Immunopathogenesis of hepatic fibrosis in chronic liver injury induced by repeatedly administered concanavalin A

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Abstract

Liver fibrosis is commonly observed in chronic liver disease. However, the immunological mechanisms underlying hepatic fibrosis due to chronic inflammation are not well defined, mainly because suitable experimental models have not been established. We have found that weekly i.v. administration of concanavalin A (Con A) in BALB/c mice brought about a striking alanine aminotransferase increase, resulting in piecemeal necrosis with bridging fibrosis in the parenchyma. Using this fibrosis model, we demonstrated the kinetics of cytokine mRNA expression in liver. Transforming growth factor (TGF)-β1, TGF-α, basic fibroblast growth factor (bFGF) and hepatocyte growth factor mRNAs were up-regulated after each Con A administration. Furthermore, either anti-IFN-γ, anti-tumor necrosis factor (TNF)-α or anti-TGF-β mAb given together with Con A markedly inhibited the development of hepatic fibrosis. Treatment with either anti-IFN-γ or anti-TNF-α mAb also completely prevented hepatic injury; in contrast, treatment with anti-TGF-β mAb did not. The treatment with anti-TGF-β mAb did not affect the levels of hepatic mRNAs for either IFN-γ or TNF-α after Con A injection. Treatment with either anti-IFN-γ or anti-TNF-α did not affect the expression levels of TGF-β in the liver. In conclusion, the continuous presence of both severe liver damage and up-regulation of TGF-β synthesis is necessary to induce hepatic fibrosis in this model.

Introduction

Hepatic fibrosis is a common feature of chronic hepatitis and chronic liver injury leads to liver cirrhosis. Regardless of the causes, the fibrosis is characterized by an increase in extracellular matrix constituents that collectively form hepatic scars (1). Liver cirrhosis, represented for hepatic fibrosis, is clinically very important as high risk conditions underlie the development of hepatocellular carcinomas (HCC), but the immunological mechanisms for hepatic fibrosis are not well defined. For prevention of HCC, an understanding of such mechanisms is essential. In human cases, it has been shown that hepatic fibrosis is caused after the death of hepatocytes by immunological mechanisms with infection with hepatitis viruses.

Although there have been reports of experimental fibrosis models established by repeated administration of carbon tetrachloride (2), ligation of the bile duct (3) and porcine serum (4), these experimental models are generally unsuitable for analyzing the mechanisms of human hepatic fibrosis, since these models do not involve immunological responses, such as lymphocyte infiltration and IFN-γ synthesis, compatible for human cases.

Recently it has been shown that hepatitis B surface antigen (HBsAg)-specific cytotoxic T lymphocytes (CTL) cause a fatal necroinflammatory liver disease in HBsAg transgenic mice (5,6). This model revealed that antigen-non-specific amplification mechanisms play a major role in spreading the liver necrosis (6,7). It has been reported that hepatitis B virus (HBV)-specific CTL are actually induced in patients with acute
Methods

transforming growth factor (TGF)-β. The mechanisms are mainly based on the up-regulation of gene expression at the indicated time points after Con A injection. Total RNAs were isolated by the guanidinium isothiocyanate–phenol–chloroform method using RNAzol (Tel-Test, Friendswood, TX). In brief, tissue samples were homogenized with RNAzol and 0.2 ml chloroform was added to 2 ml of homogenate. The samples were shaken vigorously for 15 s and incubated for 5 min on ice, and then centrifuged at 12,000 g for 15 min. The aqueous phase was transferred to a fresh tube, mixed with an equal volume of isopropanol and placed for 15 min on ice. After centrifugation at 12,000 g for 15 min, the RNA pellet was washed once with ice-cold 75% ethanol with vortexing, centrifuged at 7500 g for 5 min, dried for 10 min and dissolved in diethylpyrocarbonate (Sigma)-treated RNase-free solution. Total RNA contents of the samples were estimated spectrophotometrically by absorbance at 260 nm. Purity of RNA was determined from the 260/280 absorbance ratio.

