Oncostatin M reduces the synthesis of macrophage-colony stimulating factor stimulated by TGF-β via suppression of p44/p42 MAP kinase and JNK in osteoblasts

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ABSTRACT
Bone fracture is an important trauma frequently encountered into emergency medicine as well as orthopedics reflecting an aging society. Oncostatin M, an inflammatory cytokine produced by osteal macrophages, has been considered to play a crucial role in fracture healing. Macrophage colony-stimulating factor (M-CSF) secreted from osteoblasts is essential in osteoclastogenesis, and the secretion is stimulated by transforming growth factor-β (TGF-β). The aim of this study is to elucidate the effects of oncostatin M on the TGF-β-induced M-CSF synthesis in osteoblast-like MC3T3-E1 cells and the underlying mechanisms. Oncostatin M attenuated the TGF-β-stimulated M-CSF release and the mRNA expressions. SMAD3 inhibitor SIS3, p38 MAP kinase inhibitor SB203580, and SAPK/JNK inhibitor SP600125 significantly suppressed the M-CSF release. Oncostatin M suppressed the TGF-β-induced phosphorylation of p44/p42 MAP kinase and SAPK/JNK, but failed to affect the phosphorylation of SMAD3 and p38 MAP kinase. Oncostatin M attenuated the TGF-β-stimulated vascular endothelial growth factor (VEGF) release and the TGF-β-induced mRNA expressions of VEGF. These results strongly suggest that oncostatin M downregulates TGF-β signaling upstream of p44/p42 MAP kinase and SAPK/JNK, but not SMAD 2/3 and p38 MAP kinase, in osteoblasts, leading to the attenuation of M-CSF synthesis. Our findings might provide a new therapeutic strategy for the acceleration of fracture healing process.

INTRODUCTION
Bone fracture is an important trauma frequently encountered into emergency medicine as well as orthopedics reflecting an aging society (Gioffre-Florio M et al. 2018). Usual bone healing enables early re-

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Abbreviations:
M-CSF, macrophage colony-stimulating factor; TGF-β, transforming growth factor-β; VEGF, vascular endothelial growth factor; RANK, receptor activator of nuclear factor-κB; RANKL, receptor activator of nuclear factor-κB ligand; MAP kinase, mitogen-activated protein kinase; SAPK/JNK, stress-activated protein kinase/c-Jun N-terminal kinase; ELISA, enzyme-linked immunosorbent assay; RT-PCR, reverse transcription-polymerase chain reaction; α-MEM, α-minimum essential medium; FBS, fetal bovine serum; GAPDH; Glyceraldehyde-3-phosphate dehydrogenase
habilitation and return to the society, however, delayed union and non-union of bone, the former means an impairment which shows slower healing rate than expected, and the latter means a severe impairment in which healing is no longer expected without additional treatment, have been reported in 5–10% of fracture patients especially in old persons (Gomez-Barrena E et al. 2015). It is important for the improvement of therapies to address the process of bone fracture healing adequately, which requires understanding the cellular and molecular mechanisms of bone fracture healing.

The process of bone fracture healing is composed of multiple steps: the recruitment of mesenchymal progenitor cells to the fracture site, differentiation into bone-forming osteoblasts to repair, and subsequent remodeling initiated by activated bone-resorbing osteoclasts (Alexander et al. 2011; Einhorn et al. 2015). Once fracture happens, hematoma formed at the injury site induces inflammatory cytokines such as interleukin-6 and tumor-necrosis factor-α leading to recruitment of inflammatory cells, which further potentiate the inflammation and recruit mesenchymal stem cell populations including osteoblast progenitor cells (Zuscik 2013). The intensive cross-talk is recognized between bone cells and immune cells such as lymphocytes and macrophages (Danks et al. 2013). Regarding the inhibition of bone healing under the reduction of macrophages (Toben et al. 2011), it is recognized that macrophages play a pivotal role in the osteogenesis in the process of fracture healing in addition to osteoblasts (Einhorn et al. 2015).

