ENHANCED EFFECT OF SOLUBLE TRANSFORMING GROWTH FACTOR-β RECEPTOR II AND IFN-γ FUSION PROTEIN IN REVERSING HEPATIC FIBROSIS

H. Yao1, J. Pan2, Y. Qian3, Z. Pei4, A. Bader4, N. H. Brockmeyer4, P. Altmeyer4, L. Zhang3

1State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, the First Affiliated Hospital, Zhejiang University School of Medicine, China, 2Department of Microbiology and Parasitology, 3Institute of Immunology, Department of Dermatology and Allergy, Ruhr-University, Bochum, Germany

Abstract
Objective: To examine the in vivo anti-fibrotic effect of rat soluble transforming growth factor β receptor II (RstβRII) and IFN-γ fusion protein (RstβRII-IFN-γ) in rat hepatic fibrosis model.

Methods: Model rats were divided into five groups and treated i.m. for 8 weeks: 1) fibrotic model group (each rat, 100µl of 0.9% NaCl day⁻¹); 2) RstβRII-IFN-γ treatment group (each rat, 0.136 mg·day⁻¹); 3) IFN-γ treatment group (each rat, 7.5 MU·day⁻¹); 4) RstβRII treatment group (each rat, 0.048 mg·day⁻¹); and 5) mixture of IFN-γ and RstβRII treatment group (each rat, IFN-γ 7.5 MU·day⁻¹ + RstβRII 0.048 mg·day⁻¹). After treatment, hepatic fibrogenesis was evaluated by histopathological analysis and measurement of collagen III, α-smooth muscle actin (α-SMA), TGF-β1, TGF-βRII and their mRNA.

Results: Immunohistochemistry, Western blot and real-time RT-PCR showed that RstβRII-IFN-γ treatment significantly inhibited liver expression of collagen III, α-SMA, TGF-β1 and TGF-βRII at both protein and mRNA levels. Histopathological analysis also showed that the enhanced anti-fibrotic effects were achieved in model rats treated with RstβRII-IFN-γ.

Conclusion: Our results confirmed that RstβRII-IFN-γ has the enhanced effects in reversing hepatic fibrosis.

Key words: TGF-βRII; IFN-γ; Recombinant fusion protein; Hepatic fibrosis

Abbreviations: ECM: extracellular matrix; HSC: hepatic stellate cells; TGF-β: transforming growth factor-β; IFN-γ: interferon-γ; TGF-βRII: TGF-β type II receptor; sTGF-βRII: soluble TGF-β type II receptor; RstβRII: rat soluble TGF-β type II receptor; RstβRII-IFN-γ: rat soluble TGF-β type II receptor and IFN-γ fusion protein; VSV: vesicular stomatitis virus; Col III: collagen III; α-SMA: α-smooth muscle actin

INTRODUCTION
Liver fibrogenesis represents the common response of the liver to toxic, infectious, or metabolic agents and is characterized by increased synthesis and deposition of newly formed extracellular matrix (ECM) components [1]. Activated hepatic stellate cells (HSC) are known to be the primary ECM-producing cells in hepatic fibrogenesis [2]. An important role in fibrogenesis has been assigned to transforming growth factor (TGF)-β. TGF-β is in turn found in increased quantities in the injured liver, particularly after chronic liver injury [3]. The TGF-β superfamily consists of multiple family members, including the highly homologous TGF-β1 (TGF-β1, TGF-β2, and TGF-β3) isoforms, among of which TGF-β1 has prominent fibrogenic properties [4, 5], because TGF-β1 plays an important role in the activation of HSC which is the critical and fundamental event in hepatic fibrogenesis [6, 7]. Stellate cell activation is associated not only with enhanced production of all forms of ECM but also with up-regulation of cytoskeletal proteins, cytokines, and various cell surface receptors [8]. TGF-β stimulates collagen I mRNA transcription and increases its stability in activated stellate cells [9].

