Metallothionein gene transfection reverses the phenotype of activated human
hepatic stellate cells

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ABBREVIATIONS: α-SMA, α-smooth muscle actin; COL1A1, collagen type I alpha 1; COL3A1, collagen type III alpha 1; DMEM, Dulbecco’s modified Eagle’s medium; ECM, extracellular matrix; EDTA, Ethylene Diamine Tetraacetic Acid; FBS, fetal bovine serum; HEPES, 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid; hMT-IIA, human metallothionein IIA; HSC, hepatic stellate cells; MMPs, matrix metalloproteinases; MT, Metallothionein; MT-KO, Metallothionein knockout; PMSF, phenylmethanesulfonyl fluoride; TBS-T, Tris-buffered saline/Tween 20; TGF-β1, transforming growth factor-β1; TIMPs, tissue inhibitors of metalloproteinases;
Cellular and molecular section
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 (HSC) contributes to the fibrinolysis effect of MT. Human HSC LX-2 cells were activated after cultured for 24 hrs, 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 of protein levels of α-SMA and collagen-I, and the decrease in their mRNA levels. Importantly, MT gene transfection resulted in upregulation of MMP-1, -8 and -13, which are importantly 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 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 as well as metabolic stress (Ikejima, et al., 2001; Monto, et al., 2004; Pietrangelo, 1996). 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 (Inagaki, et al., 2003; Kim, et al., 1999; Masuda, et al., 1994). 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 make a critical contribution to the deposition of collagen proteins (Lee, et al., 2001; Watanabe, et al., 2000).

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 wks developed a reversible
liver fibrosis upon removal of the chemical, correlating with a high level of hepatic MT; but for 8 wks developed an irreversible liver fibrosis along with low levels of hepatic MT. The same carbon tetrachloride treatment for 4 wks resulted in an irreversible liver fibrosis in MT knockout (MT-KO) mice. Adenoviral delivery of hMT-IIA gene therapy reversed the fibrosis along with increased hepatocyte regeneration in both WT and MT-KO mice with irreversible fibrosis.

MT is an important zinc-binding protein and is involved in zinc metabolism and homeostasis (Kang, 2006; Martinez, et al., 2004; Spahl, et al., 2003; Vallee, 1995). The interaction of MT with a number of oxidants causes a release of zinc bound to the protein (Feng, et al., 2006; Lee, et al., 2003; Maret, 2003; Spahl, et al., 2003), and the released zinc would thus affects 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 (HSCs). Human HSC (LX-2) cells were obtained as a generous gift from 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 (FBS, 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-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 (Invitrogen, Auckland, NZ) according to the manufacturer’s instructions. In brief, LX-2 cells were plated into 6-well dishes (1 × 10⁵ cells/well) or 24-well dishes (3 × 10⁴ cells/well) in DMEM containing 10% (v/v) FBS. 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) : 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 hrs after culturing in the fresh media for further analyses.
**Immunocytochemical analysis of HSC activation.** The LX-2 cells were fixed in 4% paraformaldehyde in PBS (pH 7.4) for 15 min at room temperature, and then permeabilized with PBS containing 0.25% Triton X-100 for 10 min. After blocking with 10% goat serum in PBST for 30 min, 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, USA) was used to detect the target protein. Nuclei were counterstained with 0.5 μg/ml Hoechst (Sigma, St. Louis, MO) for 10 min. 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 (PMSF). 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 min at 100°C and cooled. Equal amounts of 30 μg protein from each sample were separated by 10% SDS-polyacrylamide electrophoresis gel. Proteins were electrophoretically transferred to a polyvinylidene fluoride membrane (Bio-Rad Laboratories, Hercules, CA). Transferred proteins were blocked with 5% non-fat dry milk in tris-HCl buffer solution containing tris-HCl (50
mM), NaCl (150 mM), and Tween-20 (0.1%) (TBS-T) for 1 hr at room temperature. The blots were then incubated with respective primary antibodies (anti-α-SMA and anti-collagen I, Abcam, Cambridge, MA) in blocking solution according to the vendor’s recommendations. After incubation, the blots were washed with TBS-T six times for 5 min each. The blots were incubated for 1 hrs with appropriate secondary antibody. After washing six times (5 min each), target proteins were visualized using a chemiluminescence horseradish peroxidase substrate (Millipore Corporation, Billerica, MA) and analyzed by densitometry using a Quantity One Software.

**Real-time quantitative RT-PCR.** Total RNA was extracted from LX-2 cells collected after treatment for 24 hrs with TRIzol reagent (Life Technologies, Carlsbad, CA) according to the manufacturer’s instruction. RNA concentration was quantified with a Gene Quant pro (Amersham Biosciences, GE healthcare, USA). Complementary DNAs (cDNAs) were synthesized using an iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA) in MJ mini personal thermal cycler (Bio-Rad Laboratories, Hercules, CA). The amount of cDNA corresponding to 50 ng of RNA was amplified using a SYBR green PCR kit (Bio-Rad Laboratories, Hercules, CA) with the primers for α-SMA, COL1A1, COL3A1, MMP-1, MMP-8, MMP-13, TGF-β1, TIMP-1, TIMP-2 and β-actin. The primer sequences (Table 1) were designed and synthesized by Invitrogen. Real-time RT-PCR reactions were 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 Figure 1 were analyzed by
Student’s t-test. One-way ANOVA was used for initial analysis of the results presented in Figure 2, 3, and 4, followed by Dunnett’s T3 test for Figure 2 and Bonferroni t-test for Figure 3 and 4 for comparison among multiple groups. All data were calculated using the software program SPSS (Chicago, IL) for Windows, version 19.0. P values < 0.05 were considered statistically significant.
Results

