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Osteopontin Promotes Oncostatin M Production in Human Osteoblasts: Implication of Rheumatoid Arthritis Therapy

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Accumulating evidence indicates that subchondral bone might play an essential role in rheumatoid arthritis (RA). Osteopontin (OPN) induces the production of an important proinflammatory cytokine involved in the pathogenesis of RA. This study evaluated the activation of oncostatin M (OSM) by OPN in human primary osteoblasts to understand RA pathogenesis and characterized the intracellular signaling pathways involved in this activation. Quantitative PCR, ELISA, and Western blot results indicated that stimulation of human primary osteoblasts with OPN induces OSM expression through αvβ3 integrin/c-Src/platelet-derived growth factor receptor transactivation/MEK/ERK. Treatment of osteoblasts with OPN also increased c-Jun phosphorylation, AP-1 activation, and c-Jun binding to the AP-1 element on the OSM promoter, as demonstrated using chromatin immunoprecipitation assay. Moreover, inhibition of OPN expression using lentiviral-OPN short hairpin RNA resulted in the amelioration of articular swelling, cartilage erosion, and OSM expression in the ankle joint of mice with collagen-induced arthritis as shown using microcomputed tomography and immunohistochemistry staining. Our results imply that OSM expression in osteoblasts increases in response to OPN-induced inflammation in vitro. Finally, lentiviral-OPN short hairpin RNA ameliorates the inflammatory response and bone destruction in mice with collagen-induced arthritis. Therefore, OPN may be a potential therapeutic target for RA.

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Rheumatoid arthritis (RA) is an autoimmune disease, which results in progressive articular cartilage erosion and synovial hyperplasia. Although the pathogenesis of RA have not been completely elucidated, it is clear that both structural damage and infiltration of proinflammatory cytokines occur at the articular capsule microenvironment (1). In fact, it is known that excessive production of proinflammatory mediators is a determining factor for RA pathogenesis. Activated immune cells infiltrating the synovial tissue secrete large quantities of cytokines such as IL-6, which is a potent effector in RA and generates enzymes to degrade cartilage and bone (1, 2). Oncostatin M (OSM), a member of the IL-6 family, is capable of activating osteoblasts and stimulating mesenchymal progenitor differentiation toward the osteoblastic cell lineage (3). OSM stimulates bone formation and is produced by osteocytes and macrophages (4). OSM secretion has been associated with the modulation of other inflammatory diseases, including RA, osteoarthritis, and hepatic cellular carcinoma (5).

Osteopontin (OPN) is an extracellular matrix glycoprotein that is present in the extracellular fluid surrounding the sites of mineralized tissues and bone remodeling (6). OPN is an important mediator of the immune response as it is involved in cell migration and Th1-mediated immunity (7, 8). OPN was found to selectively induce expression of proinflammatory chemokines and acted as a crucial mediator in the rheumatoid synovium (9, 10). Accumulating evidence suggests that higher OPN levels could be observed in the synovial fluid and synovial membrane in RA patients (11, 12). OPN-deficient mice were found to attenuate articular cartilage erosion and reduce arthritic scores in collagen-induced arthritis (CIA) (13). In addition, the arginine–glycine–aspartate (RGD) motif in OPN acts as a multifunctional cytokine that interacts with multiple ubiquitously expressed cell surface receptors, including various integrins (14). Thereby, OPN-induced effects on osteoblasts might be implicated in RA.

During the development of RA, subchondral bone plays an essential role in the mechanisms of bone remodeling that involves OSM (3). A recent study has reported OSM to be constitutively expressed in the subchondral bone and possibly associated with osteoblasts (15). Nevertheless, the mechanism of interaction between OPN and OSM during osteoblast inflammation has not yet been elucidated.

Our results showed that OPN induces upregulation of OSM expression in primary osteoblasts. Similarly, in vivo results showed that lentiviral knockdown of OPN significantly abolishes bone erosion and inflammation in mice with CIA. These results provide new insights into the mechanisms of OPN action that may have therapeutic value for RA.