In mammalian cells, TGF-β response is mediated by type I and type II cell surface receptors that signal via cytoplasmic serine/threonine kinase domains [10]. TGF-β type II receptors (TGF-βRII) are constitutively active kinases that lead to ligand-binding specific signaling. TGF-βRII docking leads to TGF-β type I receptor recruitment, phosphorylation, and subsequent cellular signaling. TGF-βRII mediated signaling leads to pleiotropic effects in the wound healing response [11, 12].

Because the prominent role of TGF-β in hepatic fibrogenesis, a number of approaches have been used to abrogate the effect of TGF-β. Qi et al. used an adenoviral vector expressing a truncated TGF-βRII that can competitively bind to free TGF-β with membrane surface receptors to inhibit the effect of TGF-β on HSC [13]. Yata et al. used soluble TGF-βRII (sTGF-βRII) protein to treat hepatic fibrosis in mice and elucidated a dose-response relationship between the degree of inhibition of TGF-β and fibrogenesis [14]. Nakamura et al. found that remote delivery and expression of sTGF-βRII in muscle prevented hepatic fibrosis in rats [15]. Additionally, development of a chimeric soluble TGF-β receptor, which binds free TGF-β and
thus inhibits its effects, has been shown to reduce fibrogenesis acutely after liver injury in rats [16].

It was found that IFN-γ can induce the expression of Smad7, inhibit the interaction between TGF-β complex and Smad3, which indirectly inhibits TGF-β activity and HSC activation in vitro [17, 18]. Because sTGF-βRII and IFN-γ act at different sites to block the activity of TGF-β and the activation of HSC, combined application of them may produce a synergic effect and in turn reduce their required dose to lower the side effect.

In our previous study, we constructed an recombinant plasmid expressing a bioactive fusion protein (RstTβRII-IFN-γ) that comprises rat sTGF-βRII (RstTβRII) and IFN-γ in mammalian cells and showed that the purified RstTβRII-IFN-γ could antagonize the proliferation-inhibitive effect of TGF-β1 on Mink lung epithelial (Mv1Lu, CCL-64) cells and inhibit HSC activation in vitro [19]. In the present study, we investigated the in vivo anti-fibrotic effects of RstTβRII-IFN-γ fusion protein in a rat model with hepatic fibrosis.

**MATERIALS AND METHODS**

**AMPLIFICATION AND PURIFICATION OF THE RsTβRII-IFN-γ FUSION PROTEIN**

Clones expressing the highest levels of RstTβRII-IFN-γ fusion protein were selected for production of the fusion protein. RstTβRII-IFN-γ fusion protein was purified from culture supernatant by protein A-Sepharose affinity chromatography under sterile and endotoxin free conditions. The protein is greater than 95% pure as judged by sodium dodecyl sulfate/polyacrylamide gel electrophoresis and contains less than 1 unit endotoxin per milligram protein.

**RstTβRII-IFN-γ FUSION PROTEIN ANTIVIRAL ASSAY**

Antiviral assay was performed as described by Pan et al. [20] with minor modification. 1x10^6 L929 cells (100μl) per well were added to a 96-well microtiter tissue culture plate. The cells were incubated at 37°C in 5%CO2 condition and 100μl serial dilutions of RstTβRII-IFN-γ fusion protein in which RstTβRII activity has been blocked by its neutralizing antibody were added onto each well. The cells were incubated at 37°C for 72 hours and 10μl MTT (5mg/ml) were added into each well 4 hours before the end of the culture. After centrifugation at 1000rpm for 5 minutes, supernatants were removed. The cells were then lysed in 150μl DMSO. The absorbance which reflects cell proliferation was measured at 570 nm. Each assay was carried out in triplicate.

**CONSTRUCTION, PURIFICATION, AND ACTIVITY OF THE RstTβRII PROTEIN**

RstTβRII genes were amplified from rat liver cDNA with specific primers as previously described [19]. The RstTβRII fragment was digested with HindIII/BamHI restriction enzymes, then cloned into the pSecTag2 expression vector. The ultimate construct was screened by ampicillin and identified by double enzyme digestion and DNA sequencing. Transfection of the pSecTag2/RstTβRII into CHO was conducted with liposomes and the stable expression strains were screened with Zeocin®. The supernatants of transfected CHO were examined by Western blotting for RstTβRII expression. The purification and activity testing of the RstTβRII protein were performed as described for RstTβRII-IFN-γ fusion protein.

