Up-regulation of Matrix Metalloproteinase-9 in Smooth Muscle Cell Undergoing Death

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This study investigated whether matrix metalloproteinases (MMPs) can be modified in apoptotic smooth muscle cell (SMC) using the SMC that undergoes apoptotic death by expressing Fas-associated death domain containing protein (FADD) when they are grown without tetracycline in culture medium. In the absence of tetracycline, FADD-SMC lost adherence and showed the fragmentation of the nuclei. In proportion to duration of tetracycline removal, phosphorylated form of p38 MAPK and of ERK increased, whereas phosphorylation of protein kinase B (PKB) was not changed very much in response to tetracycline. The levels of cyclin A and cyclin D were also decreased in a time dependent manner. Up-regulation of MMP-9 expression and activity was observed when the SMC were grown without tetracycline. Immunoreactivity of MMP-9 was detected from both attached and floating FADD-SMCs grown without tetracycline. An inhibitor of MAPK kinase, PD098059, and an inhibitor of p38 MAPK, SB203580, inhibited the up-regulation of MMP-9. Treatment of the SMC with a synthetic MMP inhibitor, BB94, attenuated death occurring in the absence of tetracycline. These results indicate that SMC undergoing death is able to up-regulate MMP-9 and that the enzyme can affect cell viability.

Key words – Metalloproteinase, MAPK, smooth muscle cell

Introduction

Matrix metalloproteinases (MMPs), a major group of enzymes that regulate cell-matrix composition by using zinc for their proteolytic activities, are essential for various normal biological processes such as embryonic development, morphogenesis, and tissue remodeling. Matrix metalloproteinase-9 (MMP-9), a secreted multi-domain enzyme that regulates cell-matrix composition, is produced by selected cell types, including keratinocytes, monocytes, tissue macrophages, polymorphonuclear leukocytes, and vascular smooth muscle cells (VSMCs)[19]. The main function of MMP-9 is the regulation of cell matrix composition. As MMP-9 belongs to the gelatinase subfamily of the MMPs, it cleaves denatured collagen (gelatins) and type 4 collagen, which is the major component of the basement membranes. This cleavage helps lymphocytes, and other leukocytes to enter and leave the blood and lymph circulations. Therefore, expression and secretion of MMP-9 by activated lymphocytes, monocytes and smooth muscle cell is tightly regulated by cytokines, chemokines, eicosanoids and peptidoglycans[4,15,17].

VSMCs together with their synthetic products elastin, collagen and extracellular matrix components comprise the medial layer of adult arteries. Although VSMCs also accumulate in atherosclerosis and restenosis after vessel injury, VSMC death by apoptosis is increasingly recognized in vascular disease. VSMC death rates increase as atherosclerotic plaques develop and increase in unstable versus stable lesions, implying the involvement of VSMC apoptosis in plaque rupture[2,12]. Atherosclerosis complicated by plaque rupture or thrombosis is a major cause of potentially lethal acute coronary syndromes and stroke. Rupture occurs frequently in plaques containing a soft, lipid-rich core that is covered by a thin and inflamed cap of fibrous tissue. Compared to the intact plaques, the ruptured caps are usually thinner, contain less collagen, and have fewer smooth muscle cells and more macrophages. Therefore, major determinants of plaque vulnerability and rupture are progressive lipid accumulation, ongoing inflammation, and cap weakening that is associated with increased collagen degradation and impaired healing and repair process by VSMCs[2,12,13]. The MMPs have been shown to be associated with atherogenesis and plaque rupture. In human atherosclerotic plaques, several MMP system components including MMPs 1, 2, 3, 9, 13, and 14 have been shown to

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contribute to the instability of plaques. In addition, targeted gene disruption of MMP-9 suppresses development of experimental abdominal aortic aneurysms and arterial remodeling[8,9,11,20,23]. Thus, increased MMP activity may be associated with plaque rupture and aneurysm formation, whereas impaired MMP activity may contribute to the growth and stabilization of atherosclerotic plaques. These findings indicate that activation of MMP-9 and VSMC death concur to cause plaque destabilization in atherosclerosis. Thus, this study was undertaken to investigate whether MMP-9 was modified during VSMC death using the rat smooth muscle cell that undergoes death by expressing FADD (FADD-SMC) when they are grown without tetracycline in the culture medium.

