Arch Pharm Res Vol 28, No 11, 1257-1262, 2005

Archives of Pharmacal Research
http://apr.psk.or.kr

MAP Kinase Activation is Required for the MMP-9 Induction by TNF-Stimulation

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(Received September 8, 2005)

MMP-9 is a metalloproteinase capable of basement membrane degradation *in vivo*. Expression of MMP-9 can be found in normal conditions such as trophoblasts, osteoclasts, and leukocytes and their precursors. They also occur as well as in pathological conditions, such as the invasive growth of primary tumors, metastasis, angiogenesis, rheumatoid arthritis, and periodontal diseases. MMP-9 upregulation can be highly induced by a wide range of agents. These agents include growth factors, cytokines, cell-cell, and cell-ECM adhesion molecules, and agents altering cell shape. Here, we observed that TNF-α stimulated human monocytic cell line, HL-60 produced MMP-9 in a dose and time dependent manner. Real time PCR results indicated transcriptional upregulation of MMP-9 as early as 3 h post TNF-α stimulation. To investigate the signaling pathway underlined in TNF-α induced MMP-9 expression, three MAP kinase inhibitors were added to cells 1 h prior to TNF-α treatment. The ERK inhibitor completely abolished MMP-9 expression by TNF-α. But neither p38 MAP kinase nor JNK inhibitor had an effect on TNF-α induced MMP-9 expression, suggesting that ERK activation is required for the MMP-9 induction by TNF-α. Taken together, we found that TNF-α stimulation facilitates ERK activation, which results in the transcriptional upregulation of MMP-9 gene and subsequent MMP-9 production and secretion.

Key words: TNF-α, MMP-9, MAP Kinase, Signaling pathway

INTRODUCTION

The matrix metalloproteinases (MMPs) are zinc dependent endopeptidases responsible for the degradation of extracellular matrix (ECM) constituents. These include collagen, gelatin, fibronectin, laminin and proteoglycan, as well as non-matrix proteins (Reichardt and Tomaselli, 1991). They comprise a large family of proteinases that share common structural and functional elements and are products of different genes (Massova *et al.*, 1998). Nowadays, 24 members of this enzyme family are known to exist (Woessner, 1999; Woessner and Gunja-Smith, 1991). MMPs play a role in the huge field of physiological processes. They are assumed to be participated in collagenolyses in, for example, blastocyst implantation, embryonic development, nerve growth, endometrial cycling, mammary gland morphogenesis, bone remodeling, wound healing angiogenesis, and apoptosis. Among the pathologic processes, they are assumed to induce tissue destruction, for example, rheumatoid arthritis and dilated cardiomyopathy (Ishikawa *et al.*, 2005; Kassiri *et al.*, 2005; Wang *et al.*, 2004).

MMP-9 is another metalloproteinase capable of basement membrane degradation *in vivo*. MMP-9 expression normally occurs in trophoblasts, osteoclasts, and leukocytes and their precursors (Borregaard *et al.*, 1995; Harvey *et al.*, 1995; Witty *et al.*, 1996). MMP-9 transcription can be highly induced by a wide range of agents. These agents include growth factors, cytokines, cell-cell, and cell-ECM adhesion molecules, and agents altering cell shape (Dong *et al.*, 2001; Martin *et al.*, 2001).

TNF-α has been demonstrated to upregulate the expression of MMPs (Hanemaaijer *et al.*, 1997; Migita *et al.*, 1996) contributing to the complex process of target tissues remodeling which involves focal degradation of the basement membrane and extracellular matrix (ECM). A number of studies have suggested that TNF-α induced MMP expression may play a role in various diseases, such as rheumatoid arthritis, periodontal diseases (Drynda

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et al., 2005; Kim *et al.*, 2005; Tervahartiala *et al.*, 2000), and malignant cell invasion & migration (Tanimura *et al.*, 2005). However, the underlying regulatory mechanism has not fully been explained. We undertook studies to determine the signaling pathway that mediates the effects of TNF-α on MMP-9 production.

MATERIALS AND METHODS

Materials

Cell culture media and reagents were from Gibco BRL (Gaithersburg, MD). Recombinant human TNF-α was purchased from R&D Systems (Minneapolis, MN). MAP kinase inhibitors U0126 (MEK1/2 inhibitor), SB203580 (p38 MAPK inhibitor), SP600125 (JNK inhibitor) were from Calbiochem (San Diego, CA).