[Note: The rest of the text contains detailed scientific discussion and experimental results but is not necessary for the natural text representation.]
Materials and Methods

**Materials**

Anti-mouse and anti-rabbit IgG-conjugated HRP, rabbit polyclonal Ab specific for β-actin, phospho-c-Src, c-Src, platelet-derived growth factor receptor (PDGFR)-β, phospho-MEK, MEK, phospho-ERK, ERK, phospho-c-Jun, c-Jun, IgG, and OSM were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal Ab specific for phospho-PDK1 was purchased from Cell Signaling Technology (Danvers, MA). Mouse monoclonal Abs specific for OSM or ovβ3 integrin were purchased from R&D Systems (Minneapolis, MN). An OSM enzyme immunoassay kit was purchased from R&D Systems. Tanshinone IIA was purchased from Biomol (Butler Pike, PA). The AP-1 luciferase plasmid was purchased from Stratagene (La Jolla, CA). The c-Src dominant-negative (DN) mutant was a gift from Dr. S. Parsons (University of Virginia, Charlottesville, VA). MEK-1 DN mutant was a gift from Dr. W. M. Fu (National Taiwan University, Taipei, Taiwan). ERK-2 DN mutant was a gift from Dr. M. Cobb (Southwestern Medical Center, Dallas, TX). pSβ-β-galactosidase vector and the luciferase kit were purchased from Promega (Madison, WI). All other chemicals or inhibitors were obtained from Sigma-Aldrich (St. Louis, MO).

**Cell cultures**

Human primary osteoblasts were isolated from bone chips obtained from 20 donors (ages 40–60 y) who were generally healthy and had no bone disorder other than hip dysplasia, for which they had undergone hip arthroplasty at China Medical University Hospital. Informed consent was obtained from each donor. The protocol for this study was approved by the Institutional Review Board at China Medical University Hospital (16). The osteoblasts were cultured in DMEM containing 100 μg/ml ascorbic acid, nonessential amino acids, penicillin/streptomycin, and 10% FBS (Invitrogen, Carlsbad, CA). Cultures were maintained in a humidified atmosphere of 5% CO₂ at 37°C. The human osteoblast-like cell line MG-63 was purchased from the American Type Culture Collection (Manassas, VA). MG-63 cells were cultured in DMEM supplemented with 10% FBS and antibiotics (100 U/ml penicillin G and 100 μg/ml streptomycin).

We obtained approval for this study from the local ethics committee and all animals gave written informed consent to participate. Abnormal RA synovial fibroblasts (RASFs) were obtained from six patients (ages 40–60 y) during total knee arthroplasty for RA. Human primary synovial fibroblasts were isolated from RA patients and incubated in a solution of type II collagenase for 18 h and then filtered through 70-μm nylon filters. Cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FCS (Life Technologies, Carlsbad, CA) and were seeded at a concentration of 3 × 10⁵ cells/T55 flask. After 1 wk, nonadherent cells were removed and the adherent RASFs were expanded in vitro. RASFs from passages 3 to 9 were used for the experiments.

**OSM quantification**

Human primary osteoblasts were cultured in 24-well culture plates. Cells were treated with OPN and then incubated at 37°C for 24 h. To examine the downstream signaling pathways responsive to OPN treatment, cells were pretreated with various inhibitors for 30 min before the addition of OPN (100 ng/ml). After incubation, the supernatant medium was collected and stored at −80°C until the assay was performed. OSM in the medium was assayed using an OSM ELISA kit, according to the manufacturer’s instructions.