RT-PCR analysis

Total RNA (1 µg) was reverse transcribed into cDNA by using AMV reverse transcriptase (Boehringer, Mannheim, Germany) and the cDNAs were amplified by PCR. Briefly, 1 µg of total RNA was denatured by heating for 10 min at 70°C and added to a mixture containing 2 µl of oligo-dT (0.5 mg/ml) (Boehringer), 6 µl of 5-fold concentrated reverse transcriptase buffer, 2 µl of DTT (Boehringer; 0.1 mol/l), 1 µl of dNTP (Boehringer; 10 mmol/l) and 1 µl of reverse transcriptase. Each cDNA synthesis was performed for 1 h at 42°C and stopped by incubation for 10 min at 98°C. PCR was performed in a volume of 50 µl containing 400 pmol/l of each primer, 2 µg of cDNA, Tris–HCl (10 mmol/l, pH 8.3), KCl (50 mmol/l), MgCl2 (1.5 mmol/l), dNTP (0.2 mmol/l) and 2 U of Taq DNA polymerase (Boehringer). Thermal cycle conditions were 94°C for 45 s for denaturing, 60°C for 45 s for annealing and 72°C for 2 min for extension. The number of amplification cycles was 28 for each set of primers. For each set of primers, dilutions of cDNA were amplified for 20, 23, 25, 28, 30, 32, 34, 36 and 38 cycles to define optimal conditions for linearity and to permit semiquantitative analysis of signal strength. After the last cycle of amplification, the samples were incubated for 7 min at 72°C. The PCR products were electrophoresed on 1.2% agarose gels and stained with ethidium bromide. To ensure that contaminating DNA had not been amplified, PCR without reverse transcriptase was simultaneously demonstrated. Sense and anti-sense primers were based on Clontech (Palo Alto, CA) Amplimer sets for mouse IFN-γ, TNF-α, TGF-β1 and G3PDH. In addition, TGF-α, acidic fibroblast growth factor (aFGF), basic FGF (bFGF) and hepatocyte growth factor (HGF) were investigated. The sense and anti-sense primers were 5′-TGG CCA GAT TCC CAC ACT-3′ and 5′-TGG ATC AGC ACA CAG GTG for TGF-β1, 5′-CCG GGG CCA CTT CTT GAG GA-3′ and 5′-ACC GGG AGG GGC AGA AAC AA-3′ for aFGF, 5′-TTC CCA CCA GGC CAC TTC A-3′ and 5′-TGG CCA GTT CTT ACT ACG GC-3′ for bFGF, and 5′-CGG AGC CCA TGG TGC TAC A-3′ and 5′-CTC GGA TGT TTG CAG CAG TTG-3′ for HGF respectively. Quantitation of IFN-γ, TNF-α and TGF-β1 mRNAs was performed by

Methods

Mice

Female BALB/c mice (7–10 weeks old; weight 25–30 g) were obtained from Japan SLC (Shizuoka, Japan).

Disease model

Con A (Sigma, St Louis, MO) was dissolved in pyrogen-free PBS (Sigma) at a concentration of 1 mg/ml and i.v. injected into BALB/c mice at a dose of 0.3 mg/mouse once a week. Serum samples from individual mice were obtained from the retro-orbital sinus 1 week after the final injection of Con A. Liver tissues were fixed in 10% neutral-buffered formalin and embedded in paraffin. For the histopathological evaluation, mice were sacrificed 1 week after the i.p. injection of hamster mAb H22 and TN3 19.12 specific for murine IFN-γ and TNF-α respectively. The serum levels of IFN-γ and tumor necrosis factor (TNF)-α were monitored using commercially available ELISA kits (Gibco/BRL, Gaithersburg, MD and Genzyme, Cambridge, MA respectively). The detectable lower limits of sensitivity of the kits were 125 and 35 pg/ml respectively. To evaluate the role of IFN-γ and TNF-α in the fibrosis process, Con A was administered at 24 h after the i.p. injection of hamster mAb H22 and TN3 19.12 specific for murine IFN-γ and TNF-α respectively, which were generously provided by Dr Robert D. Schreiber (Department of Pathology, Washington University School of Medicine, St Louis, MO). Purified hamster IgG (Organon, West Chester, PA) was used as a control antibody.

Serum levels of IFN-γ and tumor necrosis factor (TNF)-α

The serum levels of IFN-γ and TNF-α were measured by ELISA kits as described above. The results were expressed as picograms per milliliter.

