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Mitogen-Activated Protein Kinases Mediate Matrix Metalloproteinase-9 Expression in Vascular Smooth Muscle Cells

Aesim Cho, Jonathan Graves, Michael A. Reidy

Abstract—Expression of matrix metalloproteinase (MMP)-9 has been linked to the progression of plaque rupture and intimal formation in arterial lesions. In this study, we determined which factors and signaling pathways are involved in regulating the MMP-9 gene. Rat carotid arterial smooth muscle cells treated with tumor necrosis factor (TNF)-α showed a marked increase in MMP-9 activity and mRNA level, whereas platelet-derived growth factor (PDGF) showed a slight induction of the MMP-9 mRNA level. TNF-α treatment caused an increase in c-Jun N-terminal kinase (JNK), p38 mitogen-activated protein kinase (p38 MAPK), and extracellular signal–regulated kinase (ERK) activities, whereas PDGF treatment caused an increase in ERKs and p38 MAPK activities without any effect on JNK activity. Treatment with either SB203580 (inhibitor of p38 MAPK) or U0126 (inhibitor of the ERK pathway) downregulated the TNF-α–induced MMP-9 expression in a dose-dependent manner. Treatment of cells with TNF-α and PDGF together stimulated the MMP-9 expression at a level higher than that observed with either factor alone, suggesting that TNF-α and PDGF have a synergistic effect on MMP-9 expression in arterial smooth muscle cells. Furthermore, suboptimal inhibitory concentrations of SB203580 and U0126 together almost completely inhibited the MMP-9 expression. These results suggest that p38 MAPK and ERK pathways contribute to the transcriptional regulation of MMP-9 in arterial smooth muscle cells. (Arterioscler Thromb Vasc Biol. 2000;20:2527-2532.)

Key Words: mitogen-activated protein kinases • p38 mitogen-activated protein kinase • extracellular signal–regulated kinase • matrix metalloproteinase-9 • tumor necrosis factor

Degradation of extracellular matrix by matrix metalloproteinases (MMPs) is thought to be important in the progression of atherosclerosis and plaque rupture. MMP-1, MMP-3, and MMP-9 have been identified in human atherosclerotic lesions, and the enhanced expression of MMP-9 at the shoulders of these lesions has been linked to plaque rupture. In experimental animal models, MMPs are also shown to be important for smooth muscle cell migration into the intima. In a rat arterial injury model, after the initial medial smooth muscle cell replication, medial smooth muscle cells migrate and first appear in the intima 4 days after injury. MMP-9 is expressed within 6 hours after injury in rat carotid arteries and continues to be expressed up to 6 days, whereas MMP-2 activity is markedly increased after 4 days of injury. The importance of these MMPs in the migration of smooth muscle cells is illustrated by the finding that the administration of MMP inhibitor almost completely inhibits the number of smooth muscle cells migrating into the intima. Although MMPs play an important role in lesion growth, little is known about the signaling mechanism involved in regulating MMP expression in vascular smooth muscle cells.

Several growth factors and cytokines, such as basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), tumor necrosis factor (TNF)-α, and interleukin (IL)-1, induce MMPs, including interstitial collagenase (MMP-1), stromelysin (MMP-3), and type IV collagenase (MMP-2 and MMP-9) in a variety of cell types. Likely intracellular signaling transduction pathways activated by these factors are mitogen-activated protein kinase (MAPK) pathways, of which there are 3 distinct groups: extracellular signal–regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK. The ERK pathway is mainly activated by growth factors and has been linked to cell proliferation, cell growth, and differentiation. JNK and p38 MAPK are known as stress-activated protein kinases, inasmuch as they are strongly activated in response to stressful stimuli, such as osmotic shock, UV light, and cytokines. JNK and p38 MAPK have been linked to apoptosis, cell survival, transformation, development, cell migration, and immune activation. These MAPKs are activated by sequential kinase cascade; eg, ERKs are activated by MAPK kinase kinase (MKK)1 and MKK2, JNK is activated by MKK4 and MKK7, and p38 MAPK is activated by MKK3 and MKK6. Once activated, ERKs act on p90rks, Elk-1, c-Fos, and cAMP response element binding protein, and JNK phosphorylates activating transcription factor (ATF)-2 and c-Jun.
vated p38 MAPK can phosphorylate ATF-2, C/EBP-homologous protein (CHOP), and MAPK-activated protein kinase (MAPKAPK)-2, which activates small heat shock proteins and cAMP response element binding protein.12,13

In the present study, we investigated the signaling pathways involved in the regulation of MMP-9 expression in vascular smooth muscle cells, and our data show that the activation of ERKs and p38 MAPK contributes to the induction of MMP-9 expression in vascular smooth muscle cells.