**ANIMAL MODEL WITH HEPATIC FIBROSIS AND TREATMENT**

Sprague-Dawley rats (n = 210, provided by Zhejiang University Experimental Animal Center) weighing 135g-150g were used. Principles of laboratory animal care (NIH publication No. 86-23, revised 1985) were followed.

Hepatic fibrosis was induced by subcutaneous injection of CCI4 as described previously [21] with minor modification. Rats (n = 200, in addition, 10 rats were chosen as the normal control group, named group Nor.) were subcutaneously administered dissolved CCI4 in camellia oil (a proportion of 1:1) at 0.3ml/100 g of body weight, twice a week for 8 weeks. Five rats were killed at the end of each week to examine pathological changes of livers. According to histological examination, hepatic fibrosis appeared at the end of the eighth week from the start of the study. 156 rats with hepatic fibrosis could be used for the study. Model rats were randomly divided into five groups: 1) fibrotic model group (n = 10) (group E, each rat, 10μl of 0.9% NaCl day⁻¹, i.m.); 2) RstTβRII-IFN-γ fusion protein treatment group (group Δ, each rat, 0.136mg (containing 7.5 MU IFN-γ and 0.048mg RstTβRII)·day⁻¹, i.m.); 3) IFN-γ treatment group (group B, each rat, 7.5MU·day⁻¹, i.m.); 4) RstTβRII protein treatment group (group C, each rat, 0.048mg·day⁻¹, i.m.); and 5) mixture of IFN-γ and RstTβRII treatment group (group D, each rat, IFN-γ 7.5MU·day⁻¹ + RstTβRII0.048mg·day⁻¹, i.m.).
The latter four groups were given, every two days for totally 8 weeks, RsTβRII-IFN-γ fusion protein, IFN-γ, RsTβRII protein, mixture of IFN-γ and RsTβRII protein, respectively. Rats in the fibrotic model group were given 0.9% sodium chloride by intramuscular injection. Rats in the normal control group didn't receive any treatment.

Four weeks after the finish of treatment, rats of each group were killed, and histological sections of their livers were evaluated by hematoxylin and eosin and Sirius red stains. Expressions of collagen III (Col III), α-smooth muscle actin (α-SMA), TGF-β1 and TGF-βRII were examined with immunohistochemistry, Western blotting and real-time RT-PCR.

**Histopathological Analysis**

Livers were fixed in 10% neutral formalin, embedded in paraffin, sectioned, and stained with hematoxylin-eosin and Sirius red respectively. Sirius red staining was quantitated in the sections that were randomly chosen (under 20× magnification; 10 fields each from 8 different rats for a total of 80 fields for each group) with Meta-View software (Universal Imaging Corp., Downington, PA). These sections were observed under polarization microscope (Leica DMLB, Leica Wetlar, Germany) to distinguish type I and III collagen [22].

**Immunohistochemistry Analysis**

For measurement of Col III, α-SMA and TGF-β1, liver tissues were fixed in formalin, embedded in paraffin wax, and 5-µm sections were cut and stained using the immunohistochemical Streptavidin-biotin-peroxidase complex (SABC) method as described previously with minor modification [23, 24].