RNA preparation

Frozen liver tissue was mechanically pulverized and total hepatic RNA was isolated for the analysis of cytokine mRNA expression at the indicated time points after Con A injection. The samples were homogenized with RNAzol and 0.2 ml chloroform was added to 2 ml of homogenate. The aqueous phase was transferred to a fresh tube, mixed with an equal volume of isopropanol and placed for 15 min on ice. After centrifugation at 12,000 g for 15 min, the RNA pellet was washed once with ice-cold 75% ethanol with vortexing, centrifuged at 7500 g for 5 min, dried for 10 min and dissolved in diethylpyrocarbonate (Sigma)-treated RNase-free solution. Total RNA contents of the samples were estimated spectrophotometrically by absorbance at 260 nm. Purity of RNA was determined from the 260/280 absorbance ratio.

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hepatitis B (8–11) and thus the HBsAg transgenic mouse model may have essential similarities to human hepatitis. However, since adoptively transferred HBsAg-specific CTL abolish the HBV gene and antigen expression in the liver (12,13), a 4 week interval of CTL administration is required to induce an equal level of liver injury in this model. Thus, it has been difficult to establish liver fibrosis using this mouse model, although it is speculated that continuous hepatocellular injury may induce liver fibrosis. With another approach, it has been reported that liver-specific expression of IFN-γ in transgenic mice causes chronic active hepatitis, in which initial necroinflammatory changes are followed by lymphoid cell infiltration in the portal areas (14). However, since the mouse liver has a remarkable regenerative capacity for individual hepatocytes, a mouse model of hepatic fibrosis following immunologically induced hepatocellular injury has not yet been produced. According to these results, it has been thought to be difficult to establish a liver fibrosis model after hepatocellular injury mediated by immunological mechanisms.

We now report that concanavalin A (Con A)-induced hepatitis develops hepatic fibrosis after repeated liver injury and the mechanisms are mainly based on the up-regulation of transforming growth factor (TGF)-β1 expression in the liver.
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Role of TGF-β1 in hepatic fibrosis
To evaluate the role of TGF-β1 in the development of liver fibrosis, the monoclonal neutralizing antibody to TGF-β1, 2 and 3, purchased from Genzyme (Cambridge, MA), was simultaneously administered (200 μg/mouse/each injection) i.p. at the time of each Con A injection. Sera and liver tissues were collected at several time points after the administration of Con A and anti-TGF-β mAb for analysis of both hepatic mRNA expressions and serum levels of IFN-γ and TNF-α. Histopathological examination was also performed using the liver tissues.

Statistical analysis
Values were expressed as mean ± SD. Differences between experimental and control groups were analyzed by the Kruskal–Wallis test followed by Scheffé’s F-test.

Results
The evaluation of hepatocellular injury induced by repetitive Con A injections
After Con A was i.v. injected into BALB/c mice at a dose of 0.3 mg/mouse, sALT activities were measured at indicated time points (Fig. 1A). sALT levels began to rise at 4 h (434 ± 138 IU/l) and reached a peak at 24 h (4840 ± 1194 IU/l). The levels then rapidly decreased within 48 h (490 ± 130 IU/l). With weekly administrations of Con A, as shown in Fig. 1(B), striking increases of sALT were observed 24 h after each injection.

Histopathological analysis of hepatic fibrosis
To assess the evolution of pathological changes in livers due to weekly Con A injections, liver tissues were derived from mice before Con A injection, 24 h after the first injection, 1 week after the second injection and 1 week after the fourth injection. Twenty-four hours after first Con A injection, hepatocellular necrosis had become widespread throughout the lobule (Fig. 2B). No evidence of hepatic fibrosis was observed at this period. No hepatocellular necrosis and hepatic fibrosis were found 1 week after the second Con A injection (Fig. 2C). However, as shown in Fig. 2(D), bridging fibrosis in the parenchyma with hepatocellular necrosis was detectable 1 week after the fourth Con A injection by Azan and silver staining (inset).

Role of IFN-γ and TNF-α
Since it has been reported that both IFN-γ and TNF-α play critical roles in Con A-induced liver injury (15, 16), we investigated changes in both serum levels and hepatic mRNA expression of IFN-γ and TNF-α at several time points after Con A injection. Serum levels of IFN-γ gradually increased and reached a peak 12 h after a single Con A injection (4328 ± 1109 pg/ml). Serum levels of TNF-α strikingly increased and reached a peak within 2 h (1122 ± 105 pg/ml) (data not shown). Quantitative RT-PCR performed using liver tissues also revealed up-regulation of hepatic mRNA expression of both IFN-γ and TNF-α, reaching peaks within 4 h after Con A injection (data not shown).

