Changes of Gelatinolytic Activity in Human Amniotic Membrane-Derived Mesenchymal Stem Cells during Culture in Hepatogenic Medium

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ABSTRACT

The present study was conducted to investigate gelatinolytic activities in HAM and to determine whether there are any changes in gelatinolytic activity profiles when the cells are cultured in hepatogenic medium. Placenta was obtained during caesarean section of the volunteers, with informed consent. HAM were isolated from amniotic membrane using collagenase type A. HAM were cultured in hepatogenic medium for 3 weeks and the conditioned media were obtained at day 7, 14 and 21. The zymographic pattern of gelatinolytic activity of the HAM did not undergo a change during passages. When the HAM were cultured in a fibronectin-coated dishes in a hepatogenic medium, there was no significant difference of the gelatinase pattern between before and after culture. However, when bFGF was added to the culture, a dramatic increase of 62kDa and 59kDa gelatinases was observed. Interestingly, when ITS instead of FN was present, HAM-conditioned medium also showed a similar increase of both gelatinases. Immunoblotting analysis demonstrated that both 62kDa and 59kDa gelatinases were the active form of MMP-2 resulting from the turnover of MMP-2 proform. Futher study will be necessary to determine the relationship between bFGF and active MMP-2 during hepatogenesis of HAM.

(Key words : HAM, Gelatinolytic activity, bFGF, Zymography)

INTRODUCTION

Gelatinases are members of the matrix metalloproteinase (MMP) family responsible for the degradation of extracellular matrix (ECM) and basement membrane components during remodeling of most tissues. Two forms of gelatinases have been identified, namely a 72-kDa gelatinase A and a 92-kDa gelatinase B, referred to as MMP-2 and MMP-9 respectively. Both gelatinases have a broad spectrum of substrate specification, such as type IV collagen, laminin and fibronectin, as well as gelatin (Birkedal-Hansen *et al.*, 1993). The activity of MMPs is regulated at several levels including gene transcription, proenzyme activation and inhibition of activated enzymes by tissue inhibitor of MMP (TIMP) (Basbaum and Werb, 1996). MMPs are though to be mainly involved in the ECM degradation, which could also control cell proliferation via integrin signaling and growth factor activation. Recent studies demonstrated that proteinsases play important role in not only ECM remodeling bul also hepatocyte proliferation (Mars *et al.*, 1996).

The amnion is of fetal origin and is considered to protect the fetus from mechanical injury by enveloping it in the amniotic fluid. The amnion consists of a single layer of epithelial cells on a thicker basement membrane and a collagen spongy layer containing mesenchymal cells. The epithelial layer of amnion lines the amniotic cavity. The amnion has been applied clinically, e.g., in the treatment of burn lesions (Trelford *et al.*, 1979), to cover surgical wounds to avoid collusion (Trelford *et al.*, 1979), and in ocular surface reconstitution (Koizumi *et al.*, 2000), since human amniotic epithelial cells (HAE) seem to be relatively resistant to rejection even after allotransplantation. They have been shown not to express immuno-suppressive factors, such as CD59 (Rooney *et al.*, 1992) and soluble HLA-G (Rebmann *et al.*, 1992). Under the appropriate culture conditions, HAE could differentiate into a variety of somatic cells including pancreatic beta cells (Wei *et al.*, 2003), neural cells (Kakishita *et al.*, 2000), or hepatocytes *in vitro* based on the phenotypes and functions (Seiji *et al.*, 2004). Human amniotic mesenchymal cells (HAM) also express phenotypes of neuroglial progenitor cells (Sakuragawa *et al.*, 2005) and exhibit some characteristics of cardiomyocytes (Zaho *et al.*, 2005). Because the amniotic

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tissue is disposed after parturition, it is easy to obtain and overcomes the ethical issues associated with use of fetal tissue. Taken together, HAM might be a useful cell source for cell therapy.