Cell culture

Human monocytic HL-60 cells were obtained from American Type Culture Collection (Manassas, VA) and grown in RPMI 1640 supplemented with 10% fetal bovine serum (HyClone), 2 mM L-glutamine, 10 unit/mL penicillin, and 10 g/mL streptomycin at 37°C in 5% CO_2. The cells were subcultured twice a week and seeded in either 24- or 6-well plates. They were stimulated with TNF-α in the presence or absence of several inhibitors for various time periods ranging from a few minutes to 24 h. Inhibitors were treated 1 h prior to TNF-α stimulation.

Measurement of MMP-9 activity

Zymogram analysis was preformed to assess MMP-9 activity. Cell-free culture medium was mixed with a loading buffer containing 62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 4% SDS, and 0.01% bromophenol blue, and loaded onto 10% SDS-PAGE gel containing 1 mg/mL gelatin. After electrophoresis, gels were washed three times with 2.5% Triton X-100 in water, then incubated overnight at 37°C in 0.2% Brij 35, 5 mM $CaCl_2$, 1 mM NaCl, and 50 mM Tris, pH 7.4, in a closed container. Gels were then stained for 30 min with 0.25% Coomassie R-250 in 10% acetic acid and 45% methanol and destained for 30 min using an aqueous mix of 20% acetic acid, 20% methanol, 17% ethanol. Areas of protease activity appeared as clear bands.

Western blot analysis of MMP-9

The conditioned medium of the HL-60 cells was concentrated by precipitation with trichloroacetic acid. They were quantified with the Bio-Rad protein assay system and different amounts of protein aliquots were adjusted to 15 μL with 4 × sample buffer comprising 62.5 mM Tris-HCl, 20% glycerol, 0.032% bromophenol blue, 5% mercaptoethanol and 2% SDS. Along with the prestained broad-range molecular mass standards (Bio-Rad), samples were fractionated using a 4% stacking and 10% SDS-PAGE mini gel (Bio-Rad, Mississauga, ON) and transferred to a nitrocellulose membrane by electroblotting at 200 mA in a buffer comprising 25 mM Tris-HCl, 192 mM glycine, 0.04% SDS and 20% ethanol. The membranes were rinsed with distilled water, incubated for 1 h in PBS pH 7.4 with 5% non-fat milk to block non-specific interactions, and washed five times (twice for 5 min, once for 15 min and twice for 5 min) with PBS containing 0.1% Tween. They were then reacted overnight sequentially in the same buffer at 4°C with 1 to 2 μg/mL anti-human MMP-9 antibody (R&D systems, Mineapolis, MN). Subsequently, membranes were washed five times at 22°C with PBS containing 0.1% Tween. They were then incubated with the anti-rabbit or anti-mouse secondary peroxidase-conjugated IgG (300 mU/mL), and washed seven times with PBS containing 0.1% Tween. To reveal the MMP-9 bands, membranes were incubated with 100 μL of solution A and 100 μL of solution B of the chemiluminescence detection system (Amersham, Piscataway, NJ) for 1 min and exposed to film for 2 to 15 min.

RT-PCR

To examine if TNF-α stimulation elicits transcriptional activation of MMP-9, mRNA levels of MMP-9 were measured using a conventional RT-PCR, then quantified by real-time PCR. In brief, total RNA was isolated from the cells treated with 500 pg/mL TNF-α for various hrs using Trizol (Gibco). After reverse transcription of RNA to cDNA, samples were subjected to PCR reactions. The primer designs and PCR reactions were performed as described by other reports (Ho *et al.*, 2005). The consensus primers for MMP-9 were 5'-CAC AGC TTT CCT CCA CTG CTG CTG C-3' (sense) and 5'-GGC ATG GTC CAC ATC TGC TCT TGG C-3 (antisense). For beta-actin, they were 5-CCA CCC ATG GCA AAT TCC ATG GCA-3' (sense) and 5'-TCT AGA CGG CAG GTC AGG TCC ACC-3' (antisense). The PCR condition was 30 cycles of denaturation (95°C/1 min), annealing (61°C/30 sec) and extension (72°C/1 min) in the presence of 2.5 mg $MgCl_2$, followed by a final 5 min extension at 72°C. The PCR products were separated by electrophoresis through a 1% agarose gel containing ethidium bromide. The results of the real-time PCR were expressed by fold of MMP-9 induction and was calculated using the level of β-actin, which was amplified in the same condition, as an internal control.

Mitogen-activated protein (MAP) kinase activation

To address the involvement of MAP kinase activation in TNF-α-induced MMP-9 expression, HL-60 cells were pretreated with several MAPK inhibitors for 1 h and then stimulated with 500 pg/mL TNF-α for various periods. For

zymogram and Western blot analysis, conditioned media of 24 h stimulation were used. For RT-PCR analysis, cells were treated with TNF-α for 6 h and then mRNA was prepared from cells and subjected to reverse transcription following PCR.

