Oncostatin M Stimulates the Growth of Dermal Fibroblasts Via a Mitogen-Activated Protein Kinase-Dependent Pathway

Hironobu Ihn and Kunihiko Tamaki

*J Immunol* 2000; 165:2149-2155; doi: 10.4049/jimmunol.165.4.2149

http://www.jimmunol.org/content/165/4/2149

References

This article cites 48 articles, 27 of which you can access for free at:
http://www.jimmunol.org/content/165/4/2149.full#ref-list-1

Subscription

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Oncostatin M Stimulates the Growth of Dermal Fibroblasts Via a Mitogen-Activated Protein Kinase-Dependent Pathway

Hironobu Ihn$^2$ and Kunihiko Tamaki

Oncostatin M (OSM), a member of the hemopoietic cytokine family, has been implicated in the process of fibrosis and dermal wound healing. As a part of an ongoing study of the mechanisms of fibrosis and dermal wound healing, we have investigated the mechanism of the growth regulation of dermal fibroblasts by OSM. OSM stimulates the mitogenesis of dermal fibroblasts in a dose-dependent manner. This effect was completely blocked by anti-OSM IgG, but not by anti-IL-6 IgG. Furthermore, OSM induction was abolished by genistein, a tyrosine kinase inhibitor, or by PD98059, a specific mitogen-activated protein (MAP) kinase pathway inhibitor, but not by calphostin C, a protein kinase C inhibitor. Immunoblotting analysis using a specific Ab against phosphorylated MAP kinase (Thr$^{202}$/Tyr$^{204}$) showed that OSM induces phosphorylation of MAP kinase in dermal fibroblasts. Furthermore, transient transfection of the dominant-negative mutant MAP kinase into dermal fibroblasts abolished the OSM induction. These results strongly suggest that OSM stimulates the growth of dermal fibroblasts via a MAP kinase-dependent pathway. The Journal of Immunology, 2000, 165: 2149–2155.

Dermal wound healing is a complex biological process involving an acute inflammatory response. Transient activation of dermal fibroblasts to proliferate and produce elevated quantities of extracellular matrix is also essential to normal fibrotic repair. Transient fibroblast activation is likely regulated by a variety of cytokines produced by infiltrating platelets, monocytes, T lymphocytes, and other inflammation-associated cells (1). Numerous in vitro and in vivo studies have suggested that some cytokines such as TGF-β, platelet-derived growth factor, epidermal growth factor, IL-1α and -β, TNF-α and -β, IL-4, and IL-6 regulate dermal fibroblast proliferation and extracellular matrix deposition (2–9). Dermal fibroblasts derived from patients with systemic sclerosis or keloid appear to be persistently activated and produce elevated levels of extracellular matrix components in tissue culture. It is postulated that this persistent fibroblast activation occurs as a result of chronic exposure to various cytokines, such as TGF-β and platelet-derived growth factor (10–12).

Oncostatin M (OSM)$^3$ is a member of the hemopoietic cytokine family and produced by activated T cells and monocytes (13, 14). OSM belongs to a subfamily of hemopoietic cytokines that also includes IL-6, IL-11, leukemia inhibitory factor (LIF), and ciliary neurotrophic factor. Members of this family bind receptor complexes containing a signal transducing subunit, gp130 (15, 16). OSM utilizes a dual receptor system (17). A heterodimeric receptor complex consisting of gp130 and OSM receptor β is activated by OSM only. As a consequence, some biological effects are shared by OSM and LIF, whereas others are OSM-specific. OSM is known to be a growth regulator which stimulates the growth of fibroblasts (18), vascular smooth muscle cells (19), and Kaposi’s sarcoma cells (20).