**Quantitative real-time PCR**

Total RNA was extracted from osteoblasts using a TRIzol kit (MDBio, Taipei, Taiwan). RNA quality and yield were determined by absorbance at 260-nm measurements performed with a Nanovue Spectrophotometer (GE Healthcare, Madison, WI). cDNA was synthesized from 1 μg total RNA using a Moloney Murine Leukemia Virus Reverse Transcription kit (Invitrogen) following the manufacturer’s recommendations. Quantitative real-time PCR (qPCR) analysis was carried out with TaqMan One-Step RT-PCR Master Mix (Applied Biosystems, Foster City, CA). All target gene primers and probes were purchased from Applied Biosystems. β-Actin was used as an internal control. qPCR assays were carried out in triplicate using a StepOnePlus sequence detection system (Applied Biosystems), according to the manufacturer’s instructions.

**Western blot analysis**

Cellular lysates were prepared as described previously (17). Proteins were resolved using NaDodeSO₄-PAGE and transferred to Immobilon polyvinylidene fluoride membranes (Millipore, Billerica, MA). The membranes were blocked with 4% BSA for 1 h at room temperature and then probed with rabbit Ab against human phospho-ERK, ERK, phospho-MEK, MEK, phospho-c-Jun or c-Jun (1:1000) for 1 h at room temperature. After three washes, the blots were incubated with a donkey anti-rabbit peroxidase-conjugated secondary Ab (1:10000) for 1 h at room temperature. The blots with HRP-labeled substrate were detected by ECL and visualized by using a Fujifilm LAS-3000 chemiluminescence detection system (Fujifilm, Tokyo, Japan).

**Transfection and reporter gene assay**

Control ON-TARGETplus small interfering RNA (siRNA) and ON-TARGETplus siRNAs against c-Jun and PDGFR-β were purchased from Dharmacon Research (Lafayette, CO). Transient transfection of siRNA (0.5 nM) was carried out using DharmACECT 1 transfection reagent (Thermo, Waltham, MA) for 24 h, according to the manufacturer’s instructions. For the reporter assay, cells were cotransfected with AP-1 luciferase (0.5 μg) and β-galactosidase expression vector (0.5 μg) for 24 h using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s protocol.

**Chromatin immunoprecipitation assay**

Chromatin immunoprecipitation (ChIP) analysis was performed as described previously (18). DNA, immunoprecipitated with an anti-c-Jun antibody, was eluted and chromatin and extracted with phenol/chloroform using PCR. PCR products were resolved by electrophoresis in a 1.5% agarose gel and visualized under UV light. Primers 5'-AACCTCCTCTTCTTGCCTGT-3' and 5'-CTGTCGACGACATCCTCCAG-3' were used to amplify across the OSM promoter region (19).

**Lentiviral production**

Recombinant lentiviruses were produced by transient cotransfection of 293T cells with short hairpin (sh)RNA-expressing plasmid (TRCN0000004877) along with the packaging plasmid pCMVΔR8.91 and the VSV-G envelope glycoprotein expression plasmid pMD.G, all of which were obtained from the National RNAi Core Facility at the Academia Sinica in Taiwan. After 48 h, lentiviral particles carrying OPN shRNA (Lenti-shOPN) were isolated from the supernatant of 293T cells. A plaque assay using serial dilution was performed in human osteoblasts to determine virus titers (in plaque forming units) of LentishOPN. The viral titer of Lentish-opN was determined to be 7.1 × 10⁹ PFU.

