Essential Role of Matrix Metalloproteinases in Interleukin-1-induced Myofibroblastic Activation of Hepatic Stellate Cell in Collagen*

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Liver fibrosis represents chronic wound repair after diverse insults including viral infections, toxic damage, as well as autoimmune reactions (1). The ultimate outcome of liver fibrosis is the formation of nodules encapsulated by fibrillar scar matrix (2). During fibrogenesis, there is a shift in the composition of extracellular matrix (ECM) from the normal basement membrane-like matrices of types IV and VI to one rich in fibrillar collagens and other ECM (3–5). Located within the space of Disse between the hepatocytes and sinusoidal endothelial cells, hepatic stellate cells (HSC) play a key role in liver fibrogenesis (6). The bona fide markers for HSC in normal livers and the freshly isolated “quiescent” cells are intracellular vitamin A droplets and desmin (7, 8). In the process of fibrosis, HSC undergo dramatic morphological and functional changes called “activation” during which the star-shaped HSC are converted to myofibroblast-like cells with induced expression of α-smooth muscle actin and stress fibers but decreased retinoid storage. Liver fibrogenesis is initiated by hepatocyte damage and subsequent recruitment and activation of Kupffer cells, platelets, and inflammatory cells via the release of fibrogenic cytokines and growth factors (2). These soluble factors and the surrounding extracellular matrix are the two major classes of mediators that determine the fate of HSC. The morphological and metabolic changes associated with HSC activation are reproduced by culturing isolated HSC on plastic (9, 10). Such in vitro activation is also achieved and accelerated by the addition of Kupffer cell-conditioned medium or soluble factors identified to be pivotal in stimulating HSC. Of these, TGF-β (11–14) and PDGF (15, 16) are the two most potent mediators. Whether TNF-α has a direct role in the activation remains unclear (17). However, it induces HSC proliferation (18) and expression of intercellular adhesion molecule-1 and chemokines in a nuclear factor-κB-dependent manner (17, 19). Although insufficiently documented for HSC activation, IL-1 is accepted as a potent cytokine for fibrosis of other organs such as the lung, (20) bone marrow (21), and kidney (22, 23).

An intact ECM is essential for the tissue homeostasis and proper functions, differentiation and morphology of cells (24). On the other hand, excessive remodeling of ECM occurs in chronic diseases such as tumor metastasis, unhealed trauma, and arthritis (25). For liver fibrosis, the intracellular events that occur in activation of HSC have been characterized (26), and changes in the matrix substratum were shown to affect the behavior of HSC (27–29). However, the precise role of the ECM remodeling in HSC activation is yet to be explored. In particular, there are few investigations on ECM remodeling and HSC

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The abbreviations used are: ECM, extracellular matrix; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; HSC, hepatic stellate cell(s); MMP, matrix metalloproteinase; PDGF, platelet-derived growth factor; α-SMA, α-smooth muscle actin; TGF, transforming growth factor; TIMP, tissue inhibitor of metalloproteinase; TNF, tumor necrosis factor.

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activation in a three-dimensional ECM culture model, which more closely mimics HSC in vivo (30, 31). Within the perilobular space, HSC are in direct contact with the loose matrix composed of basement membrane constituents on the endothelial side and with type I and III collagen fibers on the parenchymal side (32). These ECMs support the three-dimensional cell structure and morphology of HSC in the quiescent state in vivo.

Matrix remodeling is mostly carried out by the balance between activities of proteinases and their inhibitors (33). Matrix metalloproteinases (MMPs) are a family of zinc metalloendopeptidases and responsible for the turnover of all of the ECM components (34). In a rat model of bile duct ligation-induced hepatic fibrosis, MMP-2 and MMP-9 are elevated during liver fibrosis (35). Earlier investigations have shown that primary cultures of rat HSC produce pro-MMP-2 and that the proteolytic activation of the zymogen is facilitated by type I collagen (36, 37). In a mouse model of acute hepatitis caused by TNF-α, the lethality, hypothermia, and influx of leukocytes into the liver are all prevented by a broad spectrum MMP inhibitor, suggesting a role for MMPs even in acute hepatitis (38).

MMP-9, also called gelatinase B and type IV collagenase, was originally identified as a secreted factor of alveolar macrophages (39). Based on the substrate preference, MMP-9 is believed to participate in remodeling of basement membrane and cell migration (40). MMP-9 is secreted as a zymogen and maintained in the latent form presumably by the interaction of a conserved cysteine in N-terminal prodomain with the zinc atom in the catalytic sites. Activation by cleavage of the prodomain is essential for the enzymatic activity and is observed in many chronic diseases (41). For example, the active form of MMP-9 is present in hepatocellular carcinoma, suggesting a prognostic value (42), and overexpression of MMP-9 correlates with the growth of small hepatocellular carcinoma (43). However, the regulatory mechanisms underlying pro-MMP-9 activation still remain elusive.

