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Stimulation of Pro-MMP-2 Production and Activation by Native Form of Extracellular Type I Collagen in Cultured Hepatic Stellate Cells

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ABSTRACT. **Cultured hepatic stellate cells (HSCs) are known to change their morphology and function with respect to the production of extracellular matrices (ECMs) and matrix metalloproteinases (MMPs) in response to ECM components. We examined the regulatory role of the native form of type I collagen fibrils in pro-MMP-2 production and activation in cultured HSCs. Gelatin zymography of the conditioned media revealed that pro- and active form of MMP-2 was increased in the HSCs cultured on type I collagen gel but not on type I collagen-coated surface, gelatin-coated surface, type IV collagen-coated surface, or Matrigel, suggesting the importance of the native form of type I collagen fibrils in pro-MMP-2 production and activation. The induction of active MMP-2 by extracellular type I collagen was suppressed by the blocking antibody against integrin β1 subunits, indicating the involvement of integrin signaling in pro-MMP-2 activation. RT-PCR analysis indicated that MMP-2, membrane type-1 MMP (MT1-MMP) and tissue inhibitor of metalloproteinase-2 (TIMP-2) mRNA levels were elevated in HSCs cultured on type I collagen gel. The increased MT1-MMP proteins were localized on the cell surface of HSCs cultured on type I collagen gel. In contrast to the expression of MMP-2, HSCs showed a great decline in MMP-13 expression in HSCs cultured on type I collagen gel. These results indicate that the native fibrillar (polymerized) but not monomeric form of type I collagen induced pro-MMP-2 production and activation through MT1-MMP and TIMP-2 in cultured HSCs, suggesting an important role of HSCs in ECM remodeling in the hepatic perisinusoidal spaces.**

Key words: MMP-2/Hepatic stellate cell/type I collagen/extracellular matrix/gelatin zymography/MT1-MMP

Introduction

The interaction between cells and extracellular matrices (ECMs) is essential for cell behaviors such as morphology, proliferation, motility, differentiation, and gene expression (Bissell *et al.,* 1982; Birk de Silver and Trelstad*.,* 1991; Jones *et al.,* 1993). Of the ECM components, type I collagen is the most abundant one in the human body, and the 3-dimensional structure of native type I collagen fibrils has been shown to induce expression of matrix metalloproteinases (MMPs) including MMP-2 (gelatinase A) and MMP-13 (collagenase-3) in several cell types (Azzam and Thompson, 1992; Boyd and Balkwill, 1999; Gilles *et al.,* 1997; Lee *et al.,* 1997; Preaux *et al.,* 1999; Theret *et al.,* 1999; Seltzer *et al.,* 1994; Tomasek *et al.,* 1997; Knäuper *et al.,* 1996; Knäuper *et al.,* 2002). MMPs can degrade most of the ECM components and play a pivotal role in the remodeling of connective tissue under both physiological and pathological conditions (Nagase and Woessner, 1999; Yu *et al.,* 1997; Kähäri and Saarialho-Kere, 1999). MMP-2 is able to digest type IV collagen, a major basement membrane component, and has been implicated in organ growth, endometrial cycling, wound healing, bone remodeling, tumor invasion and metastasis, arthritis, and periodontal disease (Woessner, 1994). MMP-2 is produced as a latent pro-MMP-2 and the activation is a unique process mediated by membrane type-1 MMP (MT1-MMP) and tissue inhibitor of metalloproteinases-2 (TIMP-2) rather than by soluble proteinase cascades (Gilles *et al.,* 1996; Ellerbroek *et al.,* 1999; Kurschat *et al.,* 1999; Ellenrieder *et al.,* 2000; Hotary *et al.,* 2000). Pro-MMP-2 is thought to form a trimolecular complex with

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Abbreviations: CM, conditioned medium; DAPI, 4',6-diamidino-2-phenylindole dilactate; ECM, extracellular matrix; FBS, fetal bovine serum; HSC, hepatic stellate cell; MMP, matrix metalloproteinase; MT1-MMP, membrane type-1 matrix metalloproteinase; TBS, Tris-buffered saline; TIMP, tissue inhibitor of metalloproteinase; TRITC, tetramethylrhodamine-5-isothiocyanate.

MT1-MMP and TIMP-2, and then to be cleaved by an adjacent MT1-MMP molecule on the cell surface (Strongin *et al.,* 1995). Different from the gelatinase-type MMP-2, MMP-13 is a member of the collagenase subfamily of MMPs, is expressed in breast tumors, hypertrophic chondrocytes, and skin fibroblasts *in vivo* (Freije *et al.,* 1994; Mitchell *et al.,* 1996; Ravanti *et al.,* 1999), and has been reported to be an essential enzyme for degrading type I collagen in liver fibrosis (Seyer *et al.,* 1977; Rojkind *et al.,* 1979).