Materials and Methods

Cell Culture

Smooth muscle cells were grown in Dulbecco's modified Eagle's medium-high glucose (DMEM) (Life Technology, Grand Island, NY) supplemented with 10% fetal bovine serum, 5 mM L-glutamine, 50 units/ml penicillin, 50 ug/ml streptomycin and 10 ug/ml tetracycline in a humidified atmosphere of 5% CO_2.

Antibodies

Antibodies for phospho-p38 MAPK and phospho-ERK were from New England Biolabs Inc. (Beverly, MA). Antibodies for cycline A and cycline D were from Santa Cruz Biotechnology. (Santa Cruz, CA). Anti-MMP9 antibody was from Dr. Moon (Institute of Biotechnology, Euven, Belgium).

Zymography

The culture media were subjected to zymography. Equal volumes of conditioned media were loaded onto 8% polyacrylamide gels containing 0.1% of type I gelatin (Sigma Chemical Co) and electrophoresed at a constant voltage. After electrophoresis, gels were rinsed in 2.5% Triton X-100 for 30 minutes, incubated for 16 to 18 hours at 37°C in a buffer (50 mM Tris [pH 8.0], 2.5 mM $CaCl_2$, and 0.02% sodium azide), rinsed in 10% trichloroacetic acid, and stained in rapid Coomassie stain. The stained gels were visualized by Eagle-Eye Image.

Western blot analysis

Cells were lysed in a lysis buffer (50 mM TrisCl, pH 7.8, 150 mM NaCl, 1% NP40, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride). The protein content was determined using BCA Protein Assay Reagent. Twenty micrograms of protein was separated on SDS-PAGE gels and transferred to polyvinylidine difluoride membrane (Millipore Corp.). Nonspecific binding sites were blocked in T-TBST (50 mM TrisHCl, pH 7,4, 150 mM NaCl, 0.1% Tween 20) containing 5% nonfat dry milk for 2 hours at room temperature. The membrane was incubated with primary antibodies in T-TBST at 4C overnight. After 3 washes with T-BST, the membrane was incubated with horseradish peroxidase-conjugated secondary antibody. Being washed 3-4 times with T-TBST, membrane was incubated with Enhanced Luminol Reagent. The chemiluminescent signal was imaged on the X-ray film.

Measurement of oligonucleosomes

Oligonucleosomes in cells treated with appropriate reagents were quantitatively determined with the cell-death detection ELISA^{PLUS} kit following manufacturer's instructions (Roche, Indianapolis, IN). Cells were collected, resuspended in the lysis buffer provided in the kit, and incubated for 30 minutes at room temperature. The resultant supernatants after centrifugation at 200 g for 10 minutes were transferred into the streptavidin coated microplate with the Immunogen reagent. After incubation for 2 hours with gentle shaking at 300 rpm, each well was washed three times with the incubation buffer in the kit. The developing solution was added to each well and incubated on a plate shaker at 250 rpm until the color development was sufficient for a photometric analysis. The absorbance was measured at 405 nm with a reference wavelength of 492 nm.

Results

Phosphorylation of MAPKs in SMC destined for death

FADD-SMCs were grown in the absence or presence of tetracycline, and the cells were visualized after staining with propidium iodide (Fig. 1A). The number of attached cells were decreased in proportion to duration of tetracycline removal, as the cells lost adherence. Fragmented or condensed nuclei were observed in the cells grown without tetracycline. The mophological changes did not occur when cells were grown in the presence of tetracycline.