In this study we examined the IL-1-mediated regulation of MMPs and its role in HSC activation in the three-dimensional ECM environment. This culture model simulates the morphologic characteristics of the quiescent HSC in vivo (31). Under this condition, no MMP-9 is generated. When HSC are embedded in three-dimensional Matrigel, IL-1α induces pro-MMP-9 expression without its activation. In contrast, IL-1α causes massive induction and proteolytic activation of pro-MMP-9 by HSC grown in three-dimensional type I collagen gel, whereas IL-1α or three-dimensional type I collagen individually fails to do so. On the other hand, HSC grown in three-dimensional fibrin lattices do not generate MMP-9 in response to the cytokine stimulation. IL-1α also induces MMP-13, an interstitial collagenase, and MMP-3 (stromelysin-1), a wide spectrum proteinase, by HSC grown in the collagen matrix. Parallel to the IL-1α-induced expression and activation of the multiple MMPs in three-dimensional collagen, HSC undergo activation and cause the breakdown of the collagen matrix. To support a link between MMP-9 activities with HSC activation, we show that inhibition of MMP-9 by TIMP-1 or depletion of the proteinase by gelatin-Sepharose 4B attenuates the IL-1α-induced HSC activation. Further, we demonstrate colocalization of MMP-9 and α-smooth muscle actin in human liver fibrosis specimens. Taken together we propose that MMP activities including that of MMP-9 are essential for IL-1-induced trans-differentiation of HSC during the early phase of liver fibrogenesis.

MATERIALS AND METHODS

Isolation Rat HSC—HSC were isolated from male Wistar rats (Charles River) by the Non-Parenchymal Liver Cell Core of the Research Center for Alcoholic Liver and Pancreatic Diseases. The normal Wistar rats were treated by in situ sequential digestion with Pronase and collagenase followed by arabinogalactan gradient ultracentrifugation as described previously (10, 44). Briefly, the liver was sequentially digested with Pronase (Roche Applied Science) and type IV collagenase (Sigma) by in situ perfusion. Parenchymal cells were removed by centrifugation of the digest at 50 × g for 2 min, and nonparenchymal cells were layered on top of the four density gradients of arabinogalactan (Sigma). The gradients were centrifuged at 2,400 rpm for 40 min at 25 °C using a Beckman SW41 Ti rotor (Beckman Instruments). A pure fraction of HSC was recovered from the interface between the medium and the density of 1.035. The purity of HSC was assessed by phase-contrast microscopy and ultraviolet excited fluorescence microscope, and the viability was examined by the trypan blue exclusion test. Both the purity and viability of the collected cell fractions were always higher than 95%.

Cell Culture and Embedding in Three-dimensional Matrix—The model used in the most of the experiments described below is based on a comparison of morphologic and biochemical parameters of HSC cultured in three-dimensional matrices (type I collagen gel, Matrigel, fibrin) and those on plastic. Freshly isolated HSC were plated on 10-cm culture dishes with 10% FBS in DMEM for 2 days. During this period, HSC retained the quiescent state as monitored by star-shaped morphology with long, prominent dendritic processes and autofluorescence of vitamin A-containing droplets. Then cells were detached by a trypsin/EDTA treatment followed by neutralization by 10% FBS in DMEM. After washing with serum-free DMEM the cells were suspended in 4 ml of DMEM. The cell density was adjusted to 2 × 10⁵/cm². One plate of cells was preincubated on plastic, 2 ml of the cell suspension was mixed with 18 ml of DMEM containing 10% FBS and seeded of 1 ml in each well of a 24-well plate. Another 2 ml cell suspension was preincubated with type I collagen gel mixture as described previously (45). Briefly, this mixture was prepared by mixing 0.6 ml of 10 × minimal essential medium, 0.6 ml of 0.1 × NaOH, 1.4 ml of DMEM, and 4.8 ml of Vitrogen (Cohesion Technologies). An aliquot of 0.4 ml was loaded in the wells of a 24-well plate and incubated at 37 °C for 2 h. After forming of lattices, 1 ml of DMEM and 10% FBS were added on top and cultured in 5% CO₂ at 37 °C for 6 h. Then the monolayers or lattices were washed gently by DMEM followed by culturing in 1 ml of DMEM (0.5% FBS) with cytokeratin (K8 K18 K19) or F4/80 (System) as mentioned in the text. To make the fibrin clot, a stock was prepared by mixing 3 ml of 10 mg/ml fibrinogen in DMEM and 1 ml of cell suspension. Aliquots of 5 μl of thrombin (250 units/ml) were preloaded in the wells followed by adding 0.4 ml of the fibrinogen cell mixture. To make Matrigel gel lattices, the stock (BD Biosciences) was diluted twice with cold DMEM followed by mixing with the cell suspension. For some experiments, a sandwich gel was made by placing an additional 0.2 ml matrix layer before loading the collagen lattices on the top. For inhibition experiments, purified recombinant TIMP-1 (CC3328, Chemicon) was applied to the culture.

