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Regulatory Role of Extracellular Matrix Components in Expression of Matrix Metalloproteinases in Cultured Hepatic Stellate Cells

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ABSTRACT. **Hepatic stellate cells (HSCs) were changed in their morphology, proliferative activity, and functions by culturing on type I collagen gel, as compared to the culture on polystyrene surface. HSCs have been found to produce extracellular matrix components and matrix metalloproteinases (MMPs). In this study, we have assessed the effects of several types of substrata on the expression of MMPs in HSC culture. MMP-1 expression was detectable in HSC culture on polystyrene surface and on type I collagen gel by immunofluorescence staining and reverse transcriptase-polymerase chain reaction (RT-PCR). The results from in situ zymography revealed the presence of interstitial collagenase activity around HSCs and along their cellular processes. Although proMMP-2 and proMMP-9 were detectable by gelatin zymography in the conditioned medium from both cultures using type I collagen gel and Matrigel as substratum, an active form of MMP-2 but not of MMP-9 was detected only in the culture using type I collagen as a substratum. Tissue inhibitor of metalloproteinase-2 expression was observed by RT-PCR in HSCs cultured on or in type I collagen gel, suggesting the suppression of MMP-2 activity detected in HSC culture using type I collagen. These results indicate a differential expression of MMP activity, hence the remodeling of extracellular matrix components is dependent on the substratum used for HSC culture. The HSC culture using several types of substrata appears to be a useful in vitro model to study the mechanism of extracellular matrix remodeling.**

Key words: hepatic stellate cell/matrix metalloproteinase/tissue inhibitor of metalloproteinase/extracellular matrix/zymography

Cellular interaction with extracellular matrix (ECM) affects a diverse range of cellular functions including cell differentiation, migration, proliferation, and survival. Information from ECM can regulate processes of embryonic growth and differentiation, and tissue remodelling and repair (33). Collagen turnover and ECM remodeling that occur during various physiological and pathological processes including tissue repair, wound healing, fibrosis, and tumor invasion are largely dependent on the regulation of activities of matrix metalloproteinases (MMPs) and membrane-type MMPs (MT-MMPs), which are Zn^{2+}-dependent neutral proteases and degrade distinct types of collagen and gelatin (3, 8). Their activity is regulated at transcription, by proenzyme activation, and finally by tissue inhibitor of metalloproteinase (TIMP), which is coexpressed and present at the tissue sites of enzyme activity (20). MMP-1 attacks collagen types I, II, III, VII, and X, and MMP-1 also degrades casein and cartilage proteoglycan. MMP-2 (gelatinase A) readily degrades gelatins, but also has activity on collagen type IV, V and VII, elastin and proteoglycan. Increasing attention has been focused on the effects of ECM components on cellular expression of MMPs, since ECM plays an important role in regulating the behavior of the cells that contact it (11, 13, 18, 23).

Hepatic stellate cells (HSCs), which are localized in the perisinusoidal space of the hepatic lobule, store 80%–90% of vitamin A in the body, and have multifunctional characteristics in: (a) retinoid metabolism; (b) synthesis and secretion of ECM components; (c) production of MMPs and TIMPs; and (d) synthesis and secretion of cytokines (6, 10, 19, 21, 32). HSCs also ap-

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Abbreviations: DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; FITC, fluorescein isothiocyanate; HSC, hepatic stellate cell; MMP, matrix metalloproteinase; RT-PCR, reverse transcription-polymerase chain reaction; TIMP, tissue inhibitor of metalloproteinase; TRITC, tetramethylrhodamine isothiocyanate.

pear to play an important role in the development of hepatic fibrosis. It has been shown that in the early stages of hepatic fibrosis there is a remodelling of basement membranes and increased expression of MMPs. HSCs develop a myofibroblast-like phenotype, proliferate, and secrete high amounts of ECM components (9). However, the effect of ECM components on MMP expression is still enigmatic. In previous studies, we demonstrated that ECM components can regulate morphology, proliferation and functions of the cultured HSCs (24, 26, 28, 30).

