ABSTRACT
Metallothionein (MT) gene therapy leads to resolution of liver fibrosis in mouse model. The present study was undertaken to test the hypothesis that reversal of the phenotype of activated hepatic stellate cells (HSCs) contributes to the fibrinolysis effect of MT. Human HSC LX-2 cells were activated after they were cultured for 24 hours, as indicated by expression of α-smooth muscle actin (α-SMA) and collagen-I and depressed expression of collagenases. Transfection with a plasmid containing human MT-IIA gene in the activated HSCs effectively increased the protein level of MT. The expression of MT was accompanied by the reduction in protein levels of α-SMA and collagen-I and a decrease in their mRNA levels. Of importance, MT gene transfection resulted in upregulation of matrix metalloproteinases 1, 8, and 13, which are involved in the resolution of liver fibrosis. This study demonstrates that reversal of the phenotype of activated HSCs, particularly the upregulation of collagenases, is likely to be involved in the resolution of liver fibrosis observed in MT gene therapy.

Introduction
Liver fibrosis resulting from excessive accumulation of extracellular matrix (ECM) proteins represents a uniform response of the liver to chronic insults of toxic and infectious agents and metabolic stress (Pietrangelo, 1996; Ikejima et al., 2001; Monto et al., 2004). Collagens, predominately type I and III, are the major fibrous proteins in ECM, and their synthesis increases and their degradation decreases in the liver undergoing fibrogenesis (Masuda et al., 1994; Kim et al., 1999; Inagaki et al., 2003). The enzymes that are involved in the ECM accumulation include lysyl oxidase, lysyl hydroxylase, and prolyl hydroxylase (Bickel et al., 1998). The degradation of ECM is dependent on the activity of matrix metalloproteinases (MMPs) (Okazaki et al., 2000). According to their substrate specificity, MMPs fall into five categories: collagenases (MMP-1, -8, -13), gelatinases (MMP-2, -9), stromelysins (MMP-3, -7, -10, -11), membrane type MMPs (MMP-14, -15, -16, -17, -24, -25), and metalloelastase (MMP-12) (Okazaki et al., 2000). The activities of these enzymes are altered during the processes of fibrogenesis and fibrinolysis (Knittel et al., 2000; Okazaki et al., 2000; Watanabe et al., 2001), accompanied by changes in the activities of tissue inhibitors of metalloproteinases (TIMPs). The imbalance in the activities between MMPs and TIMPs makes a critical contribution to the deposition of collagen proteins (Watanabe et al., 2000; Lee et al., 2001).

Our early studies using human metallothionein IIA (hMT-IIA) as a therapeutic agent observed that adenovirus-mediated hMT-IIA gene transfection significantly reversed liver fibrosis induced by carbon tetrachloride in a mouse model (Jiang and Kang, 2004). Wild-type (WT) mice treated with carbon tetrachloride for 4 weeks developed reversible liver fibrosis after removal of the chemical, correlating with a high level of hepatic MT; however, for 8 weeks, they developed irreversible liver fibrosis along with low levels of hepatic MT. The same carbon tetrachloride treatment of 4 weeks resulted in irreversible liver fibrosis in MT knockout mice. Adenoviral delivery of hMT-IIA gene therapy reversed the fibrosis along with increased hepatocyte regeneration in both WT and MT knockout mice with irreversible fibrosis.

MT is an important zinc-binding protein and is involved in zinc metabolism and homeostasis (Vallee, 1995; Spahl et al., 2003; Martinez et al., 2004; Kang, 2006). The interaction of MT with a number of oxidants causes a release of zinc bound to the protein (Lee et al., 2003; Maret, 2003; Spahl et al., 2003; Feng et al., 2006), and the released zinc would thus affect the activities of enzymes involved in fibrogenesis and fibrinolysis in the liver. Therefore, the present study was undertaken to test the hypothesis that hMT-IIA transfection changes the profile of expression of metalloproteinases in human hepatic stellate cells, a cell population critically involved in the initiation and progression of liver fibrosis.