Deprivation of MMP-9 during IL-1α-induced Activation of HSC—Isolated rat HSC were embedded into three-dimensional type I collagen in the bottom chamber of a Transwell (BD Biosciences). 400 μl of fibrin/ gelatin-Sepharose 4B (Amershaw Biosciences) or the control beads were loaded into the insert chambers. After culture with 20 ng/ml IL-1α for 6 days the cells were fixed for immunostaining of α-SMA. The conditioned medium was examined for gelatinase activities.

Zymography—Prior to gel electrophoresis the conditioned medium was briefly centrifuged and mixed with a SDS-PAGE sample buffer in the absence of reducing agents. The conditioned medium was resolved by 10% polyacrylamide separation gel with 0.1% (w/v) gelatin (Sigma). Because some components in fetal bovine serum were found to be tightly associated with the collagen matrix and recognized by the secondary antibody in Western blot analysis, quiescent HSC were cultured in serum-free basal DMEM, and a minimal concentration of PDGF at 0.2 ng/ml was added in the basal medium for culturing fully activated HSC to prevent apoptosis. The conditioned medium was resolved by 12.5% SDS-PAGE, and the protein was transferred to Immobilon-P (Millipore). After blotting with 10% nonfat milk,
the individual membranes were probed with either anti-rat MMP-13 (mAB13426, Chemicon), MMP-3 (mAB19150, Chemicon), TIMP-1 (MS-570, NeoMarkers), or MMP-9 (AB805, Chemicon) overnight. After incubation with horseradish peroxidase-conjugated secondary antibodies, the membranes were developed by SuperSignal West Pico Chemiluminescent substrate (Pierce).

**MMP-9 Activity Assay**—The fluorogenic peptide substrate, DNP-Pro-Leu-Gly-Met-Trp-Ser-Arg (Calbiochem), was dissolved in 15% dimethyl sulfoxide. Assays were carried out by incubation of 5 μl of conditioned medium, 45 μl of H2O, 50 μl of 36 μM peptide dilution, and 50 μl of buffer containing 100 mM NaCl, 30 mM CaCl2 in 50 mM Tris, pH 7.4, at 37 °C for 4 h. The hydrolysis of the peptide was measured by the fluorescence at λex = 280 nm and λem = 380 nm in a Hitachi F-2000 spectrofluorometer.

**Immunohistological Staining**—After cultivation the HSC in matrix lattices and on plastic were washed by phosphate-buffered saline and fixed in cold methanol for 20 min for three-dimensional gel and 10 min for monolayers. After three washes the cells were permeabilized by incubation with cold 0.1% Triton X-100 with 0.1% bovine serum albumin (fraction V from Sigma) in phosphate-buffered saline for 15 min. After treatment with blocking solution containing 5% bovine serum albumin for 60 min, the staining was performed by incubation of 1:200 dilution of fluorescein isothiocyanate-conjugated monoclonal antibody against α-smooth muscle actin (α-SMA, Sigma) in phosphate-buffered saline with 5% bovine serum albumin for 16 h at 4 °C. After three washes, the nuclei were stained by 0.1 μg/ml TOPRO-3 (Invitrogen) for 10 min. For some experiments the colorimetric staining with horseradish peroxidase-conjugated secondary antibodies and AEC substrates (Labvision) was used. The cells were viewed using a Nikon microscope equipped with digital camera available at the Imaging Core of the USC Research Center for Liver Diseases. To examine the colocalization of MMP-9 and α-SMA, a dual immunofluorescence staining was performed onto human fibrotic liver biopsies by the Morphology Core of the Research Center for Alcoholic Liver and Pancreatic Diseases with approved IRB protocol. Briefly, a paraffin block was sectioned at a thickness of 5 μm followed by a dewaxing process. After hydration, the slides were treated with 0.1% trypsin at 37 °C for 30 min and then blocked by a solution containing 5% skim milk (BD Biosciences), 0.25% serum, and 1% bovine serum albumin (EM Science). The slides were incubated with rabbit antibodies for human MMP-9 (AB805, Chemicon) (1:300) and mouse monoclonal antibody for α-SMA (1:300) in the blocking solution for 16 h at 4 °C. After washing of the slides the fluorescein isothiocyanate-conjugated anti-mouse IgG and Cy3-conjugated anti-rabbit IgG were applied at a dilution of 1:400. Before mounting, the slides were stained with 1 μg/ml DAPI.

