Matrix Metalloproteinase-13 Promotes Recovery from Experimental Liver Cirrhosis in Rats

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Key Words

Fibrosis • Matrix metalloproteinase(s) • Interstitial collagenase • Adenoviral gene transfer • Extracellular matrix • Hepatocyte growth factor

Abstract

Objective: To evaluate the role of matrix metalloproteinase (MMP)-13 gene expression in the early phase of recovery from liver fibrosis/cirrhosis. **Methods:** Liver fibrosis was induced in male Wistar rats by administration of carbon tetrachloride (CCl₄) for 10 weeks. Recombinant adenovirus-mediated human MMP-13 gene transfer (RAdMMP-13) was performed via the femoral vein on day 3 after the last CCl₄ injection. The role of MMP-13 in stably expressing cell lines was also analyzed. **Results:** Fibrous deposition in the liver was decreased in RAdMMP-13-injected rats by day 3 after gene transfer compared with empty vector RAd66-injected rats. Furthermore, MMP-2 and MMP-9 enzymatic activity was markedly enhanced in the liver of RAdMMP-13 injected rats. Hepatocyte growth factor (HGF) induction was also increased in RAdMMP-13 injected rats. In established stable

HT-1080 cells transfected with MMP-13, HGF- α expression and MMP-2 and MMP-9 enzymatic activity were increased. The conversion of precursor HGF into mature HGF was also increased in the MMP-13 expressing cell lines. **Conclusion:** Forced MMP-13 expression effectively accelerated recovery from liver cirrhosis via the effects of MMP-13-mediated HGF, MMP-2, and MMP-9 expression, which induced the degradation of collagen fibers and promoted hepatic regeneration.

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Introduction

Liver fibrosis/cirrhosis is the end stage of most liver diseases regardless of the etiology. Despite remarkable improvements in the treatment of chronic hepatitis C, more than 200 million patients suffer from liver cirrhosis worldwide [1, 2]. Liver failure and portal hypertension are frequently observed in end-stage cirrhosis [3], which is characterized by a high accumulation of extracellular matrix (ECM) [4, 5]. These ECM deposits consist mainly of type I and type III interstitial collagens with fibrous

septa that surround regenerating nodules [4, 6]. Recent advances toward understanding the molecular mechanisms of liver fibrogenesis indicate that liver injury activates hepatic stellate cells which then secrete several cytokines and enzymes that induce liver fibrosis [4, 5]. On the other hand, hepatic stellate cells are also the main contributors to resolving fibrillar collagen deposits [4, 6]. Matrix metalloproteinases (MMPs) specifically degrade ECM proteins and are involved in tissue remodeling during fibrotic and/or inflammatory processes [6–10]. During progressive fibrogenesis, tissue inhibitor metalloproteinases (TIMPs) inhibit MMP activity, causing the accumulation of ECM [11, 12].

MMP-13, the main interstitial collagenase in rodents, is a key enzyme for ECM resolution in liver fibrosis/cirrhosis in rats [13, 14]. We previously reported transient but prominent MMP-13 gene expression during the early phase of recovery from experimental rat liver fibrosis induced by chronic carbon tetrachloride (CCl₄) administration [15, 16]. In addition, MMP-13 mRNA expression is induced in hepatic scar-associated macrophages during spontaneous regression of liver fibrosis [17]. This transient upregulation of MMP-13 occurs during liver fibrogenesis, whereas MMP-13 gene expression is very limited during the recovery phase in liver cirrhosis [15, 16, 18]. It is difficult to ameliorate the pathologic conditions of severe liver fibrosis compared with mild liver fibrosis [19]. Indeed, we previously reported that removing the causative agents of chronic CCl₄-induced liver cirrhosis in rats results in only moderate regression of the fibrosis [16, 20]. Therefore, liver cirrhosis therapy must generate dynamic changes in the balance between the production and degradation of ECM, and the balance must be aimed at ECM resolution.

In the present study, we investigated the effects of MMP-13 on recovery from liver cirrhosis. Overexpression of the MMP-13 gene by recombinant adenovirus transfer dramatically attenuated the extent of experimental liver cirrhosis in vivo via hepatocyte growth factor (HGF) activation and MMP-2 and MMP-9 induction. Our findings reconfirm the importance of interstitial collagenase activation in the current therapeutic strategies for liver cirrhosis.

