**Oncostatin M, an Interleukin-6 Family Cytokine, Upregulates Matrix Metalloproteinase-9 Through the Mitogen-Activated Protein Kinase Kinase–Extracellular Signal–Regulated Kinase Pathway in Cultured Smooth Muscle Cells**

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**Objectives**—Matrix metalloproteinase (MMP)-9 is implicated in extracellular matrix (ECM) degradation of atherosclerotic lesions. Oncostatin M (OSM) regulates ECM metabolism in various kinds of cells. Thus, we sought to investigate whether OSM regulates MMP-9 expression in cultured rat aortic smooth muscle cells (SMCs) and, if so, to determine the signaling pathway for MMP-9 induction by OSM.

**Methods and Results**—Competitive reverse transcriptase polymerase chain reaction showed that OSM upregulated MMP-9 mRNA expression, peaking at 4 hours and returning to unstimulated levels by 24 hours. Gelatin zymography revealed that MMP-9 activity was increased in the conditioned medium after the 24-hour OSM treatment. Immunoblot analysis demonstrated that OSM transiently induced extracellular signal–regulated kinase (ERK)1/2 and STAT3 phosphorylations with a peak at 15 and 5 minutes, respectively. A MEK1 inhibitor, PD98059, not only blocked ERK1/2 phosphorylation but also abolished the OSM-induced MMP-9 upregulation, whereas the MMP-9 induction was not affected by overexpressing dominant-negative STAT3. In addition, OSM slightly upregulated MMP-2 and downregulated tissue inhibitors of MMP-1 and -3 through different mechanisms from that in case of MMP-9.

**Conclusions**—OSM upregulates MMP-9 expression in SMCs through the MEK-ERK but not STAT3 pathway. (Arterioscler Thromb Vasc Biol. 2003;23:1111–1116.)

**Key Words:** oncostatin M ■ matrix metalloproteinase ■ muscle ■ smooth ■ signal transduction

Oncostatin M (OSM) is a member of the interleukin (IL)-6 superfamily cytokines and is produced by activated macrophages and T lymphocytes.1 The IL-6 superfamily cytokines share a common signal transducer receptor component, glycoprotein 130 (gp130), and induce biological actions through gp130 by activating bifurcating signaling pathways, that is, mitogen-activated protein kinase kinase–extracellular signal–regulated kinase (MEK-ERK) pathway and JAK-STAT pathway.2 Although OSM was originally recognized as a proliferation inhibitor of tumor cells, OSM has a wide variety of actions against many types of cells. Several lines of evidence indicate that endothelial cells (ECs) are one of the primary targets of OSM: OSM not only stimulates EC proliferation and migration but also upregulates expressions of IL-6, P-selectin, and plasminogen activator.3–6 In addition, it has been shown that OSM acts on cultured human smooth muscle cells (SMCs) and promotes the proinflammatory and prothrombotic processes by inducing expressions of IL-6, cyclooxygenase-2, and tissue factor.7,8 Because activated macrophages and T lymphocytes are found in the atherosclerotic lesions,9 it is conceivable that OSM is produced by these activated inflammatory cells in the vascular lesions. It was noteworthy that immunoreactive OSM was detected in macrophages infiltrating human aortic aneurysm.5 These observations raised the possibility that OSM is secreted by macrophages within the atherosclerotic lesions and in turn contributes to development of atherosclerosis.

Extracellular matrix (ECM) degradation is an extremely important biological process with profound clinical implications. Initial degradation of the ECM is an inevitable step for vascular cells to hypertrophy, proliferate, and migrate. Vascular cells, including SMCs, secrete matrix metalloproteinases (MMPs), enzymes that selectively digest the individual components of the ECM. Among MMPs, MMP-2 and MMP-9 regulate SMC migration and proliferation by acting especially on basement membrane components that modulate the cell-to-cell communication with activated surrounding cells.10 It has been shown that MMP-2 is constitutively expressed in SMCs in normal artery and that in addition to increased MMP-2 expression, MMP-9 expression is induced in SMCs and macrophages in atherosclerotic artery, espe-
cially atheroma prone to rupture.\textsuperscript{11,12} Also, we have documented elevation of plasma MMP-9 in patients with acute coronary syndromes but not with stable effort angina.\textsuperscript{13} These findings indicate that these MMPs play an important role in formation and destabilization of atherosclerotic lesions.