In order to reveal the role of IFN-γ and TNF-α in hepatic fibrosis, we investigated the protective effects of passive immunization with anti-IFN-γ mAb and anti-TNF-α mAb in this model. Anti-IFN-γ mAb or anti-TNF-α mAb (150 μg/mouse) were administrated i.p. 24 h before each Con A injection. As shown in Fig. 3(A), the serum ALT levels were markedly suppressed by the administration of either anti-IFN-γ mAb or anti-TNF-α mAb after every Con A injection. Histopathological examination similarly revealed inhibition of hepatocellular injury and hepatic fibrosis (Fig. 3B and C) at 1 week after the fourth Con A injection. To evaluate whether the inhibitory effects were due to independent pathways, the serum levels of IFN-γ or TNF-α were monitored after Con A injection with anti-TNF-α mAb or anti-IFN-γ mAb. As shown in Fig. 4(A and B), the levels of IFN-γ showed a 84% reduction in the peak after Con A injection with anti-TNF-α mAb, as compared with the Con A injection alone. Similarly, an 87% reduction in the peak of TNF-α was observed after Con A injection with anti-TNF-α mAb, as compared with the Con A injection alone.

Cytokine gene expression in liver after Con A administration
To determine whether cytokines contribute to the hepatic fibrosis, we analyzed the mRNA expression levels of TGF-α, bFGF, HGF, aFGF and TGF-β1, reported to affect tissue fibrogenesis (17–22) by either semiquantitative or competitive RT-PCR after Con A administration. As shown in Fig. 5(A), mRNA expression of TGF-α, bFGF and HGF was up-regulated after Con A injection (peak = 4, 24 and 24 h respectively). In contrast, aFGF was negatively regulated. Competitive competitive PCR using the PCR Mimic Protocol (Clontech). IFN-γ, TNF-α and TGF-β1 competitor primers yielding product sizes of 500, 500 and 390 bp respectively were used in each reaction. Furthermore, G3PDH competitor primers was constructed using a PCR Mimic construction kit (Clontech).
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Fig. 2. Histopathological evaluation of the livers of Con A-treated mice. BALB/c mice were i.v. injected with Con A at a dose of 0.3 mg/mouse once per week. Histopathological characteristics of liver without Con A injection (A), 24 h after the first injection (B), 1 week after the second injection (C) and 1 week after the fourth injection (D). Hematoxylin & eosin staining was performed for (A) and (B), Azan staining for (C), and Azan staining and silver staining (inset) for (D). Note the presence of hepatic fibrosis (arrowheads) in (D). Original magnification ×200. Representative figures derived from analyses of four mice are shown in each time point.

RT-PCR revealed that mRNA expression of TGF-β1 was up-regulated and reached a peak 48 h after Con A injection (Fig. 5B). These results indicated that most of the cytokine’s mRNAs except for aFGF were up-regulated after Con A injection.

Prevention of Con A-induced hepatic fibrosis by anti-TGF-β mAb

Since it has been recently reported that the hepatic expression of TGF-β1 mRNA is markedly up-regulated in patients with liver cirrhosis, as compared with both normal control and chronic hepatitis (23), we focused on the role of TGF-β1 among the cytokines studied above in the development of hepatic fibrosis in this mouse model.

As a first step to determine whether TGF-β1 affects the liver injury induced by Con A, we monitored sALT activities 24 h after each Con A injection either with or without administration of anti-TGF-β mAb from weeks 1 to 4. There was no significant difference in sALT levels between the two groups at any time points (Fig. 6A). In contrast, histopathological examination revealed that the administration of anti-TGF-β1 mAb markedly inhibited hepatic fibrosis (Fig. 6C).