Materials and Methods

Construction of Recombinant Adenoviral Vectors

The replication-deficient (E1–/E3–) adenovirus RAdMMP-13, harboring the entire cDNA sequence (1,637 bp) coding for human

Fig. 1. Schematic representation of the experimental protocol. To generate rat liver cirrhosis, 30% CCl₄ dissolved in olive oil (1 ml/kg) was administered intraperitoneally at equal intervals twice a week. After 10 weeks of CCl₄ administration, rats were injected via the femoral vein with RAdMMP-13 adenovirus, RAdLacZ control virus, empty vector RAd66, or saline 3 days after the last CCl₄ injection. Rats were sacrificed on day 3, 7, 14, or 30 after the adenoviral gene transfer.

MMP-13, was previously described [21]. Recombinant replication-deficient adenovirus RAdLacZ, which contains the *Escherichia coli* β-galactosidase (*lacZ*) gene regulated by the cytomegalovirus immediate early promoter and the corresponding empty control adenovirus RAd66 were kindly provided by Dr. Gavin W. Wilkinson (University of Cardiff, UK). The dose of recombinant viruses used in the present study (1×10^{10} PFU/rat) was determined based on the number of β-galactosidase-expressing cells in the liver (data not shown).

Induction of Liver Cirrhosis and Adenoviral Injections

All animals used in the present study received humane care, and the experiments were approved by the Animal Experiment Committee of Tokai University. Male Wistar rats weighing 150 g were purchased from CLEA Japan, Inc. (Tokyo, Japan) and injected intraperitoneally with 1 ml/kg of 30% (v/v) CCl₄ in olive oil twice a week for 10 weeks. The CCl₄-injected rats were then divided into the following 4 groups: MMP-13, LacZ, RAd66, and saline ($n = 5$ at each time point in each group). The 4 groups were injected via the femoral vein with adenovirus RAdMMP-13, RAdLacZ control virus, empty virus RAd66, and saline, respectively, on day 3 after the last CCl₄ injection (fig. 1). A subgroup of each group was killed on days 3, 7, 14, and 30 after virus injection and then subjected to morphologic and molecular biologic analyses.

Histologic Examination and Morphometric Analysis of Liver Fibrosis

Conventional histologic examination was performed by hematoxylin and eosin or Azan-Mallory staining of excised liver sections as described previously [16]. Expression of β-galactosidase in the liver tissue was assessed by X-Gal staining using a commercially available kit according to the manufacturer's instructions (Invitrogen, Carlsbad, Calif., USA). The degree of liver fibrosis was semiquantified by morphometric analyses of the Azan-Mallory-stained sections as previously described [16]. The mean area of fibrosis was calculated as the mean percentage for 3 fields ($\times 25$) from each animal.

Table 1. Primers and PCR conditions used for detecting gene expression

Gene (species)	Strand	Oligonucleotide sequences	Annealing temperature, °C	Cycles, n
MMP-13 (human)	sense	5'-GGGTTCTGATGTGGGTGAA-3'	58	35
	antisense	5'-GCTTTTGCCGGTGTAGGTGA-3'		
MMP-9 (rat)	sense	5'-AAGGATGGTCTACTGGCAC-3'	58	35
	antisense	5'-AGAGATTCTCACTGGGGC-3'		
HGF (rat)	sense	5'-CCCGGTGCTGCAGCATGTCCT-3'	58	35
	antisense	5'-TCCCCTCGAGGATTCGACAG-3'		
c-met (rat)	sense	5'-GCATCTGTGTTGTGTACGGT-3'	58	35
	antisense	5'-GCGAACTAATCACTGCCCA-3'		
TNF- α (rat)	sense	5'-GTAGCCCACGTCGTA1GCAAT-3'	58	35
	antisense	5'-CCCTTCTCCAGCTGGGAGAC-3'		
GAPDH (rat)	sense	5'-ACCACAGTCCATGCCATCAC-3'	62	20
	antisense	5'-TCCACCACCCTGTTGCTG TA-3'		

Immunohistochemical and Immunofluorescence Analysis

Immunohistochemical analysis was performed as previously described [16, 20]. Primary antibodies against MMP-9 (Chemicon, Limburg, Germany) and HGF- α (Santa Cruz Biotechnology, Santa Cruz, Calif., USA) were used.