Oncostatin M is a cytokine belonging to the gp130-related cytokine superfamily produced by macrophages (Zarling et al. 1986). It has been reported that physiological stimuli such as prostaglandin E2 induces the synthesis of oncostatin M in macrophages, which is secreted via exocytosis (Repovic et al. 2002). On the other hand, the functional receptor of oncostatin M composed of gp130 and oncostatin M receptor-complex is recognized to express in a variety of cells including osteoblasts (Gómez-Lechón 1999; Walker et al. 2010; Guihard et al. 2015). The oncostatin M reportedly activates osteoblasts and inhibits bone resorption (Jay et al. 1996). It has recently been reported that macrophage-associated oncostatin M is overexpressed during the initial inflammatory phase of tibia injury model in mice, promoting osteoblastic bone formation (Guihard et al. 2015). However, the molecular mechanism whereby oncostatin M modulates osteoblast function remains to be clarified. On the other hand, the bone remodeling is a regenerative process consisting of bone resorption by osteoclasts and bone formation by osteoblasts (Kular et al. 2012). Osteoclasts derived from mononuclear precursors in the myeloid lineage of hematopoietic cells play a central role in bone resorption, but the process is crucially regulated by osteoblasts (Kular et al. 2012). Osteoblasts secrete macrophage colony-stimulating factor (M-CSF), a hematopoietic growth factor which induces the proliferation of osteoclast progenitor cells (Fixe et al. 1998). M-CSF further promotes differentiation of the progenitor cells into osteoclasts cooperatively with the receptor activator of nuclear factor-κB (NF-κB) RANK ligand (RANKL), which is expressed on the osteoblasts stimulated by a variety of bone resorbing stimuli such as hormones and cytokines to bind RANK on the surface of osteoclast precursors (Walsh et al. 2006; Amarasekara et al. 2018). The osteoblast-released M-CSF and the osteoblast-expressed RANKL coordinately regulates the formation of active osteoclast and bone resorption. Thus, it is now recognized that the fine communication between macrophages and osteoblasts is essential for the complete of bone healing to maintain the quality and quantity of bone tissue (Kim et al. 2020).

Transforming growth factor-β (TGF-β) is well known to promote osteogenesis of osteoblasts in an autocrine or paracrine fashion (Guo et al. 2009). During bone remodeling, TGF-β embedded in the bone matrix is released via bone resorption, and then promotes the proliferation and the differentiation of osteoblast progenitor cells into osteoblasts for bone formation, thereby combining the resorption and the formation of bone to maintain bone mass (Tang et al. 2009; Zuo et al. 2012). In the process of fracture healing, TGF-β is highly synthesized in the periosteum and upregulates the proliferation and differentiation of periosteal mesenchymal cells to induce extracellular matrix production and chemotactic to bone cells (Wang et al. 2017). Regarding the intracellular signaling, TGF-β acts classically through SMAD-dependent canonical pathways such as SMAD2 and SMAD3 (ten Dijke et al. 2004), whereas SMAD-independent non-canonical pathways such as mitogen-activated protein kinase (MAP) kinases are also recognized to be involved (Moustakas et al. 2005). We have previously reported that the synthesis of vascular endothelial growth factor (VEGF), a specific growth factor for vascular endothelial cells with a potent angiogenic effect (Ferrara 2004), is stimulated by TGF-β, and that the synthesis is exerted via p44/p42 MAP kinase, p38 MAP kinase, and stress-activated protein kinase/
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Reagents. Oncostatin M, TGF-β, the mouse M-CSF enzyme-linked immunosorbent assay (ELISA) kits and VEGF ELISA kits were purchased from R&D System, Inc. (Minneapolis, MN). SIS3 was obtained from EMD Millipore Corp. (Darmstadt, Germany). SB203580, PD98059 and SP600125 were obtained from Calbiochem-Novabiochem Co. (La Jolla, CA). Phospho-specific SMAD3 antibodies, phospho-specific p38 MAP kinase antibodies, p38 MAP kinase antibodies, phospho-specific p44/p42 MAP kinase antibodies, p44/p42 MAP kinase antibodies, phospho-specific SAPK/JNK antibodies and SAPK/JNK antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). GAPDH was purchased from Santa Cruz Biotechnology, Inc. (CA, USA). The ECL Western blot detection system was obtained from GE Healthcare Life Sciences (Buckinghamshire, UK). Other materials and chemicals involved in the study were purchased from commercial sources. SIS3, SB203580, PD98059 and SP600125 were dissolved in dimethyl sulfoxide. The maximum concentration of dimethyl sulfoxide was 0.1%, which did not affect M-CSF and VEGF assays, detection of the mRNA level using reverse transcription-polymerase chain reaction (RT-PCR) analysis, or the detection of the protein level using western blotting.

