Fibronectin dependent upregulation of matrix metalloproteinases in hepatic stellate cells

P Pranitha and P R Sudhakaran*
Department of Biochemistry, University of Kerala, Karyavattom, Trivandrum 695 581, India

Received 13 June 2003; revised 29 October 2003

Activation and transition of hepatic stellate cells (HSCs) to myofibroblast (MFB)-like cells is influenced by growth factors, cytokines and matrix proteins like fibronectin (FN). To examine whether the FN-dependent transition of HSCs is mediated through FN receptor, a marker function, such as matrix metallo-proteinase (MMP) production by HSCs in primary culture was studied. An upregulation of MMP production by HSCs maintained on FN was observed. FN-dependent upregulation of MMPs was significantly reduced when cells were pre-treated with antibodies to α5β1 integrin. Treatment of cells with genistein, a protein kinase C inhibitor completely blocked the gelatinase production by HSCs, indicating that the FN-dependent upregulation of MMPs is mediated through integrins and it involves tyrosine phosphorylation dependent signaling pathways.

Keywords: Hepatic stellate cells, fibronectin-dependent upregulation, matrix metalloproteinase

Hepatic stellate cells (HSCs) in liver are normally quiescent, but are activated during injury and change to a myofibroblast (MFB)-like phenotype. MFBs are responsible for the progression of fibrosis and also accumulation of matrix components. Collagen I (Col I) and glycosaminoglycans (GAGs) are the major extracellular matrix (ECM) components that accumulate during fibrosis. Cytokines, growth factors and matrix protein like fibronectin (FN) play a major role in fibrogenesis as they influence the transition of HSCs to MFB-like cells. HSCs, apart from their role in the production of ECM components, may also play a role in the pathogenesis of liver fibrosis through the secretion of neutral matrix metallo-proteinases (MMPs). MMP2, MMP3 and MMP9 are the three major MMPs expressed by HSCs. Activation through plasminogen activator mediated proteolytic cleavage and inhibition by tissue inhibitor of metallo-proteinase (TIMP), constitute two possible mechanisms for the control of the matrix/cell mass ratio. HSCs, by controlling the expression and release of TIMP-1 play a role in the pathogenesis of liver fibrosis. TIMP 1 and TIMP 2 expression increased during fibrosis. Several growth factors and cytokines induce the expression of MMPs. Further, matrix substrata with which the cells are in contact with, influences the expression of MMPs in human peripheral blood monocytes. HSCs are activated by both the growth factors/ cytokines and by matrix proteins to MFB-like cells in fibrosis. In order to study whether MMP production by HSCs is dependent on matrix protein substrata, HSCs were maintained in culture on different matrix protein substrata and examined for the production of MMPs in primary culture.

Materials and Methods

Eagles minimal essential medium (MEM), penicillin, streptomycin, percoll, nycodenz, collagenase, gelatin, genistein, horseradish peroxidase (IgG-HRP) and antibodies to MMP2 and MMP9 were purchased from Sigma Chemical Co., USA. Tissue culture plates were obtained from NUNC, Denmark. Collagen I was kindly provided by Dr J Rauterberg (Munster, Germany). Fibronectin was isolated from human plasma by affinity chromatography over gelatin sepharose.

HSC isolation and culture

HSCs were isolated from rat (Sprague-Dawley strain) livers by collagenase perfusion by the
procedure as described\textsuperscript{9} and modified\textsuperscript{10}. The average yield of the cells was 0.34 mg protein/liver. The viability of the cell preparation was checked by trypan blue exclusion and only the preparations having more than 90\% viability were used for the experiments. HSCs were suspended in MEM containing penicillin (100 mg/L), streptomycin (100 mg/L) and NaHCO\textsubscript{3}. Culture dishes were passively coated with 1.5 ml each of Col I (50 $\mu$g/ml) and FN (50 $\mu$g/ml) for 2 hr. Proteins were then removed and the dishes were washed repeatedly with phosphate buffered saline (PBS). Cells (2×10\textsuperscript{6} cells/ml) were seeded on culture dishes and were incubated at 37°C in a CO\textsubscript{2} incubator (Forma) at 95\% air and 5\% CO\textsubscript{2}. The cells were allowed to attach for 4-5 hr. The medium and unattached cells were removed, fresh medium (MEM) was added and maintained in culture for various experiments.