The aim of this study is to investigate the molecular mechanism of ECM remodeling, as well as the regulation of MMP expression using HSC culture as a model. We investigated the expression of MMPs in HSCs cultured using several types of substrata including polystyrene surface, type I collagen-coated surface, type I collagen gel, and Matrigel containing the basement membrane components. Such a culture system using extracellular matrix components as a substratum appears to be useful to study the mechanism of ECM remodeling.

Materials and Methods

Isolation and culture of HSCs using several types of substrata

Human HSCs were isolated by the collagenase shaking method (2, 25), and then purified by differential centrifugation over the Percoll density gradient, as previously described (27, 28). HSCs were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and antibiotics at 37°C in a humidified atmosphere of 95% air and 5% CO_2. HSCs were subcultured at 1:3 split ($2–4 \times 10^4$ cells/ml), and used during 5–10 passages in this study. To prepare type I collagen-coated dishes, culture dishes were soaked in 0.3 mg/ml of porcine type I collagen (Nitta Gelatin, Osaka, Japan) solution in 0.001 N HCl, pH 3.0 for 30 min, and then were air-dried at room temperature after removal of the collagen solution. For cultivation of cells on or in 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 or a 1:1 mixture of cell suspension and 1.5 mg/ml of type I collagen solution in DMEM containing 10% (v/v) FBS and antibiotics was inoculated on the polymerized type I collagen gel. For cultivation of cells on Matrigel (the basement membrane matrix, Becton Dickinson Labware, Bedford, MA, USA), 2.8 mg/ml of Matrigel solution in DMEM were placed and gelled in polystyrene culture dishes, followed by the inoculation of cell suspension.

Preparation of conditioned media and gelatin zymography

HSCs were seeded on each substratum in DMEM containing 10% FBS. After 18 hours, the medium was removed, and the cells were washed three times in serum-free medium and cultured in the serum-free medium. Then, the conditioned medium was collected after 4 hours, clarified from cells and collagen debris by centrifugation at 10,000 rpm ($9,000 \times g$) for 5 min, and analyzed by gelatin zymography.

Immunofluorescence staining for MMP-1

Monoclonal anti-MMP-1 was a kind gift from Dr. K. Iwata (Fuji Chemical Industries, Ltd., Toyama, Japan). After fixation with 4% paraformaldehyde in 0.1 M sodium phosphate-buffer, pH 7.4, the cells were reacted with the primary antibody and then fluorescein isothiocyanate (FITC)- or tetramethylrhodamine isothiocyatnate (TRITC)-labeled secondary antibody (Sigma, St. Louis, MO, USA). FITC- or TRITC-labeled phalloidin (Sigma) was used for staining of fibrillar actin (F-actin). The fluorescence signals were observed by using an Inverted Laser Scanning Microscope LSM 410 (Carl Zeiss, Germany).

Gelatin zymography of conditioned media from HSC culture

The conditioned media from HSC culture were analyzed for MMP-2 and MMP-9 by gelatin zymography (14). Proteins were separated by SDS-PAGE under the non-reducing condition on 10% polyacrylamide gels containing 1 mg/ml gelatin. SDS was extracted with Triton X-100 from the gels, which were then incubated overnight at 37°C in 50 mM Tris-HCl, pH 7.8, containing 150 mM NaCl and 5 mM $CaCl_2$. The gels were stained with Coomassie Brilliant Blue R-250 and destained in 45% methanol and 10% acetic acid.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total cellular RNA was isolated by the method using guanidinium isothiocyanate (5). The quality of the isolated RNA was confirmed by sharp ribosomal bands on 1% agarose gel after electrophoresis. Each total RNA sample, prepared as described above, was used for the first-strand cDNA synthesis using the cDNA synthesis kit (Pharmacia Biotech, Cambridge, England). Amplification reaction was performed with Super Tag Premix Kit (Sawady Tech. Inc., Tokyo, Japan) according to the manufacturer's instructions. The sequences of upstream and downstream primers for MMP-1 were 5′-TAG CTGGTTCAACTGCAGGA-3′ and 5′-ATCCCTTGCCTAT CTAGGGT-3′ at positions 1451 to 1470 and 1886 to 1905, respectively (12). The sequences of upstream and downstream primers for MMP-2 were 5′-TGGGCAACAAATATGAGAG AGC-3′ and 5′-CGGCATCCAGGTTATCGGGG-3′ at posi-