Materials and Methods
Cultures of Human Hepatic Stellate Cells. Human hepatic stellate (HSC) (LX-2) cells were obtained as a generous gift from

ABBREVIATIONS: α-SMA, α-smooth muscle actin; ECM, extracellular matrix; hMT-IIA, human metallothionein IIA; HSC, hepatic stellate cells; MMPs, matrix metalloproteinases; MT, metallothionein; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; TGF-β1, transforming growth factor-β1; TIMPs, tissue inhibitors of metalloproteinases.
Dr. Scott L. Friedman at Mount Sinai School of Medicine, New York.

The cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen, Carlsbad, CA) supplemented with 10% (v/v) fetal bovine serum (Invitrogen, Auckland, NZ) and 100 U/ml penicillin, 100 μg/ml streptomycin, 3.7 mg/ml NaHCO₃, and 2.4 mg/ml HEPES. Cells were routinely maintained in a standard culture incubator with humidified air containing 5% CO₂ at 37°C. Twenty-four hours after culturing, the cells displayed a myofibroblast-like cell phenotype, including strong α-smooth muscle actin (α-SMA) staining and collagen I production.

**Transfection with hMT-IIA Gene.** A pEGFP-N1-hMT-IIA plasmid was transfected into LX-2 cells with Lipofectamine 2000 reagent according to the manufacturer’s instructions. In brief, LX-2

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**TABLE 1**
The primer sequence for real-time reverse-transcription PCR

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
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<tbody>
<tr>
<td>β-actin</td>
<td>CCACGAAACTACCTTCAACTCC</td>
<td>GTGATCTCCTTGTGCATCTCTGT</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>TGTTGGAACCCCAACACGAA</td>
<td>GAGCAACACCGGTTCAGGTAA</td>
</tr>
<tr>
<td>MMP1</td>
<td>CCAGCCCACTGGCCCCACAAA</td>
<td>GCCGTTCAAGCCCTTTGCTGA</td>
</tr>
<tr>
<td>MMP8</td>
<td>TGGCCATCTTGGGGCTGC</td>
<td>TGGGGTCACAGGGTGTTGTTG</td>
</tr>
<tr>
<td>MMP13</td>
<td>ATTGGGCTGGGCTTGCGCTT</td>
<td>TGGAGGCTTCATGTCATGAGC</td>
</tr>
<tr>
<td>α-SMA</td>
<td>CAGCCAAGGACGTAGGAAT</td>
<td>TTGCTCTCTGTCGTCAC</td>
</tr>
<tr>
<td>COL1A1</td>
<td>CATGCTACGTTTGGACTTC</td>
<td>TCAACATGGGTCAC</td>
</tr>
<tr>
<td>COL5A1</td>
<td>CTCTCCTCCAGGGGACTTC</td>
<td>GTATGTGTCAGGGCCTTCG</td>
</tr>
<tr>
<td>TIMP1</td>
<td>CTCGTCACTGGCCAAAGTT</td>
<td>GGACCTGGGAAATTCGCG</td>
</tr>
<tr>
<td>TIMP2</td>
<td>CTTCGAGGGAAATCGGGTA</td>
<td>TCACTGTTGTCAGGGG</td>
</tr>
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COL1A1, collagen type I α1; COL3A1, collagen type III α1.

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**Fig. 1.** The expression of α-SMA and collagen I in human hepatic stellate LX-2 cells cultured for varying times. (A) The immunostaining of α-SMA (upper, red) appeared to be positive, with increasing intensity after the cells were cultured for 16 hours. The expression of collagen I (bottom, red) increased markedly after the cells were cultured for 24 hours (bar = 50 μm). (B) Western blot analysis of the changes in protein contents of α-SMA (left) and collagen I (right) in LX-2 cells cultured for different times. The expression of α-SMA and collagen I increased gradually with the increase in culturing times. The semiquantitative data were obtained from three independent experiments and expressed as mean ± S.E.M. The expression of collagen I was quantified as the sum of intensity of collagen I and mature collagen I (M-Collagen I). *Significantly different from 8 hour (P < 0.05).
cells were plated into 6-well dishes (1 × 10^5 cells/well) or 24-well dishes (3 × 10^5 cells/well) in Dulbecco’s modified Eagle’s medium containing 10% (v/v) fetal bovine serum. After cell cultures reached 90–95% confluence, the constructed pEGFP-N1-hMT-IIA expression vector and the negative control pEGFP-N1 vector were respectively transfected into LX-2 cells with the ratio of DNA (μg) to lipofectamine (μl) at 1:1. Four hours after the transfection, the plasmid/lipofectamine solution was replaced by cell growth medium with antibiotics. The cells were collected 12 hours after culturing in the fresh media for further analyses.