Zymography

Activity of MMPs secreted by cells maintained on different matrix protein substrata into the medium was determined by zymography\textsuperscript{11,12}. Medium equivalent to the same amount of cell protein was subjected to substrate gel electrophoresis. Zymographic gels consisted of 7.5\% polyacrylamide gel impregnated with gelatin (1 mg/ml). Electrophoresis was carried out in mini gel at a current of 1 mA/cm\textsuperscript{2}. At the end of the run, the gel was taken, washed twice (30 min each) with Triton-X-100 (2.5\%) and incubated with substrate buffer (50 mM Tris/HCl, 5 mM CaCl\textsubscript{2}, pH 7.5) at 37°C for 24-48 hr. The gels were then stained with Coomassie brilliant blue and destained with water. Clear white bands in the gel against blue background indicate gelatinolytic activity. The pH dependence of these enzymes was determined by incubating the zymographic gels in substrate buffer having pH ranging from 5-9 and developed as above. Cation specificity of these enzymes was assessed by incubating zymographic gels in substrate buffer containing EDTA and CaCl\textsubscript{2} (5 mM), MgCl\textsubscript{2} (5 mM), and ZnCl\textsubscript{2} (5 mM). Substrate buffer containing EDTA (1 mM) served as the negative control.

ELISA

MMPs secreted into the medium by HSCs maintained on different matrix protein substrata were quantitated by ELISA\textsuperscript{13}. Culture medium equivalent to the same amount of cell proteins pre-coated on ELISA plates by incubating at room temperature for 3 hr served as the antigen. The plates were then washed with Tween-PBS and blocked with gelatin (0.2\%) for 1 hr, washed again with Tween-PBS and incubated with antibodies against MMP2 and MMP9 for 2 hr. Anti-MMP2 (catalogue no. 6302 aminoterminal end) developed in rabbit reacts with bands at 72 kDa and 68 kDa, Anti-MMP9 (catalogue no. 5177 carboxyterminal end) developed in rabbit reacts with bands at 92 kDa and 88 kDa. The plates were then treated with secondary antibody conjugated to HRP. The colour was developed using o-dianisidine as the substrate for HRP and the optical density was measured at 400 nm.

Succinylated gelatin assay

MMPs were also assayed using succinylated gelatin as substrate\textsuperscript{14,15}. The reaction mixture (150 $\mu$l) contained 200 $\mu$g of succinylated gelatin, sample and borate buffer (50 mM, pH 8.5) for MMP2 and borate buffer (50 mM, pH 8.5) for MMP9. Blank reaction mixtures contained enzyme and buffer, but without substrate. The reaction mixture was incubated at 37°C for 30 min and then 50 $\mu$l of trinitro benzene sulphonic acid (TNBSA) (0.03\%) was added to the mixture and incubated further for 20 min at room temperature before measuring the optical density at 450 nm.