It was examined whether phosphorylation of MAPK

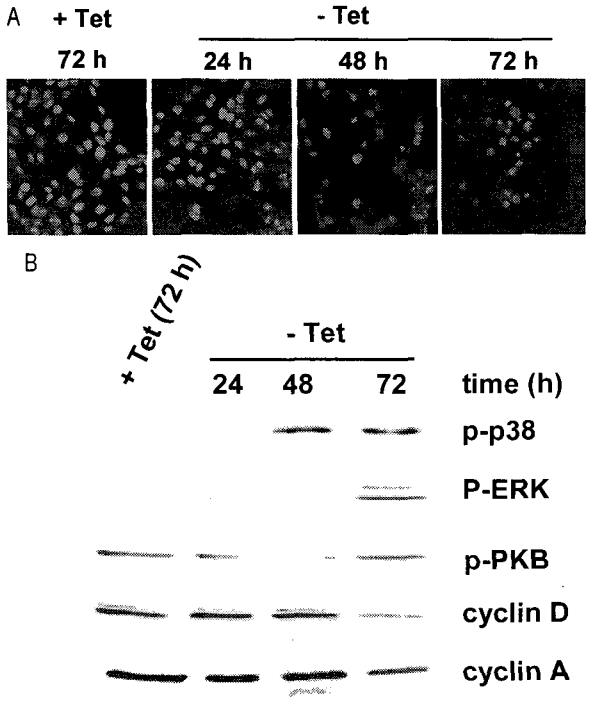

Fig. 1. (A) FADD-SMCs were grown for indicated time period in the absence (- Tet) or presence (+ Tet) of tetracycline. Cells were fixed with 4% paraformaldehyde containing propodium iodide. Cells were visualized by fluorescent microscopy. (B) FADD-SMCs were grown for indicated time period in the absence (- Tet) or presence (+ Tet) of tetracycline. Cell lysates were subjected to Western blot analysis with appropriate antibodies.

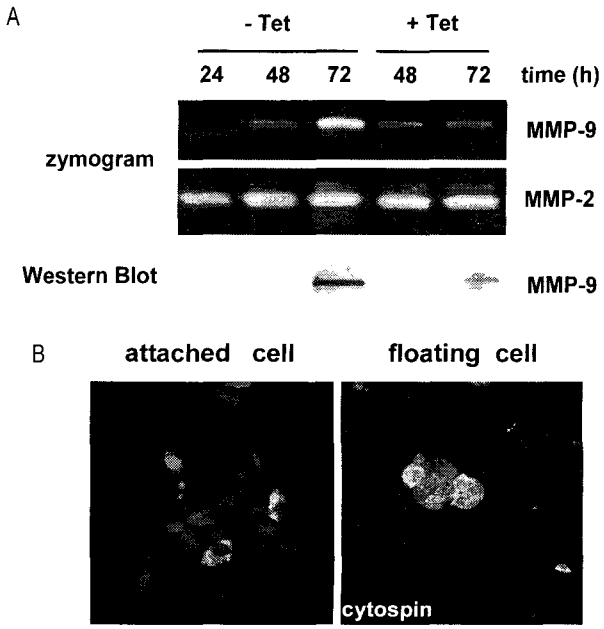

Fig. 2. (A) FADD-SMCs were grown for indicated time period in the absence (- Tet) or presence (+ Tet) of tetracycline. Activities of MMP-9 and -2 were examined by zymography. Also, MMP-9 protein was blotted in the identically processed cells. (B) FADD-SMCs were grown for 72 hours in the absence of tetracycline. MMP-9 immunoreactivity in floating and adherent cells were probed using FITC-conjugated secondary antibody. The cells were visualized by confocal microscopy. 400X.

was changed in FADD-SMC. The cells were grown with or without tetracycline, and phosphorylated forms of MAPK was detected by Western blot analysis (Fig. 1B). Phosphorylated form p38 MAPK and ERK were noticeably increased at 3 days post removal of tetracycline. The levels of cycline A and cycline D were decreased in FADD-SMC grown without tetracycline.