**Real-time PCR**

Frozen rat's liver tissue, stored at -80° C, were shattered in liquid nitrogen and total RNA was isolated by the RNeasy isolation kit (Qiagen) according to the manufacturer's instructions. First-strand cDNA was synthesized. Real-time reverse transcriptase (RT)-PCR was performed as described by Li et al. [25]. Briefly, a real-time PCR was performed using Light Cycler FastStart DNA Master Mix, 1.8μl of MgCl2 (25mM), 0.4μl of each primer (10μM), 2μl of 1:10 diluted cDNA, 9.3μl of H2O. The reactions started at 95° C for 10 minutes followed by 50 cycles at 94° C for 10 seconds, 65° C for 15 seconds, 72°C for 15 seconds. PCR products were denatured for 1min at 95°C before rapidly chilling to 55°C for 30 seconds. Melting peaks of PCR products were determined by heat-denaturing them over a 40°C temperature gradient at 0.1°C/s from 55°C to 95°C. Reaction specificity was confirmed by 1.5% gel electrophoresis of products after real-time PCR and melting curve analysis. Ratios of Col III/β-actin, α-SMA/β-actin, TGF-β1/β-actin, TGF-βRII/β-actin, and mRNA were calculated for each sample and expressed as the means ± SEM.

**Western blot Analysis**

Western blot analysis of Col III, α-SMA, TGF-β1, TGF-βRII and β-actin in the liver was performed as previously described [26].

**Results**

**RsTβRII-IFN-γ Fusion Protein Can Protect L929 from Infection of VSV**

Anti-viral activity is one of the biological characteristics of IFN-γ [14, 27]. We tested if RsTβRII-IFN-γ fusion protein could protect L929 cells from infection of VSV. 1×10⁴/ well L929 cells were added into a 96-well plate. Serial dilutions of RsTβRII-IFN-γ fusion protein were added into the assay plate except for the normal controls after the cells adhered to wells. 3 hours later, 25µl of VSV was added into each well except for the normal controls. The cells were incubated for 24 hours totally and 10µl MTT (5mg/ml) were added into each well 4 hours before the end of the culture. The cells were lysed in 150µl DMSO. The absorbance at 570nm was detected and percent protection of L929 from infection of VSV was calculated. Each assay was carried out in triplicate. 1: original RsTβR-IFN-γ fusion protein; 2~10: 2xserial dilutions of 1; VSV Control: L929 challenged by VSV.

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**Fig. 1.** RsTβRII-IFN-γ dose-dependently protects L929 cells from infection of VSV. 1×10⁴/ well L929 cells were added into a 96-well plate. Serial dilutions of RsTβRII-IFN-γ fusion protein were added into the assay plate except for the normal and viral controls after the cells adhered to wells. 3 hours later, 25µl of VSV was added into each well except for the normal controls. The cells were incubated for 24 hours totally and 10µl MTT (5mg/ml) were added into each well 4 hours before the end of the culture. The cells were lysed in 150µl DMSO. The absorbance at 570nm was detected and percent protection of L929 from infection of VSV was calculated. Each assay was carried out in triplicate. 1: original RsTβR-IFN-γ fusion protein; 2~10: 2xserial dilutions of 1; VSV Control: L929 challenged by VSV.
of VSV by anti-viral assay. We found that L929 cells quickly exhibited typical signs of VSV infection in the absence of RstβRII-IFN-γ, however, in the presence of RstβRII-IFN-γ, L929 cells became insensitive to VSV challenge, and this protective effect is dose-dependent (Fig. 1), indicating that RstβRII-IFN-γ fusion protein retains the anti-viral activity of IFN-γ.

**Recombinant RstβRII-IFN-γ Fusion Protein Can Antagonize the Activity of TGF-β1 in Vitro**

It was demonstrated that stTGF-βRII could antagonize the bioactivity of TGF-β1 to inhibit the proliferation of mink lung epithelial cells [14]. We tested if RstβRII-IFN-γ has the bioactivity of stTGF-βRII by a mink lung epithelial cell proliferation assay. As shown in Figure 2, when TGF-β1 was added to the media, proliferation of CCL-64 was inhibited. While different concentration of RstβRII-IFN-γ fusion protein and same quantity of TGF-β1 were incubated together with CCL-64 cells for 72 hours, TGF-β1 mediated inhibition of proliferation was blocked by RstβRII-IFN-γ fusion protein in a dose-dependent manner, suggesting that RstβRII-IFN-γ retains RstβRII bioactivity.