**Immunocytochemical Analysis of HSC Activation.** The LX-2 cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4) for 15 minutes at room temperature and then permeabilized with PBS containing 0.25% Triton X-100 (Millipore Corporation, Billerica, MA) for 10 minutes. After blocking with 10% goat serum in PBS containing 0.1% Tween-20 for 30 minutes, the cells were incubated with primary antibodies (anti-α-SMA and anti-collagen I; Abcam, Cambridge, MA) diluted in blocking solution, according to the manufacturer’s instructions, overnight at 4°C. Then, the fluorescent-conjugated secondary antibody (Jackson Immunoresearch Laboratories, West Grove, PA) was used to detect the target protein. Nuclei were counterstained with 0.5 μg/ml Hoechst (Sigma-Aldrich, St. Louis, MO) for 10 minutes. All photographs were taken using an Olympus microscope.

**Western Blotting.** The protein contents of α-SMA and collagen I in LX-2 cells were determined by Western blot. Cells scraped in PBS were washed 3 times and lysated using RIPA buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 0.5% sodium deoxycholate, 1% NP-40, and 0.1% SDS) supplemented with 1% complete EDTA-free protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) and 1 mM phenylmethanesulfonyl fluoride. Equal loading of proteins was assured by prior quantitation using the Bio-Rad assay procedure (Bio-Rad Laboratories, Hercules, CA). Protein samples were mixed with 5× loading buffer, boiled for 10 minutes at 100°C and cooled. Equal amounts of 30 μg protein from each sample were separated by 10% SDS-PAGE. Proteins were electrophoretically transferred to a polyvinylidene fluoride membrane (Bio-Rad Laboratories). Transferred proteins were blocked with 5% nonfat dry milk in Tris-HCl buffer solution containing Tris-HCl (50 mM), NaCl (150 mM), and Tween-20 (0.1%) for 1 hour at room temperature. The blots were then incubated with respective primary antibodies (anti-α-SMA and anti-collagen I; Abcam) in blocking solution, according to the vendor’s recommendations. After incubation, the blots were washed with Tris-buffered saline/Tween 20 six times for 5 minutes each. The blots were incubated for 1 hour with appropriate secondary antibody. After washing six times (5 minutes each), target proteins were visualized using a chemiluminescence horse-radish peroxidase substrate (Millipore Corporation) and analyzed by densitometry with use of a Quantity One Software (Bio-Rad Laboratories).

**Real-Time Quantitative Reverse-Transcription Polymerase Chain Reaction (PCR).** Total RNA was extracted from LX-2 cells collected after treatment for 24 hours with TRIzol reagent (Life Technologies, Carlsbad, CA) according to the manufacturer’s instructions. RNA concentration was quantified using a Gene Quanti pro (GE Healthcare, Waukesha, WI). Complementary DNAs (cDNAs) were synthesized using an iScript cDNA Synthesis Kit (Bio-Rad Laboratories) in MJ mini personal thermal cycler (Bio-Rad Laboratories). The amount of cDNA corresponding to 50 ng of RNA was amplified using a SYBR green PCR kit (Bio-Rad Laboratories) with the primers for α-SMA, collagen type I α1, collagen type III α1, MMP-1, MMP-8, MMP-13, transforming growth factor-β1 (TGF-β1), TIMP-1, TIMP-2, and β-actin. The primer sequences (Table 1) were designed and synthesized by Invitrogen. Real-time reverse-transcription PCR was performed, recorded, and analyzed by the iCycler.