tions 1020 to 1041 and 1791 to 1810, respectively (7). The sequences of upstream and downstream primers for TIMP-2 were 5′-GTGGACTCTGGAAACGACAT-3′ and 5′-CCAGG AAGGGATGTCAGAGC-3′ at positions 417–436 and 981–1000, respectively (4). PCR reactions were performed as follows: 1 cycle at 94°C for 1 min; and then 30 cycles at 55°C for 1 min, 72°C for 1 min, and 94°C for 1 min. PCR products were separated and visualized on a 1% agarose gel containing ethidium bromide.

Results

MMP-1 expression in HSC culture

To study the effect of ECM components on the expression of MMPs, human HSCs were cultured overnight on polystyrene surface or on type I collagen gel. The cultured HSCs were induced to elongate long, multipolar processes in the presence of type I collagen gel used as a substratum, as compared to flattened or round cell shapes on polystyrene surface (24). Human HSCs cultured either on polystyrene surface or on type I collagen gel expressed MMP-1, as detected by immunofluorescence staining (Fig. 1A). Small dots indicating MMP-1 localization were distributed diffusely in HSCs cultured on polystyrene surface (Fig. 1A-a), whereas the dots were condensed in the cellular processes of HSCs cultured on type I collagen gel (Fig. 1A-b). When non-immune mouse IgG, as a control, was used instead of primary antibody, no fluorescence signals were detected (data not shown). RT-PCR analysis was performed using total RNA isolated from cells cultured on polystyrene surface and on type I collagen gel. MMP-1 transcripts were detected in both cells cultured on polystyrene surface and on tyep I collagen gel (Fig. 2).