Methods

Cell Culture
Arterial smooth muscle cells, isolated from carotid arteries of Sprague-Dawley rats, were grown in Waymouth complete media supplemented with 10% calf serum, 200 U penicillin/mL, and 200 mg streptomycin/mL. At confluence, cells were serum-starved for 24 hours and then treated with TNF-α (R & D Laboratories) transforming growth factor (TGF)-β1 (R & D Laboratories), PDGF (gift from Hoffman La Roche, Basel, Switzerland), bFGF (gift from Scios Nova, Sunnyvale, Calif), SB203580 (p38 MAPK inhibitor, Calbiochem), U0126 (MEK1 inhibitor, Promega), and anisomycin (Sigma Chemical Co) as indicated in each experiments. At the end of treatment, conditioned media were collected for zymography and Western blot, and cells were rinsed once with ice-cold PBS and collected for RNA extraction, kinase assays, and Western blot analysis. Cells were used between passages 5 and 25.

Zymography
Conditioned media were subjected to zymography according to the procedure previously described.14 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 mmol/L Tris [pH 8.0], 2.5 mmol/L CaCl₂, 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 (Stratagene), and the relative amounts of MMP-9 and MMP-2 were quantified by NIH Image Analysis software.

Northern Blot Analysis and Quantitative PCR
Cells from culture experiments were scraped and lysed in Trizol (GIBCO-BRL), and RNA was extracted by chloroform extraction and isopropanol precipitation. Fifteen micrograms of total RNA was separated on a 0.5% formaldehyde–1.2% agarose gel in 1× MOPS buffer and transferred to nylon membranes. The membranes were hybridized with cDNA probe for rat MMP-9 (800 bp, a gift from Dr Birkedal-Hansen, University of Alabama, Birmingham)15 labeled with [32P]dCTP by random primer extension (Multi-Prime, Amersham). Two micrograms of total RNA from each sample was run on a 10% SDS-PAGE gel and separated on a 0.5% formaldehyde–1.2% agarose gel in 1× MOPS buffer and transferred to nylon membranes. The membranes were hybridized with cDNA probe for rat MMP-9 (800 bp, a gift from Dr Birkedal-Hansen, University of Alabama, Birmingham)15 labeled with [32P]dCTP by random primer extension (Multi-Prime, Amersham). Two micrograms of total RNA from each sample was run on a 10% SDS-PAGE gel 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 mmol/L Tris [pH 8.0], 2.5 mmol/L CaCl₂, 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 (Stratagene), and the relative amounts of MMP-9 and MMP-2 were quantified by NIH Image Analysis software.

Effect of Growth Factors and Cytokines on MMP-9 Expression
To determine which MAPK pathways might contribute to the regulation of MMP-9 gene expression in vascular smooth muscle cells, we examined the effect of growth factors and cytokines known to be present in injured arteries when MMP-9 is expressed. Rat arterial smooth muscle cells were treated with various concentrations of PDGF-BB, bFGF, TNF-α, or TGF-β1, and with the exception of TNF-α, the conditioned media from smooth muscle cells showed no detectable MMP-9 gelatinolytic activity (Figure 1A). This induction of MMP-9 activity by TNF-α was completely abrogated in the presence of a soluble receptor for TNF-α (data not shown). The gelatinolytic activity of MMP-2 was not significantly affected by any of the factors tested. Northern blot analysis showed that mRNA transcripts corresponding to MMP-9 mRNA level
Figure 1B. Interestingly, TNF-α-elevated when cells were treated with bFGF and PDGF mRNA level by TNF-α-shown). These data suggest that the induction of the MMP-9 activities of p38 MAPK, JNK, and ERK directly and increased the phosphorylation of ERKs. We also measured TNF-α-phosphorylation was markedly upregulated with PDGF and significantly, increased with bFGF treatment, whereas its Figure 1B). The phosphorylation of p38 MAPK was slightly, but not very little increase in the phosphorylation of JNK, whereas TNF-α markedly increased JNK phosphorylation (Figure 1B). The phosphorylation of p38 MAPK was slightly, but not significantly, increased with bFGF treatment, whereas its phosphorylation was markedly upregulated with PDGF and TNF-α (Figure 1B). ERK phosphorylation was markedly elevated when cells were treated with bFGF and PDGF (Figure 1B). Interestingly, TNF-α treatment also moderately increased the phosphorylation of ERKs. We also measured the activities of p38 MAPK, JNK, and ERK directly and observed a result similar to that shown in Figure 1B (data not shown). These data suggest that the induction of the MMP-9 mRNA level by TNF-α and PDGF may be regulated by elevated activities of ERK, JNK, and p38 MAPK.