Fibroblast growth factors (FGFs) constitute one of the largest families of growth and differentiation factors for cells of mesodermal and neuroectodermal origin. The family comprises two prototypic members, acidic FGF (aFGF) and basic fibroblast growth factor (bFGF), as well as 23 additionally related polypeptide growth factors that have been identified to date. FGFs are involved in many biological processes during embryonic development, wound healing, hematopoiesis, and angiogenesis. In prostate, bladder, and renal cancers, FGFs regulate the induction of MMP that degrade ECM proteins, thus facilitating tumor metastasis. Probably due to their potent angiogenic properties, aFGF and bFGF have received the most attention (Cronauer *et al.*, 2003). bFGF, the prototypic member of a large family of heparin-binding polypeptides and a potent angiogenesis inducer, modulates vascular endothelial cell proliferation, migration, and proteinase production (Miyake *et al.*, 1996). bFGF initiates intracellular signaling in a variety of types, including endothelial cells and smooth muscle cells, through a dual receptor system consisting of receptor tyrosine kinases (FGFRs) and heparin sulfate proteoglycans (Collolly *et al.*, 1987). Also bFGF has known to a potent inducer during hepatogenesis (Seiji *et al.*, 2004; Saji et al., 2004), while its action mechanism how to differentiate into hepatic lineage cells from non-hepatogenic cells is not known yet.

The present study was conducted to investigate the profile of MMP-2 and MMP-9 gelatinases secreted from human amniotic mesenchymal cells by using zymography and immunoblotting methods during culture of the cells in hepatocyte-inducing medium.

MATERIALS AND METHODS

Isolation of HAM

Placenta was obtained at cesarean section from the volunteers with the informed consent. The amnion was peeled off mechanically from the chorion and washed in phosphate-buffered saline (PBS) to remove blood and cellular debris. The amniotic epithelial cells, which line the surface of the amnion that bathed amniotic fluid *in vivo*, were isolated as described previously (Casey *et al.*, 1996). Briefly, amnion tissue was minced and placed in a spinner flask with 150 mL Dulbecco's Modified Eagle Medium (DMEM-LG; Gibco, Grand Island, NY, USA) containing 0.25% trypsin (Gibco). The flasks were maintained 37°C, with continuous stirring for 30 min. The first digestion supernatant, consisted primarily of red blood cells, was discarded. The tissue was minced again and incubated at 37°C with trypsin for 30 min with stirring. Then the epithelial cells were pelleted by centrifugation and suspended in DMEM-LG supplemented with 100 U/mL penicillin, 0.1 mg/mL streptomycin, 3.7 mg/mL sodium bicarbonate and 10% fetal bovine serum (FBS). The remaining tissue pieces were minced and incubated again with trypsin for 30 min two more times, and the dispersed epithelial cells were isolated from amnion. Human amniotic-derived mesenchymal cells were isolated from the amnion tissue pieces after complete (>98%) removal of epithelial cells (which was evaluated microscopically). The tissue pieces were place in PBS containing 2 mg/mL collagenase A (Roche Diagnostics, Rotkreuz, Switzlerland) and 0.05 mg/mL DNase (Roche) and were incubated at 37°C for 120 min with stirring. The dispersed mesenchymal cells were collected by centrifugation. After several washes with DMEM-LG, the cells were plated in 75 mL-size culture flask (Nunc, Rosktlde, Denmark) containing DMEM-LG supplemented with 10% FBS. Cell culture media were changed twice a week after 7 days culture. When achieving confluence, cells were treated with 0.125% trypsin and 1 mM ethylenediaminetetraacetic acid (EDTA) for 3 min. Released cells were collected and subcultured again. Morphologically homogeneous population of fibroblast- like cells, namely HAM, was obtained after 1 or 2 subcultures.

Hepatic Differentiation of HAM

Total 10^4 cells/mL of HAM at passage 5 were seeded on Lab-Tek® chamber slide™ (Nalge Nunc Int, IL, USA) coated with 10 μg/mL fibronectin (FN) or not. Hepatogenic media were consisted of basic medium (BM) consisting of DMEM-LG, 10% FBS, 20 ng/mL hepatocyte growth factor (HGF, Peprotech, Princeton, NJ, USA), 10 ng/mL oncostatin M (OSM), 10^{-6}M dexamethasone (DEX) supplemented with either aFGF, bFGF, ITS premix (1.0 g/L insulin, 0.55 g/L trasnferrin, 0.67 mg/L selenious acid; Gibco) alone or in combination. Medium was changed twice a week, and the culture medium was obtained on days 7, 14 and 21 for gelatin zymography and immunoblotting. Glycogen deposition was visualized using periodic acid schiff (PAS) staining. Cultivated cells were fixed with 4% paraformaldehyde for 30 min at room temperature. They were incubated with 1% periodic acid for 5 min at room temperature. Then, cells were treated with Schiff's reagent for 15 min at room temperature followed by washing twice with distilled water. After haematoxylin counterstaining, cells were observed for the staining using a light microscope (LSM410; Carl Zeiss, Oberkochen, Germany). Hepatic differentiation was assessed by immunocytochemical staining against the human albumin. Cultivated cells were fixed with 4% paraformaldehyde in PBS at 4°C for 2 h, rinsed with PBS. Cells were permeablized with PBS consisting of 0.5% Triton X for 10 min at room temperature. After several