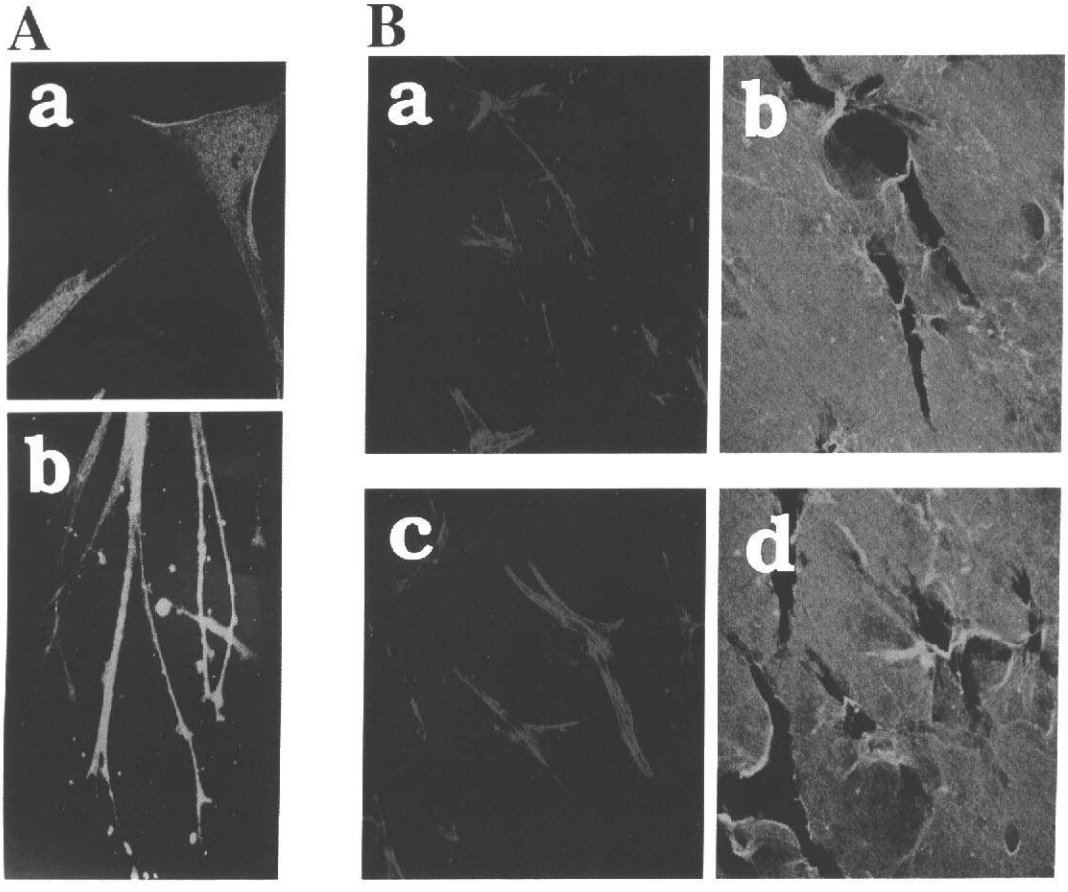

Fig. 1. MMP-1 expression in HSC culture. A: Human HSCs were cultured overnight on polystyrene surface (a) or on type I collagen gel (b), and stained with monoclonal anti-human MMP-1 antibody and then FITC-labeled anti-mouse IgG antibody. B: In situ zymography for detection of interstitial collagenase activity in HSC culture on type I collagen gel. Human HSCs were cultured for a week on type I collagen gel, and then the cells were stained for F-actin with TRITC-labeled phalloidin (a and c), and for type I collagen gel used as substratum with anti-type I collagen and FITC-labeled secondary antibodies (b and d).

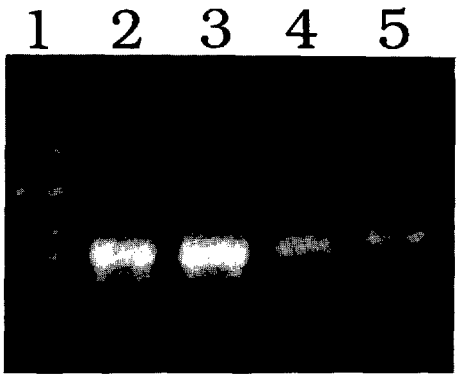

Fig. 2. MMP-1 mRNA expression in HSC culture. RT-PCR analysis was performed using primers, and total RNA isolated from human HSCs on polystyrene surface (lanes 2 and 3) or on type I collagen gel (lanes 4 and 5), as described under Materials and Methods. Lane 1: DNA size markers.

In situ zymography indicating degradation of type I collagen gel in HSC culture

To determine the presence of interstitial collagenase activity, human HSCs were cultured for a week on type I collagen gel, and then the cells were stained for F-actin in HSCs with TRITC-labeled phalloidin (Fig. 1B, a and c), and for type I collagen gel used as substratum with anti-porcine type I collagen and FITC-labeled secondary antibodies (Fig. 1B, b and d). Many cracks of type I collagen gel were detectable around HSCs and frequently along their processes, suggesting the presence of interstitial collagenase activity such as MMP-1 or MT1-MMP activity. HSCs were seen in the cracks and under the collagen gel.