Cell culture. Clonal osteoblast-like MC3T3-E1 cells derived from newborn mouse calvariae (Sudo et al. 1983) were maintained as previously described (Kozawa et al. 1992). Briefly, MC3T3-E1 cells were cultured in α-minimum essential medium (α-MEM) supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere with 5% CO2/95% air. The cells were seeded onto 35-mm diameter dishes (5 × 10^4 cells/dish) for M-CSF and VEGF assays and RT-PCR analysis, or 90-mm diameter dishes (2 × 10^5 cells/dish) for western blot analysis in α-MEM supplemented with 10% FBS. After 5 days, the medium was replaced with α-MEM supplemented with 0.3% FBS. After 48 h, the cells were utilized for experiments.

Assay for M-CSF. The cultured cells were pretreated with 0, 1, 3, 10, 30 or 50 ng/mL of oncostatin M at 37°C for 60 min, and then stimulated by 7 ng/mL of TGF-β or vehicle for 1 mL α-MEM supplemented with 0.3% FBS at 37°C for 48 h. The conditioned medium was then collected and the concentration of M-CSF was measured using the mouse M-CSF ELISA kit, according to the manufacturer’s protocol (Hioki et al. 2021).

Assay for VEGF. The cultured cells were pretreated with 0, 1, 3, 10, 30 or 50 ng/mL of oncostatin M at 37°C for 60 min, and then stimulated by 7 ng/mL TGF-β or vehicle for 1 mL α-MEM supplemented with 0.3% FBS at 37°C for 48 h. The conditioned medium was collected following the incubation periods and the concentration of VEGF was measured using the mouse VEGF ELISA kit, according to the manufacturer’s protocol (Yamamoto et al. 2015).

Real-time RT-PCR. The cultured cells were pretreated with 50 ng/mL of oncostatin M or vehicle for 60 min, and then stimulated by 7 ng/mL of TGF-β or vehicle in α-MEM, containing 0.3% FBS for 6 h. Total RNA was isolated and reverse transcribed into complementary DNA using TRizol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Heysham, Lancashire, UK) and Omniscript Reverse Transcriptase kit (Qiagen Inc., Valencia, CA), respectively. RT-PCR was performed in capillaries using a LightCycler system with the LightCycler FastStart DNA
and plotted as the fold-increase compared to that of control cells treated without stimulation (Yamamoto et al. 2015).

Statistical analysis. The data were analyzed by an analyzed by of variance (ANOVA) followed by Bonferroni method for multiple comparisons between pairs, and $P < 0.05$ was statistically significant. The data are presented as means ± standard error of mean (SEM) of at least triplicate determinations from independent cell preparations (Yamamoto et al. 2015).

RESULTS

Effect of oncostatin M on the TGF-β-induced release and mRNA expression of M-CSF in MC3T3-E1 cells

We first examined the effect of oncostatin M on the TGF-β-induced M-CSF release in osteoblast-like MC3T3-E1 cells. Oncostatin M, which by itself did not affect M-CSF release, significantly suppressed the TGF-β-stimulated M-CSF release in a dose-dependent manner observed at 1, 3, 10, 30 and 50 ng/mL (Fig. 1A). The maximum effect of oncostatin M on the M-CSF release stimulated by TGF-β was observed at 50 ng/mL, which almost completely inhibited the TGF-β effect. To investigate whether the oncostatin M suppression for the release of M-CSF stimulated by TGF-β was mediated through transcriptional events or not, we examined the effects of oncostatin M on the TGF-β-induced mRNA expression levels of M-CSF with RT-qPCR. Although oncostatin M by itself did not have any significant effect on the levels of M-CSF mRNA, it significantly suppressed the TGF-β-upregulated mRNA expression levels of M-CSF observed at a dose of 50 ng/mL (Fig. 1B).

Effects of SIS3, SB203580, PD98059 and SP600125 on the TGF-β-induced M-CSF release in MC3T3-E1 cells