In fibroblasts, OSM stimulates the production of extracellular matrix components such as collagen and glycosaminoglycan production (21, 22). OSM has been reported to stimulate the synthesis of tissue inhibitor of metalloproteinases 1 and 3 and plasminogen activator (23, 24). Recent studies with transgenic mice overexpressing OSM in a tissue-specific manner demonstrated its association with visceral fibrosis (25). Furthermore, OSM has been reported to be a mitogen for murine NIH 3T3 cells and human foreskin and synovial fibroblasts (26). These results suggest that OSM plays some role in extracellular matrix deposition.

In this study, we investigated the mechanism of the growth regulation of dermal fibroblasts by OSM. The results suggest that OSM stimulates the growth of dermal fibroblasts via a mitogen-activated protein (MAP) kinase-dependent pathway.

Materials and Methods

Cytokines and other materials

Recombinant human OSM, polyclonal rabbit anti-human OSM, and polyclonal rabbit anti-human IL-6 were obtained from Genzyme Diagnostics (Cambridge, MA), and recombinant fibroblast growth factor-2 (FGF-2) was purchased from R&D Systems (Minneapolis, MN). Genistein, PD98059, and calphostin C, which were purchased from Calbiochem (La Jolla, CA), were dissolved in DMSO. Controls were incubated with an equal concentration of DMSO. The p44/42 MAP kinase and phospho-specific MAP kinase (Thr$^{202}$/Tyr$^{204}$) rabbit polyclonal Abs were obtained from New England Biolabs (Beverly, MA). Anti-phosphotyrosine Ab (4G10) was purchased from Upstate Biotechnology (Lake Placid, NY).

Fibroblast cultures

Human adult skin fibroblasts were grown from explants of forearm skin biopsies from six healthy donors, with institutional approval and informed consent. Cells were cultured in MEM supplemented with 10% FCS. Fibroblasts cultures independently isolated from different individuals were maintained as monolayers at 37°C in an atmosphere of 5% CO₂ and 95% air. Fibroblasts under the fifth subpassage were used for the experiments. Cell viabilities was determined by trypan blue stain.

Department of Dermatology, Faculty of Medicine, University of Tokyo, Tokyo, Japan.

Received for publication June 22, 1999. Accepted for publication May 30, 2000.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

$^1$This work was supported by a grant for scientific research from the Ministry of Science, Japan (10770391) and by the Lydia O’Learly Memorial Foundation, Japan.

$^2$Address correspondence to and reprint requests to Dr. Hironobu Ihn, Department of Dermatology, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113, Japan. E-mail address: IN-DER@h.u-tokyo.ac.jp

$^3$Abbreviations used in this paper: OSM, oncostatin M; MAP, mitogen-activated protein; FGF, fibroblast growth factor; MEK, MAP kinase kinase; ERK, extracellular signal-related kinase; JNK, c-Jun N-terminal kinase.

Copyright © 2000 by The American Association of Immunologists
DNA synthesis

The cells (2 × 10⁴/well) were plated in 24-well plates in MEM with 10% FCS and grown to confluency, then incubated for 24 h in MEM with 0.1% BSA. Next the cells were stimulated with cytokines for 24 h in the absence of serum and were labeled with [³H]thymidine (final concentration, 1 μCi/ml; New England Nuclear, Boston, MA) for 2 h. The cell layers were washed three times with cold PBS and five times with ice-cold 5% TCA and dissolved in 500 μl of 0.1 N NaOH/0.1% SDS. An aliquot of this extract was measured in a Beckman (Fullerton, CA) scintillation counter.

Transfections and constructs

Transient transfections were performed as described previously (27). Fibroblasts were transfected by the lipofection technique (FuGene 6 Transfection Reagent, Boehringer Mannheim, Indianapolis, IN) with various amounts of constructs. The plasmid used encodes the extracellular signal-related kinase 2 (ERK2) (p42 MAP kinase) cDNA in which Thr183 and Tyr185 that are required to be phosphorylated for activity were replaced with either glutamic acid or alanine and phenylalanine thus rendering the protein inactive (28, 29) (kindly provided by Dr. Dennis Templeton). pSV-β-galactosidase control vector (Promega, Madison, WI) was transfected to visualize for transfection efficiency. After incubation overnight, the medium was replaced with serum-free MEM and 0.1% BSA. The cells were stimulated with cytokines for 24 h in the absence of serum, and the DNA synthesis was determined as described above.