**RstβRII Can Neutralize the Growth-Inhibitory Effect of TGF-β1 on Mink Lung Epithelial Cells**

To identify the bioactivity of the RstβRII, we performed a mink lung epithelial cell proliferation assay. As shown in Figure 3, RstβRII dose-dependently inhibited the growth-inhibitory effects of TGF-β1 on CCL-64 cells, implying that RstβRII we prepared could competitively bind to TGF-β1 with the membrane TGF-βRII, hereby inhibiting the bioactivity of TGF-β1.

**Fig. 3. RstβRII blocks the growth-inhibitory effect of TGF-β1 on CCL-64 cells.** Serial dilutions of RstβRII were incubated with 800 pg/ml TGF-β1 for one hour in assay medium in a 96-well plate. 1x10^6 cells of CCL-64 per well were added into each well. The cells were incubated at 37 °C for 72 hours and 10µl MTT (5mg/ml) were added into each well 4 hours before the end of the culture. The cells were lysed in 150µl DMSO and the absorbance at 540 nm was measured and ratios of inhibition were calculated. Mv1Lu: Untreated CCL-64 cells; 1: Original purified RstβRII protein; TGF-β1: CCL-64 cells treated with TGF-β1 but without RstβRII protein.

**RstβRII-IFN-γ Fusion Protein Treatment Reverses Hepatic Fibrosis**

Histological examination of livers from normal rats (group Nor) demonstrated that ECM deposition was present only in portal and central areas (Fig. 4Nor). Hematoxylin-eosin staining of liver sections from untreated rats with hepatic fibrosis (group E) exhibited perportal, pericentral, and central-central, portal-portal, and portal-central matrix deposition and numerous granulomas containing a diffuse array of inflammatory cells, within an invasive collagen network (Fig. 4E). In contrast, livers from rats with hepatic fibrosis treated with RstβRII-IFN-γ fusion protein, IFN-γ, RstβRII, and mixture of IFN-γ and RstβRII, respectively (group A, B, C and D) showed much less granulomas in which only a few cells were found, and markedly decreased collagen (Fig. 4A, B, C and D).

Quantitative morphometric assessment of fibrosis (identified by Sirius red staining) showed a significant reduction in fibrogenesis after treatment of rats with RstβRII-IFN-γ fusion protein, IFN-γ, RstβRII, and mixture of IFN-γ and RstβRII, respectively (Figs. 4A, B, C and D). Sirius red staining showed that a little collagen deposition in the portal tracts and lobules was seen in livers from rats with hepatic fibrosis treated by RstβRII-IFN-γ fusion protein. Furthermore, only collagen fibers around the terminal hepatic veins were observed (Fig. 4A). Collagen depositions in liver sections from rats treated with IFN-γ, RstβRII or RstβRII+IFN-γ, respectively were particularly evident, but only thin bands of collagen which formed short, incomplete septum could be seen (Figs. 4B, C and D).

Taken together, the most effective anti-fibrotic effect was achieved in model rats treated with RstβRII-IFN-γ fusion protein.
Fig. 4. Histopathological analyses of livers in rats from different groups. 4 weeks after the finish of treatment, rats with hepatic fibrosis in each group were killed and livers were harvested, fixed in 10% neutral formalin, sectioned, and stained with hematoxylin-eosin (left column) and Sirius red (right column) respectively. Representative micrograph in each group was shown (original magnification ×200). A, RsTβRII-IFN-γ fusion protein treatment group; B, IFN-γ treatment group; C, RsTβRII treatment group; D, mixture of IFN-γ and RsTβRII treatment group; E, fibrotic model group; Nor, normal control group.