**RESULTS**

**Induction and Activation of MMP-9 during IL-1α-induced Activation of HSC in ECMs**—A general design of this study was to establish a three-dimensional culture of HSC by embedding isolated quiescent HSC in ECM matrix and to examine cytokine regulation of MMPs and HSC activation. We first tested type I collagen gel because this model was shown to recapitulate best the quiescent morphology of HSC in vivo (31). HSC were isolated from rats and plated on a plastic dish with medium containing 10% FBS for 2 days. More than 95% of the cells retained the quiescent phenotype of HSC as judged by perinuclear retinoid droplets and star-shaped morphology with prominent dendritic protrusion. Then the cells were either reseeded on plastic as a monolayer or embedded in the reconstituted fibrillar type I collagen. The cells were cultured in basal medium with an increasing concentration of IL-1α. After an additional 6 days of culture in the absence of IL-1α, the cells in three-dimensional collagen retained a quiescent state with intracellular vitamin A droplets and the absence of α-SMA immunoreactivity (Fig. 1A). Treatment of HSC with IL-1α induced apparent trans-differentiation of the quiescent HSC into myofibroblast-like cells with the formation of α-SMA-positive fibers. The initial induction of α-SMA for monolayer and three-dimensional-collagen was evident at 0.2 and 2 ng/ml, respectively. At the concentration of 20 ng/ml, IL-1α brought formation of heavy bundles of stress fiber in the cells.

Gelatinolytic activities in the conditioned medium were resolved and are shown in Fig. 1B. To our surprise, robust gelatinase activities with a molecular mass of 105–95-kDa were produced by IL-1α-treated HSC in three-dimensional collagen. In contrast, IL-1α or three-dimensional collagen matrix alone was without effect. The 105-kDa gelatinase represents the rat pro-MMP-9 with a prodomain of ~100 amino acid residues. The 95-kDa gelatinase is regarded as the active form of rat MMP-9 generated by the cleavage of the prodomain. In fact, only pro-MMP-9 was generated when the cells were treated with a low concentration of IL-1α (0.2 ng/ml). Further, after a continued culture for 6 days with IL-1α, the collagen gel was completely degraded, suggesting that fibrillar collagenase activities were induced by IL-1α (Fig. 1C). We also assessed serine proteinase activities by casein zymography. As shown in Fig. 1D, a major serine proteinase with an approximate molecular size of 75-kDa was expressed at the basal state in the collagen lattice, and the treatment with IL-1α induced the generation of a lower molecular mass serine proteinase, presumably through an activation process.

To address the concern that cellular contaminants such as Kupffer cells may provide the source of MMP-9, we performed detailed histological analysis of the three-dimensional cultures. The identity and purity of HSC were readily confirmed by UV excited autofluorescence of intracellular retinoids. By this standard, more than 95% of cells were found to be vitamin A-positive. After a 2-day culture in the collagen lattice, the cells were immunostained by the anti-rat MMP-9 monoclonal antibody. As shown in Fig. 1E, MMP-9 immunoreactivity was present only in the cells stimulated with IL-1α. We also confirmed the type IV collagenase activity as MMP-9 by a Western blot analysis of the 6-day cultured medium (Fig. 1F).

These results indicate that IL-1α-induced HSC activation in three-dimensional collagen is tightly associated with cytokine-provoked expression and proteolytic activation of multiple ECM proteinases. They also suggest that the breakdown of the ECM which helped maintain the quiescent state of HSC is essential for IL-1α-induced activation of HSC. Conversely, in the absence of ECM framework the HSC grown on plastic readily undergo activation without the need of MMPs.