**Statistics.** Data were obtained from three separate experiments and presented as mean values ± S.E.M. The results presented in Fig. 1 were analyzed using Student’s t test. One-way analysis of variance was used for initial analysis of the results presented in Figs. 2–4, followed by Dunnett’s T3 test for Fig. 2 and Bonferroni t test for Figs. 3 and 4 for comparison among multiple groups. All data were calculated using the software program SPSS (SPSS, Chicago, IL) for Windows, version 19.0. P values <0.05 were considered to be statistically significant.

**Results**

The activation of human hepatic stellate LX-2 cells was observed after these cells were cultured for 24 hours, as indicated by the expression of α-SMA and collagen I (Fig. 1). The fluorescent staining of α-SMA in LX-2 cells appeared to be positive, with increasing intensity after the cells were cultured for 16 hours. Western blot analysis of the proteins isolated from these cells showed a significant increase in the protein contents of α-SMA and collagen I. Along with this activation, gene expression profile underwent significant changes. As shown in Fig. 2, the mRNA levels for α-SMA and collagen I significantly increased as a function of time, and MMP-1, MMP-8, and MMP-13 were gradually decreased. Other genes measured, including TGF-β1, collagen III, TIMP-1, and TIMP-2, were not changed.

Transfection with the pEGFP-hMT-IIA plasmid significantly increased the protein levels of the transgene product (Fig. 2). Of importance, the expression of human MT-IIA significantly reversed the phenotype of the activated LX-2 cells. The levels of α-SMA and collagen I significantly decreased, as demonstrated by both fluorescent staining of LX-2 cells in cultures and Western blot analyses of proteins (Fig. 3). With this phenotype reversal, the gene expression profile also changed. The expression of α-SMA and collagen I and III were suppressed; the expression of MMP-1, MMP-8, and MMP-13 were significantly increased; and the expression of TGF-β1 and TIMP-1 and -2 were not affected (Fig. 4).
**Discussion**

The data obtained from this study reveal that effective transfection of human stellate LX-2 cells with hMT-IIA gene resulted in the production of hMT-IIA in the cells. The hMT-IIA expression changed the phenotype of the LX-2 cells from its pro-fibrogenic state to pro-fibrolytic state. This was evidenced by the suppression of myofibroblast-like cell markers, such as the increased expression of α-SMA and collagen I and the decreased expression of MMP-1, -8, and -13. All of these changes, however, were not only suppressed, but also reversed by hMT-IIA expression.

We previously demonstrated that MT gene therapy reversed liver fibrosis induced by carbon tetrachloride in a mouse model (Jiang and Kang, 2004). It is important to understand the mechanism by which MT gene therapy made the irreversible liver fibrosis become reversible. Interstitial collagenases, including MMP-1, -8, and -13, are important metalloproteases involved in the liver fibrinolysis. For this reason, several studies have determined the role of collagenases in the recovery of established liver fibrosis. For instance, human MMP-8 delivered by an adenovirus vector (AdMMP-8) was shown to effectively reverse liver fibrosis (Garcia-Banuelos et al., 2002). The same AdMMP-8 system was also shown to be effective in reducing liver fibrosis in rats with bile duct–ligated cirrhosis (Siller-Lopez et al., 2004). A similar approach but using MMP-1 delivered by an AdMMP-1 construct was also
accompanied by the decrease in elevated in the hMT-IIA results, the mRNA levels for MMP-1, -8, and -13 were all effect of hMT-IIA gene transfection on the expression of fibrolytic activity in HSC cells. Therefore, we examined the 2003). type I Fig. 2. *Significantly different from control (P 0.05). COL1A1, collagen a-SMA and collagen-I proteins. Their mRNA levels were also depressed. The combination of the increased collagenases and the depressed expression of collagen mRNAs would contribute to the decreased protein levels of collagens, although the mechanisms by which the hMT-IIA expression suppressed the expression of collagens and increased the expression of collagenases were unknown.

