12 h before adding different naringenin concentrations ranging from 0 to 50 μM for 12 h incubation for RT-PCR analysis or 24 h incubation for Western blot. (a) The level of Smad3 mRNA was determined using RT-PCR. (b) The level of Smad3 protein was measured by Western blot. (c) The level of phosphor-Smad3 protein was measured by Western blot. (d) Values of densitometric scan are means ± SD of three experiments. (e) Ratio of phosphor-Smad3 to total Smad3. Experiments were performed in triplicate and data presented are representative of three independent experiments.

We further examined whether naringenin can inhibit TGF-β1-induced phosphorylation of Smad3 in HSC-T6 cells. Naringenin caused reduction in TGF-β1-induced Smad3 phosphorylation, and this inhibiting effect was parallel to the dosages used in treatment of cells (Fig. 4c and d).

To test whether naringenin can also inhibit Smad3 at both mRNA and protein levels at basal state in HSC-T6 cells, the cells were treated with various concentrations of naringenin in the absence of TGF-β1. Naringenin had a relatively slight influence on Smad3 at both mRNA and protein levels even when concentration was increased up to 100 μM (Fig 5a). TGF-β1 induced a significant increase in the level of phospho-Smad3, but exerted no change in Smad3 expression in HSC-T6 cells (Fig 5b). On the other hand, SB-431542, a recently developed and potentially specific inhibitor of type I receptor of TGF-β1, acts as a competitive ATP binding site kinase inhibitor and was shown to inhibit the *in vitro* phosphorylation of Smad2/Smad3 (23). SB-431542 failed to affect either the expression of Smad3 mRNA (Fig. 6a) or the level of Smad3 protein (Fig. 6b) induced by TGF-β1 even at a high concentration (10 μM). However, it effectively reduced the phosphorylation of Smad3 (Fig. 6c and d). The data suggest that naringenin has a different mechanism from SB-431542 for inhibiting TGF-β1-mediated biological effects through TGF-β signaling.

Naringenin is a Specific Smad3 Inhibitor

In the above experiment, we assessed the effect of naringenin on the expression of Smad3 and Smad3 phosphorylation. Naringenin not only inhibits the phosphorylation of Smad3, but also reduces the expression of Smad3 mRNA and protein. Is naringenin then a Smad3-specific inhibitor? To address this question, we performed RT-PCR to examine the gene expression involved in the regulation of TGF-β signaling, including TβRI, TβRII, Smad2, Smad3, Smad4, and Smad7 mRNAs. Cells were preincubated with TGF-β1 for 12 h, followed by treatment with or without naringenin for 12 h. The levels of TβRI, TβRII, Smad2, Smad3, Smad4, and Smad7 mRNA were monitored by RT-PCR. As shown in Fig. 7, except for Smad3, naringenin had no influence on the mRNA levels of TβRI, TβRII, Smad2, Smad4, and Smad7, even at high concentrations (50 μM), indicating that naringenin is a selective inhibitor for Samd3.

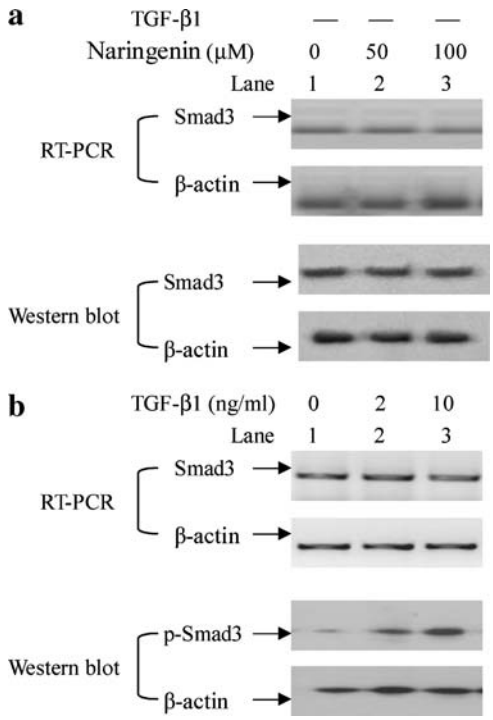

Fig. 5. Effect of naringenin on the expression of Smad3 without TGF-β1stimulation in HSC-T6 cells (a) and effect of TGF-β1 on expression of Smad3 and the phosphorylation of Smad3 (b). Cells were treated with different concentrations of naringenin for 24 h or treated with different concentrations of TGF-β1 for 12 h, respectively.