Hepatic stellate cells (HSCs), referred to also as Ito cells, vitamin A-storing cells, lipocytes, or fat-storing cells, are located in the hepatic perisinusoidal spaces between sinusoidal endothelial cells and parenchymal cells in the liver. HSCs function as major vitamin A-storing cells, containing over 80% of the total vitamin A in the body to maintain retinoid homeostasis, and also are the major cell type producing ECM components including type I, III, IV, and VI collagens, fibronectin, laminin, and proteoglycans in the hepatic lobules. Several types of MMPs including MMP-1, MMP-2, MMP-3, MMP-9, and MMP-11 (Milani *et al.,* 1994; Takahara *et al.,* 1997; Geisler *et al.,* 1997; Iredale *et al.,* 1992) are expressed in hepatic lobules, and HSCs produce virtually all of the key MMPs, including MMP-2 and MMP-3 that degrade the normal ECM components in the perisinusoidal space between parenchymal cells and endothelial cells. In addition to MMPs, TIMP-1 and -2, which can inhibit MMP activities, are expressed only in HSCs (Iredale *et al.,* 1992; Herbst *et al.,* 1997; Knittel *et al.,* 1999; Friedman and Arthur, 2002). Cultured HSCs have been found to respond to extracellular type I or type III collagen fibrils used as a substratum by displaying altered morphology and function including proliferation, ECM production, or MMP expression (Senoo *et al.,* 1996; Li *et al.,* 1999; Sato *et al.,* 2003).

In this study, the regulatory role of ECM components in the expression of MMPs, particularly pro-MMP-2 and its activation, was examined by using HSCs cultured separately on several ECM components including a polystyrene surface, type I collagen-coated surface, gelatin-coated surface, type I collagen gel, type IV collagen-coated surface, and Matrigel. We found that the active form of MMP-2 (gelatinase A) was specifically induced by the native form of extracellular type I collagen fibrils, probably through MT1-MMP and TIMP-2, in cultured HSCs but that MMP-13 (collagenase 3) expression was suppressed by these fibrils.

Materials and Methods

Cell culture

Hepatic parenchymal and non-parenchymal cells were isolated from male Wistar rats weighing 250–300 g by the collagenase perfusion method (Berry and Friend, 1969; Seglen, 1976). HSCs were separated from parenchymal cells by low speed centrifugation and from other non-parenchymal cells by differential centrifugation over a Percoll density gradient, as previously described (Senoo *et al.,* 1993; Senoo and Hata, 1994), and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS) and antibiotics at 37°C in a humidified atmosphere of 95% air and 5% CO_2. For preparation of type I or type IV collagen-coated dishes, the bottom of each culture dish was covered with 0.3 mg/ml of porcine type I or type IV collagen (Nitta Gelatin, Osaka, Japan) solution in 0.001 N HCl, pH 3.0, for 30 min, and then air-dried at room temperature after removal of the collagen solution. For preparation of gelatin-coated dishes, the bottom of each culture dish was covered with 0.3 mg/ml of porcine gelatin (Nitta Gelatin) for 30 min and then air-dried at room temperature after removal of the gelatin solution. For cultivation of cells on type I collagen gel, 1.5 mg/ml porcine type I collagen solution in DMEM was polymerized in polystyrene culture dishes, and then a cell suspension in DMEM containing 10% (v/v) FBS was inoculated onto the polymerized type I collagen gel. For cultivation of cells on Matrigel (a basement membrane matrix, Becton Dickinson, Bedford, MA, USA), Matrigel solution (2.8 mg/ml) in DMEM was placed and gelled in polystyrene culture dishes, and then the cell suspensions were inoculated onto the gel.