RESULTS

Dose-dependent induction of MMP-9 by TNF-α stimulation

Since the expression of MMP-9 by pro-inflammatory cytokines has been well known, we examined the effect of TNF-α treatment on MMP expression in HL-60, human monocytic cells. HL-60 cells were incubated with 100-500 pg/mL TNF-α for 24 h and activities of MMP-9 secreted into the conditioned media were measured by gelatin zymography and immunoblotting. A zymographic analysis of samples revealed the presence of the proform of MMP-9, but not the active form (Fig. 1A). TNF-α stimulation induced MMP-9 expression and in proportion as TNF-α concentration increased, MMP-9 levels were increased, suggesting that TNF-α treatment induced MMP-9 expression in a dose dependent manner (Fig. 1B).

Time-dependent induction of MMP-9 by TNF-α stimulation

To examine the kinetics of MMP-9 production, HL-60 cells were stimulated with 500 pg/mL TNF-α for 3, 6, 12, 24 h and conditioned media were used to test the MMP-9 level by gelatin zymography. MMP-9 expression was detectable within 12 h and increased up to 24 h after TNF-α treatment (Fig. 2A). Immunoblot analysis also revealed that MMP-9 expression was upregulated by TNF-α treatment, indicating that TNF-α stimulation induced MMP-9 expression in a time-dependent manner (Fig. 2B). In contrast to MMP-9, we did not detect MMP-2 activity from the same conditioned media (data not shown).

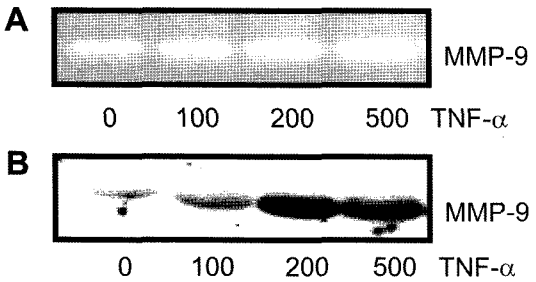

Fig. 1. Effect of TNF-α on MMP-9 induction from HL-60 cells, human monocytic cells. HL-60 cells were treated with the indicated concentrations of human recombinant TNF-α. Conditioned media were harvested 24 h later, and were subjected to gelatin zymography and Western blot analysis with antibody against MMP-9. The data shown are representative of three independent experiments.

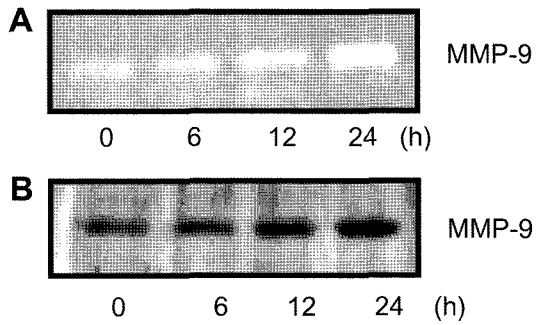

Fig. 2. Kinetics of TNF-α-mediated MMP-9 induction from HL-60 cells. HL-60 cells were treated with 500 pg/mL TNF-α for the indicated time periods. Conditioned media were harvested at different time point, and were used for the gelatin zymography and Western blot analysis. The data shown are representative of three independent experiments.

TNF-α activated transcriptional upregulation of MMP-9

To further support TNF-α mediated MMP-9 activation, we determined the mRNA levels of MMP-9 after TNF-α stimulation *via* real-time PCR analysis. HL-60 cells were treated with various concentrations of TNF-α for different time periods. Total RNA was isolated from those stimulated cells and used in the subsequent reverse transcription and PCR with specific primers. As shown in Fig. 3A