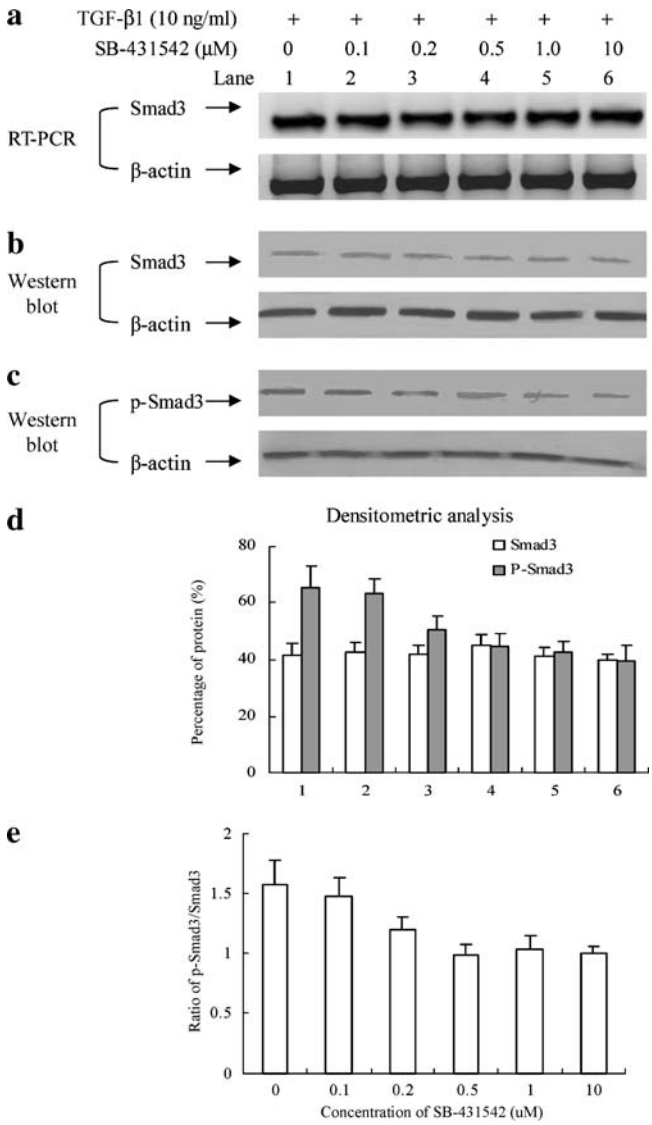

Fig. 6. SB-431542 inhibits the expression of Smad3 and the phosphorylation of Smad3. Cells were treated with 10 ng/mL TGF-β1 for 12 h before adding different SB-431542 concentrations ranging from 0 to 10 μM for 12 h incubation for RT-PCR analysis or 24 h incubation for Western blot. (a) The level of Smad3 mRNA was determined using RT-PCR. (b) The level of Smad3 protein was measured by Western blot. (c) The level of phosphor-Smad3 protein was measured by Western blot. (d) Values of densitometric scan are means ±SD of three experiments. (e) Ratio of phosphor-Smad3 to total Smad3. Experiments were performed in triplicate and data presented are representative of three independent experiments.

DISCUSSION

In this study, we have proven that naringenin has a potential to inhibit TGF-β1-induced production of Smad3, PAI-1, Col Iα1, and FN in HSC-T6 cells, suggesting that naringenin may inhibit the accumulation of ECM components via disruption of TGF-β1-Smad3 signaling pathway, at least partially. The present study for the first time showed that naringenin could reduce the TGF-β1-induced accumulation of ECM in cultured HSC-T6 cells. One mechanism was put forward that naringenin disrupts TGF-β1 signaling by selectively targeting Smad3.

First of all, we investigated the cytotoxicity of naringenin to HSC-T6 cells. MTT assay showed that naringenin has lower cytotoxicity with an LC_{50} value of 250 μM. The antiproliferative action of TGF-β has been documented particularly in HSCs and rats, disrupting TGF-β signaling leads to increased HSCs proliferation (24). At lower concentrations without toxicity, naringenin slightly promoted HSC-T6 cells propagation (Fig. 1), indicating that naringenin could be an antagonist of TGF-β signaling.

HSCs are regarded as the primary target cells for hepatic fibrogenesis, and activated HSCs have been identified as the key source of excess deposition of ECM proteins in liver fibrosis (5,25). Increasingly extracellular TGF-β1 initiates TGF-β1-Smad3 signaling in liver fibrogenesis, then activated Smad3/Smad4 complexes translocate into the nucleus, and immediately bind and activate targeting genes, including Col Iα1, TIMP-1, PAI-1, FN, and other ECM (13,16,26–29). As a specific targeting gene of Smad3, PAI-1 was usually used as one of the markers and developed the reporter gene in activated TGF-β1-Smad3 signaling (22,28). Naringenin significantly inhibited the expression of PAI-1 mRNA and protein induced by TGF-β1 (Fig. 2), demonstrating that naringenin may directly or indirectly intervene in TGF-β1-Smad3 signaling. In addition, the decrease in PAI-1 protein could cause indirect increase in matrix degradation through increased plasmin activity (24).