Effect of MAPK Activities on MMP-9 Expression
To examine directly whether MAPKs regulate MMP-9 expression, we determined the activities of 3 members of the MAPKs (ERKs, JNK, and p38 MAPK) in vascular smooth muscle cells treated with TNF-α, bFGF, and PDGF. The kinase activities were assessed by measuring their degree of regulatory phosphorylation by using phosphospecific antibodies. bFGF and PDGF treatment showed very little increase in the phosphorylation of JNK, whereas TNF-α markedly increased JNK phosphorylation (Figure 1B). The phosphorylation of p38 MAPK was slightly, but not significantly, increased with bFGF treatment, whereas its phosphorylation was markedly upregulated with PDGF and TNF-α (Figure 1B). ERK phosphorylation was markedly elevated when cells were treated with bFGF and PDGF (Figure 1B). Interestingly, TNF-α treatment also moderately increased the phosphorylation of ERKs. We also measured the activities of p38 MAPK, JNK, and ERK directly and observed a result similar to that shown in Figure 1B (data not shown). These data suggest that the induction of the MMP-9 mRNA level by TNF-α and PDGF may be regulated by elevated activities of ERK, JNK, and p38 MAPK.

Activities of MAPKs in Vascular Smooth Muscle Cells
To determine which signaling pathway is involved in the activation of MMP-9 expression, we examined the activities of 3 members of the MAPKs (ERKs, JNK, and p38 MAPK) in vascular smooth muscle cells treated with TNF-α, bFGF, and PDGF. The kinase activities were assessed by measuring their degree of regulatory phosphorylation by using phosphospecific antibodies. bFGF and PDGF treatment showed very little increase in the phosphorylation of JNK, whereas TNF-α markedly increased JNK phosphorylation (Figure 1B). The phosphorylation of p38 MAPK was slightly, but not significantly, increased with bFGF treatment, whereas its phosphorylation was markedly upregulated with PDGF and TNF-α (Figure 1B). ERK phosphorylation was markedly elevated when cells were treated with bFGF and PDGF (Figure 1B). Interestingly, TNF-α treatment also moderately increased the phosphorylation of ERKs. We also measured the activities of p38 MAPK, JNK, and ERK directly and observed a result similar to that shown in Figure 1B (data not shown). These data suggest that the induction of the MMP-9 mRNA level by TNF-α and PDGF may be regulated by elevated activities of ERK, JNK, and p38 MAPK.

Figure 2. Quantitative PCR amplification of MMP-9 mRNA level of arterial smooth muscle cells pretreated with SB203580 (0.1, 0.3, 0.75, 1.25, 2.5, 5, 10, and 20 μmol/L) or dimethyl sulfoxide (0.1%, 0 μmol/L SB203580) for 45 minutes and then incubated with 100 ng/mL TNF-α (A) or 10 μg/mL anisomycin (B) for 24 hours. Data are representative of 3 independent experiments, and values are expressed as mean (±SE) number of MMP-9 cDNA copies normalized to number of 18S cDNA copies.

at a concentration of 50 ng/mL (please see Figure I, published online at http://atvb.ahajournals.org).

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U0126 compound, a selective inhibitor of MEK1/MKK1, almost completely inhibited ERK activity at a concentration of 2.5 μmol/L (Figure 4A) and decreased the TNF-α-induced MMP-9 gelatinolytic activity in a dose-dependent manner (Figure 4D). This suggests that the ERK activity contributes to the upregulation of the MMP-9 gene in arterial smooth muscle cells. U0126 had no effect on activation of c-Jun, but the phosphorylation of p38 MAPK was slightly decreased to the same extent with all concentrations of the inhibitor (Figure 4B and 4C).

Additive Role of p38 MAPK and ERKs on MMP-9 Expression

Because inhibition of either ERK or p38 MAPK activity downregulated the TNF-α-induced MMP-9 gene expression, we were interested in examining whether ERK and p38 MAPK cooperate in the regulation of MMP-9. Thus, cells were pretreated with suboptimal inhibitory concentrations of SB203580 and U0126, and the gelatinolytic activity of MMP-9 in response to TNF-α was measured. SB203580 (5 μmol/L) and U0126 (2.5 μmol/L) individually partially decreased the MMP-9 gelatinolytic activity, but together these inhibitors almost completely blocked MMP-9 (Figure 5).