Gelatin zymography

HSCs were cultured on each substratum in DMEM containing 10% FBS for 18 hours, and then the cells were washed 3 times with serum-free DMEM and further cultured in serum-free DMEM for 24 hours. Aliquots (100 μl) of each conditioned medium (CM) were treated with 4 volumes of cold acetone, and the precipitates were dissolved in Laemmli's sample buffer without 2-mercaptoethanol, and then used for gelatin zymography as previously described (Gilles *et al.,* 1997; Li *et al.,* 1999). In brief, acetone-precipitates from the CMs were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% polyacrylamide gel containing 1 mg/ml gelatin. The gels were washed twice each time for 30 min in 2.5% (w/v) Triton X-100 solution at room temperature, and then incubated at 37°C for 15–18 hours in developing buffer (50 mM Tris-HCl, pH 7.8, 5 mM $CaCl_2$, 0.15 M NaCl, and 1% (w/v) Triton X-100). The gels were then stained with a solution containing 0.1% (w/v) Coomassie Brilliant Blue R-250, 12.5% (v/v) ethanol and 7.5% (v/v) acetic acid, and thereafter destained in 45% methanol and 10% acetic acid. For preparation of cell lysates, the cultured HSCs were washed with Tris-buffered saline (TBS; 20 mM Tris-HCl, pH 7.4, containing 150 mM NaCl), and lysed directly with RIPA buffer (50 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 1 mM EDTA, 1% (w/v) Nonidet P-40, 0.25% (w/v) sodium deoxycholate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM PMSF). Aliquots of the cell lysates were analyzed by gelatin zymography as described above.

For the integrin blocking experiment, trypsinized HSCs were resuspended and cultured for 18 hours on a polystyrene surface, type I collagen-coated surface, type I collagen gel, or Matrigel in

serum-supplemented DMEM containing 0, 3, or 10 μg/ml of anti-integrin β1 antibody. Then, the cells were washed 3 times with serum-free DMEM and further cultured in serum-free DMEM containing 0, 3, or 10 μg/ml of anti-integrin β1 antibody for 12 hours, after which gelatin zymography of the CMs was performed as described above.

Immunoblot analysis

HSCs were cultured for up to 48 hours on each substratum, washed with TBS, and lysed directly with RIPA buffer. Aliquots of the cell lysates having equal amounts of proteins were resolved by SDS-PAGE using 10% polyacrylamide gel, and then transferred onto a PVDF membrane (Immobilon-P Transfer membrane, Millipore Co., Bedford, MA, USA). The blot was washed and blocked for 30 min at room temperature in TBS containing 1% (w/v) BSA and 0.1% (w/v) Tween 20, and then incubated with anti-MT1-MMP or anti-MMP-13 antibody for 1 hour at room temperature (Sigma, St. Louis, MO, USA). Then, following incubation with horseradish peroxidase-conjugated secondary antibody (New England Bio-Labs, Beverly, MA, USA) for 30 min at room temperature, the color was developed by using a Konica Immunostaining HRP-1000 kit (Seikagaku Co., Tokyo, Japan).

Immunofluorescence staining

HSCs were cultured on each substratum for 24 hours, fixed with 4% (w/v) paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4), washed with TBS, and blocked with 1% BSA, 0.1% (w/v) Triton X-100 in TBS for 15 minutes. The cells were then sequentially reacted with rabbit anti-MT1-MMP antibody for 1 hour, biotin-labeled mouse anti-rabbit IgG (Sigma) for 30 minutes, and Alexa Fluor 488-labeled streptavidin (Molecular Probes, Inc., Eugene, OR, USA) for 30 minutes at room temperature, stained for F-actin with tetramethylrhodamine-5-isothiocyanate (TRITC)-labelled phalloidin (Sigma), and then counterstained nuclei with 4',6-diamidino-2-phenylindole dilactate (DAPI, Molecular Probes, Inc.) for 15 minutes. The fluorescence signals were analyzed by using a laser-scanning microscope LSM510 (Carl Zeiss, Germany).

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total cellular RNA was extracted by the method using guanidinium isothiocyanate from HSCs cultured for 48 hours on each substratum (Chomczynski and Sacchi, 1987). First-strand cDNA was synthesized by using the total RNA as a template with oligo (dT) primer and AMV reverse transcriptase according to the manufacturer's instructions (Pharmacia Biotech, Cambridge, UK), and PCR amplification was done with a super Taq premix Kit (Sawady Tech. Inc., Tokyo, Japan) and the following specific sets of upstream and downstream primers: 5'-TGACTATGCGTGGCTGGAA-3' and 5'-AAGCTGAAATCTTGCCTTGGA-3' for MMP-13, 5'-GATACCCCAAGCCACTG-3' and 5'-TCCAAACTTCACGCTCTT-3' for MMP-2, 5'-TCTGGGCAACAAGTATGAG-3' and 5'-CCACGGATCTGAGCAAT-3' for MT1-MMP, and 5'-AGCGAGAAGGAGGTGGAG-3' and 5'-CCAGGGCACAATAAAGTCT-3' for TIMP-2, and 5'-ATCGGAACCGCTCATTGCC-3' and 5'-TTGTAACCAACTGGGACGATA-3' for β-actin as an internal control, respectively. PCR was performed as follows: 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 56°C for 15 seconds, and extension at 72°C for 30 seconds. The PCR products were resolved on 1.5% agarose gel, visualized by ethidium bromide staining, and quantified by image analysis using NIH Image version 1.61 (NIH, Bethesda, MD, USA).