**Specific ECM and Cytokines Are Both Required for Pro-MMP-9 Expression and Activation by HSC**—We addressed next the specificity of ECM required for the IL-1α-mediated MMP-9 induction and activation. We tested Matrigel, which contains the basement membrane components similar to those found in the space of Disse between HSC and sinusoidal endothelial cells. We also tested fibrin clot, which is frequently present in the early stage of acute wounds. These two matrices were compared with fibrillar type I collagen, which is normally present between HSC and hepatocytes and becomes abundant in fibrotic conditions. Quiescent HSC were embedded in the ECM followed by cytokine stimulation. We found very interesting patterns of cytokine-mediated regulation of MMP-9 expression/activation by the HSC in these matrices. In Matrigel, IL-1α and TNF-α but not TGF-β induced expression of pro-MMP-9. However, the 105-kDa pro-MMP-9 remained as a zymogen without being converted to the active form. When HSC were grown in type I collagen, IL-1α induced pro-MMP-9 expression followed by conversion to the active 95-kDa form, whereas TNF-α failed even to induce pro-MMP-9. In fibrin clot HSC did not secrete MMP-9 in response to all of these cytokines. Thus these results highlight that the ECM, presumably through the specific cellular receptors, can drastically determine the responsiveness of HSC for their cytokine-mediated regulation of MMP-9 expression and activation.

**IL-1α and Type I Collagen Serve as Costimulatory Signals for Induction of MMP-9 and MMP-13, whereas Collagen Is Not Required for MMP-3 Expression**—We evaluated cytokines and
ECM on other MMPs represented in fibrosis. As shown in Fig. 3A, we confirmed that dual signals from IL-1α/H9251 and type I collagen are required for induction and activation of MMP-9. After a prolonged development of the zymogram, we occasionally observed weak induction of pro-MMP-9 by the IL-1α/H9251-treated HSC on plastic.

We also examined cytokine-mediated regulation of MMP-3 and MMP-13 in this model. MMP-1, an interstitial collagenase (collagenase-1) is absent in the rat and mouse genome, whereas MMP-13 (collagenase-3) is regarded as its counterpart in these species (46). Very similar to the mode of MMP-9 induction, dual signals from IL-1α/H9251 and type I collagen are required to induce MMP-13 by HSC (Fig. 3B). A 60-kDa MMP-13 and a fragment of 25 kDa were recognized by a monoclonal antibody for the MMP. TNF-α/H9251, TGF-β/H9252, and PDGF individually or in combination with collagen all failed to induce MMP-13. This IL-1α-specific induction of MMP-13 paralleled the breakdown of the fibrillar collagen lattices (Fig. 3C). Conversely, the absence of MMP-13 in response to the stimulation by TNF-α, TGF-β, and PDGF is consistent with their incompetence for induction of collagen breakdown. Thus the global meltdown of fibrillar collagen gel is likely attributable to the participation of MMP-13.

MMP-3 (stromelysin-1) is a wide spectrum proteinase for many ECM components. As shown in Fig. 3D, of the panel of cytokines, only IL-1α induced secretion of MMP-3. In contrast to MMP-9 and MMP-13 induction, collagen signal seemed not required for the MMP-3 induction. Finally, MMP-2 was constitutively expressed by HSC, and these cytokines seemed without effect on its expression or activation. Thus, these differential requirements of ECM for IL-1α-mediated induction of MMPs may be of pathophysiological importance because the ECM environment surrounding HSC dynamically changes in liver fibrogenesis, and this may in turn determine the responsiveness of HSC in MMP expression. These results also suggest that similar intracellular signaling may be involved in the regulation of MMP-9 and MMP-13 expression, whereas MMP-3 induction is collagen-independent.

**Inactivation of MMP-9 by TIMP-1 or Depletion of MMP-9 by Gelatin-Sepharose 4B Prevents IL-1α-induced HSC Activation in Three-dimensional Collagen**—Pro-MMP-9 forms a complex with TIMP-1, which inhibits both the catalytic activity and the zymogen activation (47). Because of the association of HSC activation with pro-MMP-9 expression/activation in IL-1α-treated HSC in three-dimensional collagen, we assessed the causal role of MMP-9 in HSC activation with the use of TIMP-1. Primary HSC were embedded in three-dimensional collagen or plated on plastic as a monolayer followed by treatment with IL-1α in the presence or absence of purified recombinant TIMP-1. At the basal state (control), the perinuclear fat droplets were evident in HSC grown in collagen matrix even
after culture for 5 days (difficult to observe at this magnification, Fig. 4A). On the treatment of IL-1α, the expression of α-SMA became prominent, and fat droplets were reduced markedly in HSC in three-dimensional collagen and more so in those on plastic. TIMP-1 at 10 nM decreased expression of α-SMA in IL-1α-treated HSC cultured in collagen but not in those on plastic. With a higher concentration of TIMP-1 (30 nM), IL-1-induced expression of α-SMA was conspicuously inhibited. HSC cytoplasm was retracted, and the morphological features of quiescent HSC were restored, including more abundant lipid droplets (Fig. 4A). In contrast, TIMP-1 even at this high concentration had no effect on the IL-1α-induced HSC activation on plastic. This is important because it indicates that the TIMP-1-mediated inhibition of HSC activation in collagen gel is not likely mediated via its direct effects on the cells, but rather through inhibition of extracellular proteinase(s). Indeed, the TIMP-1 treatment inhibited the type IV collagenase activities in the conditioned medium as measured by zymography (Fig. 4B). At 30 nM TIMP-1 also prevented IL-1α-induced conversion of the 105-kDa pro-