Discussion

Previous studies have shown that MMPs, specifically MMP-2 and MMP-9, are important for smooth muscle cell migration in balloon catheter arterial injury lesions.3,20 However, the factors and signaling mechanisms involved in the expression of MMPs in vascular smooth muscle cells are poorly understood. In the present study, we demonstrated that TNF-α induces the expression of MMP-9 in vascular smooth muscle cells and that this induction is regulated by activation of the TNF-α and PDGF individually activate p38 MAPK and ERKs and induce MMP-9 in vascular smooth muscle cells (Figure 1). Therefore, we asked whether MMP-9 expression in smooth muscle cells stimulated with TNF-α and PDGF together could still be blocked by these inhibitors of the p38 MAPK and ERK pathways. The addition of TNF-α and PDGF together induced MMP-9 gelatinolytic activity to a higher level than did treatment with TNF-α or PDGF alone (Figure 6A). This induction of MMP-9 by TNF-α and PDGF together was only slightly inhibited by SB203580 or U0126 alone but was almost completely blocked when both inhibitors were added together (Figure 6B). Together, these data suggest that p38 MAPK and ERKs are the most important signaling pathways and that both are required for the synergistic induction of MMP-9 by cytokines and growth factors.
Individually the ERK and the p38 MAPK pathways have been shown to regulate MMP-9 expression. For example, Gum et al.\textsuperscript{11} showed that overexpression of dominant-negative MEK1 inhibits the MMP-9 expression in PMA-treated carcinoma cells. In a separate study, Simon et al.\textsuperscript{22} showed that p38 MAPK is involved in PMA-induced MMP-9 secretion with use of the SB203580 compound. However, none of these studies addressed the potential role of ERK and p38 MAPK acting together in the regulation of MMP-9 gene expression. Our data show that complete inhibition of either p38 MAPK or ERK alone or partial inhibition of both pathways is able to totally downregulate MMP-9 expression. This means that when there is a complete inhibition of one pathway, activation of the other pathway is not sufficient to induce MMP-9 gene. Presumably, therefore, each pathway might contribute to different transcription factors necessary for activation of the MMP-9 promoter. If correct, this would explain why the absence of signal from either pathway would result in the complete inhibition of MMP-9. Thus, we believe that both ERK and p38 MAPK are necessary for MMP-9 expression. One caveat with this conclusion is the finding that anisomycin, a potent activator of p38 MAPK and JNK, does not activate ERK and yet strongly induces MMP-9 expression. This might suggest that activation of p38 MAPK to superphysiological levels can by itself stimulate MMP-9 expression; however, ERK as well as p38 MAPK signals are required when a physiological activator such as TNF-\(\alpha\) is used.

It has been widely accepted that SB203580 is a highly selective inhibitor of p38 MAPK.\textsuperscript{18,19} A recent study by Clerk and Sugden\textsuperscript{23} demonstrated that SB203580 is able to inhibit JNK2-related isoforms in cardiac myocytes with 50% inhibition at concentrations of 3 to 10 \(\mu\)mol/L and thus raises a question concerning the specificity of this compound. However, these authors observed that even at 30 \(\mu\)mol/L SB203580, JNK activity was not completely inhibited (\(\approx 70\%\) inhibition), whereas the MAPKAPK-2 activity was completely blocked at a concentration of 10 \(\mu\)mol/L, similar to previous observations by others.\textsuperscript{18,19} This shows that SB203580 is a more effective inhibitor of p38 MAPK than of JNK2. In the present study, SB203580 had no effect on JNK activity induced by TNF-\(\alpha\) or anisomycin, as demonstrated by its phosphorylation level and the activation of c-Jun (specific target of JNK). Furthermore, a similar range of SB203580 concentrations (\(\approx 10 \mu\)mol/L) completely inhibited MMP-9 expression and p38 MAPK activity. Thus, p38 MAPK activity, but not JNK activity, correlates with the regulation of MMP-9 gene expression.

U0126, an inhibitor of the ERK pathway, has been reported to be a potent and specific inhibitor of MEK1 (an upstream kinase of ERK 1/2); however, Favata et al.\textsuperscript{24} reported a 50% inhibition of p38 MAPK at 20 \(\mu\)mol/L U0126 in PMA-treated COS cells. We found U0126 to be a very potent inhibitor of the ERK pathway in arterial smooth muscle cells, as demonstrated by the complete inhibition of ERK phosphorylation; however, we did note a small inhibitory effect (\(\approx 40\%\)) on TNF-\(\alpha\)-induced p38 MAPK activity. We do not think this finding significant because U0126 completely inhibits ERK activity and MMP-9 expression when p38 MAPK activity is still significantly.