Activation of proMMP-2 in HSC culture on type I collagen gel

To study the effect of ECM components on activation of proMMP-2 in HSC culture, HSCs were cultured on polystyrene surface, type I collagen-coated surface, type I collagen gel, or Matrigel. Like most other MMPs, MMP-2 is secreted as an inactive proenzyme and requires the proteolytic removal of the N-terminal profragment for its activation. Conditioned media from HSC culture on different substrata and FBS prior to cell culture were analyzed by gelatin zymography. FBS used for cell culture contained proMMP-2 and proMMP-9, but not the active form of MMP-2 (Fig. 3B, lanes 5–10). Although a majority of proMMP-2 and proMMP-9 in the conditioned medium seemed to be derived from FBS used for cell culture and remained in collagen gel, an active form of MMP-2 was specifically detectable in HSC culture on type I collagen gel (Fig. 3A and A′, lanes 7–9, and Fig. 3B and B′, lanes 3 and 4). Unknown gelatinase activity was also observed on the bands with slower mobility than proMMP-9 (Fig. 3A and A′, lanes 5–9, and Fig. 3B and B′, lanes 2–4). After overnight incubation in DMEM containing 10% FBS in the absence of cells, type I collagen gel trapped serum proMMP-2 and proMMP-9, and then released them into the serum-free medium, but no active form of MMP-2 was detectable (data not shown), ruling out a direct role of type I collagen gel in proMMP-2 activation. In contrast to the HSC culture on type I collagen gel, HSC culture on Matrigel containing laminin and tyep IV collagen but lacking type I collagen, did not induce the proMMP-2 activation (Fig. 3A, lanes 10 and 11). HSC culture on type I collagen-coated surface (monomeric type I collagen, Fig. 3A, lanes 5 and 6) showed the presence of a small amount of active form of MMP-2. These results indicated that induction of MMP-2 activation was specific to HSC culture in the presence of type I collagen. However, MMP-9, a member of the gelatinases, was not activated under similar conditions.

Expression of TIMP-2 in HSC culture

The activity of MMP-2 is exquisitely regulated by the levels of TIMP-2 on the extracellular surface. We analyzed the effect of culture substratum on TIMP-2 expression in HSC culture. TIMP-2 transcripts in HSCs cultured on different substrata were detected by using RT-PCR. TIMP-2 mRNA was expressed in HSCs cultured on or in type I collagen gel (Fig. 4), as well as HSC cultures on polystyrene surface and on Matrigel, suggesting the suppression of MMP-2 activity detected in HSCs cultured using type I collagen gel.

Discussion

The pivotal role of HSCs in the remodeling of ECM in the liver has been widely documented (10). Primary cultured HSCs have been described to synthesize several members of the MMP family. In early primary culture, HSCs produce interstitial collagenase (MMP-1 in humans, MMP-13 in rats) (15, 16). In contrast, MMP-2 expression is not detectable in the early HSC culture, but elevates from days 3–5 and then becomes a prominent feature of HSC culture (1). HSC primary cultures have been proposed as a model of hepatic fibrogenesis. However, even in physiological situations in the perisinusoidal space, HSCs exist in an ECM component-rich environment, comprising type I, III, IV, V and VI collagen, laminin, fibronectin and proteoglycans (22). During liver fibrosis, there is an increased deposition and altered composition of ECM. Thus, HSCs cultured on different ECM components as substratum is an attractive system to study the effect of ECM on MMP expression and ECM remodeling.