washing, the cells were incubated in 3% hydrogen peroxide for 15 min to quench the endogenous peroxidase activity. They were rinsed with PBS and incubated in blocking solution consisting of 2% bovine serum albumin (BSA) for 1 h at room temperature. Cells were then incubated with 1 μg/mL mouse monoclonal antibody against human albumin. Primary antibody incubation lasted for 17 h at 4°C, after which the slides were rinsed three times with PBS and incubated with biotinylated goat anti-mouse and rabbit IgG (DAKO, CA, USA) for 20 min at room temperature. The slides were then rinsed with PBS, and then incubated with horseradish peroxidase conjugated streptavidin (DAKO) for 20 min at room temperature. Immunoreactivity for each protein was visualized utili- zing the 3,3'-diaminobenzidine tetrahydrochloride (DAB) and counterstained with Mayer's haematoxylin. Finally the sections were photographed under a microscope using bright-field illumination.

Gelatin Zymography

Gelatin substrate sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used with the addition of 1 mg/mL bovine skin gelatin (type B) to 8% resolving gel, as described previously (Kim *et al.*, 2003). Briefly, samples were dissolved in the SDS sample buffer in the absence of reducing agent without boiling. Unless specified otherwise, 10 μL of conditioned media and 30 μg protein in 1 μL human follicular fluid (hFF) was loaded onto a single well. After gelatin-SDS electrophoresis, gels were soaked with 2.5% Triton X-100 in 50 mM Tris-HCl buffer (pH 8.0) for 30 min and wash in 100 mL incubation buffer (5 mM $CaCl_2$, 0.02% NaN_3, 50 mM Tris-HCl, pH 8.0). Then, gels were incubated in fresh incubation buffer overnight at 37°C for the development of gelatinolytic activity in the presence or absence of proteinase inhibitors. Reacted gels were stained with Coomassie brilliant blue R-250 dye and clear bands on a blue background were regarded as gelatinases bands because gelatinases degrade the gelatin present in the acrylamide gel. Wide-range molecular makers (M-3788; Sigma Chemical Co., St Louis, MO, USA) were used as standards for the SDS-PAGE gel. 5 mM EDTA and 5 mM phenylmethylsulphonyl fluoride (PMSF) was added to the incubation buffer as a protease inhibitor. All zymographic results were confirmed experiments two or more times.

Immunoblotting

Proteins were separated by non-reducing electrophoresis on 8% acrylamide gel in parallel with a prestained protein molecular marker. Subsequently, gels were soaked in a transfer buffer composed of 25 mM Tris (pH 8.4), 192 mM glycine and 10% methanol for 15~30 min. To hydrate polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore, Bedford, MA, USA), they were soaked in absolute methanol for 15 s, soaked in distilled water for 2 min and then equilibrated in transfer buffer for 5 min. Proteins on the gel were electrotransfered onto a PVDF membrane for 1 h at 4°C and 100 V. To saturate non-specific binding sites after transfer, membranes were incubated at 37 °C for 1 h in a washing buffer (PBS; 10 mM, pH 7.4, containing 0.8% NaCl, 0.02% KCl, 0.144% $Na_2HPO_4 \cdot 2H_2O$, 0.02% KH_2PO_4, 0.2% Tween 20, 10 mM sodium azide and 0.1% BSA) containing 5% BSA. Membranes were then incubated for 1 h in washing buffer containing 1% normal goat serum and 1 μg/mL mouse monoclonal antibody against human MMP-2 or MMP-9. Following several washes with the washing buffer containing 0.1% BSA, membranes were incubated for 2 h in washing buffer containing 1:100 diluted gold-labeled goat anti-rabbit IgG. After reaction, the signal was visualized using an IntenSE BL kit (Amersham, Buckinghamshire, England) according to the manufacturer's instructions. Immunoblotting results were confirmed by three independent experiments.