SB-431542, a well-known small molecular inhibitor of type I TGF-β receptor through specific and competitive ATP binding site kinase, only inhibits the phosphorylation of Smad2/Smad3 by binding TβRI (Fig. 6c) (22), which in turn, inhibits the syntheses of FN, PAI-1, and Col Iα1 induced by exogenous TGF-β1. In this work we showed that naringenin inhibited not only the expression of Smad3 mRNA and protein, but also the phosphorylation of Smad3 in HSC-T6

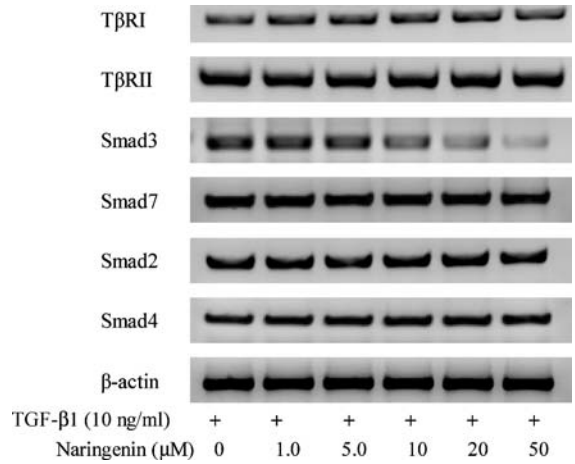

Fig. 7. Naringenin specifically down-regulates transcription of Smad3 gene. Cells were treated with 10 ng/mL TGF-β1 for 12 h before different naringenin concentrations were added ranging from 0 to 50 μM for 12 h incubation. mRNA levels of TβRI, TβRII, Smad2, Smad3, Smad4, and Smad7 were determined by RT-PCR. Details of reactions and primer pairs are described in Materials and Methods.

cells induced by TGF-β1 (Fig. 4). This suggests that naringenin could have a different molecular mechanism from SB-431542 for reduction of ECM components.

Naringenin has been found to increase PI3K and MAPK erk (p 38) activity in HepG2 cells, which paralleled that of insulin. However, in contrast to insulin, naringenin did not induce phosphorylation of insulin receptor substrate (IRS) (30,31). Insulin and insulin-like growth factor-I mediated signaling pathways have been shown to modulate TGF-β signaling (32,33). However, the molecular details of how these pathways control TGF-β signaling are not clear. One simple mechanism by which growth and antiapoptotic signaling, mediated by PI3K-PKB, thwarts TGF-β signaling is by inducing an interaction between PKB and Smad3, thus reducing the pool of Smad3 available for TGF-β signaling. This model is based on all the suppression of TGF-β-regulated gene expression by insulin and insulin-like growth factors, and can be reversed using highly specific inhibitors of PI3K, LY294002, or wortmannin. The phosphorylation of Smad3, but not Smad2, induced by TGF-β1 was significantly inhibited with insulin, whereas the levels of total Smad3 and Smad2 protein did not change. However, naringenin reduced both the levels of Smad3 protein and the phosphorylation of Smad3 in HSC-T6 cells induced by TGF-β1, suggesting that naringenin also has a different molecular mechanism from insulin and insulin-like growth factors for reduction of ECM components.

To investigate the specificity of naringenin to Smad3, we used RT-PCR to examine the effects of naringenin on other Smads, including Smad2 (high homology to Smad3), Smad4 (involving in the signaling transduction), and Smad7 (inhibiting TGF-β1-Smad3 signaling response) (22). Except for Smad3 mRNA, which was decreased, the mRNA levels of Smad2, Smad4, and Smad7 were not changed in HSC-T6 cells treated with naringenin (Fig. 7), indicating that naringenin specifically inhibits the expression of Smad3.

Both naringenin and SB-431542 reduced the level of phosphorylated Smad3 protein, decreased the formation and translocation into nucleus of the Smad3/Smad4 complex, and then had the same terminal effects resulting in the reduction of ECM components. The data demonstrate that the level of phosphorylated Smad3 protein plays a critical role in the TGF-β1 signaling pathway. Compared with SB-431542, which had no effect on the total Smad3 protein expression, naringenin significantly decreased the total Smad3 protein expression, suggesting that naringenin's attenuating impact on the level of phosphorylated Smad3 protein might be through the reduction of the total Smad3 protein expression. However, the presence of TGF-β1 is necessary for naringenin to exert its inhibitory effect on the total Smad3 protein expression. Reasons why naringenin exhibits inhibitory effect on the expression of Smad3 in the presence of TGF-β1 in HSC-T6 cells are unknown, seeming to be involved in crosstalk between signaling pathways as mentioned above.

In conclusion, naringenin disrupts TGF-β1-Smad3 signaling via reducing the formation of Smad3 directly or indirectly for its antiliver fibrosis. This further verifies Smad3's capacity as a pivotal mediator for TGF-β in modulating fibrogenic function and implicated that inhibition of Smad3 protein function may be a potential and effective therapeutic strategy to antifibrogenesis.

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