For the immunofluorescence examination, liver tissues were embedded in OCT compound (Sakura, Tokyo, Japan) and snap-frozen in liquid nitrogen. To detect the expression of MMP-13 and MMP-9 in liver tissue, mouse anti-human MMP-13 (Daiichi Fine Chemical, Takaoka, Japan) and rabbit anti-rat MMP-9 (Chemicon) antibodies were used, respectively, together with the appropriate secondary antibodies conjugated with Qdot® 605 or Qdot 655 (Quantum Dot, Hayward, Calif., USA). Antibodies recognizing granulocytes (BD Biosciences, San Jose, Calif., USA) and ED1 (BMA Biomedicals, Augst, Switzerland) were used to identify granulocytes and macrophages, respectively. Nuclei were stained with TOTO-3 (Invitrogen). Fluorescent signals were observed and analyzed using a confocal laser-scanning microscope (LSM 510 META; Carl Zeiss, Jena, Germany). Colocalization signals were confirmed by 3-dimensional analysis using Imaris software version 6.2.1 (Biplane, Zurich, Switzerland).

In general, inflammatory tissues such as cirrhotic liver have abundant autofluorescence signals that can confound analysis [22]. Therefore, specific and nonspecific autofluorescence signals should be separated. We distinguished specific fluorescent signals from background autofluorescence using a linear unmixing analysis as previously described [23, 24] because the traditional method using band-pass filter technology to detect a specific fluorescent signal was not effective (see fig. 2).

Reverse-Transcriptase Polymerase Chain Reaction Analysis

Total RNA was isolated from liver tissue and reverse transcribed as previously described [16]. Table 1 summarizes the polymerase chain reaction (PCR) primers and conditions used to detect expression of human MMP-13, rat MMP-9, rat HGF, its

receptor c-met, rat tumor necrosis factor- α (TNF- α), and rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNAs.

Western Blot Analysis

Western blot analysis was performed as described previously [25, 26]. Immunoblotting was performed using specific antibodies in Tris-buffered saline with 0.05% Tween 20. The primary antibodies were as follows: α -smooth muscle actin (α -SMA), β -actin (Sigma), c-Met and phospho-Met (Cell Signaling, Beverly, Mass., USA), HGF- α (Santa Cruz Biotechnology), MMP-9 (Chemicon), and MMP-13 (Daiichi Fine Chemical). To examine the conversion of pro-HGF into active HGF by MMP-13 expression, anti-rhHGF antibody [which possesses reactivity for both the pro- and active form of HGF (Sigma)], was used as previously described [27, 28].

Zymography

The enzymatic activity of MMPs in the liver tissue and culture supernatant was detected by gelatin and collagen zymography as previously described, with minor modifications [20]. Frozen liver tissue was homogenized in lysis buffer, and the protein concentration was determined using a DC protein Assay Kit (Bio-Rad). To examine the gelatinolytic and collagenolytic activity, SDS-polyacrylamide gels were copolymerized with 1 mg/ml gelatin (Sigma) or type I collagen (Sigma).

Cell Cultures, Plasmids, and Stable Transfections

The human fibrosarcoma cell line HT1080 was cultured in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum, 50 U/ml penicillin, 50 μ g/ml streptomycin, and nonessential amino acids (Gibco BRL, Paisley, UK).

Full-length human MMP-13 cDNA in the pcDNA3.1MycHis vector (Invitrogen) was kindly provided by Dr. Michael Byrne (Partners Health Care, Massachusetts General Hospital, Boston,

Fig. 2. Unmixing of specific fluorescent signals from nonspecific signals and autofluorescence by confocal laser scanning microscopy. **a** Selection of emission signals using a traditional band-pass filter technique. It is impossible to separate specific fluorescent signals from nonspecific signals with autofluorescence. Distinct band-pass emission spectra (green, red, and blue), indicated by the green, red, and blue square regions, respectively, include sev-

eral crossover signals. As a result of crossover signals, many cells become positive when the images obtained by this method are merged. Colors refer to the online version only. **b** Abundant autofluorescence of unstained cirrhotic liver sections was observed using a traditional band-pass analysis. **c** Spectral data obtained by the lambda stack for the autofluorescence present in the unstained specimens were used as reference spectra.