Chemicals

Acrylamide, bisacrylamide and N,N,N',N'-tetramethylethylenediamine were purchased from Bio-Rad (Hercules, CA, USA). Mouse monoclonal antibodies of anti-human MMP-2 and MMP-9 were purchased from Chemicon (Temecula, CA, USA). All other chemicals were obtained from the Sigma Chemical Co. (St. Louis, MO, USA). The EDTA was prepared as a stock solution of 500 mM in distilled water. PMSF were dissolved in dimetyl sulphoxide (DMSO) as 500 mM stock solution.

RESULTS

Morphology of HAM before and after Culture in Hepatogenic Medium

Searching for alternative sources of MSC, we attempted to isolate cells exhibiting fibroblast-like morphology typical of MSC from placental amnion. After two weeks from the primary culture, fibroblast-like cell colony appeared among the various types of cell population. However, during a few subsequent passages, most other types of cells disappeared but a rather homogenous population of fibroblast-like cells remained and proliferated actively. Fig. 1 shows the morphology of these MSC (HAM) before and after cultivation in hepatogenic medium. Well established fibroblastoid morphology of HAM continued until cells reached 16th passage. However, when HAM were cultured in hepatogenic medium for 21 days, their appearance turned into round shape typical of heapatocyte-like cell. Regardless of the presence of hepatogenic additives such as aFGF, bFGF, ITS alone or

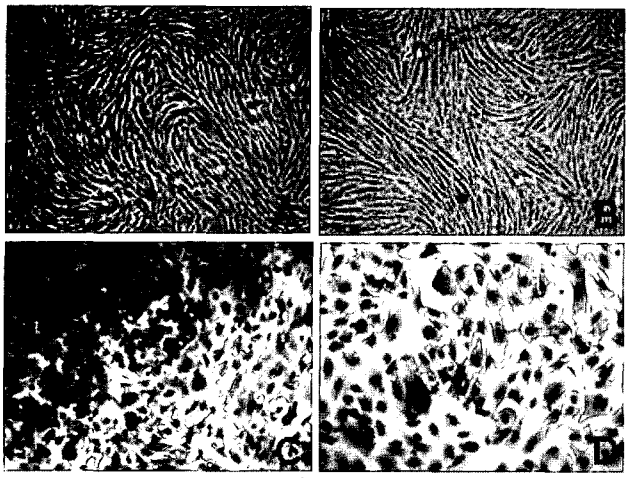

Fig. 1. Morphology of HAM before and after culture in hepatogenic medium. Hepatogenic medium was consisted of BM (DMEM+10% FBS+HGF+OSM+DEX+ITS) supplemented with aFGF and bFGF. Culture dishes were coated with fibronectin. A, after 31 day culture (passage 2); B, after 72 day culture (passage 8); C, PAS staining of HAM differentiated into hepatocyte-like cells; D, immunocytochemistry of HAM differentiated into hepatocyte-like cells using antibodies against human albumin. Magnification: A, B, ×40; C, ×100; D, ×200.

in combination, PAS staining and immunocytochemical staining against human albumin demonstrate that HAM cultivated under one of these culture conditions consistently might have differentiated into hepatocyte-like cells (Fig. 1. C, D). However, HAM cultured in a basic medium without any of the above hepatogenic inducers did not show positive PAS staining, anti-albumin staining or hepatocyte-like morphology.

Gelatinolytic Activity of HAM during Subculture

To investigate a possible change of the gelatinase profile of HAM during passages, cells were cultured in DMEM supplemented with 10% FBS and subcultured when they were confluent. The HAM-conditioned medium was obtained at 5, 8, 11, 15 and 16 passages, respectively.

As seen in fresh unconditioned medium which included 10% FBS, three gelatinases of 92kDa, 86 kDa and 62 kDa size were consistently observed in every HAM-conditioned media (Fig. 2). Among these 62 kDa one was most pronounced. When gelatinolytic activity of HAM-conditioned media was examined, interestingly, distinct gelatinolytic activity having molecular weight of 120 kDa appeared demonstrating that it was HAM-specific gelatinase. Gelatinase of 72 kDa-size also newly appeared indicating it also being HAM-specific. However, its gelatinolytic activity was not as strong as that of 120 kDa gelatinase. Based upon the relative position in comparison to the gelatinase profiles of human follicular fluid,[20] 92 kDa and 86 kDa gelatinases corresponded to gelatinase B known as MMP-9 while 72 kDa and 62 kDa