There is increasing evidence that OSM regulates the ECM metabolism by inducing MMPs and tissue inhibitors of metalloproteinases (TIMPs) in various types of cells other than SMCs.\textsuperscript{14,15} However, there is no available information regarding the effects of OSM on MMP expression in SMCs. Accordingly, we sought to examine whether OSM affects MMP-9 expression in cultured SMCs from rats and, if so, to determine the signaling pathway responsible for the MMP-9 expression.

**Methods**

**SMC Culture and Cell Preparation**

Primary cultures of SMCs were obtained from the aortic media of male Sprague–Dawley rats by using an enzymatic dissociation method,\textsuperscript{16} and the cells were passaged in DMEM (Sigma) containing 10% fetal bovine serum (Dainippon Seiyaku). SMCs from passages 7 to 15 were seeded on 100-mm dishes, fed every other day, and used when just before confluency. For all experiments, SMCs were growth-arrested in serum-free DMEM for 48 hours. Mouse OSM (R&D Systems) or the vehicle (0.1% bovine serum albumin, control) was applied to SMCs (37 °C). After incubation of denoted periods, the reaction was terminated by aspirating the medium and by washing three times with ice-cold PBS on ice. In some experiments, 10 μmol/L PD98059 (Calbiochem), a MEK-1 inhibitor, was administered to SMCs 30 minutes before the OSM treatment.

**Competitive Reverse Transc-ta-ase Polymerase Chain Reaction (RT-PCR) for MMP-9**

For quantitative estimate of rat MMP-9 mRNA, competitive RT-PCR was performed using Abe et al’s method\textsuperscript{17} with minor modifications. The nucleotide sequences of primer pairs for MMP-9 and GAPDH\textsuperscript{18} were as follows:

- **MMP-9**: 5' -TGTCATCCAGTTTGGTGT-3' 3'-primer 5' -TAGGGCAGAAGCCATACAGT-3'
- **GAPDH**: 5' -TTCTTGTGCAGTGCCAGCCTCGTC-3' 3'-primer 5' -TAGGAACACGGAAGGCCATGCCAG-3'

Nonhomologous DNA fragment containing primer template sequences as internal control (PCR MIMIC cDNA) was created according to the manufacturer’s instructions (PCR MIMIC construction kit, Clontech). Two-fold dilutions of each PCR MIMIC cDNA between 10\textsuperscript{-2} and 10\textsuperscript{-5} attomol were added to the PCR amplification reaction mixture (Taq DNA polymerase core kit, Qiagen) containing 10 pg of sample cDNA. Both MMP-9 and GAPDH expressions were analyzed by 30 cycles of amplification (denaturation at 94°C for 45 seconds, primer annealing at 50°C for 45 seconds, and primer extension at 72°C for 45 seconds). A constant amount of each PCR product was electrophoresed on a 4% polyacrylamide gel stained with SYBR Gold (Molecular Probes Inc), and the densitometric values of the target and internal control were analyzed using a digital image analyzer. There was a good correlation between the amount of internal control and the target/internal control ratio in each experiment (Figure 1A). The amount of target gene was determined as amount of the internal control at the point where the densitometric value of the internal control was equal to that of target. The relative amount of MMP-9 cDNA was corrected by the amount of GAPDH cDNA. Additionally, the relative changes of MMP-9 amount were finally expressed as percent changes from the value of the vehicle-treated control cells.

**Real-Time RT-PCR for Other MMPs and TIMPs**

To screening mRNA expressions of other MMPs and TIMPs, real-time TaqMan RT-PCR was performed with the relative standard curve method.\textsuperscript{19} An aliquot (25 ng) of total RNA was reverse-transcribed and amplified in triplicate with TaqMan EZ RT-PCR kit (PE Biosynthesis). The primer nucleotide sequences for PCR primers and TaqMan probes for rat MMP-2, TIMP-1, and TIMP-3 were as follows:

- **MMP-2**: 5' -primer 5' -GGACAGTGACACCCAGCGA-3'
Gelatin Zymography
Growth-arrested SMCs were incubated in the serum-free DMEM containing 30 ng/mL OSM or the vehicle for 24 hours (37°C). Gelatinase activity in conditioned medium was measured by zymography. An equal amount of the conditioned medium (25 μL) was subjected to electrophoresis containing 0.1% gelatin, and the gel was incubated in the reaction solution at 37°C for 72 hours (37°C) to evaluate activities of MMP-9 and MMP-2, respectively, using a Gelatin Zymo-Electrophoresis kit (Yagai, Yamagata, Japan). The gel was stained with Coomasie blue. For quantitative analysis of MMP-9 activity, the resultant bands were scanned and analyzed with a digital image analyzer. In each experiment, standard sample solution containing 1.5 x 10^5 U/lane proMMP-9 was also run on the gel. The band densities of proMMP-9 and activated MMP-9 in each sample were normalized by the density of the proMMP-9 standard band. The relative changes in proMMP-9 and activated MMP-9 were expressed as the arbitrary unit.

Immunoblotting
After cell homogenization and protein extraction, equal amount of protein sample was separated by 10% SDS-PAGE, and then subjected to immunoblotting with phospho-specific antibody against ERK1/2 or STAT3 (New England Laboratories). The signals were quantified using a chemiluminescence detection system (Amersham Pharmacia Biotech). The membrane was then reprobed with anti-ERK1/2 antibody or anti-STAT3 antibody (Santa Cruz). The signal levels of phosphorylated ERK1/2 and phosphorylated STAT3 were normalized by the levels of total ERK1/2 and total STAT3, respectively. The relative changes in ERK1/2 or STAT3 phosphorylation were expressed as percent changes relative to the vehicle-treated control cells.

Overexpression of Dominant-Negative STAT3
A dominant negative form of STAT3 (dnSTAT3) was generated by converting Tyr to Phe and was proven to have dominant-negative activity of STAT3, as described elsewhere. Adenoviral vector encoding dnSTAT3 (AxdnSTAT3) or β-galactosidase (AxLacZ) was constructed, purified, and concentrated as described previously. Just before confluency, SMCs were washed with PBS three times and incubated in PBS with AxdnSTAT3 or AxLacZ for 2 hours at room temperature under gentle agitation. After three washes with PBS, the cells were cultured in serum-free DMEM for 48 hours (37°C) and then used for experiments. Infection efficiency of adenovirus (30 moi) was almost 100% as determined by the staining of the β-galactosidase expressed by AxLacZ (data not shown). Viability of SMCs transfected with AxdnSTAT3 (up to 30 moi) was more than 95% as determined by the trypan blue exclusion test.

Statistical Analysis
Unless otherwise indicated, statistical analysis was performed using ANOVA followed by Scheffe’s F test. In Figure 2, unpaired t test was performed for comparison between control and OSM-treated groups. A P<0.05 was considered significant.

Results
MMP-9 Expression and Activation
Competitive RT-PCR demonstrated that OSM induced a transient upregulation of MMP-9 mRNA (Figure 1B). MMP-9 expression levels was elevated by OSM (30 ng/mL)
after 2 hours and peaked at 4 hours, returning to the unstimulated levels by 24 hours. The OSM-induced MMP-9 upregulation was dose dependent when cells were stimulated by OSM for 4 hours, and the minimal dose required to induce the maximal effect was 30 ng/mL (Figure 1C).

Gelatin zymography showed an evident 82-kDa band for active form of MMP-9 and a faint 92-kDa band for proenzyme form of MMP-9 (ProMMP-9) in the conditioned medium obtained from control SMCs (Figure 2A). The major 82-kDa band for active MMP-9 and the minor 92-kDa band for proMMP-9 were increased by 2.0- and 1.7-fold versus control SMCs, respectively, after the 24-hour treatment with 30 ng/mL OSM (Figure 2B). Activated MMP-9 accounted for 81±7% and 89±6% of total (proMMP-9+activated MMP-9) in controls and OSM-stimulated cells, respectively, and there were no significant difference in the two groups.