Results

Stimulation of active MMP-2 production in cultured HSCs by native extracellular type I collagen

To investigate the effects of ECM components on pro-MMP-2 production and activation, we cultured HSCs for 18 hours in FBS-supplemented DMEM and then for 24 hours in serum-free DMEM on a polystyrene surface, type I collagen-coated surface, gelatin-coated surface, type I collagen gel, type IV collagen-coated surface, or Matrigel (Fig. 1). Interestingly, the active form of MMP-2, seen as an apparent 62 kD band, as well as a faint intermediate form, was detectable only in the CM from HSCs cultured on type I collagen gel (Fig. 1A). Although the type I collagen gel or Matrigel used for cell culture contained no detectable amount of MMPs (data not shown), FBS contained a significant amount of pro-MMP-2 but no active form of MMP-2 (Fig. 1A). As the HSCs had been cultured overnight in FBS-supplemented DMEM prior to serum-free culture on each substratum to ensure cell survival, we checked to see whether or not the FBS remaining in the type I collagen gel or Matrigel used as a substratum affected the gelatin zymographic data (Fig. 1B). We prepared control CMs collected from the culture dishes, which were treated with type I or type IV collagen solution, gelatin solution, or Matrigel as described in Materials and Methods, and incubated them with FBS-supplemented DMEM for 12 hours and then with serum-free DMEM for 24 hours in the absence of HSCs (Fig. 1B, –HSCs), in parallel with HSC cultures (Fig. 1B, +HSCs) on each substratum. Zymographic analysis of the control CMs indicated that a small amount of pro-MMP-2 and pro-MMP-9 but no active form of MMP-2 remained in the type I collagen gel (Fig. 1B, –HSCs). The cell lysates from HSCs cultured on the type I collagen gel also showed an increase in pro-MMP-2 and active form of MMP-2 as well as a low level of pro-MMP-9, as compared with those from HSCs cultured on other substrata by gelatin zymography (Fig. 1C), also suggesting the induction of pro-MMP-2 production and its activation in cultured HSCs by extracellular type I collagen fibrils.

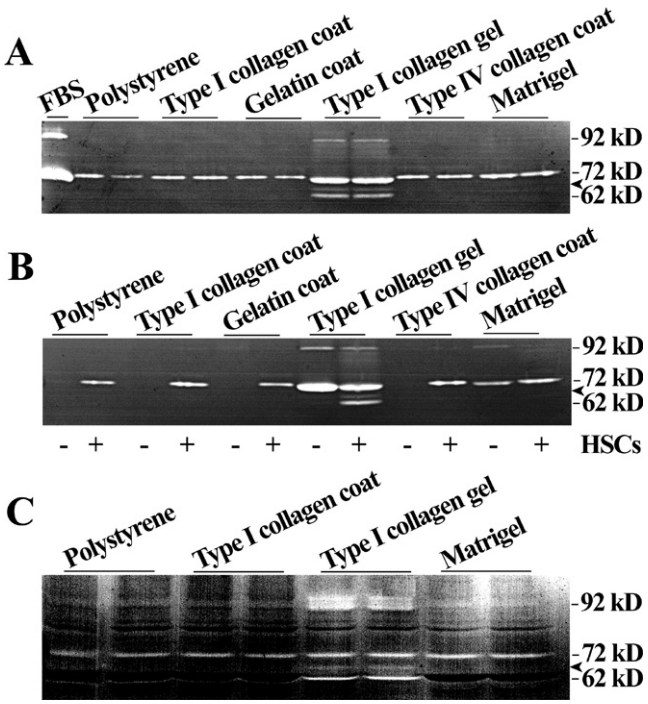

Fig. 1. Activation of pro-MMP-2 in rat HSCs cultured on type I collagen gel. A) After an 18-hour culture in FBS-supplemented DMEM on polystyrene surface, type I collagen-coated surface, gelatin-coated surface, type I collagen gel, type IV collagen-coated surface, or Matrigel, HSCs were further cultured in serum-free DMEM for 24 hours on each substratum. Aliquots of the CMs from the duplicate cultures of HSCs on each substratum, as well as 0.5 μl of FBS used for cell culture, were analyzed by gelatin zymography. B) For evaluation of the contribution of the FBS remaining in each substratum to zymographic data, control CMs were prepared as follows: culture dishes were treated with type I collagen solution, gelatin solution, or Matrigel, and incubated for 18 hours with FBS-supplemented DMEM and then with serum-free DMEM for 24 hours but in the absence of HSCs (–HSCs), in parallel to the HSC cultures (+HSCs) on each substratum. Gelatin zymography was then performed with the control CMs and CMs from the corresponding HSC cultures. C) The cell lysates from the duplicate cultures of HSCs on each substratum were analyzed by gelatin zymography.