Fig. 5. Immunohistochemistry of α-SMA, Col III, TGF-β1. Paraffin sections of liver were incubated overnight at 4°C with anti-α-SMA (Fig. 5A), anti-type III collagen (Fig. 5B), anti-TGF-β1 (Fig. 5C) monoclonal antibodies (1:100 dilutions). This was followed by incubation in biotinylated goat anti-mouse antibodies (1:200 dilutions) for 40 min and treated with SABC for 30 min at 37°C. The peroxidase activity, which resulted in a brown staining of cytoplasmic reaction sites, was visualized by incubation for 30 sec–1 min in dimethylbenzidine and staining lightly with hematoxylin.
RstßRII-IFN-γ FUSION PROTEIN TREATMENT REDUCES EXPRESSION OF α-SMA, COL. III, TGF-β1 AND TGF-βRII

Immunohistochemistry analyses of the liver further revealed the striking differences. As shown in Figures 5A, in the normal control group (Group Nor), α-SMA positive cells were detected mainly in the portal space as elements of vascular walls or as fibroblastlike cells scattering in the connective tissue or near bile ductules. In lobule, α-SMA positive cells were present on the walls of large and medium sized terminal hepatic veins. The pattern of distribution of α-SMA positive cells was modified in liver injured rats as compared with the normal control rats. In rats with hepatic fibrosis (group E), α-SMA positive cells were detected in portal space, sinusoid, lobule and areas where fibrotic septum appeared. Distributions of α-SMA positive cells in liver from rats with hepatic fibrosis treated with RstßRII-IFN-γ fusion protein, IFN-γ, RstßRII or RstßRII +IFN-γ respectively were all reversed when compared with those of hepatic fibrosis model rats. After 8 weeks of RstßRII-IFN-γ fusion protein treatment (group A), only thin and incomplete parenchymal α-SMA positive septum joining thickened centrilobular veins were observed in liver sections. α-SMA positive cells were mainly found in portal space and areas around fibrotic septum. Few α-SMA positive cells were present in sinusoid and lobule (Fig. 5A). Moreover, it was obvious that the greatest anti-fibrotic effect was achieved by treatment with RstßRII-IFN-γ fusion protein in accordance with the histopathological analysis (Fig. 4A). The deposition and distribution range of positive staining for COL III in livers from rats with hepatic fibrosis treated by RstßRII-IFN-γ fusion protein mirrored the same trend as α-SMA (Fig. 5B).

As TGF-β1 is an profibrogenic cytokine that signals through a receptor consisting of type I and type II components [28], the expression of TGF-β1 in livers from different groups was also examined by immunohistochemistry. As shown in Figure 5C, normal rat livers showed light, positive staining which was scattered in the perivascular spaces, around portal tracts. Livers from rats with hepatic fibrosis showed a marked increase in positive staining for TGF-β1, with the reactivity primarily occurring in the portal tract area and granulomas. However, livers from rats with hepatic fibrosis treated with RstßRII-IFN-γ fusion protein, IFN-γ, RstßRII, and mixture of IFN-γ and EuRoPEAN JouRNAL OF MEdIcAL RESEaRcH April 8, 2010

Fig. 6. Western blotting analysis of COL III, α-SMA, TGF-β1 and TGF-βRII. Liver tissues from rats in different groups were lysed in the lysis buffer. Samples were centrifuged to pellet cell debris, mixed with SDS-PAGE sample buffer. After boiled for 5~10 min, 40 μg of protein was electrophoresed on a 10% SDS-polyacrylamide gel. The protein was transferred to PVDF membrane. The blots were incubated with the primary antibody against COL III, α-SMA, TGF-β1, TGF-βRII and β-actin (1:400) at 4°C overnight respectively and subsequently with corresponding secondary antibody (1:1000) at room temperature for 2 h. Protein bands were visualized with an ECL Western blotting detection system kit and signal was captured on X-ray film.

Fig. 7. Real-time RT-PCR for COL III, α-SMA, TGF-β1 and TGF-βRII mRNA expression. Total RNA was isolated from frozen liver tissues. Real-time RT-PCR was performed using Light Cycler FastStart SYBR Green I Master according to the manufacturer’s instructions. (A) Gel electrophoresis of PCR products. (B) Calibrator normalized relative ratios. A: RstßRII-IFN-γ fusion protein treatment group; B: IFN-γ treatment group; C: RstßRII protein treatment group; D: mixture of IFN-γ and RstßRII treatment group; E: fibrotic model group; Nor: normal control group.
RsTβRII respectively (group A, B, C and D) showed
decreased expression of TGF-β1. Moreover, liver sec-
tions from rats with hepatic fibrosis treated with RsT-
βRII-IFN-γ fusion protein (group A) showed marked
decrease in expression of TGF-β1 when compared
with those from rats with hepatic fibrosis treated with
IFN-γ, RsTβRII and mixture of IFN-γ and RsTβRII,
respectively (group B, C and D) although the distribu-
tion pattern of positive cells was similar to that of
the hepatic fibrotic model group (group E).