Relationship between TGF-β and IFN-γ or TNF-α in Con A-induced hepatic fibrosis

To determine the relationships between the cytokines preventing hepatic fibrosis in this model, serum levels and hepatic mRNA expression of IFN-γ and TNF-α were analyzed after Con A injection, with or without simultaneous anti-TGF-β mAb treatment. As shown in Fig. 7(A), serum levels of IFN-γ, but not TNF-α, were significantly increased by the simultaneous treatment of anti-TGF-β mAb 4 h after Con A injection. Reflecting these results, hepatic mRNA expression of IFN-γ was enhanced; in contrast, that of TNF-α was not enhanced by the simultaneous treatment of anti-TGF-β mAb (Fig. 7B). Treatment with either anti-IFN-γ or anti-TNF-α mAb did not affect the expression levels of TGF-β1 mRNA in liver after Con A injection (Fig. 7B).

Discussion

Fibrosis is an important component of advanced chronic inflammatory liver disease. Although the changes in hepatic
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Fig. 3. Role of IFN-γ and TNF-α in hepatocellular injury and hepatic fibrosis. Sera and liver tissues were sampled from BALB/c mice 1 week after each weekly Con A injection (0.3 mg/mouse). Control rat-IgG, anti-IFN-γ or anti-TNF-α mAb (150 µg/mouse) were administered i.p. 24 h before weekly Con A injections. Hepatocellular injury was monitored by analyzing sALT levels. Pretreatment with either anti-IFN-γ or anti-TNF-α mAb completely inhibited hepatocellular injury as compared with that with control rat-IgG. Each value represents the mean ± SD of results in three mice. The point marked (**P < 0.001) is significantly lower than a group of mice treated with control antibody (A). Histopathological examinations revealed that administration of either anti-IFN-γ (B) or anti-TNF-α mAb (C) (150 µg/mouse) completely inhibited the development of hepatic fibrosis 1 week after the fourth weekly Con A injection. Azan staining was performed for (B) and (C). Original magnification ×100. Representative figures derived from the analysis of three mice are shown in each group.

structures that occur in liver cirrhosis have been described in detail (24), the immunopathogenesis of fibrosis in this process is largely unknown. In order to elucidate the underlying mechanisms, an appropriate animal model with essential similarities to patients with chronic hepatitis should be established. Ideally, the animal model should have the following features: (i) it should be based upon immunological mechanisms, (ii) hepatic fibrosis should be induced following continuous liver injury and (iii) the inducer of hepatic fibrosis is should not be a direct hepatotoxin. However, no animal model that satisfies these criteria has been established.

As cited above, the animal model of liver injury induced by HBV-specific CTL in HBV transgenic mice has similarities to human cases (6). However, liver injury cannot be continuously induced, since the expression of HBsAg is down-regulated by the CTL.

It has been recently described that Con A-induced experimental liver injury in mice is mediated by activated T cells (25,26). Con A is a lectin from jack bean and is known as a T lymphocyte mitogen in vitro. Tiegs et al. reported that acute liver injury was induced within 8 h after i.v. injection of Con A in BALB/c mice. The organ specificity was attributed to the preferential binding of Con A in the liver. Because immunodeficient mice (SCID or athymic mice) or mice treated with immunosuppressive drugs such as cyclosporine, FK506 and corticosteroids do not develop hepatitis after Con A injection, it is accepted that the model requires T cell activation. Cell depletion experiments identified CD4 T cells as the effector cells (25). Furthermore, neutralization experiments revealed that TNF-α and IFN-γ play critical roles in the development of Con A-induced hepatitis (15,16). Since these results indicate that Con A-induced hepatitis is based upon immunological mechanisms and has similarities to chronic inflammatory liver disease in man, Con A-induced hepatitis model was thought to be one of the potent models to induce liver fibrosis mediated by hepatocellular injury.

In the present study, we established a hepatic fibrosis model with repeated administrations of Con A (Fig. 2). Hepatocellular injury was consistently observed in terms of sALT elevation 24 h after each Con A injection (Fig. 1A and B) following an
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Fig. 4. The effect of anti-TNF-α mAb and anti-IFN-γ mAb on the kinetics of serum levels of IFN-γ (A) and TNF-α (B) after Con A injection. Control rat IgG, anti-IFN-γ or anti-TNF-α mAb (150 µg/mouse) were administered i.p. 24 h before a single Con A injection (0.3 mg/mouse). Hepatocellular injury was monitored by analyzing sALT levels. Each value represents the mean ± SD of results in five mice. The point marked (**P < 0.001) is significantly higher than a group of mice treated with either anti-TNFα mAb or anti-IFNγ mAb.