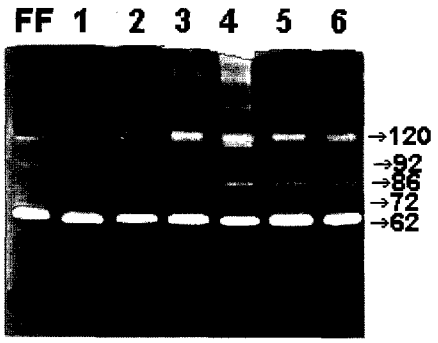

Fig. 2. Gelatinolytic zymogram of HAM. HAM were cultured in MEM supplemented 10% FBS. FF, human follicular fluid for the comparison; lane 1, fresh medium; lane 2, HAM at 5th passage; lane 3, HAM at 8th passage; lane 4, HAM at 11th passage; lane 5, HAM at 15th passage; lane 6, HAM at 16th passage.

gelatinases corresponded to gelatinase A known as MMP-2. All gelatinolytic activity disappeared upon incubation of the gels in the presence of EDTA, but were unaffected by PMSF, a serine/threonine proteinase inhibitor, implying that they might be MMPs or derivatives (data not shown).

Gelatinolytic Activity of HAM during Culture in Hepatogenic Medium

To see if there is any difference of the gelatinase profiles of HAM-conditioned media among the type of hepatogenic media, HAM at passage 5 were cultivated for 3 weeks in one of the hepatogenic media. After culture, the HAM- conditioned media were collected and analyzed by zymography.

As shown in Fig. 3A, there was little difference among the gelatinase profiles of HAM-conditioned medium containing either aFGF, aFGF+ITS or aFGF+bFGF. However, HAM-conditioned medium containing bFG+ITS, interestingly, showed an intense band of 59 kDa gelatinase that did not appear in fresh media or follicular fluid and appeared only faintly in other hepatogenic media. A weak 45 kDa gelatinase band was also observed. When aFGF was added to the same medium containing both bFGF and ITS, intensity of the 59 kDa band became very weak similar to that of bFGF medium or aFGF+bFGF medium. Since fibronectin molecule have also been reported to support the differentiation of mouse bone marrow or human multipotent adult progenitor cells into hepatocytes-like cells *in vitro* (Schwartz *et al.*, 2002), the effect of fibronectin on the gelatinase profile of HAM-conditioned media was examined. When HAM were cultivated plastic dishes precoated with fibronectin in the presence of BM, aFGF alone or both aFGF and ITS, gelatinase profiles of the conditioned media did not show any difference compared to those obtained from the fibronectin-free

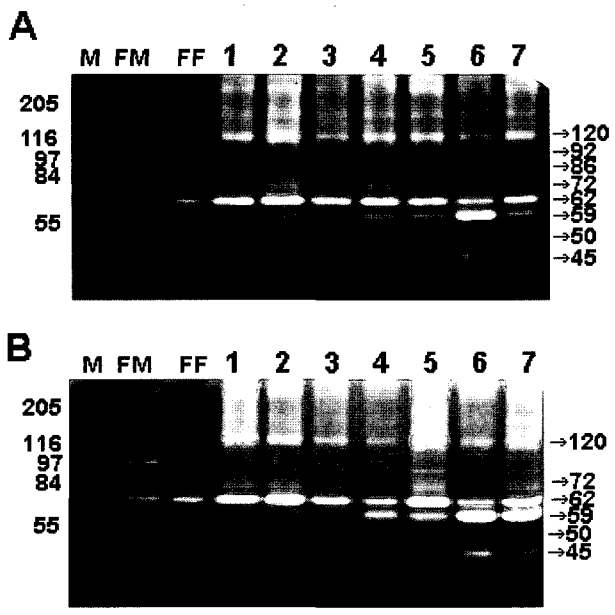

Fig. 3. Gelatinolytic zymograms of HAM cultured in hepatogenic medium for 21 days. The medium was consisted of BM (DMEM +10% FBS+HGF+OSM+DEX) supplemented aFGF, bFGF or ITS. HAM were cultured in a non-coated (A) or fibronectin-coated (B) dishes. M, molecular marker; FM, fresh media; FF, human follicular fluid; lane 1, DMEM+10% FBS; lane 2, BM+aFGF; lane 3, BM+ aFGF+ITS; lane 4, BM+bFGF; lane 5, BM+bFGF+aFGF; lane 6, BM+ bFGF+ITS; lane 7, BM+bFGF+aFGF+ITS.