Western blotting analyses of collagen III, α-SMA,
TGF-β1 and TGF-βRII content in livers of different
groups also substantiated the histological impression,
showing decreased contents of Col III, α-SMA, TGF-
β1 and TGF-βRII in livers from rats with hepatic fi-
brosis treated by RsTβRII-IFN-γ fusion protein, IFN-
γ, RsTβRII, and mixture of IFN-γ and RsTβRII, re-
spectively (Fig. 6). However, the most effective therapeu-
 tic effect was achieved again by treatment with
RsTβRII-IFN-γ fusion protein.

Finally, we investigated the mRNA expression of
Col III, α-SMA, TGF-β1 and TGF-βRII in livers of differ-
ent groups. As shown in Figure 7, 4 weeks after
the stop of treatment, there was an increase in TGF-
β1 mRNA expression in livers from rats with hepatic
fibrosis, while only detectable level of TGF-β1 mRNA
was shown in livers from normal control rats.
This increase in TGF-β1 steady-state mRNA levels
approximately paralleled the increase in Col III, α-
SMA and TGF-βRII mRNA levels. Moreover, livers
from rats with hepatic fibrosis treated with RsTβRII-
IFN-γ fusion protein (group A) showed markedly de-
creased mRNA expression of Col III, α-SMA, TGF-
β1 and TGF-βRII compared with untreated, hepatic
fibrotic rats. In contrast, RsTβRII, IFN-γ or RsT-
βRII+IFN-γ treatment did not significantly alter
steady-state mRNA levels for TGF-β1 and TGF-βRII
(Fig. 7A and B). These data substantiated again the
histological analyses, and demonstrated that RsTβRII-
IFN-γ fusion protein treatment did inhibit collagen
deposition.

**DISCUSSION**

Hepatic fibrosis is a pathological process shared by
a variety of causes in response to hepatocyte necrosis
and inflammatory insults, which is mainly character-
ized by the excessive deposition of ECM in Disse
space in the liver. Activated HSC are known to be the
primary ECM-producing cells in hepatic fibrogenesis.
The activation of HSC is largely mediated by TGF-β,
which is actually the most potent profibrogenic cy-
tokine. Thus the TGF-β/HSC axis is widely consid-
ered a potential target for anti-hepatic-fibrosis therapy
by many investigators. TGF-β exhibits its biological
function via the TGF-β receptors, including type I, II
and III receptors. Both the dominant-negative TGF-
βRII and TGF-βRII have been proved to be anti-fi-
brrosis, they can bind to TGF-β but lack the intracellu-
lar kinase domain which is essential to initiate signal
transduction[29, 30]. IFN-γ inhibits the interaction be-
tween TGFBR complex and Smad3 by blocking
TGF-β signaling via JAK/STAT pathway, thus antago-
nizing the fibrogenic effect[31]. Neutralizing the effect
of TGF-β in vivo leads to reduced ECM deposition in
both the liver [13~16] and other tissues [32]. Thus, the
evidence linking TGF-β and fibrogenesis is substantial
and, moreover, raises important possibilities for therape-
utics targeted at this pathway.

Cytokine recombinant fusion protein refers to a
category of artificial protein products produced by ge-
etically fusing the encoding fragments of cytokines
and/or other proteins with specific biological func-
tions. This kind of protein exerts strongly enhanced
biological activities when compared with the original
individual proteins or mixed proteins. Because sTGF-
βRII and IFN-γ act at different sites to block the ac-
tivity of TGF-β and the activation of HSC, combined
application of them may produce a synergetic anti-fi-
brotic effect and in turn reduce their required dose to
lower the side effect. So we prepared a novel RsT-
βRII-IFN-γ fusion protein by gene recombinant, and
tested the bioactivity and the in vivo anti-fibrotic ef-
fects of RsTβRII-IFN-γ fusion protein.