Up-regulation of MMP-9 in dying SMC

It was investigated whether MMP-9 was modified in dying SMC. FADD-SMCs were grown in the absence or presence of tetracycline, and activity and protein level of MMP-9 were examined (Fig. 2A). Whereas MMP-9 activity was low in the presence of tetracycline, the enzyme activity was increases in cells grown without tetracycline. In contrast MMP-9, activity of MMP-2 was not changed in response to tetracycline. It was examined whether the enzyme was modified at the protein level. MMP-9 protein was increased in cells grown in the presence of tetracycline. Immunoreactivity of MMP-9 was examined with FADD-SMC after immunofluorescence staining. Immunoreactivity of MMP-9 was detected from both attached and floating FADD-SMCs. The results indicate that MMP-9 was modified at its activity and at the protein level in FADD-SMC in the absence of tetracycline.

Active roles of MAPKs in the up-regulation of MMP-9

To investigate role of MAPKs in the up-regulation of MMP-9, an inhibitor of MAPK kinase, PD098059, and an inhibitor of p38 MAPK, SB203580, were employed (Fig. 3A). FADD-SMCs were grown with the inhibitors in the absence of tetracycline, and MMP-9 activity was examined. PD098059 and SB203580 inhibited MMP-9 activation in a concentration dependent manner, indication involvement of both inhibitors in the up-regulation of MMP-9.

To have an idea whether MMP-9 up-regulation is associated with SMC death, FADD-SMCs were grown with a synthetic MMP inhibitor, BB94, in the absence of tetracycline, and then DNA fragmentation was examined by ELISA (Fig. 3B). In comparison with the cells grown with

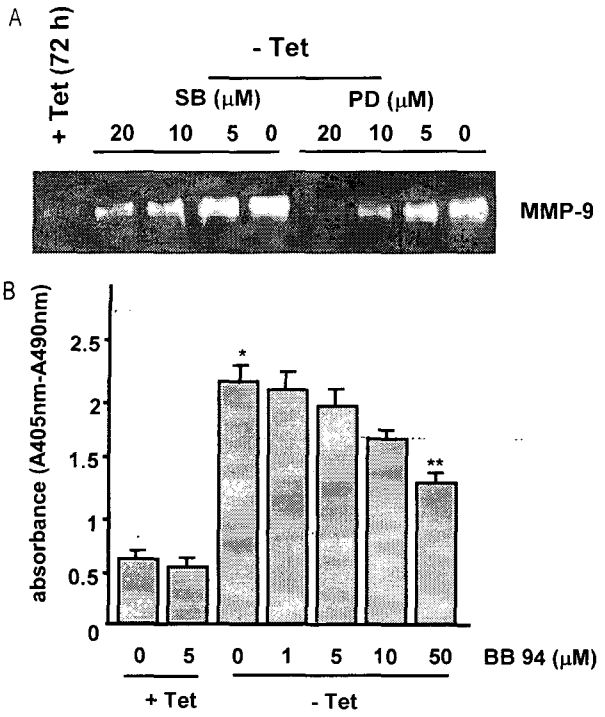

Fig. 3. (A) FADD-SMCs were grown for indicated time period in the absence (- Tet) or presence (+ Tet) of tetracycline with or without indicated inhibitors. MMP-9 activity in the cell was examined by zymography. (B) FADD-SMCs were grown for indicated time period in the absence (- Tet) or presence (+ Tet) of tetracycline with or without indicated inhibitor. Then, nuclesome formation was determined. Data are the representative of three independent experiments in triplicate. Values are mean±SD. *P<0.01 vs. +Tet; **P<0.05 vs. -Tet without BB94.

tetracycline, oligonucleosomes were increased when the cells were grown in the absence of tetracycline (P<0.01). The data indicate cell death in the absence of tetracycline. The addition of MMP inhibitor, however, attenuated oligonucleosome formation in cells grown without tetracycline (P<0.05).