condition. However, HAM were cultivated in the fibronectin-coated dishes in the presence of bFGF alone, aFGF+bFGF, bFGF+ITS or aFGF+bFGF+ITS, every medium exhibited intense band of 59 kDa gelatinase. Again 45 kDa gelatinase appeared only in the media containing bFGF+ITS or aFGF+bFGF+ITS (Fig. 3B). Gelatinolytic activity of 59 kDa gelatinase did not diminish in the medium containing aFGF+bFGF+ITS.

Taken together, in the absence of fibronectin, treatment of bFGF and ITS together resulted in the enhanced gelatinolytic activity of 59 kDa and the appearance of 45 kDa gelatinases. Addition of aFGF prevented this effect. However, in the presence of fibronectin, bFGF alone could increase the activity of 59 kDa gelatinase and aFGF did not affect the enhancement.

Effects of Various Cytokines on the Gelatinolytic Activity of HAM-Conditioned Hepatogenic Medium

In the above experiment, we obtained two different results. In the presence of ITS, aFGF showed the inhibitory effect of bFGF-enhanced 59 kDa gelatinase activity, whereas in the presence of fibronectin, aFGF showed little inhibitory effect. Thus it was further examined whether the inhibitory effect of aFGF might be due to the presence of

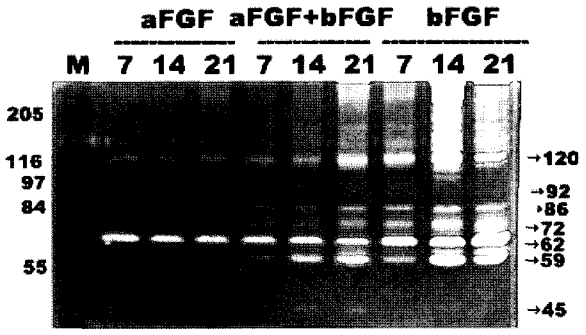

Fig. 4. Gelatinolytic zymogram of HAM cultured in hepatogenic medium day at 7, 14 and 21. Hepatogenic medium was consisted of BM (DMEM+10%FBS+HGF+OSM+DEX+ITS) supplemented with aFGF or bFGF. Culture dishes were coated with fibronectin. Each 7, 14 and 21 presents day from the culture. M, molecular marker.

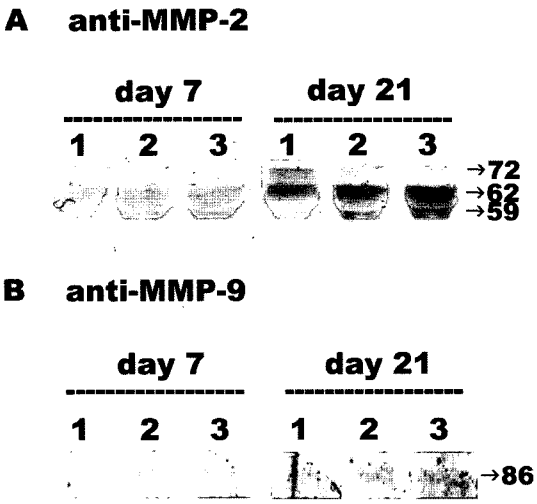

Fig. 5. Immuoblotting analyses of HAM cultured in hepatogenic medium day at 7 and 21. Hepatogenic medium was consisted of BM (DMEM+10% FBS+HGF+OSM+DEX+ITS) supplemented with aFGF or bFGF. Culture dishes were coated with fibronectin. Each 7 and 21 presents day from the culture. Mouse monoclonal antibodies against human MMP-2 (A) or MMP-9 (B) were used. Lane 1, BM+aFGF; lane 2, BM+aFGF+bFGF; lane 3, BM+bFGF.

ITS or might be prevented by fibronectin. To do this all conditioned media were obtained using fibronectin-coated dishes and contained ITS in common.