Results

Production of MMPs by rat HSCs in primary culture

Production of MMPs by HSCs was studied by maintaining cells in culture. After culturing for 6 hr, the medium was collected and subjected to zymography. The results showed gelatinolytic activity at 92 kDa and 72 kDa regions (Fig. 1a). Analysis of pH dependence of these enzymes showed the maximum activity at pH 7.5 (Fig. 1b). The metal ion dependence of these gelatinases was studied by incubating the zymographic gels in substrate buffers containing 5 mM each of CaCl\textsubscript{2}, MgCl\textsubscript{2} and ZnCl\textsubscript{2} and 1 mM EDTA. Parallel control without EDTA was taken. EDTA inhibited the activity of 92 kDa and 72 kDa gelatinases, while Ca\textsuperscript{2+} and Zn\textsuperscript{2+} ions reversed the inhibitory effect of EDTA on gelatinases; Mg\textsuperscript{2+} ions did not have any significant effect. In presence of cations, particularly Zn\textsuperscript{2+} ions, the activation of latent enzymes appeared to take place as evidenced by additional bands on zymogram (Fig. 1c). These results indicate that the 72 kDa and 92 kDa gelatin degrading activities are metal ion-dependent, having neutral pH optima and act on matrix proteins, suggesting that these are neutral MMPs.
Fig. 1 — Production of gelatinases by primary cultures of HSCs. (a): Isolated HSCs were maintained in culture on plates passively coated with Col I (50 µg/ml) substrata in a CO₂ incubator for 6 hr. The culture medium was subjected to zymography as described in the Text. Zymographic gel was incubated with substrate buffer (50 mM Tris·HCl, 5 mM CaCl₂, pH 7.5) at 37°C for 24-48 hr. Gels were then stained with Coomassie brilliant blue and destained with water. Clear zones in the gel indicate gelatinolytic activity; (b): pH dependence [After electrophoresis as described in the Text, zymographic gel was incubated with substrate buffer (50 mM Tris·HCl, 5 mM CaCl₂, pH 5-9) at 37°C for 24-48 hr. Gels were then stained with Coomassie brilliant blue and destained with water. Clear zones in the gel indicate gelatinolytic activity]; (c): Cation dependence [The culture medium was subjected to zymography as described in the Text. Zymographic gel was incubated with substrate buffer (50 mM Tris·HCl, pH 7.5) at 37°C for 24-48 hr containing different cations viz; CaCl₂, MgCl₂, and ZnCl₂ (5 mM each) with EDTA (1 mM). Substrate buffer containing Ca²⁺ without EDTA and that with EDTA (1 mM) alone served as controls. Gels were then stained with Coomassie brilliant blue and destained with water. Clear zones in the gel indicate gelatinolytic activity]. (1, EDTA; 2, Ca²⁺; 3, EDTA+Ca²⁺; 4, EDTA+Mg²⁺; and 5, EDTA+Zn²⁺)

Fig. 2 — Effect of matrix protein substrata on gelatinase production by HSCs. (A): Isolated HSCs were maintained in culture plates passively coated with BSA (1), Col I (50 µg/ml) (2) and FN (50 µg/ml) (3) substrata in a CO₂ incubator for 24 hr. The culture medium equivalent to the same amount of cell protein was taken and subjected to multi well zymography (a) to determine the total gelatinase activity and substrate gel zymography (b) to determine the activity of individual gelatinases as described in the Text. The gels were incubated with substrate buffer (50 mM Tris·HCl, 5 mM CaCl₂, pH 7.5) at 37°C for 24-48 hr. Gels were then stained with Coomassie brilliant blue and destained with water. Clear zones in the gel indicate gelatinolytic activity; (B): [ELISA-Isolated HSCs were maintained in culture plates passively coated with Col I (50 µg/ml) (■) and FN (50 µg/ml) (□) substrata in a CO₂ incubator for different time intervals. The culture medium equivalent to the same amount of cell protein coated on ELISA plates served as the antigen. After washing with PBS-Tween, the wells were treated with antibody against MMP2 (a) and MMP9 (b) and developed using o-dianisidine as described in the Text. Values given are the average of duplicate experiments]
Effect of substratum on the production of matrix metallo proteinases (MMPs)

To study whether the MMP production was influenced by the nature of the matrix protein substrata with which the cells are in contact, HSCs isolated from normal rat liver were maintained on petridishes previously coated with matrix proteins Col I and FN for different periods. The medium was subjected to zymography for the analysis of MMPs. Total gelatinase activity secreted into the medium was determined by multiwell zymography and the results are given in [Fig. 2(a)]. Activity of MMP was significantly high in the medium from the cells maintained on FN compared to cells cultured on Col I at all the time intervals tested. Analysis of individual MMPs by zymography showed activity at 72 kDa and 92 kDa regions [Fig. 2A (b)]. In certain experiments, besides the latent forms of 72 kDa and 92 kDa, the respective active forms at 68 kDa and 88 kDa were also found. Activities of both these MMPs were high in cells maintained on FN coated plates, compared to cells cultured on Col I.