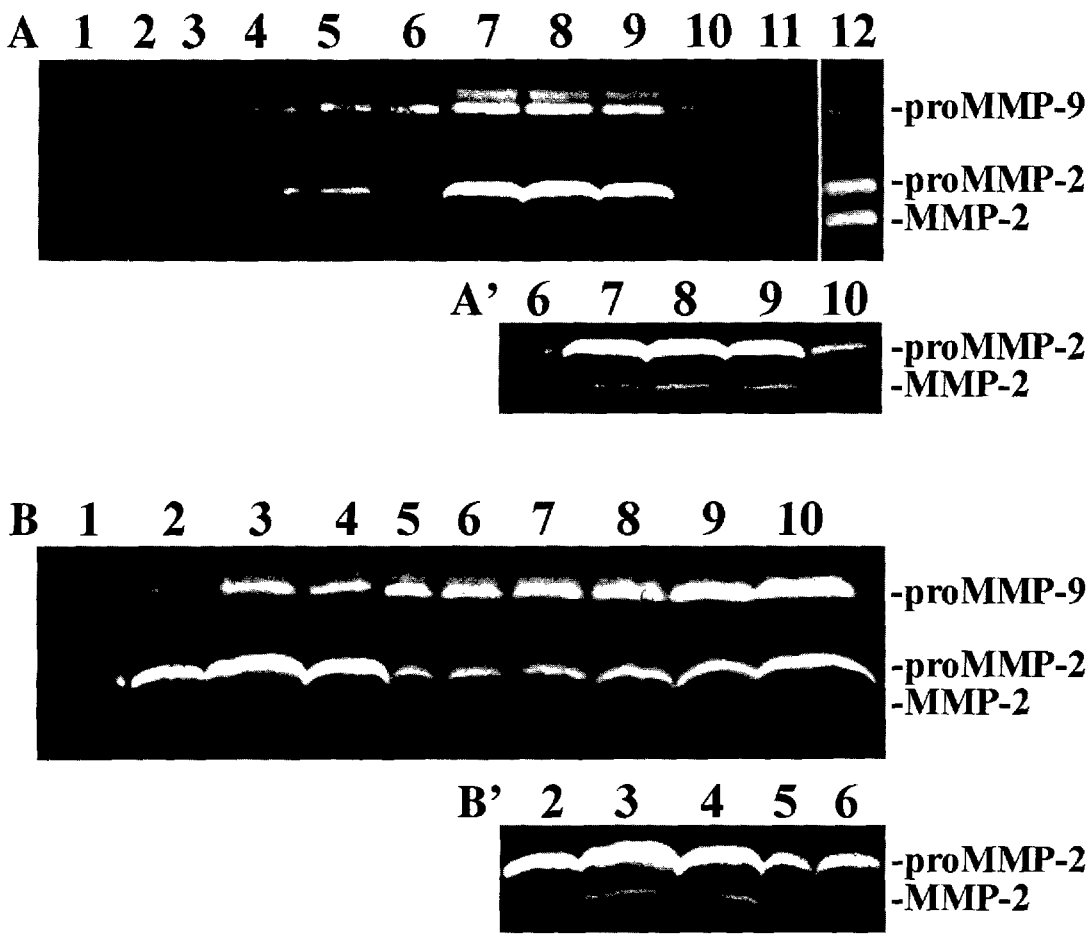

Fig. 3. Zymographic detection of MMPs in the conditioned medium from human HSC culture. A: Conditioned medium from HSC culture on several substrata including polystyrene (lane 1–4), monomeric type I collagen-coated dish (lanes 5 and 6), polymeric type I collagen gel (lanes 7–9) and Matrigel (lanes 10 and 11). Lane 12: authentic MMP markers. A' indicates an enlarged picture of lanes 6–10 in Fig. 3A, displaying pro-MMP-2 and an active form of MMP-2. B: Conditioned medium from HSC culture on polystyrene surface (lane 1), monomeric type I collagen-coated surface (lane 2), or polymerized type I collagen gel (lanes 3 and 4), as well as 1 μl (lanes 5 and 6), 2 μl (lanes 7 and 8) and 5 μl (lanes 9 and 10) of FBS prior to use for cell culture were analyzed by gelatin zymography. B' indicates an enlarged picture of lanes 2–6 in Figure 3B, displaying proMMP-2 and an active from of MMP-2.