Suppression of pro-MMP-2 activation by blocking of integrin β1 subunit in HSCs cultured on type I collagen gel

Since integrin α1β1 and α2β1 are known to be major receptors for type I collagen and to be expressed in HSCs (Carloni *et al.,* 1996), we examined the possible involvement of integrin signaling in pro-MMP-2 production and activation in HSCs cultured on type I collagen gel by using an integrin β1 blocking study. When HSCs were cultured in the presence of 0, 3, or 10 μg/ml of anti-integrin β1 blocking antibodies, pro-MMP-2 production and activation by extracellular type I collagen were dose-dependently suppressed by these antibodies (Fig. 2A), suggesting the involvement of integrin signaling in pro-MMP-2 production and activation processes in cultured HSCs by extracellular type I collagen fibrils. Since, in the presence of the blocking antibody, no morphological effects were seen in subconfluent cultures of HSCs on either polystyrene surface or type I collagen gel by observation under a phase-contrast microscope (Fig. 2B), it was unlikely that the effects of the blocking antibody are secondary to the changes in cell attachment or cell morphology.

RT-PCR analysis of MMP-2, MT1-MMP, and TIMP-2 mRNA levels regulated by extracellular type I collagen in cultured HSCs

Since MT1-MMP and TIMP-2 have been found to be closely associated with pro-MMP-2 activation processes (Gilles *et al.,* 1996; Ellerbroek *et al.,* 1999), we next examined whether or not MT1-MMP and TIMP-2 were engaged in the enhancement of pro-MMP-2 production and activation by extracellular type I collagen. In accord with the zymographic data (Fig. 1A), the MMP-2 mRNA level was elevated in HSCs cultured on type I collagen gel but not on the type I collagen-coated surface and Matrigel (Fig. 3A and B). The MT1-MMP and TIMP-2 mRNA levels were also elevated in HSCs cultured on type I collagen gel, as compared with those for HSCs cultured on the type I collagen-coated surface or Matrigel (Fig. 3A and B). These results suggest that the native form of extracellular type I collagen fibrils induces an up-regulation of MMP-2 mRNA expression and the following pro-MMP-2 production and activation *via* MT1-MMP and TIMP-2 up-regulation.

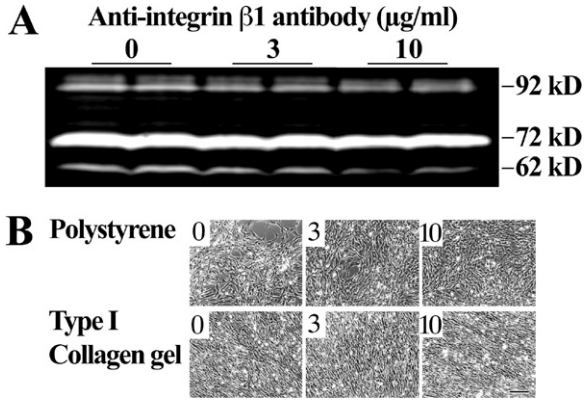

Fig. 2. Suppression of pro-MMP-2 activation by integrin β1 blocking in HSCs cultured on type I collagen gel. A) Gelatin zymography: trypsinized HSCs were resuspended and cultured in duplicate for 18 hours on type I collagen gel in FBS-supplemented DMEM containing 0, 3, or 10 μg/ml of anti-integrin β1 antibody. Then, the cells were washed 3 times with serum-free DMEM and further cultured for 24 hours in serum-free DMEM containing 0, 3, 10 μg/ml of anti-integrin β1 antibody, after which gelatin zymography of the CMs was performed as described above. B) Phase-contrast images: in the presence of 0, 3, 10 μg/ml of anti-integrin β1 antibody, no effects on cell attachment and morphology were seen in the subconfluent cultures of HSCs on either polystyrene or type I collagen gel. Scale bar indicates 200 μm.