The amount of these enzymes was also determined by ELISA, using specific antibodies against MMP2 and MMP9 and the results are given in Fig. 2B. Among the matrix protein substrata tested, higher amount of MMP2 and MMP9 protein levels was seen in those cells maintained on FN than on Col I. It was nearly 2 times higher in HSCs maintained on FN substrata compared to Col I-coated dishes. The production of MMPs was time-dependent and its expression increased with the duration of culture.

Effect of anti integrin antibody on substratum dependant upregulation of MMPs

In order to examine whether the FN-dependent upregulation of MMPs in HSCs maintained in culture was mediated through integrins, antibodies against FN receptor, α5β1 integrin was used. HSCs were maintained on FN substrata in presence of antibodies

Fig. 3—Effect of anti integrin α5 antibody on gelatinase production by HSCs. (A): [Isolated HSCs pretreated with 10 µl of anti integrin α5 antibody (1) were maintained in culture on FN (50 µg/ml) coated substrata as indicated in the text for 6 hr. Untreated cells served as the control (2). The culture medium equivalent to the same amount of cell protein was subjected to zymography as described in the Text. Gels were then stained with Coomassie brilliant blue and destained with water. Clear zones in the gel indicate gelatinolytic activity]; (B): [ELISA -HSCs were pretreated with anti integrin α5 antibody (■) and maintained in culture as described. Untreated cells served as the control (□). The culture medium equivalent to the same amount of cell protein was taken and subjected to ELISA as described in the legend to Fig. 2B. Average of duplicate experiments were taken and expressed as the percentage of the untreated controls (a-MMP2, b-MMP9)]; (C): [Succinylated gelatin assay-HSCs were pretreated with anti integrin α5 antibody (■) and maintained in culture as described. Untreated cells served as the control (□). Suitable aliquots of the medium equivalent to the same amount of cell protein were incubated with 200 µg of substrate at 37°C for 30 min to assay MMP2 (a) and MMP9 (b). 50 µl of 0.03% TNBSA was added to the reaction mixture and incubated for 20 min at room temperature. Optical density was measured at 450 nm. Values given are average of duplicate experiments]
against α5β1 integrin receptor. The medium was collected and total gelatinase activity was determined by multiwell zymography. FN-dependent upregulation of MMPs was blocked by treating cells with anti integrin α5 antibodies; a significant decrease in the amount of MMPs was observed when compared to untreated controls. Individual MMPs were characterized by zymography and the results are given in Fig. 3A. Activity of MMP2 and MMP9 were significantly reduced on treatment with anti α5 antibody.

The quantitation of individual MMPs was also done by ELISA. The cells maintained on FN, upon treatment with anti α5 integrin antibody caused a 65% reduction in MMP2 production and a 90% reduction in MMP9 production (Fig. 3B). These results were also confirmed by assay of MMP2 and MMP9, using succinylated gelatin as substrate (Fig. 3C). The cells maintained on FN, upon treatment with anti α5 integrin antibody caused a 2.5-fold reduction in MMP2 production and a 9-fold decrease in MMP9 production.

**Effect of genistein on the upregulation of MMPs by HSCs maintained on Col I and FN**

To study whether the matrix-dependent upregulation of MMPs mediated through integrins involves phosphorylation of proteins, cells were pre-treated with genistein (50 μM) for 20 min and maintained on FN. Untreated cells seeded on FN served as control. MMP production was assayed by zymography, which showed an inhibition of MMP expression upon treatment with genistein. The effect of genistein on individual MMP activity was also studied by ELISA. Genistein treatment of cells maintained on FN caused a reduction in the expression of MMP9 by 90% and MMP2 by 50% when compared to untreated controls (Fig. 4).

**Discussion**

Results of *in vitro* studies using isolated HSCs maintained in culture presented above indicate that these cells produce MMPs. Zymographic pattern, showing activity at 72 kDa and 92 kDa regions, neutral pH optima, cation-dependence and reaction with specific antibodies against MMP2 and MMP9 indicate that primary cultures of HSC produce MMP2 and MMP9 as the major gelatinases. To study whether the production of MMPs by HSCs is matrix-dependent, the cells were maintained on different matrix protein substrata and the results showed a FN-dependent upregulation of MMPs. The production of MMPs increased with time and cells maintained on FN showed a significantly higher amount of MMP production compared to cells maintained on Col I. Similar results of FN-dependent upregulation of MMPs were reported in monocytes and hepatocytes, suggesting that this is not restricted to a particular cell type. Influence of FN on MMP9 production was observed in human HL-60 myeloid leukemia cells, human peripheral blood monocytes and HL-525 cells. Our studies show upregulation of both MMP2 and MMP9 in HSCs in a FN-dependent manner.