Mass., USA) [29]. Parent HT1080 cells, which do not express endogenous MMP-13, were transfected using Lipofectamine Plus (Invitrogen) with either pcDNA3.1 alone (empty vector) or pcDNA3.1 encoding C-terminal MycHis-tagged MMP-13 cDNA. Forty-eight hours after transfection, the contents of each well from 6-well plates were transferred to 100-mm diameter dishes and cultured in the presence of 600 $\mu\text{g}/\text{ml}$ Geneticin (G418; Sigma). Approximately 12 neoresistant clones/transfectants were identified and selected using the cylinder method. Individual clones of HT1080 cells were analyzed for MMP-13 production by Western blot analysis.

Statistical Analysis

Data are expressed as means $\pm$ SD. The statistical significance of differences among groups was assessed using the Student t test or Mann-Whitney U test as appropriate. $p < 0.05$ was considered statistically significant.

Results

Distribution and Expression of Adenovirus-Mediated Gene Transduction in Rat Liver

In the early phase of recovery from liver fibrosis/cirrhosis, endogenous MMP-13 gene expression is transiently induced on days 5–7 after the last injection of CCl_4 [16]. In addition, the production of recombinant adenovirus RADMMP-13 peaks on days 2–4 after injection [21]. Therefore, we transferred the adenovirus-mediated MMP-13 gene into the liver of cirrhotic rats on day 3 after the last injection of CCl_4 to coincide with the natural course (fig. 1).

To examine the distribution of the adenovirus-transduced gene, RADLacZ (1×10^{10} PFU) was intra-

Color version available online

Fig. 2. Unmixing of specific fluorescent signals from nonspecific signals and autofluorescence by confocal laser scanning microscopy. **d** Fingerprinting of spectra to the image obtained by the lambda stack. All autofluorescence spectra were applied to each channel (AF-1, AF-2, and AF-3), respectively. **e** The autofluorescence (blue) and MMP-9 (red) was localized in the cirrhotic liver

specimen. **f** Unmixing of the specific MMP-9 signal from autofluorescence. As shown in **d**, the entire spectrum of fluorescence in the specimen was first gathered and then linearly unmixed. In the cirrhotic liver specimen, the specific MMP-9 (red) signal without autofluorescence was clearly detected by the linearly unmixed technique. Colors refer to the online version only.

venously injected into the femoral vein of control rats and rats with CCl₄-induced cirrhosis. Analysis of X-Gal staining of normal liver tissue showed many β -galactosidase-expressing cells mainly in the parenchymal and nonparenchymal cells near the central vein within the lobules. In contrast, few X-Gal-positive cells, mostly nonparenchymal cells, were observed along the fibrous septa of cirrhotic rat liver (fig. 3a). Many X-Gal positive cells were observed in the spleen and kidney (fig. 3b). In addition, the liver of RAdMMP-13 injected rats, but not saline- or empty vector RAd66-injected rats, contained human MMP-13 protein (fig. 3c). Exogenous ex-

pression of human MMP-13 mRNA was also detected by RT-PCR analysis for 7 days after RAdMMP-13 injection (data not shown). Immunofluorescence studies further revealed human MMP-13 expression in the cirrhotic liver on day 3 after RAdMMP-13 injection (fig. 3d).

MMP-13 Overexpression Improved Liver Fibrosis

Histologic changes of the cirrhotic liver were examined on days 3, 7, 14, and 30 after gene delivery. Azan-Mallory staining showed an obvious decrease in the fibrous deposition areas in RAdMMP-13-injected rats by

Fig. 3. Distribution and expression of adenovirus-mediated gene transduction in rat liver. **a** Expression of the *LacZ* gene in normal and cirrhotic liver tissues. β -Galactosidase activity was detected by X-Gal staining of liver tissues that were obtained from normal and cirrhotic rat livers on day 7 after RADLacZ injection. Original magnification, $\times 25$ or $\times 100$. **b** β -Galactosidase activity was detected by X-Gal staining. Spleen and kidney tissues were obtained from cirrhotic rats on day 7 after RADLacZ injection. Original magnification, $\times 25$. **c** Production of MMP-13 protein in rat liver

ers. Rats were injected with saline, empty vector RAD66, or RADMMP-13 (1×10^{10} PFU/rat intravenously) for 3 days and liver tissues were collected. Human MMP-13 production was examined by Western blot analysis. **d** Expression of human MMP-13 (green) was detected by immunofluorescence staining in cirrhotic liver tissue on day 3 after RADMMP-13 injection. Nuclei (blue) were stained with TOTO-3. Colors refer to the online version only. Scale bar = $10 \mu\text{m}$. Each figure represents 5 animals in each group.