**In vivo collagen-induced arthritis model**

Male C57BL/6J mice (ages 8–10 wk) were purchased from the National Laboratory Animal Center in Taiwan. Mice were maintained under conditions complying with the Guidelines of the Animal Care Committee of China Medical University. The CIA model was established according to published protocols (20). Briefly, 0.1 ml of an emulsion containing 100 μg chicken collagen type II (CII) (Sigma-Aldrich, dissolved in 10 ml of 1% sodium carbonate) was injected subcutaneously at the tail base of mice on day 0. Two weeks after the primary immunization, the second booster injection of 100 μg CII dissolved and emulsified 1:1 inIFA was administered into the hind paws. Paw swelling was measured in a blinded manner with a plethysmometer (Marsap, Mumbai, India). Once weekly for 4 wk to determine the clinical severity of arthritis. In a separate experiment, groups of 10 mice with CIA or control were injected with 7.1 × 10⁹ PFU Lentish-OPN or control-shRNA in the hind paw weekly for 4 wk, respectively, after the second CIA immunization. Upon sacrifice (day 42), the mice phalanges and ankle joints were removed immediately and fixed in 4% paraformaldehyde for microcomputed tomography (micro-CT) analysis. The micro-CT scans of the paws was performed using an in vivo micro-CT scanner (SkyScan 1176; Bruker, Kontich, Belgium) at 9 μm resolution and with a rotation step of 0.30 degree per image. Scanning was done at 50 kV X-ray voltage, 500 μA current, and 885 ms of exposure time/image with a 0.5-mm aluminum filter. Reconstruction of sections was carried out with GPU-based scanner software (NRecon; Bruker). The grayscale was based on Hounsfield units, and validated calcium standards were scanned to use as density references. Three-dimensional microstructural volumes from micro-CT scans were analyzed using SkyScan software (CTAn; Bruker). To evaluate bone focal erosion in the ankle joint, quantification of volumetric bone mineral density (BMD), bone loss (BS), and bone volume (BV) were performed in a defined bone area by manually drawn regions of interest (913 slices from the proximal calcaneus to metatarsals, 8 mm each).

**Immunohistochemistry**

Immunohistochemistry (IHC) staining was performed on serial sections of the mice ankle joints. After ankle joints from the mice were fixed in 1% formalin, they were dehydrated in a graded series of ethanol solutions and subsequently embedded in paraffin. Sections (3-μm thick) were cut and mounted on glass cover slips. Immunohistochemistry was performed by the guest on April 30, 2017 http://www.jimmunol.org/ Downloaded from
formaldehyde, the specimens were decalcified in 10% EDTA and dehydrated in ethanol/xylene as described previously (21). The sections were stained with primary rabbit anti-mouse OSM (2 μg/ml), IL-6 (0.125 μg/ml), IL-1β (5 μg/ml), TNF-α (2 μg/ml), or control IgG (5 ng/ml) (Santa Cruz Biotechnology). Biotin-conjugated goat anti-rabbit IgG was used as the secondary Ab and 3,3′-diaminobenzidine tetrahydrochloride as the substrate for color development. Some specimens were also stained with Safranin O-fast Green and H&E (21).

Statistical analysis

Data are expressed as the mean ± SEM. Parametric testing between two matched groups was performed by the paired t test. The Mann–Whitney rank sum test was used between two groups, and Kruskal–Wallis one-way ANOVA on ranks among was used for four groups. The *p value < 0.05 was considered significant.

Results

**OPN increases OSM production in human osteoblasts**

Although much information regarding RA focuses on the effects of inflammation on osteoclast differentiation and function, recent studies have begun to elucidate the impact of proinflammatory cytokines, such as OPN, on the osteoblast (22, 23). Studies regarding the inflammatory development of RA show that OSM potentially contributes to monocyte migration and macrophage activation in synovial tissue (24, 25). These findings suggest that the high concentrations of OPN involved in bone remodeling within osteoblasts might play a dynamic role promoting the secretion of inflammatory cytokines into the inflamed articular microenvironments observed in RA development. Therefore, we used human osteoblasts to investigate the signaling pathways of OPN in the production of OSM. Treatment of osteoblasts (human primary osteoblasts and MG-63 cells) with different OPN dosages (25–200 ng/ml) or performed at different time intervals induced OSM mRNA expression in concentration- and time-dependent manners (Fig. 1A, 1B, respectively). Western blot (Fig. 1C, 1D) and ELISA (Fig. 1E, 1F) analysis demonstrated that OPN stimulation results in increased OSM expression in the supernatant and cellular lysates in a concentration- and time-dependent manner. These results demonstrate that OPN induces up-regulation of proinflammatory OSM expression in osteoblasts.