As shown in Fig. 4, when HAM-conditioned medium contained aFGF alone, intense 59 kDa band was not observed. In contrast, intense 59 kDa band was distinctly seen when the medium contained bFGF alone. The intensity was discernable on day 7 of culture and became strong as the culture continued until day 21. However, when the medium contained both aFGF and bFGF, 59 kDa band began to appear on day 7 and its intensity became increased as the culture continued until day 21. However,

overall intensity was much weaker than 59 kDa of the medium containing bFGF alone. These results showed that in the presence of both ITS and fibronectin, aFGF still was capable of inhibiting the bFGF-enhanced 59 kDa activity although the effect was not as distinct as in the medium containing ITS alone.

To identify the nature of 72, 62 and 59 kDa gelatinases, an immunoblotting experiments was performed using two monoclonal antibodies against human MMP-2 and MMP-9. As seen in Fig. 5, all three gelatinase bands showed the strong immunoreactivity against the anti-MMP-2 antibody, indicating that these are MMP-2 or derivatives. Based on their molecular weight, it is rather obvious that 72 kDa gelatinase is inactive form of MMP-2, and both 62 kDa and 59 kDa gelatinases are active form of MMP-2. When the medium containing aFGF alone was examined on day 7, 62 kDa form was the predominant form and 72 kDa form was the next. The 59 kDa was not discernable. On day 21, immunoreactivity of all three bands was stronger compared to those on day 21. In contrast, when the medium containing both aFGF and bFGF was examined, active 62 kDa was the predominant form as in the medium containing aFGF alone but 59 kDa instead of inactive 72 kDa was the next on day 7. On day 21, the pattern was similar, however, intensity of both active 62 kDa and 59 kDa forms became very strong on day 21. These observations indicate that during culture in presence of aFGF, bFGF or both, HAM could actively synthesize MMP-2 and rapidly process it into active forms followed by secretion into the medium. Particularly in the presence of bFGF, HAM showed 59 kDa form in a greater amount than without bFGF.

Immunoblotting analyses identified 86 kDa gelatinase as an active form of MMP-9 secreted by HAM. However, little change of the MMP-9 secretion was observed.

DISCUSSION

To investigate whether there is any relationship between hepatic differentiation and MMP activity, HAM were treated with aFGF, bFGF, ITS or their mixture in addition to the basic hepatogenic medium containing HGF, OSM and dexamethasone, After 3 weeks of culture, results of PAS staining and immunocytochemistry demonstrated that HAM cultivated in every culture condition except the control group differentiated into hepatocyte-like cells. However, there was a great variation of the gelatinase profiles produced by HAM depending upon the type of hepatogenic media. These results showed that gelatinase profiles of HAM could not be related to the hepatic differentiation of HAM but might be related to the cytokines included in the culture medium.

In the present study, cultivation of HAM in the presence of bFGF and either ITS or fibronectin significantly increased the appearance of the active MMP-2 during the culture period from day 7 to day 21, whereas bFGF alone did not induce such increased activity. Immunoblotting results showed that there was no significant increase of 72 kDa form, inactive proMMP-2, under the same culture condition. In contrast, enhanced amount of 62 kDa and 59 kDa forms, active MMP-2, was found during the same period. These observations indicate that HAM actively synthesize proMMP-2 but most of them are rapidly converted into active MMP-2 forms, predominantly 62kDa form, only when cultivated in the presence of both bFGF and ITS or fibronectin.

The inactive proform of MMPs can be activated by proteolytic cleavage by plasmin or stromelysin, which itself belongs to the MMPs (Werb *et al.*, 1977). MMP-2 degrades type IV collagen, denaturated interstitial collagens (gelatin), and a variety of other matrix proteins. MMP-2 is involved in ECM remodeling during morphogenesis and tissue repair and in the processes of tumor invasion and metastasis. Like other members of the MMP family, the enzyme is secreted as proenzyme form, although conversion to its active form involves a unique process (Nagase, 1997).

MMP activity is controlled through three different levels: transcription, proteolytic activation of the zymogen form and inhibition of the active enzyme by a series of inhibitors. In adult tissues at rest, although MMPs are expressed at relatively low levels, interaction of cells with cytokines and growth factors stimulate the rapid induction of MMP expression (Stamekovic, 2000). The regulatory mechanism of MMPs production is still largely unclear: however, previous study showed that some growth factors and cytokines including bFGF promoted MMPs production by malignant tumors (Gohji *et al.*, 1994).