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FIG. 2. Specific ECM and cytokines are both required for pro-MMP-9 induction and activation by HSC. Isolated quiescent HSC were either plated on plastic as a monolayer or embedded in three-dimensional type I collagen, Matrigel, or fibrin gel as described under “Materials and Methods.” The cells were stimulated with 20 ng/ml TNF-α, 20 ng/ml IL-1α, and 2 ng/ml TGF-β for 6 days. The gelatinase activities were resolved by zymography.

FIG. 3. IL-1α and type I collagen serve as costimulatory signals for induction of MMP-9 and MMP-13, but collagen is not required for MMP-3 expression. Isolated quiescent HSC were seeded as a monolayer on plastic or embedded in three-dimensional collagen lattice. After treatment for 6 days with the respective cytokines or FBS, the conditioned medium was analyzed for gelatinolytic activities by zymography (A). Conditioned medium was subjected to Western blot analysis for MMP-13 (B). The breakdown of collagen lattices was recorded (C). MMP-3 in the conditioned medium was examined by Western blot (D).
MMP-9 into the active enzyme (Fig. 4C). Finally, TIMP-1 also blocked the IL-1α-mediated breakdown of collagen lattice (Fig. 4D).

Although TIMP-1 shows preferential activity toward MMP-9, it may also target other MMPs. To define the unique role of MMP-9 in HSC activation we depleted the proteinase by using gelatin-conjugated Sepharose 4B in the culture. For this, HSC were embedded into three-dimensional type I collagen in the bottom chamber of Transwell and treated with IL-1α/H9251. Gelatin-conjugated Sepharose 4B or control beads were loaded into the upper insert chambers. After a 6-day culture, the HSC with gelatin beads retained the quiescent morphology, whereas the cells with the control beads underwent activation (Fig. 5A). MMP-9 activity in the conditioned medium was reduced significantly by the use of gelatin beads but not by the control beads (Fig. 5B). Consistently, fibrillar collagen lattice was mostly intact in the culture with gelatin beads but was degraded in the control culture with carrier beads (Fig. 5C). Because MMP-2 accounts for a very small portion of the total gelatinase activities, the suppressed activation of HSC with gelatin beads is due mostly to the deprivation of MMP-9. Taken together, the results unequivocally demonstrate that the MMP-9 activity is critical for IL-1α-induced activation of HSC presumably through a dynamic remodeling of ECM. Based on these results we conclude that MMP-9 plays a pivotal role in HSC activation in this culture model.

Fully Activated HSC Reduced MMP Production—A critical biochemical feature of the progressive liver fibrosis is a relative decline in proteinase activities favoring increased deposition of collagen matrix (27). Thus we questioned how fully activated HSC would behave in response to IL-1α/H9251 in three-dimensional type I collagen. To generate fully activated HSC, the primary HSC were cultured on plastic with 10% FBS for 10 days. By then, the cells were fully trans-differentiated into myofibroblastic cells as judged by the formation of prominent stress fibers and depletion of lipid droplets. At this point, the cells were reseeded on plastic or embedded in three-dimensional collagen lattice followed by cytokine stimulation. Unlike the quiescent HSC, no MMP-9 was secreted by these fully activated cells on plastic or in three-dimensional collagen lattice under the cytokine stimulation. We also measured production of MMP-13 and MMP-3 by the fully activated HSC. As shown in Fig. 6B, no MMP-13 was detected by myofibroblastic cells grown on plastic in response to cytokine stimulation.