In a previous study, we have demonstrated that the three-dimensional structure of ECM reversibly regulates the morphology, proliferation and collagen synthesis in cultured HSCs (29). Cultured HSCs were induced to extend long, multipolar processes by interstitial collagen gel used as a substratum, as compared to the flattened or round cell shapes on polystyrene surface or on Matrigel containing the basement membrane components, respectively. The process extension was found to depend on integrin-binding to type I collagen fibers, followed by signal transduction and cytoskeleton assembly (17, 24, 29). The cell proliferation, as well as collagen synthesis, was more prominent in culture in type I collagen-coated dishes or in polystyrene culture dishes than on or in type I collagen gels (29). To elucidate a mechanism of ECM remodeling, we cultured HSCs using several types of substrata and examined the expression of MMPs and TIMP-2 in this study. MMP-1 expression was shown by immunohistochemistry and RT-PCR in HSC culture. In situ zymography indicated the presence of interstitial collagenase activity around HSCs and along cellular processes of HSCs cultured on type I collagen gel, suggesting the presence of MMP-1 or MT1-MMP activity. Active form of MMP-2 was specifically detectable in HSC culture in the presence of type I collagen gel, which suggests that the activation of MMP-2 is a response to the specific ligand type I collagen. Recently, activation of MMP-2 has been described to occur in response to type I collagen in melanoma cells and fibroblasts (11, 31), and Tomsasek *et al.* (31) demonstrated that fibroblasts produced and activated MMP-2 only

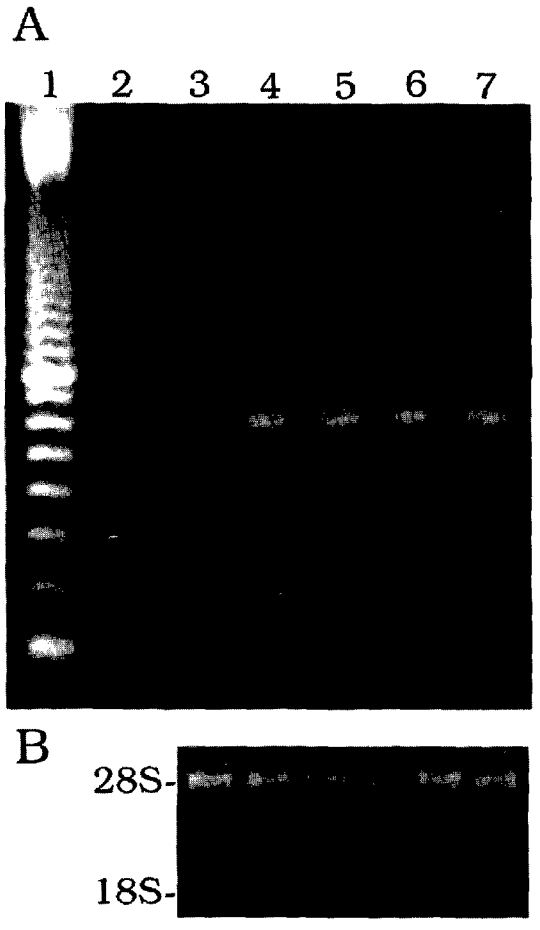

Fig. 4. Expression of TIMP-2 mRNA in HSC culture. A: TIMP-2 mRNA transcripts were detected in HSCs cultured on monomeric type I collagen-coated surface (lanes 2 and 3), on polymeric type I collagen gel (lanes 4 and 5), and on Matrigel (lanes 6 and 7) were detected by RT-PCR as described under Materials and Methods. Lane 1: DNA size markers. B: Integrity of total RNA extracted from the HSC culture was assessed by the detection of 28S and 18S ribosomal RNAs.

during three-dimensional collagen culture. However, proMMP-2 activation was not seen in HSC culture on Matrigel, which is a heterogeneous basement membrane matrix, composed largely of laminin (56%) and type IV collagen (31%), and containing small amounts of other matrix molecules. These results suggest the requirement of type I collagen, particularly the polymerized form, for the proMMP-2 activation.

In summary, we have shown the expression of MMP-1 and the activation of proMMP-2 in HSC culture in the presence of type I collagen. In previous studies, we found that the three-dimensional structure of extracellular substratum regulates the morphology, proliferation, and collagen synthesis of HSCs (24, 29, 30). We further indicated that the three-dimensional structure of ECM was able to regulate the expression of MMPs in HSCs in this study. Such a HSC culture using distinct types of substratum appears to be a useful in vitro model to study the mechanism of ECM remodeling.

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