Discussion

This study demonstrates up-regulation of MMP-9 in terms of translation and activity in dying SMC by FADD through ERK and p38 MAPK. The present study also provides evidence that MMP-9 might participate in SMC death.

We found that synthesis and activation of MMP-9 were increased in dying SMC. MMP-9 is under strict control at various levels: gene transcription, synthesis, secretion and activation. In most cell types, gene transcription of MMP-9 is inducible and occurs by cytokines and cellular interaction. Following its translation, the enzyme is immediately secreted through the normal secretory pathway. MMP-9 is secreted as zymogen (proenzyme), which remains inactive unless it is activated by the removal of the propeptide domain by proteolysis, resulting in the activation of the enzyme[4,19]. In the present study, we demonstrated that the MMP-9 up-regulation was related with p38 MAPK and ERK. The ERK and the p38 MAPK pathways were shown to regulate MMP-9 expression. For example, overexpression of dominant-negative MEK1 inhibited the MMP-9 expression in PMA-treated carcinoma cells[10]. In a separate study, p38 MAPK was involved in PMA-induced MMP-9 secretion with use of the SB203580 compound[21,22]. The contribution of both pathways to the transcriptional regulation of MMP-9 in arterial smooth muscle was reported[6].

Although MMP activities lead to degradation of extracellular matrix proteins[16], an intact matrix may be required to protect cells from apoptosis by anoikis[1,3], suggesting an association with MMP-9 in cell death. The evidence for MMP-9 relevance in apoptosis emerged from studies showing that MMP-9 deficiency affects axonal outgrowth, migration, and apoptosis in the developing cerebellum[25] and also protects against retinal ganglion cell death after optic nerve ligation[5]. We have attempted to correlate cell death with MMP-9 and this report demonstrated that activated MMP-9 accumulates intracellularly in cells undergoing apoptosis. This is compatible with earlier studies in microvascular endothelial cells in which TIMP 1-free gelatinase B is found in endothelial vesicles in response to phorbol myristate acetate[18].

The integrity of the fibrous cap, which is a critical factor for resistance to plaque rupture, depends largely on the collagenous extracellular matrix (ECM) that is produced by VSMCs or myofibroblasts. An increased inflammatory response, decreased ECM synthesis, and increased ECM degradation, all contribute to plaque instability[7,8,9]. Increased MMP activity appears to be one of the most important risk factors for the instability of vulnerable atheromatous plaques or plaque rupture. Several MMPs, including MMP-1, 2, 3, 9, 13, and 14, may be involved [7,9,24]. Among them, MMP-9 has been considered as a key molecule in mediating the destabilization of the atheromatous plaques[8,9,14]. The present study proposes that MMP-9 can be activated in dying VSMC and also contributes to the pathophysiol-

ogy of plaque instability.

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초록 : 사멸세포에서의 metalloproteinase-9의 작용

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배지에 tetracycline이 없으면 사멸하는 평활근세포 (FADD-SMC)를 이용하여 metalloproteinase (MMP)와 사멸세포의 상관관계를 조사하였다. FADD-SMC를tetracycline이 없는 배지에서 배양하는 경우 핵이 조각으로 잘라졌고 인산화한 p38과 ERK가 증가하고 MMP-9의 발현과 활성이 증가한 반면, cyclin A와 cyclin D의 발현은 감소하였다. 그리고 죽는 FADD-SMC에서 MMP-9의 발현은 immunofluorescen로 재확인하였다. MMP-9의 증가는 MAPK 억제제인 PD098059와 p38 MAPK 억제제인 SB203580에 의하여 감소하였다. 그리고 MMP 억제제인 BB94는 FADD-SMC의 사멸을 감소시켰다.