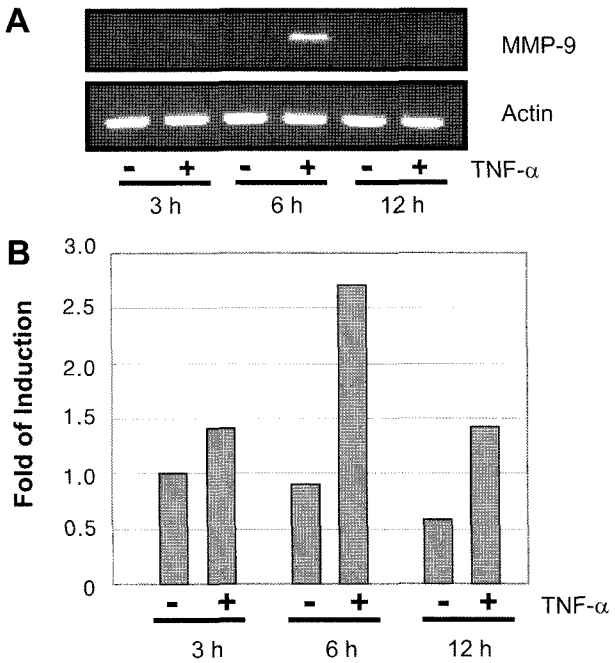

Fig. 3. Transcriptional activation of MMP-9 by TNF-α treatment in HL-60 cells. Cells were treated with 500 pg/mL TNF-α for 3, 6, 12 h. Total RNA was isolated using Trizol. After reverse transcription of mRNA, samples were subjected to PCR reactions (3A). Fold of MMP-9 induction was calculated base on the level of β-actin which was amplified in the same condition, as an internal control (3B). The data shown are representative of three independent experiments.

and 3B, MMP-9 mRNA was induced as early as 4 h after TNF-α stimulation and sustained for 12 h. The level of MMP-9 mRNA was relative to TNF-α concentration. This indicates that TNF-α mediated MMP-9 induction is upregulated at transcriptional level.

MAP kinase activation is involved in TNF-α mediated MMP-9 expression

To elucidate the signaling involved in TNF-α-dependent MMP-9 expression, we examined the effects of various inhibitors on TNF-α-dependent MMP-9 expression. HL-60 was pretreated with U0126 (1 μM, a potent MEK inhibitor), SP600125 (5 μM, a JNK inhibitor), SB203580 (5 μM, a p38 MAPK inhibitor) 1 h prior to TNF-α treatment. Conditioned media collected from stimulated cells were subjected to zymography & immunoblotting. As shown in Fig. 4A and 4B, TNF-α-dependent MMP-9 expression from HL-60 was substantially blocked by treatment with U0126, an MEK inhibitor, but not with other MAP kinase inhibitors.

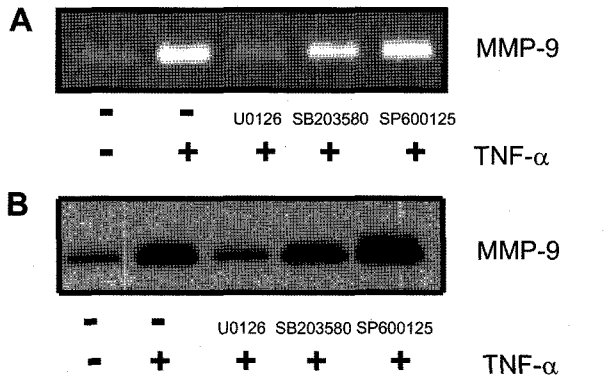

Fig. 4. Effect of MAP kinase inhibitors on TNF-α-induced MMP-9 producton from HL-60 cells. The cells were stimulated with 500 pg/mL TNF-α stimulation for 24 h in the presence or absence of inhibitors (1 mg/mL U0126, 5 mM SB203580, or 5 mM SP600125). Conditioned media were harvested and induction of MMP-9 were determined by gelatin zymography (A) and Western blot analysis (B).

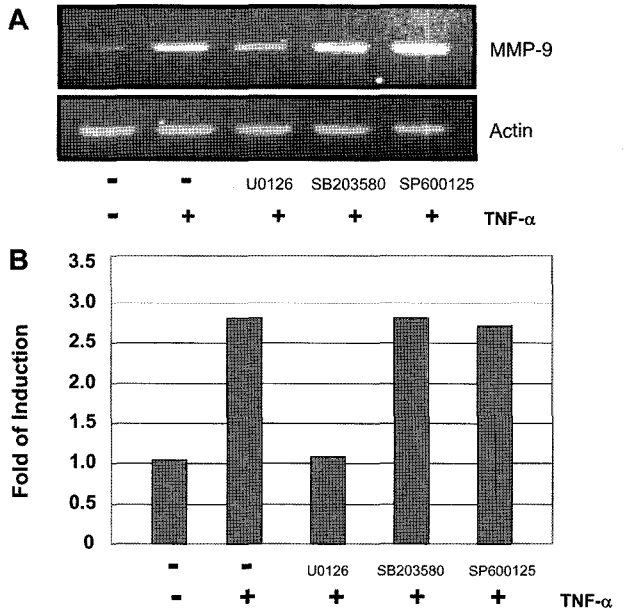

Fig. 5. Effect of MAP kinase inhibitors on transcriptional upregulation of MMP-9 by TNF-α stimulation. The cells were pretreated with the 1 mg/mL U0126, 5 mM SB203580, or 5 mM SP600125 for 1 h and then stimulated with 500 pg/mL TNF-α for 6 h. The levels of TNF-α mRNA were determined by conventional RT-PCR (5A) and real-time PCR (5B). The data shown are representative of three independent experiments.