**OPN induces OSM expression through αvβ3 integrin receptor and c-Src signaling in human osteoblasts**

Although the interaction between the RGD motif of OPN and integrins within osteoclastic bone resorption has been generally accepted (26), it has not yet been elucidated whether OPN interacts with integrins in osteoblasts during RA inflammation. Therefore, to determine the role of integrin-dependent signaling in osteoblasts, cells were treated with OPN and the mRNA expression of different integrins quantified. It was found that OPN-treatment induces mRNA expression of α1, α5, and β3 integrins, whereas expression of α3, αv, β1, and β3 integrins mRNA was not induced (Fig. 2A). Next, we specifically inhibited αvβ3 to examine its involvement in the OPN-mediated increase of OSM expression. OSM mRNA (Fig. 2B) and protein levels (Fig. 2C, 2D) in response to OPN-treatment selectively decreased in the presence of 2 μg/ml anti-αvβ3 mAb compared with control, supporting the role of αvβ3 integrin in this process.

Recently, c-Src signaling downstream of integrin has been reported (27). As such, we investigated the role of c-Src in mediating OPN-induced OSM expression. OPN-induced OSM expression was markedly attenuated by pretreatment of cells for 30 min with the specific c-Src inhibitor PP2 (10 μM) (Fig. 2E), as well as in cells expressing the c-Src DN mutant (Fig. 2F). C-Src autophosphorylation at Tyr416 results in the recruitment of other signal transduction molecules (28). Therefore, we measured the level of c-Src phosphorylation at Tyr416 in response to OPN. As shown in Fig. 2G, treatment of osteoblasts with OPN resulted in time-dependent phosphorylation of c-Src at Tyr416, which was reduced by anti-αvβ3 Ab (Fig. 2H). On the basis of these results, we concluded that OPN acts through a signaling pathway involving αvβ3 integrin and c-Src to enhance OSM expression in human osteoblasts.
FIGURE 2. Involvement of αvβ3 integrin and c-Src signaling in OPN-mediated OSM production in human osteoblasts. (A) Osteoblasts were incubated with OPN for 24 h, and mRNA expression of various integrins was examined by qPCR. (B and C) Osteoblasts were pretreated with OPN or OPN plus control IgG (1 μg/ml) or plus anti-αvβ3 mAb (2 μg/ml) for 24 h, and the expression of OSM mRNA or protein was analyzed by qPCR or Western blotting, respectively. (D) Osteoblasts were pretreated with anti-αvβ3 mAb for 30 min, followed by stimulation with OPN for 24 h. The supernatant medium was analyzed by ELISA. (E and F) Osteoblasts were pretreated with 30 min with αvβ3 mAb and PP2 (10 μM) or transfected for 24 h with c-Src DN mutant, followed by stimulation with OPN for 24 h. The expression of OSM mRNA (E) and protein (F) was analyzed by qPCR and ELISA, respectively. (G) Osteoblasts were incubated with OPN for the indicated time intervals, and c-Src phosphorylation was examined by Western blotting. (H) Osteoblasts were pretreated with αvβ3 mAb for 30 min, followed by stimulation with OPN. Bars show the mean ± SEM. *p < 0.05 versus control (A, E, and F) or IgG (B and D), #p < 0.05 versus OPN alone (B, D, E, and F).
Regulation of OPN-induced OSM expression through transactivation of PDGFR results in MEK/ERK activation