In osteoblast-like MC3T3-E1 cells, we previously reported that TGF-β activates p44/p42 MAP kinase, p38 MAP kinase and SAPK/JNK in addition to SMAD2/3, leading to the synthesis of VEGF (Tokuda et al. 2003; Kanno et al. 2005; Yamamoto et al. 2015). To investigate the intracellular signaling system involved in the TGF-β-induced M-CSF synthesis in MC3T3-E1 cells, we examined the effects of SIS3, an inhibitor of TGF-β-induced SMAD3 phosphorylation (Jinnin et al. 2006), SB203580, an inhibitor of p38 MAP kinase (Cuenda et al. 1995), Master SYBR Green I (Roche Diagnostic, Basel, Switzerland). Sense and antisense primers for mouse M-CSF mRNA and VEGF mRNA were obtained from Takara Bio Inc. (Tokyo, Japan) (primer set ID: MA171365 or MA039013, respectively). Sense and antisense primers for mouse GAPDH mRNA were synthesized based on the report of Simpson et al. (2000). The amplified products were determined using melting curve analysis. The M-CSF and VEGF mRNA levels were normalized to those of GAPDH mRNA (Yamamoto et al. 2015).
Effect of oncostatin M on the TGF-β-induced phosphorylation of SMAD3 in MC3T3-E1 cells

To clarify whether oncostatin M could affect the SMAD3 activation induced by TGF-β, the canonical pathway, or not, we examined the effect of oncostatin M on the phosphorylation of SMAD3 induced by TGF-β in osteoblast-like MC3T3-E1 cells. We confirmed that TGF-β considerably upregulated the levels of SMAD3 phosphorylation (Fig. 3). Oncostatin M at the doses up to 70 μM hardly affected the levels of SMAD3 phosphorylation with or without TGF-β (Fig. 3).

Effects of oncostatin M on the TGF-β-induced phosphorylation of p38 MAP kinase, p44/p42 MAP kinase and SAPK/JNK in MC3T3-E1 cells

To elucidate whether oncostatin M could modulate the activation of p38 MAP kinase, p44/p42 MAP kinase, and SAPK/JNK induced by TGF-β, the non-canonical pathways, or not, we next examined the effects of oncostatin M on the levels of the phosphorylation induced by TGF-β in osteoblast-like cells.
levels of the TGF-β-induced phosphorylation of SAPK/JNK dose-dependently up to 70 ng/mL with or without affecting the levels by itself (Fig. 6).

**Effects of oncostatin M on the TGF-β-induced release and the mRNA expression of VEGF in MC3T3-E1 cells**

On account of our previous reports (Tokuda et al. 2003; Kanno et al. 2005; Yamamoto et al. 2015), we further examined the effect of oncostatin M on the TGF-β-induced VEGF release in osteoblast-like MC3T3-E1 cells. Oncostatin M, which by itself did not affect the phosphorylation levels of p38 MAP kinase, p44/p42 MAP kinase and SAPK/JNK (Figs. 4–6). However, oncostatin M hardly affected the levels of p38 MAP kinase phosphorylation at the dose up to 70 ng/mL with or without TGF-β stimulation (Fig. 4). On the other hand, oncostatin M, which by itself little affected the phosphorylation levels of p44/p42 MAP kinase, markedly suppressed the levels of phosphorylation stimulated by TGF-β dose-dependently observed at 30, 50 and 70 ng/mL (Fig. 5). In addition, oncostatin M also reduced the levels of the TGF-β-induced phosphorylation of SAPK/JNK dose-dependently up to 70 ng/mL without affecting the levels by itself (Fig. 6).
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and found that the expression levels were significantly reduced by the oncostatin M in these cells. It seems that the suppressive effect of oncostatin M on the TGF-β-induced M-CSF release would be mediated through the transcriptional events in these cells. Therefore, it is most likely that oncostatin M suppresses the synthesis of M-CSF stimulated by TGF-β in osteoblast-like MC3T3-E1 cells. To the best of our knowledge, mRNA stimulated by TGF-β, and found that the expression levels were significantly reduced by the oncostatin M in these cells. It seems that the suppressive effect of oncostatin M on the TGF-β-induced M-CSF release would be mediated through the transcriptional events in these cells. Therefore, it is most likely that oncostatin M suppresses the synthesis of M-CSF stimulated by TGF-β in osteoblast-like MC3T3-E1 cells. To the best of our knowledge,
SIS3 (Jinnin et al. 2006), SB203580 (Cuenda et al. 1995), PD98059, (Alesse et al. 1995) and SP600125 (Bennett et al. 2001) of all the inhibitors suppressed the M-CSF release stimulated by TGF-β, suggesting that TGF-β elicits M-CSF synthesis via both canonical and non-canonical pathways in similar with the VEGF synthesis in these cells.

To clarify the mechanism whereby oncostatin M suppresses the TGF-β-stimulated M-CSF synthesis this is probably the first report which clearly indicates the suppressive effect of oncostatin M on the M-CSF synthesis in osteoblasts.