Additionally, we examined the transcriptional regulation of these inhibitors. Total RNA were prepared from cells after treatment, and real-time PCR analysis was performed. The level of MMP-9 mRNA was decreased by U0126, MEK inhibitor, but not by other inhibitors, indicating that TNF-α mediated upregulation of MMP-9 was abolished by the inhibition of ERK activation (Fig. 5A and 5B). Taken together, these results suggest the involvement of ERK activation in TNF-α-dependent MMP-9 expression of HL-60 cells.

DISCUSSION

The gelatinases including MMP-2 and MMP-9 are considered key enzymes in matrix component degradation and have been suggested to play a critical role in matrix remodeling. Since growth and development are associated with rapid cell movement and with the restructuring and reshaping of the extracellular matrix, a number of studies have provided evidence for the involvement of MMPs in developmentally regulated processes (Damsky and Werb, 1992). The evidence of involvement of MMPs in tumors is based on findings that indicate the expression of one or more MMPs is common among malignant tumors and the invasive growth of primary tumors, metastasis, angiogenesis are dependent on the destruction and remodeling of stromal architecture. In addition, a link between inflammatory disease progress and MMP expression is offered by several reports. These suggest the continuous or intermittent destruction of rheumatoid joints and increased collagenase activity in gingival crevicular fluid (GCF) in natural human peridontitis and in experimental peridontitis in gods and monkeys (Drynda *et al.*, 2005; Kim *et al.*, 2005; Overall *et al.*, 1991; Tervahartiala *et al.*, 2000). Analysis of GCF has revealed that mediators such as IL-1α,β and TNF-α, which are potentially capable of inducing MMP expression, are present in GCF in physiologically meaningful concentrations (Konradsson and van Dijken, 2005; Shapira *et al.*, 2003; Wei *et al.*, 2004).

TNF-α, one of the well characterized proinflammatory cytokine, shows a wide spectrum of biological activities. It is produced mainly by macrophages but can also be

produced by other cell types in response to physiological or pathological stimuli (Bazzoni and Beutler, 1996). The original interest in TNF-α was based on its antitumoral activity in transformed cell types including breast cancer cells (Carswell *et al.*, 1975). In general, nontransfomed cells are resistant to the cytotoxic or cytostatic effects of TNF-α, but there are some exceptions (Suffys *et al.*, 1989)

Many studies suggested that diverse cytokines induced MMP expression, playing a role in various pathogenic consequences, including rheumatoid arthritis, periodontal diseases (Drynda *et al.*, 2005; Kim *et al.*, 2005; Tervahartiala *et al.*, 2000), and malignant cell invasion & migration (Tanimura *et al.*, 2005). We addressed which signaling pathway might be involved in the effects of TNF-α on MMP-9 production.

The studies presented here demonstrate that TNF-α stimulation of human monocytic cell line, HL-60 induced MMP-9 expression was observed by gelatin zymography and immunoblotting. The MMP-9 level following TNF-α treatment was increased in a dose and time dependent manner. The real-time PCR results indicated that TNF-α stimulation elicits MMP-9 mRNA induction, and its mRNA can be detected as early as 3 h post-TNF-α stimulation. This finding indicates transcriptional upregulation of MMP-9 by TNF-α. To elucidate signaling pathway underlined in TNF-α induced MMP-9 expression, three MAP kinase inhibitors were added to cells 1 h prior to TNF-α treatment. The inhibition of ERK activation completely abolished MMP-9 expression by TNF-α, but neither p38 MAP kinase nor JNK inhibitor had an effect on TNF-α induced MMP-9 expression;. This indicates that ERK is a key signaling molecule in MMP-9 expression by TNF-α stimulation. In summary, we have shown that TNF-α stimulation elicits ERK activation, which results in the transcriptional upregulation of MMP-9 gene following MMP-9 production and secretion.

ACKNOWLEDGEMENTS

This study was supported by research grant from Catholic University of Daegu in 2004 and the Korea Science and Engineering Foundation (KOSEF) through the Aging-associated Vascular Disease Research Center at Yeungnam University (R13-2005-005-01003-0 (2005).

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