The activation of human hepatic stellate LX-2 cells was observed after these cells were cultured for 24 hrs, as indicated by the expression of α-SMA and collagen I (Fig 1). The fluorescent staining of α-SMA in LX-2 cells appeared positive with increasing intensity after the cells were cultured for 16 hrs. 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-MT-IIA plasmid significantly increased the protein levels of the transgene product (Fig 2). Importantly, 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 were suppressed, the expression of MMP-1, MMP-8, and MMP-13 were significantly increased, and the expression of collagen III, TGF-β1, TIMP-1 and -2 were not affected (Fig 4).
Discussion

The data obtained from this study showed 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 bile duct-ligated cirrhosis rats (Siller-Lopez, et al., 2004). A similar approach but using MMP-1 delivered by an AdMMP-1 construct was also effective in reversing liver fibrosis in rats (Iimuro, et al., 2003).
Upregulation of the collagenases would thus enhance the fibrolytic activity in HSC cells. Therefore, we examined the effect of hMT-IIA gene transfection on the expression of collagenases in human stellate LX-2 cells. As shown in the results, the mRNA levels for MMP-1, -8 and -13 were all elevated in the hMT-IIA expressing cells. This increase was accompanied by the decrease in α-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 (Friedman, 2008; Li and Friedman, 1999). 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 upon cessation of the treatment (Iredale, et al., 1998). During the recovery phase, activated HSCs (myofibroblast-like cells) underwent apoptosis, peaking at the 3rd day after the last dosing 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 metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) (Iredale, 1997; Iredale, et al., 1996; Knittel, 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 (MT1)-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 function 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 or not 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 due to 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 the advanced liver fibrosis. Up-regulation of MT production in fibrotic liver would be one of these approaches.
Authorship Contributions

Participated in research design: Kang, Xu.

Conducted experiments: Xu, Shi.

Performed data analysis: Xu, Huang, Kang.

Wrote or contributed to the writing of the manuscript: Kang, Xu.
References


Footnotes

Xu and Shi made equal contributions to this study.

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Figure Legends

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 positive with increasing intensity after the cells cultured for 16 hrs. The expression of collagen I (bottom, red) increased markedly after the cells cultured for 24 hrs. Bar = 50 μm. (B) Western blotting 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 ± SEM.

The expression of collagen I was quantified the sum of intensity of collagen I and mature collagen I (M-Collagen I), *, significantly different from 8 h (P <0.05).

Fig 2. The gene expression profile of the LX-2 cells cultured for different times. The RNA was extracted from the cells cultured for 8, 16, 24, or 48 hrs. The mRNA levels for different proteins as labeled were determined by real-time RCR. All of the data were obtained from three independent experiments, each contained triplicate samples for each gene. The data were expressed as mean ± SEM, *, significantly different from 8 hrs (P <0.05), #, significantly different from 8 hrs and 16 hrs (P <0.05).

Fig 3. The effect of the hMT-IIA gene transfection on the levels of α-SMA and collagen I in the LX-2 cells. The LX-2 cells were cultured for 24 hrs before transfection
with pEGFP-N1-hMT-IIA expression vector or the negative control pEGFP-N1 for 4 hrs, followed by culturing in fresh media for additional 12 hrs. (A) The cells were immunostained with an anti-α-SMA (upper, red) and anti-collagen I (bottom, red) antibody, and (B) the protein contents of EGFP-MT-IIA (EGFP-MT), α-SMA and collagen I were determined by Western blotting. All experiments were repeated three times. The semiquantitative data were obtained and expressed as described in Fig 1, *. significantly different from control (P <0.05).

**Fig 4.** The effect of the hMT-IIA gene transfection on the gene expression of the LX-2 cells. The LX-2 cells were cultured and treated as the same as described for Fig 3. The mRNA levels were determined from these cells at the end of experiments by real-time RCR. All experiments were repeated three times, and the data were obtained and expressed as described for Fig 2, *, significantly different from control (P <0.05).
### Table 1  The primer sequence for Real-time RT-PCR

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<th>Gene name</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<td>β-actin</td>
<td>CCACGAAACTACCTTCAACTCC</td>
<td>GTGATCTCCTTTCTGCATCCTGT</td>
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<tr>
<td>TGF-β1</td>
<td>TGGTGGAAACCCACAACGAA</td>
<td>GAGCAACACGGGTTCAGGTA</td>
</tr>
<tr>
<td>MMP1</td>
<td>CCAGCCCATCGGGCCCACAAA</td>
<td>GCAGCTTCAAGCCCATTTGGCA</td>
</tr>
<tr>
<td>MMP8</td>
<td>TGGCCATTCTTTGGGGCTCGC</td>
<td>TGGGGTCACAGGGGTGTTGGGTGT</td>
</tr>
<tr>
<td>MMP13</td>
<td>ATTGTCCGGCCCTGCCCCTT</td>
<td>TCGGAGGCTCTCTCATGACGAC</td>
</tr>
<tr>
<td>α-SMA</td>
<td>CAGCCAAGCACTGTCAGGAAT</td>
<td>TTTGCTCTGTGCTTCGTCAC</td>
</tr>
<tr>
<td>COL1A1</td>
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