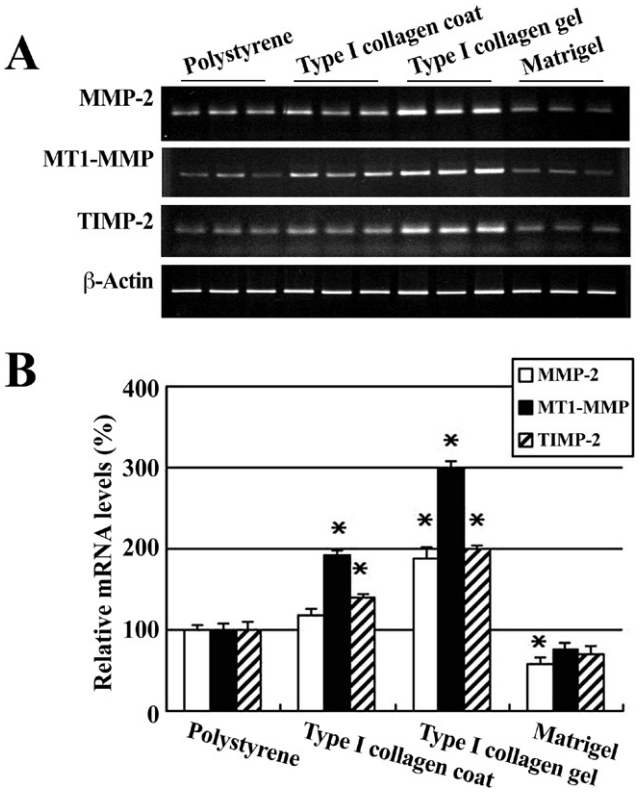

Fig. 3. RT-PCR analysis of MMP-2, MT1-MMP, and TIMP-2 mRNA levels in cultured HSCs. A) Total RNA was prepared in triplicate from the HSCs cultured on each substratum, and reverse-transcribed, which procedure was followed by PCR amplification using a set of specific primers for each of MMP-2, MT1-MMP, and TIMP-2, as well as one for β-actin as an internal control. B) MMP-2 (open bars), MT1-MMP (closed bars), and TIMP-2 (shaded bars) mRNA levels were semi-quantified by densitometry analysis of the PCR products by using NIH Image version 1.61. The MMP-2, MT1-MMP, and TIMP-2 mRNA levels normalized with the β-actin level in each total RNA sample were shown as relative to the value of HSCs cultured on the polystyrene surface (100%), from which the difference in each of mRNA levels in HSCs cultured on the type I collagen-coated surface, type I collagen gel, or Matrigel was evaluated by Student's t test (*$p<0.05$).

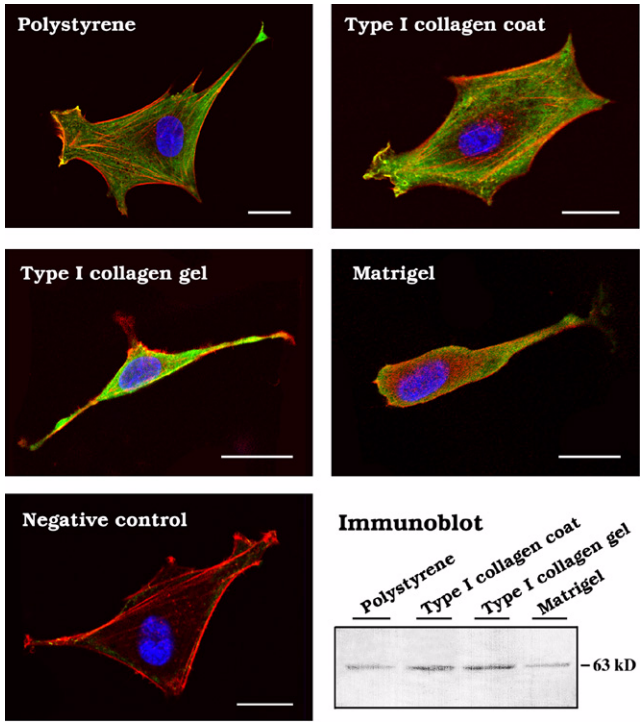

Fig. 4. Localization of MT1-MMP in HSCs cultured on type I collagen gel. HSCs were cultured overnight on each substratum, stained for MT1-MMP proteins with monoclonal anti-MT1-MMP antibody, biotin-labelled anti-mouse IgG, and Alexa Fluor 488-labelled streptavidin (green), and for F-actin with TRITC-labelled phalloidin (red), and counterstained nuclei with DAPI (blue). As a negative control, HSCs cultured on the polystyrene surface were treated as described above but used non-immune serum instead of the primary antibody. Alternatively, total cell lysates were prepared from HSCs cultured on each substratum and analyzed by immunoblotting, by which MT1-MMP proteins were seen as a single band with an apparent molecular weight of 63 kD. Scale bars, 20 μm.