At first, we showed that the growth-inhibitory ef-
fect of TGF-β on Mv1Lu epithelial cells was abrogated,
in a dose-dependent manner, by RsTβRII-IFN-γ
fusion protein, indicating that RsTβRII-IFN-γ fusion
protein retains RsTβRII bioactivity. In anti-viral assay,
L299 cells without RsTβRII-IFN-γ protection quickly
exhibits the typical signs of VSV infection, while RsT-
βRII-IFN-γ protected L299 turned out to be insensitive
to VSV challenge, and this protective effect is also
dose-dependent, indicating that RsTβRII-IFN-γ re-
tains the anti-viral activity of IFN-γ. Next, we investi-
gate the in vivo anti-fibrotic effect of RsTβRII-IFN-γ
fusion protein in rat model with hepatic fibrosis in-
duced by subcutaneous injection of CCl4.

CCl4 is one of the most widely used hepatic toxins
for experimental induction of liver fibrotic cirrhosis
in laboratory [21, 33, 34]. The model was used even
in 1936. But, disadvantages of the model, such as higher
mortality rate, were apparent. For this reason, 200 rats
were used to produce CCl4-induced hepatic fibrosis
model in our study. By histological examination of liv-
er every week, we found that hepatic fibrosis was suc-
cessfully induced at the eighth week. 156 rats in fibrot-
ic model groups showed complete fibrotic septum,
forming a pattern of micronodular cirrhosis and they
were used for the following study.

As mentioned earlier, hepatic fibrosis is a patholog-
ical process which is mainly characterized by the ex-
cessive expression and deposition of ECM (especially
Col I and III) [35] and activated HSC, characterized
by the expression of α-SMA, is the main producer of
ECM [36]. Moreover, Zhang et al. found that TGF-β1
and TGF-βRII play important roles in the pathogene-
sis of hepatic fibrosis [37]. Therefore, we used Col
III, α-SMA, TGF-β1 and TGF-βRII expressions plus
histopathological analysis as indices to evaluate the in
vivo anti-fibrotic effects of RsTβRII-IFN-γ fusion
protein. Our data showed that the pathological fibro-
sis scores were significantly lower in model rats treat-
ed with RsTβRII-IFN-γ fusion protein compared with
other groups. In the mean time, liver contents and
their distribution ranges of collagen III, α-SMA,
TGF-β1 and TGF-βRII, as well as the expression of
their mRNA decreased significantly in rats with he-
pathic fibrosis treated with RsTβRII-IFN-γ fusion protein when compared with those of hepatic fibrosis control rats. These data suggest that the most prominent anti-fibrotic effect was achieved in rats with hepatic fibrosis treated with RsTβRII-IFN-γ fusion protein.

Taken together, our findings imply that RsTβRII-IFN-γ fusion protein might be a novel potential candidate in treating hepatic fibrosis. However, whether RsTβRII-IFN-γ fusion protein may cause any side effects when used to treat hepatic fibrosis needs further detailed investigation.

Acknowledgement: This work was supported by grants from National Research and Development Plan of High Technology (2001AA215111) and Science and Technology Foundation (2009C33150) and Natural Sciences Foundation (Y207419) of Zhejiang Provincial. We appreciate Dr. Bo Wang (Department of Pathology, First Affiliated Hospital, Zhejiang University School of Medicine) for his kind help in pathological analyses.

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Received: June 20, 2009 / Accepted: November 25, 2009

Address for correspondence:
Prof. Libuang Zhang, M.D.
Institute of Immunology
Zhejiang University School of Medicine
388 Yuhangtang Road
Hangzhou 310058
P.R. China
E-mail: zhanglihuang@zucc.edu.cn