Fig. 4. TIMP-1 inhibits both MMP-9 and activation of HSC in collagen matrix. Isolated quiescent HSC were either plated on plastic as a monolayer or embedded in three-dimensional type I collagen followed by stimulation with or without IL-1α together with TIMP-1 at two concentrations. After 5-day culture the cells were fixed and immunostained for α-SMA. To visualize the intracellular fat droplets in the collagen lattice, photographs were taken under simultaneous visible light and fluorescein isothiocyanate emission (A). The type IV collagenase activities in the conditioned medium were measured by cleavage of fluorogenic peptide and plotted as percentage of the maximal activity (B). The TIMP-1 inhibition of pro-MMP-9 activation was analyzed by gelatinolytic zymography (C). Breakdown of collagen lattices was recorded by photography of the lattices after removal of the soluble fractions (D).
shown far more robust induction and even proteolytic activation of thezymogen. Although Kupffer cells are believed to be the major source for MMP-9, a recent study showed that freshly isolated stellate cells secreted MMP-9 (50). In addition, the collagen response elements in the promoter of the gene have been identified in the activated HSC. This work is the first to demonstrate: 1) the selective agonistic effect of IL-1α on fibroblast activation of hepatic stellate cells; 2) differential regulation by Matrigel and type I collagen on the cytokine-induced MMP-9 induction and the precursor activation; 3) involvement of MMP-9 in IL-1α-induced HSC activation in three-dimensional collagen; and 4) colocalization of MMP-9 and α-SMA in human fibrotic liver tissues. Of interest is the requirement of both IL-1 and type I collagen to cause MMP-9 expression/activation, HSC activation, and the matrix breakdown. These findings may explain how a dynamic switch of ECM composition is controlled by a specific proinflammatory cytokine during liver fibrogenesis.

An outstanding unanswered question is how stellate cells keep in the quiescent state in vivo. The current study indicates that three-dimensional ECM and proteinase inhibitors may represent the cellular factors in restraining the stellate cells from myofibroblastic activation even in exposure to fibrotic stimuli. In Matrigel or type I collagen, HSC retain the quiescent state, and no significant MMPs are secreted. These conditions may represent the perisinusoidal environment at the normal state. In normal liver a restrictive amount of the type I collagen fibers is also distributed between HSC and hepatocytes in the perisinusoidal space (32). Thus, the quiescent HSC in vivo may already have been primed by the ECM signals prior to pro-MMP-9 induction. Upon an acute phase response of Kupffer cells, another signal should readily stimulate HSC for matrix remodeling, activation, and potentially migration and proliferation. In this study we identified IL-1α as such a factor. In response to transient stimulation by IL-1α or TNF-α, HSC within the basement membrane components may initially express pro-MMP-9 without zymogen activation (Fig. 2). Only prolonged stimulation of IL-1α can lead to the conversion of pro-MMP-9 into the active form. By this in vitro model we found that such a transition point is at about 48–72 h, during which pro-MMP-9 is converted into active form. This delayed fashion of pro-MMP-9 activation may reflect an induction of a conversion proteinase (pro-MMP-9 activator) by the HSC upon signaling from IL-1α and collagen. Type I collagen and IL-1α together are also required by HSC to secret MMP-13 (Fig. 4). On the other hand, IL-1α seems to stimulate expression of MMP-3 by HSC regardless of surrounding ECM. This panel of MMPs may promote HSC activation through digestion of the ECM, which restrains HSC from trans-differentiation.

The basement membrane components like type IV collagen and laminin are presumably digested by active MMP-9. Breakdown of the fibrillar collagen may be mediated by a coordinated action of multiple MMPs. It is likely that the initial cleavage is executed by the interstitial collagenases (MMP-13 or MMP-1), the subsequent partial denatured collagen may be then susceptible to degradation to small fragments by active MMP-9, which is the situation of gelatin degradation. Such a notion was supported further here by the fact that deprivation of MMP-9 blocks IL-1α-induced meltdown of collagen lattice (Figs. 4 and 5). It is intriguing that once HSC are fully trans-differentiated into myofibroblastic cells by a prolonged culture on plastic, they no longer respond to the costimulatory effects of type I collagen

**DISCUSSION**

In this study we demonstrate that IL-1α is a potent inducer for the myofibroblastic activation of HSC. During the IL-1-induced trans-differentiation from the quiescent to myofibroblastic phenotype, HSC secreted large amounts of MMPs including MMP-9, MMP-3, and MMP-13. Of them, MMP-9

**Fig. 5. Depletion of MMP-9 prevents IL-1α-induced activation of HSC in collagen matrix.** Isolated quiescent HSC were embedded in type I collagen in the bottom chamber of Transwell. 400 µl of control and gelatin-conjugated Sepharose 4B were loaded into the top chamber. After culture with 20 ng/ml IL-1α for 6 days the cells were stained for α-SMA (A), and conditioned medium was resolved for gelatinase activities (B). The texture of collagen lattices was photographed after removal of medium (C).