day 3, whereas RAD66-injected rat liver showed only a slight improvement in liver fibrosis (fig. 4a). Densitometric analysis further confirmed the significant decrease in the area of fibrosis in RADMMP-13-injected rats (fig. 4b). Importantly, hepatic fibrosis was favorably improved by treatment with adenovirus-delivered MMP-13 at each time point and persisted over long periods. No prominent ascites was observed on day 3 in RADMMP-13-injected rats, but ascites was still observed on day 30 in RAD66-injected rats (data not shown), indicating that the treatment with adenovirus-delivered MMP-13 rapidly and effectively ameliorated liver cirrhosis. In contrast to the significant amelioration of severe fibrosis, the expression levels of α -SMA, a marker of activated stellate cells, were not significantly different at any time point between

RADMMP-13- and RAD66-injected rats, except on day 14 (fig. 4c, d).

MMP-9 Expression Was Induced in RADMMP-13-Injected Cirrhotic Liver

The enzymatic activity of MMP-2 and MMP-9 was enhanced in the RADMMP-13-injected liver as assessed by gelatin zymography (fig. 5a). Immunohistochemical staining confirmed the increased number of MMP-9-positive cells induced by RADMMP-13, predominantly along the fibrous septa (fig. 5b). To identify whether the cells expressing MMP-13 also expressed MMP-9, we performed double immunofluorescence staining and analyzed the tissue using confocal laser scanning microscopy. Most MMP-13-positive cells coexpressed

Fig. 4. Overexpression of human MMP-13 dramatically attenuated the established liver cirrhosis. **a** After 10 weeks of CCl₄ administration, rats were injected with RA_d66 or RA_dMMP-13 and then sacrificed at the indicated time points after gene transfer. Liver samples were subjected to Azan-Mallory staining. Original magnification, ×25. Each figure represents 5 animals in each group. **b** The fibrosis area was assessed using image analysis techniques calculating the ratio of connective tissue to the whole area

of liver sections stained with Azan-Mallory. Data are expressed as means ± SD. ** $p < 0.01$ compared with RA_d66-injected livers. **c** α-SMA expression was examined by Western blot analysis at the indicated time points. Equal amounts of total protein were isolated from adenovirus-injected cirrhotic liver tissues. **d** α-SMA expression levels were measured by densitometric analysis. β-Actin expression was used as a loading control. Data are expressed as means ± SD. * $p < 0.05$ compared with RA_d66-injected livers.

Fig. 5. Induction of MMP-9 in RadMMP-13 injected cirrhotic liver. **a** Gelatinolytic activities in cirrhotic liver tissues from representative animals for each group were measured by gelatin zymography on day 3 after adenoviral gene transfers. **b** Immunohistochemical detection of MMP-9 in cirrhotic liver sections from RAD66- or RADMMP-13 injected rats. Livers were prepared 3 days after gene transfer. Original magnification, $\times 25$. **c** Double immunofluorescence staining showed the localization of adenovirus-mediated MMP-13 (green, right column) and MMP-9 (red,

left column) in the cirrhotic liver sections on day 3 after RADMMP-13 injection. A merged image of MMP-13 and MMP-9 staining is presented in the lower right column. Nuclei (blue) were stained with TOTO-3. Colors refer to the online version only. Scale bars = 10 μ m. **d** Collagenolytic activities in cirrhotic liver tissues from representative animals for each group were measured by collagen zymography on day 3 after adenoviral gene transfer. Each figure represents 5 animals in each group.

MMP-9 in the cirrhotic livers of RADMMP-13-injected rats (fig. 5c). Further analyses indicated that most of the MMP-9-positive cells were granulocytes, and some of them were ED1-positive macrophages (data not shown).