Fig. 5. Cytokine expression in the liver after Con A administration. Hepatic TGF-α, bFGF, HGF and aFGF mRNA levels were analyzed by semiquantitative RT-PCR at indicated time points after Con A injection (0.3 mg/mouse) (A). TGF-β1 mRNA was assessed by competitive RT-PCR (B). Liver samples were obtained at the times shown. The number of amplification cycles was 28 for each set of primers. Dilutions of cDNA were amplified for 20, 23, 25, 28, 30, 32, 34, 36 and 38 cycles to define optimal conditions for linearity and to permit semiquantitative analysis of signal strength. Representative figures from the analyses of four mice are shown in each group. G3PDH was used as the internal control (A). The quantitation of TGF-β1 mRNA was performed by competitive RT-PCR using the PCR Mimic protocol (Clontech). The concentrations of competitors were as follows: a = 5×10⁻¹, b = 1.5×10⁻¹, c = 6.25×10⁻² and d = 5×10⁻² amol/µl.

increase in serum levels of both IFN-γ and TNF-α (Fig. 2A). Since hepatocellular injury was completely inhibited by pretreatment with either anti-IFN-γ or anti-TNF-α mAb (Fig. 3A), cytokine involvement could be proven in this model.

Biosynthesis and turnover of the extracellular matrix are thought to be regulated by different cytokines and growth factors, including TGF-β, FGF, insulin-like growth factor, TGF-α and HGF. In particular, TGF-β has been identified as the
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Fig. 6. Prevention of Con A-induced hepatic fibrosis by anti-TGF-β mAb. Either bovine IgG (200 µg/mouse) or anti-TGF-β mAb (200 µg/mouse) was administered i.p. into BALB/c mice simultaneously with each Con A injection (0.3 mg/mouse). Hepatocellular injury was monitored by analyzing sALT levels 24 h after each weekly Con A injection. Each value represents the mean ± SD of results in three mice (A). Histopathological evaluation of the liver 1 week after the fourth Con A injection with either control bovine IgG (200 µg/mouse, B) or anti-TGF-β mAb (200 µg/mouse, C). Azan staining was performed for (B) and (C). Note the marked inhibition of hepatic fibrosis in (C) as compared with that in (B). Original magnification x200. Representative figures derived from the analyses of three mice are shown in each group.

most potent fibrogenic mediator, contributing to immune and inflammatory responses, tissue repair, and cell growth and differentiation (27). Thus, it is speculated that TGF-β is one of the major causes of fibrosis in hepatic cirrhosis, glomerulonephritis and idiopathic pulmonary fibrosis (1,28,29).

In the present study, expression of TGF-α, HGF, bFGF and TGF-β1 mRNAs was up-regulated after each Con A administration (Fig. 5A and B). Using an experimental model of schistosomiasis in mice, Czaja et al. similarly found an increase in the content of hepatic TGF-β1 mRNA in infected animals, in parallel with an increase in type I procollagen mRNA (30). Nakatsukasa et al. also demonstrated a positive correlation between TGF-β1 mRNA, and procollagen I, III and IV mRNAs in the livers of rats with carbon tetrachloride-induced hepatic fibrosis (31). It has furthermore been reported that hepatic TGF-β1 mRNA was enhanced in patients with chronic liver disease (23).