Immunoblotting

For the preparation of cell lysates from untreated and OSM-treated fibroblasts, cells were placed in MEM and 0.1% BSA for 24 h prior to OSM treatment. After incubation with 10 ng/ml OSM for 24 h, the medium was removed and the cells were washed with PBS. The cells were lysed by scraping into solubilization buffer (50 mM Tris/Cl (pH 8), 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 1% Nonident P-40, 1 mM sodium orthovanadate, 0.2 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin). The lystate was incubated at 4°C for 30 min and then centrifuged at 5 min at 4°C. Protein concentrations of lysates were determined using a Bio-Rad (Hercules, CA) Protein Assay, as recommended by the manufacturer. In some experiments, cell lysates were dephosphorylated with calf intestinal phosphatase (20 U/20 μl of protein) (CIP) (New England Biolabs) at room temperature for 30 min (30). CIP-treated cell lysates were subsequently used for immunoblotting. Immunoblotting was performed as described previously (31). Briefly, cell lysates (20 μg) obtained from fibroblasts were subjected to electrophoresis on 10–20% gradient SDS-polyacrylamide slab gels, and then electrotransferred from the gels on to nitrocellulose sheets. The nitrocellulose sheets were then incubated overnight with primary Abs. Bound Abs were detected with HRP-conjugated anti-rabbit IgG, and immunoreactive bands were visualized by ECL (Amersham, Arlington Heights, IL) as described previously (31).

Immunofluorescence

Immunofluorescence analysis was performed as described previously (32). In brief, dermal fibroblasts were cultured in 8-well tissue culture chamber slides. Cells were placed in MEM and 0.1% BSA for 24 h prior to OSM treatment. After incubation with or without 10 ng/ml OSM for 15 min, the medium was removed and the cells were washed with PBS and fixed in 3.7% formaldehyde/PBS for 10 min. After two washes with PBS, cells were incubated for 30 min in PBS containing 10% FBS. Cells were permeabilized with 0.5% Triton X-100. The Ab against p44/42 MAP kinases (9) was used at room temperature for 30 min. CIP-treated cell lysates were subsequently used for immunofluorescence. Immunofluorescence analysis revealed that the treatment of fibroblasts with 10 ng/ml OSM for 15 min enhanced phosphorylation of substrates with molecular masses of 42–44 kDa. Densitometric analysis showed that the phosphorylation of MAP kinases with molecular masses of 42–44 kDa (3-fold) significantly. The phosphorylated forms of MAP kinases were also determined with the Ab specific for the phosphorylated site (corresponding to Thr202/Tyr204) of p44 and p42 MAP kinases. Immunoblotting of whole cell extracts revealed that p44 and p42 ratio at each concentration. As shown in Fig. 1A, OSM stimulates DNA synthesis of dermal fibroblasts in a dose-dependent manner. The mitogenic response to OSM became maximum when the concentration of OSM was 10 ng/ml; 10 ng/ml OSM induced almost 100% increases in the DNA synthesis of dermal fibroblasts. Anti-OSM IgG (500 ng/ml) completely abolished the OSM-induced mitogenic response of dermal fibroblasts (Fig. 1B). This amount of anti-OSM IgG was shown to neutralize the biological activity of OSM (data not shown). However, anti-OSM IgG had little effect on the basal mitogenic activity. OSM is known to induce endogenous IL-6 synthesis in fibroblasts (33), and IL-6 stimulates mitogenesis of fibroblasts (9). Therefore, we asked whether OSM induced mitogenesis through IL-6 production or other mechanisms. As shown in Fig. 1B, anti-IL-6 IgG (5 μg/ml) completely abolished the IL-6-induced mitogenic response, but had little effect on either OSM-induced mitogenic activity or basal mitogenic activity.