In the present experiment, the efficiency of the adenovirus-mediated gene transfer to hepatocytes was reduced in cirrhotic rat liver compared with normal rat liver (fig. 3a) because the intralobular hemodynamics are reduced by the formation of shunts between the portal and central veins [30]. Therefore, we hypothesized that not

only MMP-13 but also MMP-2 and MMP-9 expression contribute to recovery from liver cirrhosis, and MMP-13 expression serves as the initial trigger for the induction of MMP-2 and MMP-9. To confirm the increase in interstitial collagenase activity induced by RADMMP-13 injection, we further performed a zymography analysis of type I collagen and found that MMP-2 and MMP-9 collagenolytic activity was significantly enhanced in the cirrhotic liver of RADMMP-13-injected rats compared with control rats (fig. 5d).

Fig. 6. Increased HGF expression and enhanced hepatocyte proliferation in cirrhotic rat liver after RAdMMP-13 injection. **a** RT-PCR analyses showed the expression of HGF, c-met, TNF- α , and GAPDH mRNAs in cirrhotic liver on day 3 after either RAd66 or RAdMMP-13 injection from representative animals for each group. GAPDH gene expression was analyzed as a loading control. **b** Expression of HGF- α , phosphorylated c-Met, total c-Met, and β -actin in cirrhotic liver on day 3 after adenoviral injection was examined by Western blot analysis from representative animals for each group. β -Actin expression was used as a loading control. **c** Immunohistochemical detection of HGF- α in cirrhotic liver sections from RAd66- or RAdMMP-13 injected rats. Livers were prepared on day 3 after gene transfer. Original magnification, $\times 25$. A part of the upper panel is shown at a higher magnification ($\times 50$) in the lower panel. **d** Double immunofluorescence analysis shows the localization of HGF- α (red, left column) and adenovirus-mediated MMP-13 (green, right column) in the cirrhotic liver sections on day 3 after RAdMMP-13 injection. Merged images of MMP-13 and HGF- α staining are presented in the lower right column. Nuclei (blue) were stained with TOTO-3. Colors refer to the online version only. Scale bars = 10 μ m. Each figure represents 5 animals in each group.

Fig. 7. Expression of MMP-13 cleaves the HGF precursor and induces enzymatic activity of MMP-2 and MMP-9. **a** Twelve selected HT1080 cell clones (HTMMP13-1 to HTMMP13-12) stably transfected with human MMP-13 or control empty vector (con) were established. The generated clone cells were incubated in serum-free media and analyzed for the production of MMP-13 by Western blot analysis. **b** Conditioned media from established stable cell lines were examined for HGF- α and MMP-9 expression by Western blot analysis. **c** Gelatinolytic activities in conditioned

media from established stable cell lines were examined by gelatin zymography. **d** Proteolytic processing of HGF by MMP-13 expression. Conditioned media from stable cell lines were assessed by Western blot analysis using polyclonal antibodies against recombinant human HGF as described in Materials and Methods. Pro-HGF, HGF- α , and HGF- β are indicated by the arrows. The asterisk indicates a nonspecific band. Data are representative of 3 separate experiments.

MMP-13 Overexpression Increased HGF Gene Expression and Enhanced Hepatocyte Proliferation in Cirrhotic Liver

Several growth factors and cytokines induce MMP-2 and MMP-9 gene expression in liver fibrosis/cirrhosis [4, 6–8]. HGF plays a crucial role in liver regeneration and antifibrogenesis [31–34]. Furthermore, forced expression of the human MMP-8 gene induces HGF mRNA expression in experimental liver fibrosis [35]. We therefore compared the gene expression levels of growth-related signal molecules, such as HGF, its receptor c-met, and TNF- α , in liver tissues between RAdMMP-13- and RAd66-injected rats. RT-PCR analyses showed that HGF gene ex-

pression increased after RAdMMP-13 injection (fig. 6a). In addition, the gene expression levels of c-met and TNF- α tended to increase in RAdMMP-13-injected rats compared with RAd66-injected animals. Western blotting of the liver tissues revealed similar results (fig. 6b). Expression of HGF- α , an active form of HGF, and enhancement of phosphorylated c-Met protein were observed in RAdMMP-13-injected rats. Immunohistochemical staining further indicated an increase in HGF- α expression, predominantly along fibrous septa in cirrhotic livers compared to control rats (fig. 6c). Moreover, immunofluorescence studies revealed that most MMP-13-positive cells were also HGF- α -producing cells (fig. 6d). Consis-

Fig. 8. Schematic representation of the mechanisms of MMP-13-mediated HGF activation and MMP-2 and MMP-9 induction. MMP-13 expression processes ECM-bound and stromal cell-secreted pro-HGF. Pro-HGF then releases HGF- α to bind its specific receptor c-Met, thereby inducing MMP-2 and MMP-9 expression. MMP-13 is an important trigger for the recovery from fibrosis and liver regeneration.

tent with the gene and protein expression of HGF, the number of PCNA-positive cells was increased in the cirrhotic liver of RADMMP-13-injected rats compared with that of RAD66-injected animals (data not shown).