**Activation of ERK and STAT Pathways**

Phosphorylation levels of ERK1/2 and STAT3 were examined in the OSM-treated SMCs because OSM can activate the signaling pathways mediated by MEK-ERK1/2 and/or STATs, especially STAT3.2,23 The level of ERK1/2 phosphorylation was low in control SMCs (Figure 3A). OSM (30 ng/mL) induced a transient ERK1/2 phosphorylation after 5 minutes, and the ERK1/2 phosphorylation was peaked at 15 to 30 minutes, returning to the control levels by 60 minutes.

The OSM-induced ERK1/2 phosphorylations were dose dependent, and the minimal dose required to induce the maximal effect at 15 minutes was 30 ng/mL (Figure 3B). Also, OSM induced a transient STAT3 phosphorylation in SMCs after 2 minutes with a peak at 5 to 15 minutes, declining to insignificant levels by 60 minutes (Figure 4A). The STAT3 phosphorylation was dose dependent of OSM, and the minimum dose required to induce the maximal effect at 15 minutes was 30 ng/mL (Figure 4B).

**Signaling Pathway Responsible For MMP-9 Induction**

To investigate the signaling pathway responsible for the OSM-induced MMP-9 induction, a MEK1 inhibitor PD98059 and adenovirus-mediated overexpression of dnSTAT3 were used for the selective inhibition of ERK1/2 and STAT3 activation, respectively. Neither PD98059 or dnSTAT3 had effect on the basal ERK1/2 phosphorylation levels (Figure 5A). The OSM-induced ERK1/2 phosphorylation was almost abolished by the pretreatment with 10 μmol/L PD98059, whereas dnSTAT3 overexpression (30 moi) had no effect on the OSM-induced ERK1/2 phosphorylation (Figure 5A). In contrast, the OSM-induced STAT3 phosphorylation was not affected by PD98059, although it was abolished by dnSTAT3 overexpression (Figure 5B).

Figure 5C demonstrates the effects of ERK or STAT3 inhibition on MMP-9 mRNA expression. The baseline
MMP-9 expression was not affected by PD98059 or Ax-dnSTAT3 treatment. The OSM-induced MMP-9 upregulation was almost abolished by PD98059 but was not affected by dnSTAT3.

**Effects of OSM on other MMPs and TIMPs**

Currently, in addition to MMP-9, cDNAs for rat MMP-2, -3, -7, -10 and rat TIMP-1, -2, and -3 have been cloned and the nucleotide sequences available on GeneBank database. However, after vigorous efforts, we obtained specific RT-PCR bands only for MMP-2, TIMP-1, and TIMP-3 and not for other MMPs and TIMP-2. OSM increased MMP-2 mRNA by 2-fold, but the increase was not as much as that of MMP-9 (Figure 6A). Also, gelatin zymography demonstrated that gelatinase activity of MMP-2 was not significantly altered by OSM (Figure 6B). The OSM-induced MMP-2 upregulation was partially inhibited by both PD98059 and dnSTAT3 (Figure 6A). However, OSM downregulated TIMP-1 and TIMP-3 mRNAs, whereas either PD98059 or dnSTAT3 did not affect the expression levels of the TIMPs (Figure 6C).

**Discussion**

In the present study, we demonstrated that in cultured rat SMCs, OSM, a member of IL-6 superfamily cytokines, promoted MMP-9 expression and secretion of activated form of MMP-9. OSM induced time- and dose-dependent phosphorylations of both ERK1/2 and STAT3. However, the OSM-induced MMP-9 upregulation was abolished by a MEK-1 inhibitor, PD98059, but not by dnSTAT3, indicating that activation of the MEK-ERK pathway is required to OSM-induced MMP-9 expression.
most of secreted MMP-9 is active in the control state of cultured SMCs and that the induction of MMP-9 mRNA expression was associated with increased densities of the 82-kDa band and 92-kDa band, on gelatin zymography.