Localization of MT1-MMP on cell surface of HSCs cultured on type I collagen gel

The localization of MT1-MMP on the cell membrane is critical to the activation of pro-MMP-2 (Lehti *et al.,* 1998). To determine whether the ECM components used as substratum affected the intracellular localization of MT1-MMP in cultured HSCs or not, we performed immunofluorescence staining of HSCs cultured on the polystyrene surface, type I collagen-coated surface, type I collagen gel, or Matrigel for 24 h (Fig. 4). As seen by F-actin staining, HSCs spread well with stress fibers when cultured on the polystyrene surface and type I collagen-coated surface, while HSCs cultured on the type I collagen gel showed an asteroid shape with elongated processes, as previously described (Miura *et al.,* 1997; Sato *et al.,* 1998). The anti-MT1-MMP antibody used for immunostaining recognized MT1-MMP as a single band with apparent molecular weight of 63 kD on the immunoblots of cell lysates from cultured HSCs. The immunoblot showed an elevation in MT1-MMP protein expression in HSCs cultured on the type I collagen-coated surface and type I collagen gel. However, in contrast to a dispersed distribution of MT1-MMP in HSCs cultured on the type I collagen-coated surface, MT1-MMP were localized on the cell surface of HSCs cultured on type I collagen gel. No staining for MT1-MMP was seen in the negative control in which HSCs were similarly treated but used non-immune serum instead of the primary antibody against MT1-MMP.

Suppression of MMP-13 expression in HSCs cultured on type I collagen gel

To determine whether ECM components would affect the expression of the collagenase type of MMPs in cultured

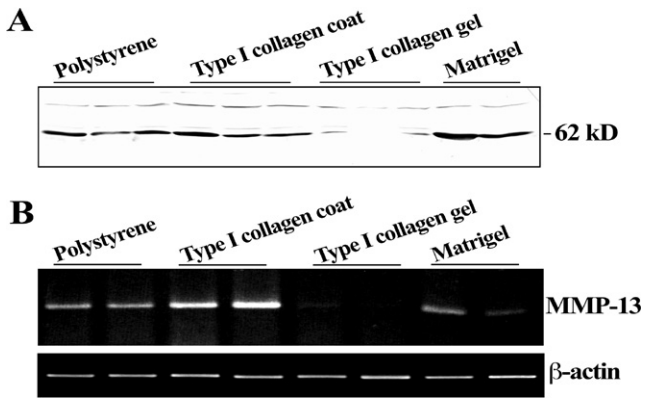

Fig. 5. Suppression of MMP-13 expression in HSCs cultured on type I collagen gel. A) Immunoblot for MMP-13 proteins in cell lysates from the HSCs cultured on each substratum in triplicate except for the duplicate cultures on Matrigel. B) RT-PCR analysis of MMP-13 mRNA level, as well as β-actin mRNA level as an internal control, in HSCs cultured on each substratum in duplicate.

HSCs, we investigated the MMP-13 expression in HSCs cultured on polystyrene surface, type I collagen-coated surface, type I collagen gel, or Matrigel. The MMP-13 expression was examined by immunoblotting (Fig. 5A) and RT-PCR analysis (Fig. 5B). Interestingly, when HSCs were cultured on the type I collagen gel, the MMP-13 protein level was markedly decreased, as compared with that of HSCs cultured on the polystyrene surface, type I collagen-coated surface, or Matrigel (Fig. 5A). RT-PCR analysis also showed a marked decline in the level of MMP-13 mRNA in HSCs cultured on the type I collagen gel, compared with that in HSCs cultured on the polystyrene surface or Matrigel, whereas the HSCs cultured on the type I collagen-coated surface showed rather an up-regulation of MMP-13, suggesting a difference between the signalings originated from unpolymerized (monomeric) and polymerized (fibrillar) type I collagen (Koyama *et al.*, 1996). These observations demonstrate that, in contrast to the up-regulation of MMP-2, the native form of type I collagen fibrils suppressed the proMMP-13 expression in cultured HSCs.

Discussion

In this study we showed that cultured HSCs responded to the native fibrillar form of extracellular type I collagen by producing and activating pro-MMP-2 through the up-regulation of MT1-MMP and TIMP-2 expression. Such a response was not seen in those HSCs cultured on type I collagen-coated (monomeric type I collagen) surface (Koyama *et al.*, 1996) or on Matrigel, which contains laminin and type IV collagen, thus suggesting that HSCs specifically recognized the native structure of type I collagen fibrils for pro-MMP-2 production and activation. A similar difference in substratum effect was previously shown with respect to a morphological change in HSCs; elongation of long processes occurred when the cells were cultured on type I collagen gel but not when a type I collagen-coated surface or Matrigel was the substratum (Senoo *et al.,* 1996; Miura *et al.,* 1997; Sato *et al.,* 1998). These results suggest that HSC morphology and function are differentially regulated by the various ECM components, such as type I and type III collagen and basement membrane components in the spaces between sinusoidal endothelial cells and parenchymal cells.