MMP-13 Expression Is a Crucial Trigger for HGF Activation and MMP-2/MMP-9 Induction

A possible mechanism for MMP-13-mediated recovery from liver cirrhosis is that the activation of HGF signaling altered MMP-2 and MMP-9 induction in this experimental model. In the last set of experiments, we first established stable transfectants of human MMP-13 constructs in HT1080 cells, an interstitial tissue-derived cell line and a source of pro-HGF (fig. 7a). Twelve clones, each established from a single cell colony, were analyzed for production of MMP-13 by Western blot analysis. Six stably transfected cell clones (HTMMP-13-1, HTMMP-13-2, HTMMP-13-3, HTMMP-13-10, HTMMP-13-11, and HTMMP-13-12) expressed high levels of MMP-13 protein in conditioned media and were chosen for further analyses. Interestingly, the established stable transfectants also produced HGF- α and MMP-9 protein (fig. 7b). Furthermore, the gelatinolytic activity of both MMP-2 and MMP-9 was also dramatically increased in the stable MMP-13 transfectants (fig. 7c). We next determined the presence of the pro- and active protein species following constitutive expression of MMP-13. The conversion of pro-HGF to mature HGF (HGF- α and HGF- β) occurred in all MMP-13-expressing cell lines (fig. 7d). These results

strongly suggest that MMP-13 expression has a critical role in the induction of MMP-2 and MMP-9 expression via HGF activation.

Discussion

The findings of the present study demonstrate that forced expression of the human MMP-13 gene in cirrhotic liver using an adenoviral gene delivery system accelerates the spontaneous regression of experimental liver cirrhosis. Recovery from liver cirrhosis was achieved by the effects of MMP-13-mediated induction of HGF- α , MMP-2, and MMP-9 to degrade collagen fibers. In addition, MMP-13 expression was involved in converting the secretion of precursor HGF to the mature HGF form (fig. 8). HGF enhances the expression of both MMP-2 and MMP-9 [36, 37]. Therefore, these findings indicate a critical role of MMP-13 in the regression process of hepatic cirrhosis. Two previous studies demonstrated that overexpression of interstitial collagenase attenuates experimental liver fibrosis. One study utilized a recombinant adenovirus expressing human MMP-1 gene [38], and the other study used overexpression of human MMP-8 [35]. In the present study, we induced expression of the human MMP-13 gene using an adenoviral vector for the following reasons. First, hepatic fibrogenesis is characterized by increased ECM deposits, especially fibrillar type I and type III collagens [4, 6]. Therefore, collagenase activity is indispens-

able for effective therapy for liver fibrosis/cirrhosis. Second, the MMP-1 gene and the MMP-1 protein, an interstitial collagenase in humans, have not been identified in rodents. Thus, adenovirus-mediated gene transfer of human MMP-1 gene may cause immune reactions, leading to necrosis of the infected hepatocytes [38]. In contrast, MMP-13, a main interstitial collagenase in rodents, has a highly specific ability to degrade insoluble fibrillar type I collagen in humans and rodents [13, 39]. In addition, MMP-13 is approximately 90% homologous between rats and humans at the amino acid level [13]. Third, MMP-13, but not MMP-8, gene expression is induced transiently in the early phase of recovery from experimental liver cirrhosis in rats [15, 16]. Hence, these previous findings prompted us to investigate the significance of the transient expression of MMP-13 in the early phase of recovery from liver cirrhosis by infecting rats with an adenovirus-mediated human MMP-13 gene in the early phase of recovery from liver cirrhosis.