Matrix-dependent upregulation of MMPs in HSCs appears to be mediated through the matrix receptor integrins, as evidenced by the blocking of FN-mediated upregulation of MMPs by treating cells with anti α5β1 antibodies. α5β1 integrin is the major receptor for FN. These results suggest that matrix-dependent production of MMPs is mediated through integrins. Our results (unpublished data) also indicate the presence of α2β1 and α5β1 integrins on HSCs. FN interacts with its receptor α5β1 integrin through a tetrapeptide sequence RGDS. Integrins might also be involved in activating specific MMPs. MMP2 is activated on the cell surface by a multimeric complex that is composed of MMP2, MT1-MMP and TIMP2 and α5β3. Carboxy terminal domain of MMP2 binds to TIMP2, which in turn, is associated with the membrane bound MT1-MMP. MT1-MMP then cleaves the amino terminal propeptide of MMP2, resulting in an intermediate form that is capable of binding α5β3 at the cell surface.

Integrin-mediated signaling events appear to involve tyrosine phosphorylation. Inhibition of
FN-dependent upregulation of MMPs in HSCs upon treatment with genistein, a protein kinase C (PKC) inhibitor indicates that the process involves signal generation through phosphorylation pathways. One of the major substrate for integrin-mediated tyrosine phosphorylation is pp125 focal adhesion kinase (pp125 FAK). Cell adhesion to FN and other stimuli can regulate pp125 FAK phosphorylation and induce the formation of complexes between pp125 FAK and other signaling molecules in vivo.

The interactions of cells with ECM ligands through integrins lead to the activation of signaling pathways, which involve the activation of transcription factors. HSCs express transcription factor Ets1 in freshly isolated cells, but its expression is diminished during activation, indicating a possible role for this factor in the early stages of activation. Other transcription factors AP-1, SP-1, CCAT binding factor, NF kappaB, and kruppel-like transcription factor Zfg were induced during HSC activation. Stress or inflammatory cytokines like tumour necrosis factor α (TNFα)-mediated jun kinase pathway and the platelet derived growth factor (PDGF) and epidermal growth factor (EGF)-mediated ERK pathway are the two major signaling pathways involved in the activation of transcription factors. The ERK 1 and 2 belong to a group of mitogen-activated kinases and are transiently activated after adhesion to FN. This activation is accompanied by translocation into the nucleus where certain genes may be transcriptionally activated.

Results of these in vitro studies appear to be significant in the activation of HSC in vivo in liver injury in the light of certain observations on changes in matrix protein and their receptors in intact liver. During liver regeneration and fibrosis, increased amounts of FN have been reported to be present at the cell surface. In another study also, an increased amount of FN and its receptor, α5β1 integrin during liver regeneration was reported. During wound repair, sinusoidal cells secrete a fetal isoform of FN that stimulates the quiescent HSCs to MFB-like cells. The production of FN by these cells is influenced by TGF β. Similarly, MMP production is also enhanced immediately after partial hepatectomy as evidenced by ELISA and immunohistological studies. The production of MMPs facilitates the migration of cells to reach the injury site to exert their action. These in vivo observations correlate with the results of in vitro studies on the FN-dependent transition of HSCs to MFB reported above. It, therefore, appears that, apart from the soluble factors like cytokines and growth factors known to activate HSCs, the matrix component like FN also plays a crucial role in the transition of HSCs to MFB-like cells. Further study, particularly on the activity of specific transcription factors is required to understand how the integrin-mediated effect of FN on transition of HSC to MFB-like cell occurs.

Acknowledgement

Financial assistance to PP in the form of SRF from Indian Council of Medical Research, New Delhi is gratefully acknowledged.

References