Figure 5. A, Effects of pretreatment with a specific MEK-1 inhibitor PD98059 (PD) or overexpression of dnSTAT3 on ERK1/2 phosphorylation levels. Top, representative immunoblots for phosphorylated (pERK1/2) and total ERK1/2. Bottom, relative changes in the ERK1/2 phosphorylation levels are presented as the percent changes relative to the vehicle-treated control (control). OSM, the 15-minute treatment with 30 ng/mL OSM. B, Effects of pretreatment with PD98059 or dnSTAT3 overexpression on STAT3 phosphorylation levels. Top, representative immunoblots for phosphorylated (pSTAT3) and total STAT3. Bottom, relative changes in STAT3 phosphorylation levels. OSM, the 15-minute treatment with 30 ng/mL OSM. C, Effects of pretreatment with PD98059 or dnSTAT3 on MMP-9 mRNA expression. Relative expression changes are presented as the percent changes in MMP-9 expression/GAPDH expression relative to control cells. OSM, the 4-hour treatment with 30 ng/mL OSM. Bar = 1 SD (n=4). *P < 0.01 vs control.

Figure 6. A, Effects of OSM on MMP-2 mRNA expression (a) and gelatinase activity (b). B, Effects of OSM on TIMP-1 (a) and TIMP-3 (b) mRNA expressions. Quantitative analysis of mRNA expression was performed by using real-time TaqMan RT-PCR analysis. Relative expression changes are presented as the percent changes in target gene expression/GAPDH expression relative to control cells. OSM, the 4-hour treatment with 30 ng/mL OSM; PD, pretreatment with PD98059; dnSTAT3, pretreatment with dnSTAT3. Bar = 1 SD (n=4). *P < 0.01 vs control cells. #P < 0.01 vs OSM-treated cells without pretreatment. Gelatinase activity was evaluated on the basis of gelatin zymography as described in Figure 2, except that the gel was incubated in the reaction solution for 24 hours (37°C). Standard, standard samples for MMP-2, ProMMP-2, and ProMMP-9.
without changing the ratio of activated MMP-9 to proMMP-9 (Figure 2), suggesting that increased MMP-9 activity in OSM-treated SMCs is mainly attributable to upregulated MMP-9 gene transcription. Accordingly, it is likely that the OSM-induced MMP-9 mRNA upregulation has physiological and/or pathophysiological significance in the ECM metabolism in the vascular wall.

Signaling Pathway Responsible for MMP-9 Induction
In various kinds of cells, OSM acts through an OSM receptor coupled to a single gp130 subunit, which potentially activates two bifurcating signal transduction pathways, that is, MEK-ERK pathway and JAK-STAT pathway. As expected, OSM induced transient phosphorylations of ERK1/2 and STAT3 in rat cultured SMCs (Figures 3 and 4), indicating that both the signaling pathways are activated by OSM. Furthermore, PD98059 not only blocked the ERK1/2 phosphorylation but also abolished the MMP-9 upregulation in OSM-treated SMCs (Figure 5). In contrast, dnSTAT3 overexpression had no effect on the OSM-induced MMP-9 induction. These findings indicate that the MEK-dependent ERK activation plays a pivotal role in promoting MMP-9 expression in OSM-treated SMCs. In the present study, the peak phosphorylation of STAT3 occurred preceding that of ERK1/2. Because the crosstalk between the ERK- and STAT-mediated pathways is documented in various kinds of cells, it is possible that ERK phosphorylation is the downstream event of STAT3 activation. However, it is not the case because dnSTAT3 had no effect on the OSM-induced ERK1/2 phosphorylation (Figure 5A).

Possible Role of OSM in Atherosclerosis
In cultured SMCs, recent studies have shown that OSM promotes IL-6 and cyclooxygenase-2 expressions in synergy with IL-1β and also upregulates the expression of tissue factor. Furthermore, OSM induces expression of plasminogen activator, IL-6, and P-selectin in ECs. Our present study provided new evidence that in cultured SMCs, OSM promotes MMP-9 mRNA expression and secretion of active MMP-9. Taken together, it is possible that OSM can contribute to the development of atherosclerotic vascular remodeling at multiple steps: the initial activation of inflammatory cytokine network, inflammatory cell recruitment to plaque, plaque destabilization, and finally plaque rupture associated with clot formation. More detailed studies are needed to determine the significance of OSM in the atherosclerotic process.

Limitation
In the present study, we focused on MMP-9 because MMP-9 is an inducible form of the gelatinases, which are implicated in vascular lesion formation and destabilization.

References


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Arterioscler Thromb Vasc Biol. published online February 13, 2003;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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