The effect of tyrosine kinase inhibitor on OSM-induced dermal fibroblast DNA synthesis

It was reported that OSM stimulated tyrosine phosphorylation in various types of cells (34). Therefore, we investigated the effects of tyrosine kinase inhibitor on OSM-induced dermal fibroblast DNA synthesis. Dermal fibroblasts were treated with a tyrosine kinase inhibitor, genistein. Pretreatment of fibroblasts with genistein (30 μg/ml for 1 h) markedly decreased the OSM-stimulated [³H]thymidine incorporation (Fig. 2A). Furthermore, pretreatment of fibroblasts with PD98059, a specific MAP kinase pathway inhibitor (30 μM for 1 h), inhibited OSM-stimulated [³H]thymidine incorporation. On the other hand, pretreatment with calphostin C, a protein kinase C inhibitor (5 μM), had no effect on OSM-induced fibroblast proliferation (Fig. 2A). Furthermore, pretreatment of fibroblasts with genistein (Fig. 2B) or PD98059 (Fig. 2C) inhibited the OSM-stimulated [³H]thymidine incorporation in a dose-dependent manner. These results suggest that OSM stimulates the growth of dermal fibroblasts via a MAP kinase-dependent pathway.

OSM induces nuclear translocation of MAP kinases

The translocation of MAP kinases by OSM was investigated by immunofluorescence analysis using serum-deprived human dermal fibroblasts. As shown in Fig. 3A, MAP kinases were located in the cytoplasm of fibroblasts without stimulation. However, MAP kinases were translocated into the nucleus after the stimulation of fibroblasts by OSM (Fig. 3B). No staining was shown without the Ab against p42/p44 MAP kinases (Fig. 3C), which indicates the necessity of the immunofluorescent staining.

OSM induces tyrosine phosphorylation of MAP kinases in dermal fibroblasts

The phosphorylation of MAP kinases by OSM was investigated using serum-deprived human dermal fibroblasts. First, the phosphorylation of MAP kinases by OSM was determined using anti-phosphotyrosine Ab (Fig. 4A). Anti-phosphotyrosine Ab immunoblotting showed that the treatment of fibroblasts with 10 ng/ml OSM for 15 min enhanced phosphorylation of substrates with molecular masses of 42–44 kDa. Densitometric analysis showed that the phosphorylation of fibroblasts with OSM also enhanced phosphorylation of several substrates (1.2–1.5-fold), but enhanced phosphorylation of substrates with molecular masses of 42–44 kDa (3-fold) significantly. The phosphorylated forms of MAP kinases were also determined with the Ab specific for the phosphorylated site (corresponding to Thr202/Tyr204) of p44 and p42 MAP kinases. Immunoblotting of whole cell extracts revealed that p44 and p42

Statistical analysis

Statistical analysis was carried out with the Mann-Whitney U test for the comparison of means. A P value <0.05 was considered significant.

Results

OSM stimulates the DNA synthesis of dermal fibroblasts

To determine whether OSM stimulates DNA synthesis of normal dermal fibroblasts, we measured the incorporation of [³H]thymidine in fibroblast cultures exposed to 1–50 ng/ml of recombinant human OSM for 24 h. The mean [³H]thymidine incorporation in serum-free MEM for each dermal fibroblast strain was arbitrarily set at 100%, and the effects of OSM were estimated as a percentage...
Thr²⁰²/Tyr²⁰⁴ phosphorylation occurs after acute treatment with 10 ng/ml OSM (Fig. 4B). OSM-induced phosphorylation of MAP kinases persisted for 3 h. Abs against MAP kinases were used to confirm that the protein concentrations of MAP kinases were maintained with or without OSM stimulation (Fig. 4B). Densitometric analysis showed that the treatment of fibroblasts with OSM enhanced phosphorylation of MAP kinases 10-fold for 15 min, 3-fold for 30 min, 2.5-fold for 1 h, and 1.5-fold for 3 h, respectively. Dephosphorylated whole cell lysates were reacted with Abs against MAP kinases (Fig. 4C, lane 1), which demonstrated that detectable bands reacted with the Ab specific for phosphorylated p42/p44 MAP kinases (Fig. 4C, lane 2) represent phosphorylated p42/p44 MAP kinases.