Conclude that ERK and p38 MAPK are both necessary for MMP-9 expression.

Our data clearly show that TNF-\(\alpha\) is a potent regulator of MMP-9 in arterial smooth muscle cells. This finding is in accord with previous studies in which TNF-\(\alpha\) increased the MMP-9 production in human saphenous vein smooth muscle cells and human microvascular endothelial cells.\textsuperscript{25,26} We also show that TNF-\(\alpha\) and PDGF in combination can synergistically upregulate MMP-9 in vascular smooth muscle cells, and a similar finding was observed in skin fibroblasts.\textsuperscript{27} This observation has an important implication to injured arteries, where these factors are likely to be present after balloon catheter injury. For instance, platelets adhering to the denuded subendothelial layer of the vessel wall immediately after injury would be a major source of PDGF release in injured arteries, and TNF-\(\alpha\) is expressed in rat arteries within 6 hours of injury and activates smooth muscle cell migration in vitro.\textsuperscript{28,29} Furthermore, several signaling kinases, including the p38 MAPK and ERKs, are activated early after injury before the onset of MMP-9 expression in rat arteries.\textsuperscript{30} Thus, these cytokines and growth factors, released by balloon catheter injury, may control MMP-9 gene expression and smooth muscle cell migration by regulating the activation of p38 MAPK and ERKs.

It is not clear how the activation of p38 MAPK and ERK pathways results in the induction of MMPs. Several studies have shown that activator protein-1, nuclear factor (NF)-\(\kappa\)B, stimulatory protein-1, Ets, and retinoblastoma binding elements are involved in the regulation of the human MMP-9 gene.\textsuperscript{21,31} We found that TNF-\(\alpha\) increases the transactivation activity of NF-\(\kappa\)B, which is inhibitable by SB203580, thus suggesting that NF-\(\kappa\)B activity may play a role in the p38 MAPK-mediated regulation of MMP-9 (data not shown). Possibly, NF-\(\kappa\)B and activator protein-1, regulated by p38 MAPK and ERK, respectively, may cooperate on the activation of MMP-9 gene. Further studies will be required to identify the full complement of the transcription factors that are involved in the p38 MAPK-mediated and ERK-mediated control of the MMP-9 gene in vascular smooth muscle cells.

In summary, we showed that PDGF and TNF-\(\alpha\) synergistically upregulate MMP-9 expression and that activation of p38 MAPK and ERK pathways is necessary in the TNF-\(\alpha\)-induced regulation of MMP-9 in arterial smooth muscle cells.

Acknowledgments

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References


Figure I. 92-kDa gelatinase (MMP-9) mRNA level of arterial smooth muscle cells treated with TNFα, TGFβ1, PDGF, and bFGF for 24 hours. RNA was extracted from cells and RNA blot was probed with a cDNA for rat 92-kDa gelatinase. The same RNA blot was stained with methylene blue for 28S ribosomal RNA to verify for even loading of RNA.

Figure II. MMP-9 protein level in conditioned media of arterial smooth muscle cells pretreated with SB203580 (0.1, 0.3, 0.75, 1.25, 2.5, 5, 10 and 20 µM) or DMSO (0.1 %, 0 µM SB203580) for 45 minutes and then incubated with TNFα (100 ng/ml) or anisomycin (10 µg/ml) for 24 hours. The data is representative of three independent experiments.
Figure III. Phosphorylation level of JNK, ERKs, p90rsk and cJun and total ERKs of arterial smooth muscle cells pretreated with SB203580 (0.1, 0.3, 0.75, 1.25, 2.5, 5, 10 and 20 µM) or DMSO (0.1 %, 0 µM SB203580) for 45 minutes and then incubated with TNFα (100 ng/ml) for 10 minutes. Activated cJUN protein is indicated by arrow.
Figure IV. Phosphorylation level of JNK, ERKs, p90rsk and cJun and total ERKs of arterial smooth muscle cells pretreated with SB203580 (0.1, 0.3, 0.75, 1.25, 2.5, 5, 10 and 20 µM) or DMSO (0.1 %, 0 µM SB203580) for 45 minutes and then incubated with anisomycin (10 µg/ml) for 45 minutes. Activated cJUN protein is indicated by arrow.