The ECM protein deposition in the fibrotic liver results primarily from the activated HSCs (Li and Friedman, 1999; Friedman, 2008). HSCs from normal liver represent about 15% of the total hepatic cell populations and show a quiescent phenotype (rich in vitamin-A fat droplets). The activation of HSCs is a key step in liver fibrogenesis; thus, experimental approaches to antifibrotic therapy have focused on manipulation of the HSCs, such as suppressing their activation. In a rat model of spontaneous resolution of liver fibrosis, it was observed that liver fibrosis induced by the treatment with carbon tetrachloride for 4 weeks was reversible after cessation of the treatment (Iredale et al., 1998). During the recovery phase, activated HSCs (myofibroblast-like cells) underwent apoptosis, peaking on the third day after the last dose and leading to a significant decrease in the number of the myofibroblast-like cells (Iredale et al., 1998). Therefore, it was concluded that apoptosis of the activated HSCs is responsible for the spontaneous resolution of the reversible liver fibrosis (Iredale et al., 1998).

The activated HSCs are a major source for MMPs and TIMPs (Iredale et al., 1996; Iredale, 1997; Knittet et al., 1999).

In the early phase of liver injury, HSCs transiently express MMP-3, MMP-13 and uroplasminogen activator, leading to degradation of ECM (Benyon and Arthur, 2001). After transdifferentiated, the myofibroblast-like cells express a combination of MMPs, such as pro-MMP-2 and membrane type 1-MMP (MMP-14), which have the ability to degrade normal liver matrix (Benyon and Arthur, 2001). In addition, there is a marked increase in the expression of TIMP-1 and TIMP-2, leading to the inhibition of the interstitial collagenases (MMP-1, -8, and -13), thus suppressing the degradation of the fibrillar collagens (Atkinson et al., 2001; Benyon and Arthur, 2001). Apoptosis of the activated HSCs will remove the cell population responsible for excessive deposition of ECM collagens and the inhibitors for interstitial collagenases, but by the same mechanism, the major source of collagenases is also removed. This may represent the scenario of the irreversibility of the advanced liver fibrosis even after the removal of the causative agent (Iredale et al., 1998).

We previously observed that MT gene therapy resulted in resolution of irreversible liver fibrosis (Jiang and Kang, 2004). This would not be simply explained by MT promotion of apoptosis of the activated HSCs. The data presented here showed that the phenotype reversal of activated HSCs would be an alternative explanation. In this context, the transfection with the hMT-IIA gene retained the life of HSCs but transformed the state of these cells from the pro-fibrogenic to the pro-fibrolytic. This change would make HSCs actively involved in the resolution of the irreversible liver fibrosis observed in the MT gene therapy. In addition, MT promotes the regeneration of hepatocytes (Cherian and Kang, 2006), making another contribution to the resolution.

As mentioned above, the limitation of this study is that the mechanism by which the transfection with the hMT-IIA upregulates the expression of collagenases remains unknown. One of the important functions of MT is its regulation of zinc homeostasis; as a zinc chaperone, MT transfers zinc to other zinc-binding proteins (Feng et al., 2005). Whether this zinc chaperone function is related to the regulation by MT of the expression of collagenases will be investigated in our future studies. In this study, we also observed that the expression of TGF-β1 was not affected by MT transfection, which was unexpected. However, it is possible that the interaction between hepatic cells and HSCs is important for this gene expression, but under current experimental condition, this interaction was eliminated because of the pure HSC population in cultures. Another limitation is that the LX2 cell culture system does not necessarily reflect culture-activated primary hepatic stellate cells; therefore, further confirmation in primary hepatic stellate cells should be included in future studies.

In summary, this study presents evidence that shows effective transfection of human stellate LX-2 cells with the hMT-IIA gene reverses the phenotype of the activated LX-2 cells from its pro-fibrogenic state to the pro-fibrolytic state. This change would be critically involved in the reversal of the advanced liver fibrosis subjected to the hMT-IIA gene therapy observed in our previous studies. Therapeutic approaches to reverse the phenotype of the activated HSCs, rather than to promote apoptosis of these cells, would be alternatively beneficial to patients with advanced liver fibrosis. Upregulation of MT production in fibrotic liver would be one of these approaches.
References


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