The expression of the dominant-negative mutant MAP kinase represses the OSM-induced dermal fibroblast DNA synthesis

To further confirm the role of MAP kinases in the growth regulation of fibroblasts by OSM, transient transfection of the dominant-negative mutant MAP kinase into dermal fibroblasts was performed. As shown in Fig. 5, transient transfection of the dominant-negative mutant MAP kinase in fibroblasts decreased basal mitogenic activity of fibroblasts by almost 50% in a dose-dependent manner. Cell viability was determined by trypan blue stain, which demonstrated that transient transfection of these amounts of dominant-negative mutant MAP kinase in fibroblasts did not have cytotoxic effect. Furthermore, transient transfection of the dominant-negative mutant MAP kinase in fibroblasts also abolished the mitogenic activity of fibroblasts induced by OSM. On the other hand, transient transfection of the dominant-negative mutant MAP kinase in fibroblasts did not abolish the mitogenic activity of fibroblasts induced by FGF-2, which was shown to induce fibroblasts proliferation without p42/p44 MAP kinases activation (35).

Discussion

OSM, a cytokine synthesized by activated human T lymphocytes and monocytes, was originally identified as a growth regulator for certain tumor- and non-tumor-derived cells (13). Although the physiological roles of this cytokine remain largely unknown, OSM has been shown to exert a variety of effects on different types of cells. Studies have shown that fibroblasts are target cells for OSM (23, 24, 36, 37) and that T-lymphocyte and monocyte/macrophage infiltration is observed in normal dermal repair as well as fibrotic lesions (38, 39). Therefore, OSM could play a role in dermal fibrotic repair or fibrosis.

This study first demonstrated that OSM stimulates the mitogenesis of human dermal fibroblasts. This result is consistent with a previous report which showed that OSM is a mitogen for murine NIH 3T3 cells and human foreskin and synovial fibroblasts (26). The mechanism of the growth regulation of fibroblasts by OSM has not been reported. OSM is known to induce endogenous IL-6 synthesis in dermal fibroblasts (33), and IL-6 stimulates mitogenesis of fibroblasts (9). Therefore, we have considered the possibility that OSM stimulates mitogenesis of dermal fibroblasts through...
endogenous IL-6 synthesis. However, our results are not consistent with this notion. The OSM-induced mitogenic response of dermal fibroblasts was completely abolished by anti-OSM IgG, but not anti-IL-6 IgG. This suggests that endogenous IL-6 synthesis is not directly involved in the OSM-induced mitogenic response of dermal fibroblasts.

**FIGURE 2.** The effect of tyrosine kinase inhibitor on OSM-induced dermal fibroblast DNA synthesis. A. The mean $[^{3}H]$thymidine incorporation (cpm) in serum-free medium for each dermal fibroblast strain (6270 cpm) was arbitrarily set at 100%, and the effects of OSM, genistein (30 μg/ml), PD98059 (30 μM), and calphostin C (5 μM) were estimated as a percentage ratio at each concentration. Pretreatment of fibroblasts with genistein (30 μg/ml for 1 h) markedly decreased the OSM-stimulated $[^{3}H]$thymidine incorporation. Furthermore, pretreatment of fibroblasts with PD98059 (30 μM for 1 h) inhibited OSM-stimulated $[^{3}H]$thymidine incorporation. On the other hand, pretreatment with calphostin C (5 μM) had no effect on the OSM-induced fibroblast proliferation. B. Pretreatment of fibroblasts with genistein (0.1–30 μg/ml) decreased the OSM-stimulated $[^{3}H]$thymidine incorporation in a dose-dependent manner. C. Pretreatment of fibroblasts with PD98059 (0.1–30 μM) decreased the OSM-stimulated $[^{3}H]$thymidine incorporation in a dose-dependent manner. The means ± SE for four separate experiments are shown. Comparisons of the $[^{3}H]$thymidine incorporation (cpm) in response to OSM were made between treated and untreated cells (*, $p < 0.01$).
OSM belongs to a family of cytokines that utilize the Janus kinase (JAK)-STAT signaling pathway (40) and MAP kinase pathway (34). Indeed, it has been reported recently that OSM activates STAT1 and STAT3 in human dermal fibroblasts (21, 41). The present study suggests that OSM stimulates the growth of dermal fibroblasts via a MAP kinase-dependent pathway.