In the present experiments, adenoviral gene transfer of MMP-13 dramatically and rapidly reduced severe fibrous bands in CCl₄-induced hepatic cirrhosis via the induction of MMP-2 and MMP-9 following an increase in exogenous MMP-13 expression (fig. 4, 5). In addition, Azan-Mallory staining dramatically declined in the areas of fibrous deposition, whereas α -SMA expression levels were not significantly altered in RAdMMP-13-injected rats by day 3 (fig. 4c). These results suggest that the comprehensive degradation system should contribute predominantly to the RAdMMP-13-mediated improvement of liver cirrhosis. Intriguingly, our previous study also showed that MMP-2 and MMP-9 gelatinolytic activities are increased after transient gene expression of endogenous MMP-13 during recovery from liver fibrosis [20]. Our findings thus suggest that MMP-13 expression is the essential trigger regulating the MMP cascade.

Forced MMP-13 expression not only accelerated the regression of liver cirrhosis but also promoted HGF induction (fig. 6). HGF, an important multifunctional mediator that was originally characterized as a potent mitogen for mature rat hepatocytes, is a potent antifibrotic cytokine that inhibits liver fibrosis [31–34]. In the present study, MMP-13 overexpression stimulated the gene expression of growth-related molecules such as HGF, its receptor c-Met, and TNF- α (fig. 6a). Similar effects on hepatocyte proliferation have been observed by overexpressing MMP-1 and MMP-8 *in vivo*, and MMP-8 expression also induces HGF gene expression [35, 38]. Another study using a null mouse lacking TIMP-1 showed that MMP expression is closely related to accelerated liv-

er regeneration via HGF activation [40]. In addition, a study in MMP-9-null mice indicated that MMP-9 has an important role in liver regeneration by affecting the expression of growth factors such as HGF and TNF- α [41]. HGF is synthesized and secreted in an inactive single-chain precursor form (pro-HGF) and remains in this form under physiologic conditions. The mature HGF forms (HGF- α and HGF- β) are released by proteolytic processing [42–44]. MMP-13 overexpression in hepatic cirrhosis and in a stably expressing cell line induces HGF- α production (fig. 5, 6). Therefore, we hypothesized that MMP-13 activates the production of mature HGF from pro-HGF. Importantly, studies of stable transfectant MMP-13 clones indicated that MMP-13 is partly responsible for pro-HGF processing, which converts pro-HGF into HGF- α . These results further indicate the novel roles of MMP-13 in the activation of HGF signaling and thus in the regression of liver cirrhosis. Furthermore, pro-HGF is observed not only in the ECM of the liver but also in the spleen and kidney bound to heparan-sulfate proteoglycan [42–45]. Adenovirus-mediated gene expression was also observed in the spleen and kidney (fig. 3b). Therefore, MMP-13-mediated HGF processing in the spleen and kidney may also contribute to the regression of liver cirrhosis and promote hepatic regeneration. Further studies are needed to clarify the MMP cascade and growth factor/cytokine network that govern the complex process of matrix degradation and liver regeneration.

Recently, Uchinami et al. [46] demonstrated, using MMP-13-null mice, that lack of MMP-13 paradoxically results in the attenuation of liver injury and fibrosis induced by ligation of the bile duct. Kim et al. [47], however, reported that the delivery of MMP-13-encoding plasmid DNA into mice inhibits the development of CCl₄-induced liver fibrosis. In contrast, Duffield et al. [48] and Fallowfield et al. [17] reported that conditional ablation of macrophages, which produce MMP-13, inhibits the recovery from hepatic fibrosis. In addition, we previously reported that MMP-13 gene expression is transiently upregulated at 2 different time points, i.e. in the early progression phase of liver fibrosis induced by CCl₄ injections and in the early phase of recovery from liver fibrosis after the cessation of CCl₄ injection [15, 16]. Therefore, MMP-13 is required not only for inducing fibrosis during the development of liver cirrhosis but also during the recovery phase. On the basis of our observations and other recent reports, MMP-13 may serve different and critical roles in matrix pathobiology depending on the stage of liver fibrosis/cirrhosis.

Work is now in progress to determine whether forced MMP-13 expression attenuates ongoing liver cirrhosis and to reveal the precise mechanisms responsible for the effects of MMP-13 on liver regeneration. These studies are expected to lead to a better understanding of the complex mechanisms underlying hepatic tissue remodeling and regeneration and eventually to the development of a new therapeutic strategy for liver fibrosis/cirrhosis based on matrix biology.

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