Pro-MMP-2 activation in HSCs cultured on type I collagen gel was associated with the up-regulation of MT1-MMP and TIMP-2 (Fig. 3). Several studies have demonstrated that pro-MMP-2 is activated by MT1-MMP through the formation of a membrane-bound ternary complex consisting of MT1-MMP, TIMP-2, and pro-MMP-2 (Strongin *et al.,* 1995; Will *et al.,* 1996; Butler *et al.,* 1998; Zucker *et al.,* 1998). MMP-2 mRNA was up-regulated in HSCs cultured on type I collagen gel, as compared with its level in the cells cultured on the polystyrene surface, type I collagen-coated surface, or Matrigel. However, even when HSCs were cultured in serum-supplemented DMEM, the active form of MMP-2 specifically appeared in HSCs cultured on type I collagen gel but not on a polystyrene surface, type I collagen-coated surface, and Matrigel (Li *et al.,* 1999). Since the serum (FBS) used for cell culture contains a significant amount of pro-MMP-2 but no active form of MMP-2 (Li *et al.,* 1999), as was shown in Fig. 1, the pro-MMP-2 activation events are specific to the HSC culture on type I collagen gel, probably through up-regulation of MT1-MMP and TIMP-2.

HSCs have been found to express several types of integrin receptors including α1β1, α2β1, αVβ1 and α6β4 (Pinzani *et al.,* 1998). In addition to the classical ECM receptors, a non-integrin receptor, discoidin domain receptor-2 (DDR-2), was shown to be expressed in HSCs (Ankoma-Sey *et al.,* 1998). The morphology and function of HSCs might be regulated by interaction with interstitial type I and III collagens and other ECM components *via* integrin and non-integrin receptors. Earlier we reported that ECM components reversibly regulated the morphology, proliferation and collagen synthesis in cultured HSCs (Sato *et al.,* 2003; Senoo *et al.,* 1998). HSCs were induced to extend long processes by the native form of interstitial type I and type III collagen used as a substratum. Many cell types utilize integrin receptors for transducing ECM signals, and HSCs also require integrin-binding to extracellular collagen fibrils and the following intracellular signaling and cytoskeleton reorganization to undergo morphological changes (Sato *et al.,* 2003; Miura *et al.,* 1997; Kojima *et al.,* 1998). Pro-MMP-2 production and activation induced by the native form of extracellular type I collagen was suppressed in the presence of blocking antibody against integrin β1 subunit (Fig. 2), indicating the HSC responded through integrin signaling. Since no such response occurred in HSCs cultured on the

type I collagen-coated surface (monomeric collagen molecules), the native form of interstitial collagen fibrils is prerequisite for inducing morphological and functional changes. Similarly, a response to the native form of collagen fibrils but not to monomeric collagen was reported with respect to morphology and function in other cell types (Grab *et al.,* 1996; Mercier *et al.,* 1996; Vogel *et al.,* 1997).

Although the reason is not clear, it has been shown that MMP-13 expression is induced by the native form of type I collagen in cultured human skin fibroblasts (Ravanti *et al.,* 1999). This opposite response of MMP-13 expression to the native form of type I collagen in the skin fibroblasts and HSCs probably depends on the difference in cell type or species. In contrast to the up-regulation of MMP-2 mRNA, MMP-13 expression was suppressed by extracellular type I collagen in HSCs, as compared with its level in the HSCs cultured on the polystyrene surface, type I collagen-coated surface, and Matrigel, as was shown in Fig. 1 in this study. These results suggest the differential effects of extracellular type I collagen signals on gelatinase and collagenase expressions, and also the difference in the effects on MMP-13 expression between the native and monomeric forms of type I collagen.

In summary, pro-MMP-2 production and activation, as well as the down-regulation of MMP-13, were specifically induced when HSCs were cultured on type I collagen gel but not on type I collagen-coated surface or on the basement membrane components, thus indicating the differential response of HSCs to ECM components for MMP expressions. Activation of pro-MMP-2 in HSCs cultured on the type I collagen gel was associated with the up-regulation of MT1-MMP and TIMP-2. These results suggest that, in addition to the regulatory effects of certain cytokines and growth factors, both production and degradation, and hence the remodeling of the ECM by HSCs are regulated by distinct ECM components, in the spaces between endothelial cells and parenchymal cells.

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