In two recent studies, TGF-β1 activity has been successfully suppressed in vivo with anti-TGF-β1 antibodies, resulting in a significant decrease in excess extracellular matrix deposition (32,33). In the present study, simultaneous administration of neutralizing TGF-β mAb with each Con A injection markedly reduced hepatic fibrosis and extracellular matrix formation (Fig. 6B), although it exerted no significant effects on hepatocellular injury. On the other hand, treatment with either anti-IFN-γ or anti-TNF-α mAb completely prevent hepatic injury (Fig. 3A). Thus, while treatment with anti-IFN-γ, anti-TNF-α or anti-TGF-β mAb markedly prevented hepatic fibrosis, the prevention was based on different mechanisms. Furthermore, serum levels of IFN-γ were significantly increased by the simultaneous treatment of anti-TGF-β mAb 4 h after Con A injection (Fig. 7A), but no equivalent influence on TNF-α was observed. Reflecting these results, hepatic mRNA expression of IFN-γ was enhanced; in contrast, that of TNF-α was not enhanced by the simultaneous treatment of anti-TGF-β mAb (Fig. 7B). Thus, the mechanism of prevention of hepatic fibrosis by anti-TGF-β mAb is clearly not through the down-regulation of either IFN-γ or TNF-α synthesis. Since, treatment with either anti-IFN-γ or anti-TNF-α mAb did not affect hepatic mRNA expression of TGF-β1 after Con A injection (Fig. 7B), as compared with Con A injection alone, their influences are separate. This result indicated that TGF-β synthesis is not
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Fig. 7. Relationships between TGF-β, IFN-γ and TNF-α in Con A-induced hepatic fibrosis. Serum levels of IFN-γ and TNF-α were analyzed after Con A injection either with or without simultaneous treatment of anti-TGF-β mAb (i.p., 200 µg/mouse) at indicated time points. Serum levels of IFN-γ were significantly increased by the simultaneous treatment of anti-TGF-β mAb 4 h after Con A injection (**P < 0.01). In contrast, serum levels of TNF-α were significantly decreased 8 h after Con A injection (*P < 0.05). Each value represents the mean ± SD of results in three mice (A). The effects of anti-TGF-β mAb on the expression of IFN-γ and TNF-α mRNA in liver after Con A injection (B, upper panel). Liver tissues were derived from BALB/c mice 4 h after Con A injection (at a time of peak expression of either IFN-γ and TNF-α mRNA; data not shown). The effect of either anti-IFN-γ or TNF-α mAb on the expression of TGF-β mRNA in liver after Con A injection (B, lower panel). Liver tissues were derived from BALB/c mice 48 h after Con A injection (at a time of peak expression of TGF-β mRNA; see Fig. 5B). The number of amplification cycles was 28 using various cytokine-specific primers and quantitation of mRNAs was performed by using the PCR Mimic protocol (Clontech). The concentrations of competitors were as follows: a = 5 × 10⁻¹, b = 1.5 × 10⁻¹, c = 6.25 × 10⁻², d = 5 × 10⁻², e = 1.5 × 10⁻², f = 5 × 10⁻¹, g = 1.5 × 10⁻¹, and h = 6.25 × 10⁻¹, attomol/µl (B). Results are from the analyses of three mice in each group.

sufficient to develop hepatic fibrosis. In addition, since treatment with anti-TGF-β mAb prevented fibrosis, repeated liver injury is also not enough to induce hepatic fibrosis.

An interesting report has recently been published regarding the correlation between TGF-β1 and liver fibrosis. (34). The authors established TGF-β1 transgenic mice and indicated that marked collagen deposition was shown localized to the sinusoids and around periportal areas. However, liver fibrosis such as bridging fibrosis with piecemeal necrosis was not detectable in parenchyma of the TGF-β1 transgenic mice. In consideration of this result, we speculate that the space caused by liver necrosis in parenchyma is necessary to induce liver fibrosis.

Furthermore, we administered Con A into female BALB/c mice until the 12th injection to examine whether Con A-induced liver fibrosis is progressive. We found that liver
fibrosis in the parenchyma was also detectable 1 week after either the eighth or 12th Con A injection by Azan stain and massive liver necrosis was detectable. However, we could not find any apparent difference in the degree of liver fibrosis among the groups 1 week after the fourth, eighth and 12th Con A injection.

We also observed untreated mice after the fourth Con A injection. Interestingly, at 3 weeks after the fourth injection liver fibrosis in the parenchyma improved. We speculate these results are caused by the regenerative capacity of mice hepatocytes and the decomposing capacity of liver fibrosis. At present the mechanism of these results has not been defined and we need to investigate this hypothesis using this mouse model.

In conclusion, the simultaneous presence of both repeated severe liver damage and up-regulation of TGF-β synthesis are essential to induce hepatic fibrosis in this model.

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Abbreviations
- aFGF: acidic fibroblast growth factor
- bFGF: basic fibroblast growth factor
- Con A: concanavalin A
- HbAg: hepatitis B surface antigen
- HBV: hepatitis B virus
- HCC: hepatocellular carcinoma
- HGF: hepatocyte growth factor
- sALTA: serum alanine aminotransferase
- TGF: transforming growth factor
- TNF: tumor necrosis factor

References
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