Colocalization of MMP-9 with α-SMA in a Subpopulation of HSC in Human Fibrotic Liver Tissues—We wanted to verify whether MMP-9 expression could be localized in HSC in liver fibrosis. To this end, we performed double immunostaining for MMP-9 and α-SMA in fibrotic liver biopsy specimens from two patients. As shown in Fig. 7, MMP-9 and α-SMA immunostaining were colocalized in clusters of the fibrotic regions. Although MMP-9 and α-SMA were largely costained in these two patients, their patterns are slightly different. In one patient most of the MMP-9-positive cells were colocalized with α-SMA staining (Fig. 7A). In the other patient, some MMP-9-positive cells were not stained for α-SMA (Fig. 7B). We speculate that these double stained cells may represent a subpopulation of HSC in the process of activation like rat HSC preceding activation in response to IL-1 in three-dimensional collagen. The MMP-9-negative/α-SMA-positive cells may be the fully activated HSC which expressed abundant α-SMA but no MMP-9. The MMP-9-positive/α-SMA-negative cells are likely to be macrophages (48) or hepatocytes (49).
and cytokines to produce inducible MMPs. This actually correlates well with the clinical observed down-regulation of MMPs in fibrotic liver.

Even though hepatic macrophages are considered as the major source of MMP-9, the MMP-9 mRNA was observed in the sinusoidal cells of the hepatic lobules and mesenchymal cells in fibrous capsules and around the necrosis tissues (49). Our study demonstrates in both the culture model and liver biopsy specimens that HSC can be a major source of MMP-9 in specific situations when multiple signals are presented. This may also have implications in tumor metastasis in addition to liver fibrogenesis. MMP-9 is expressed in most hepatocellular carcinomas (49). Colorectal cancer that metastasized to the liver shows selective expression of the active 82-kDa form of MMP-9 (51). Emerging information also indicates the role of inflammatory cytokine in tumor metastasis. For example, systemic inflammation by injection of IL-1α/H9251 is shown to enhance the metastasis of melanoma into the liver (52). A higher level of IL-1α was observed in colorectal cancer from patients with liver metastasis than in those without liver metastasis (53, 54). We speculate that the paradigm shown by the current study for IL-1α-mediated MMP-9 induction and activation by HSC may also contribute to metastasis of tumors including hepatocellular carcinomas.

Another important observation of this study is the proteolytic activation of pro-MMP-9 by HSC. The irreversible activation of pro-MMP-9 was observed only when HSC were exposed to both IL-1α and type I collagen in prolonged fashion. In Matrigel, both IL-1α and TNF-α induce pro-MMP-9, but no activation ensues. The mechanism for this differential regulation remains unknown. However, this is quite similar to our previous report of pro-MMP-9 activation observed in human skin (45). First, activation of pro-MMP-9 by both HSC and organ-cultured human skin needs a priming time between 48 and 72 h with IL-1 or TNF-α, respectively, during which pro-MMP-9 is expressed without activation (55). This may be the result of a secondary induction of a putative activator. Second, TIMP-1 inhibited the conversion of pro-MMP-9 by HSC and human skin. The fact that the type I collagen but not Matrigel is required for pro-MMP-9 activation by IL-1α should help identify the “activator” by comparing mRNA and protein expression profiles of cellular proteinases in the two models.

Another key question the present study addressed is the causal link between MMP-9 and HSC activation. Our results support the notion that MMP-9, in concert with other MMPs, may directly control activation of HSC in ECM. The first line of evidence is the association of the MMP-9 induction/activation with the cell trans-differentiation by IL-1 treated HSC in three-dimensional type I collagen. The second line of evidence is the prevention of HSC activation by inhibition or absorption of MMP-9 activity with tissue inhibitor or the gelatin-Sepharose beads. We further demonstrated that HSC activation induced by IL-1 is independent of the attachment to plastic with the use of the additional underlying matrix layer (data not shown).

The mechanistic role of MMP-9 in HSC activation is not known, although we speculate that it relates to ECM digestion achieved in concert with MMP-13, which ultimately releases the cells from the mechanical or biochemical restrain by the
MMP-9 on Activation of Hepatic Stellate Cells

Fig. 7. Colocalization of MMP-9 and α-SMA in human fibrotic livers. Biopsies from two patients with liver fibrosis were examined by dual immunostaining for MMP-9 protein and α-SMA. The anti-MMP-9 polyclonal antibodies, which do not cross-react with other MMPs, were recognized by Cy3-conjugated anti-rabbit IgG. α-SMA was detected by fluorescein isothiocyanate-conjugated anti-mouse IgG. The nucleus was stained by DAPI. Colocalization was visualized by a merger of the two stains.

ECM framework. This hypothesis may need to be proven by proteinase-resistant collagen or MMP-9 gene knock-out. The release of mediators bound to the matrix and their effects on HSC cannot be ruled out. Further, MMP-9 may activate HSC via other specific effects as exemplified by a conversion of the latent TGF-β to the active form by the cell surface-bound MMP-9 (56).

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REFERENCES
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