Regarding the intracellular signaling mechanism of TGF-β in osteoblasts, we have previously reported that SMAD2/3, p44/p42 MAP kinase, p38 MAP kinase and SAPK/JNK are involved in the TGF-β-stimulated VEGF synthesis in osteoblast-like MC3T3-E1 cells (Tokuda et al. 2003; Kanno et al. 2005; Yamamoto et al. 2015). We found here that
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Oncostatin M suppresses the TGF-β-stimulated activation of both p44/p42 MAP kinase and SAPK/JNK among non-canonical pathways in osteoblasts. Therefore, it is likely that oncostatin M suppresses the TGF-β-stimulated M-CSF synthesis via the reduction of both p44/p42 MAP kinase and SAPK/JNK activation in osteoblasts. On the other hand, we showed that oncostatin M almost completely inhibited the TGF-β-stimulated M-CSF synthesis via the reduction of both p44/p42 MAP kinase and SAPK/JNK activation in osteoblasts. On the contrary, we found that the TGF-β-induced phosphorylation of p44/p42 MAP kinase and that of SAPK/JNK were considerably suppressed by oncostatin M, suggesting that oncostatin M suppresses the TGF-β-stimulated activation of both p44/p42 MAP kinase and SAPK/JNK among non-canonical pathways in osteoblasts. Therefore, it is likely that oncostatin M suppresses the TGF-β-stimulated M-CSF synthesis via the reduction of both p44/p42 MAP kinase and SAPK/JNK activation in osteoblasts. On the other hand, we showed that oncostatin M almost completely inhibited the TGF-β-stimulated release and mRNA expression of M-CSF, whereas both SMAD2/SMAD3- and p38 MAP kinase-mediated pathways were intact. It is possible that TGF-β-stimulated M-CSF synthesis is mediated via other unknown pathway(s), which is suppressed by oncostatin M in osteoblasts.

As described above, we have shown here that the intracellular signaling mechanism of the TGF-β-stimulated M-CSF synthesis seems to be similar with that of the VEGF synthesis in osteoblast-like MC3T3-E1 cells. Thus, we further investigated whether oncostatin M suppresses the TGF-β-stimulated VEGF synthesis or not in these cells.

Fig. 7  Effects of oncostatin M on the TGF-β-induced vascular endothelial growth factor (VEGF) release and the expression of VEGF mRNA in MC3T3-E1 cells. (A) The cultured cells were pretreated with 0, 1, 3, 10, 30 or 50 ng/mL of oncostatin M for 60 min, and subsequently stimulated by 7 ng/mL of TGF-β (●) or vehicle (○) for 48 h. The identical samples in the experiment presented as Fig. 1A were analyzed. VEGF concentrations in the culture medium were determined using an ELISA. Each value is the mean ± SEM of triplicate determinations from three independent cell preparations. *P < 0.05, compared to the value of TGF-β alone. (B) The cultured cells were pretreated with 50 ng/mL of oncostatin M or vehicle for 60 min, and subsequently stimulated by 7 ng/mL of TGF-β or vehicle for 6 h. The identical samples in the experiment presented as Fig. 1B were analyzed. The respective total RNA was subsequently isolated and transcribed into cDNA. The expressions of VEGF mRNA and GAPDH mRNA were quantified by RT-PCR. The VEGF mRNA levels were normalized to those of GAPDH mRNA. Each value is the mean ± SEM of quintuple determinations from five independent cell preparations. *P < 0.05, compared to the value of control. **P < 0.05, compared to the value of TGF-β alone.
expected, the release and the mRNA expression of VEGF stimulated by TGF-β were certainly inhibited by oncostatin M, suggesting the suppression by oncostatin M of TGF-β-stimulated VEGF synthesis in addition to the M-CSF synthesis in osteoblasts. Taking our present findings into account as whole, it is most likely that oncostatin M downregulates TGF-β signaling upstream of p44/p42 MAP kinase and SAPK/JNK in osteoblasts, resulting in the attenuation of M-CSF and VEGF syntheses. The potential mechanism is summarized and presented as Fig. 8.

In conclusion, our findings strongly suggest that oncostatin M downregulates TGF-β signaling upstream of p44/p42 MAP kinase and SAPK/JNK, but not SMAD 2/3 and p38 MAP kinase, in osteoblasts and attenuates the synthesis of M-CSF.

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The authors have declared that there is no conflict of interest.

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