The effect of bFGF on MMP-2 can be viewed under various aspects. bFGF is normally found in ECM because of its strong interaction with heparin which reflects the affinity for ECM heparin sulfate proteoglycan and glycosaminoglycans and it is stored in the extracellular structures and mobilized when needed by remodeling of the basement membrane or ECM (Baird and Ling, 1987). Furthermore, bFGF mediates carotid plaque instability through MMP-2 and MMP-9 (Sapienza *et al.*, 2004). Transfection of bFGF cDNA into RenCa mouse renal cancer lines increased their invasive potential in an *in vitro* tumor cell invasion assay. Subsequent zymography showed a marked increase in MMP-2 in these cells (Miyake *et al.*, 1997). The introduction of bFGF gene into human bladder cancer cell lines stimulated not only MMP-2 production but also MMP-9 production. At higher concentration, bFGF, FGF-8 and FGF-9 also induced MMP expression (Udayakumar *et al.*, 2001).

The mechanism whereby bFGF promotes MMPs production is not known. Some studies have demonstrated

that the bFGF monoclonal antibody inhibited the cell proliferation of various cancer cell lines through an autocrine and/or paracrine pathway (Hori *et al.*, 1991); in contrast, another study suggested that bFGF promoted the cell locomotion of NIH3T3 cells transfected with the native bFGF gene through like an intracrine pathway (Taylor *et al.*, 1993). The other study showed that bFGF induced the appearance of MMP-1 activity but elicited no effect on the activity of MMP-2, MMP-3 or MMP-9 (Pickering *et al.*, 1997). Thus, the action mechanism of bFGF is still controversial. Some researchers have shown the significance of proteinases which trigger hepatocyte proliferation *in vivo* (Mars *et al.*, 1995). They suggested that the proteinases may process the proform of growth factors such as HGF, result in the production of active form which would trigger the hepatocyte proliferation. In fact, it has been reported that MMP-2 might be a growth factor in another liver cell type (Benyon *et al.*, 1999). Nevertheless a possibility can not be ruled out that the disruption of ECM by the cytokine-induced MMPs which would modify the integrin signaling may be involved in the mechanism. One possible explanation is that bFGF might trigger the MMP-2 gene expression and ITS or fibronectin might convert the inactive form into an active MMP-2 followed by secretion, or vice versa. Another possibility includes extracellular signal regulated kinases (ERK) pathway. FGFs mediate their signals through cell surface receptor tyrosine kinase, Although more than 20 FGFs with different effects on various target cells have been identified, only four FGFR types have been isolated to date (Powers *et al.*, 2000). bFGF binding to FGFRs results in activation of various intracellular signaling pathways including the ERK and phophatidyl-inositol-3 kinase (PI-3K) pathways (Schlessinger, 2000). bFGF induces MMP-3 expression in endothelial cells through activation of the ERK-1/2 pathway (Pintucci *et al.*, 2003) and MMP-9 secretion in MCF-7 cells through protien kinase C activation of the RAS/ERK pathway (Liu *et al.*, 2002).

When the HAM were cultured in a hepatogenic medium supplemented with aFGF alone or with aFGF and ITS but not with bFGF, there was no difference in the gelatinolytic pattern between FN coating plate and non-coated plate. In contrast, HAM cultivated in the medium containing bFGF and FN revealed that the dramatic increase of active 59 kDa form of MMP-2 compared to non-coated plate. Substitution of FN with ITS to hepatogenic medium also resulted in a significant increase of 59 kDa active form of MMP-2. FN is a multifunctional high molecular mass adhesive glycoprotein that is present in the ECM and plasma (Pankov and Yamada, 2002). FN contains multifunctional binding sites to many substances and is involved in cell-ECM attachment, cell migration, cell proliferation, differentiation, wound healing and oncogenic transformation (Franke *et al.*, 2003). Plasma FN is able to phosphorylate Raf and further activate Erk, although it does not alter MMP-2 protein expression or activity, indicating that MMP-2 may not be the downstream target gene of the Erk pathway (Zhang *et al.*, 2005). However, FN is suggested to be a modulator of MMPs activity and the interaction between FN and HAM might stimulate the signal pathway in HAM to produce MMP-2 active form. The present study also showed that the addition of aFGF to bFGF-containing medium appeared to inhibit the effect by bFGF and ITS. Further studies are needed to solve the question. In conclusion, HAM could produce a large amount of active MMP-2 form when they were induced to differentiate into hepatocytes by cultivating in hepatogenic medium containing both bFGF and ITS or FN.

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