MAP kinase modules are involved in the signal transduction of a wide variety of signals in all eukaryotic organisms. In mammalian cells, three well-characterized modules co-exist: p42/p44 MAP kinases, p38 MAP kinase, and JNK cascades (42). The p42/p44 MAP kinases cascade plays a pivotal role in the re-entry of fibroblasts into the cell cycle (43). Both p42 and p44 MAP kinases are activated by dual phosphorylation on threonine and tyrosine residues, achieved by the dual-specificity kinase MAP kinase kinases (MEK) 1/2. Whereas MEK1/2 remain permanently in the...
cytoplasm, p42/p44 MAP kinases are relocated from the cytoplasm to the nucleus upon stimulation (44). In fibroblasts, a correlation exists between the mitogenic potency of a stimulus and its ability to trigger p42/p44 MAP kinases translocation (44). In this study, immunofluorescent analysis revealed that MAP kinases were translocated into the nucleus after the stimulation of fibroblasts by OSM (Fig. 3). To our knowledge, this is the first evidence of OSM-mediated translocation of p42/p44 MAP kinases. Moreover, immunoblotting analyses using phosphospecific p42/p44 MAP kinase antibodies that detect only the Thr202/Tyr204-phosphorylated forms of ERK1/ERK2 demonstrated that OSM induces tyrosine phosphorylation of p44 and p42 MAP kinase in dermal fibroblasts and this effect persisted for 3 h (Fig. 4B).

We determined whether the ERK pathway was involved in the OSM-induced mitogenic response of dermal fibroblasts, using two independent approaches to block the ERK signaling pathway. First, we utilized an MEK1-specific inhibitor, PD98059, which blocks MEK1 activation by Raf, thus preventing downstream activation of p42/p44 MAP kinases, but does not inhibit JNK or p38 MAP kinase. In addition, the PD98059 has been shown to have little effect on other kinases, including cAMP-dependent kinase, protein kinase C (45, 46). In our study, pretreatment of fibroblasts with PD98059 inhibited the OSM-stimulated [3H]thymidine incorporation.

Therefore, transfection of the dominant-negative mutant MAP kinase (ERK2) into dermal fibroblasts abolished the OSM-induced mitogenic response of dermal fibroblasts.

The ERK pathway is the prototypical MAP kinase pathway induced by epidermal growth factor stimulation and implicated in the regulation of cellular proliferation (47, 48). Platelet-derived growth factor is also known to stimulate the mitogenesis of human dermal fibroblasts via a MAP kinase pathway (49). It will be of great interest to determine whether similar cytokine-induced MAP kinase-dependent signaling pathways operate in vivo to promote dermal fibroblast proliferation, and the present findings may potentially have important clinical implications in dermal wound healing and many fibrotic diseases.

Acknowledgments

We thank Dr. Dennis Templeton for providing dominant-negative mutant MAP kinase (ERK2) expression vectors.

References


terization of cytostatic lymphokines produced by activated human T lympho-
17. Mosley, B., C. De Imus, D. Friend, N. Boiani, B. Thoma, L. S. Park, and D. Costman. 1996. Dual oncostatin M (OSM) receptors: cloning and character-
ization of an alternative signaling subunit conferring OSM-specific receptor ac-