Studies have implicated transactivation of PDGFR in the mediation of cellular inflammation (29) and in the development of mineralized nodules regulated by osteoblasts (30). To test whether c-Src–dependent transactivation of PDGFR-β was involved in OPN-induced OSM expression, cells were pretreated with a PDGFR-β tyrosine kinase inhibitor AG1296. OPN-induced OSM production was reduced upon pretreatment with 1 μM AG1296, a general inhibitor of PDGFR (Fig. 3A) or in the absence of PDGFR-β expression (Fig. 3B), suggesting that PDGFR-β may play a role in OPN-mediated OSM expression of osteoblasts. In addition, upon treatment of osteoblasts with OPN, increased phosphorylation of PDGFR-β at Tyr1021 was observed (Fig. 3C). This OPN-mediated activation of PDGFR-β was abolished upon pretreatment of osteoblasts with the Ab against αvβ3 integrin or with the inhibitor PP2 (Fig. 3D). These results were consistent with our hypothesis that OPN-induced OSM production was mediated through the transactivation of PDGFR-β.

It has been reported that the activation of PDGFR-β is related to the MEK/ERK signaling pathway (31). To determine whether OPN-dependent OSM expression depends on MEK/ERK signaling downstream of PDGFR-β, osteoblasts were treated with the MEK inhibitors PD98059 (15 μM) and U0126 (10 μM) (Fig. 4A, 4B). It was observed that OPN-induced OSM expression on osteoblasts was greatly reduced upon treatment, as well as in osteoblasts expressing the MEK-1 or ERK-2 DN mutants (Fig. 4A, 4B). In addition, stimulation of osteoblasts with OPN increased MEK and ERK phosphorylation (Fig. 4C), which was markedly inhibited when cells were pretreated with anti-αvβ3 Ab, PP2, or AG1296 (Fig. 4D). In contrast, OPN-mediated PDGFR-β phosphorylation was suppressed by pretreatment of cells with anti-αvβ3 Ab or PP2 but not with PD98059 or U0126 (Fig. 4E). These results suggested that OPN-induced OSM expression may be mediated through a c-Src/PDGFR-β transactivation/MEK/ERK pathway in human osteoblasts.

Involvement of AP-1 in OPN-induced OSM expression

To examine whether AP-1 binding site is involved in OPN-induced OSM expression, cells were pretreated with the AP-1 inhibitors curcumin (20 μM) and tanshinone IIA (10 μM), leading to the reduction of OPN-mediated OSM expression (Fig. 5A, 5B). This involvement was confirmed upon measuring the levels of OSM expression after siRNA suppression of c-Jun (Fig. 5A, 5B), a component of the transcription factor complex AP-1 that binds to a specific DNA sequence (32). In fact, OPN treatment of cells resulted in marked phosphorylation of c-Jun (Fig. 5C), which was inhibited by pretreatment with anti-αvβ3 Ab, PP2, AG1296, or U0126 (Fig. 5D).

It has been reported that c-Jun is recruited to the OSM promoter, which contains an AP-1 binding site (19, 33). The binding of c-Jun to the AP-1 element on the OSM promoter after OPN stimulation was confirmed through ChIP (Fig. 5E). OPN-induced binding of c-Jun to the AP-1 element was attenuated by PD98059, PP2, AG1296, and anti-αvβ3 Ab (Fig. 5E). To further verify that the AP-1 element itself is involved in OPN-induced OSM expression, we performed transient transfections with AP-1 promoter–luciferase constructs, and it was found that osteoblasts incubated with...
OPN exhibited a 3.6-fold increase in AP-1 promoter activity. The increase in AP-1 activity by OPN was antagonized by the inhibitors of upstream signals, including anti-αvβ3 Ab, PP2, AG1296, PD98059, U0126, curcumin, and tanshinone IIA, as well as the by siRNA-mediated inhibition or the expression of DN mutants (Fig. 5F, 5G). Taken together, these data suggest that activation of the αvβ3 integrin/c-Src/PDGFR transactivation/MEK/ERK/c-Jun/ AP-1 pathway is required for the OPN-induced increase in OSM expression in human osteoblasts.

Knockdown of OPN attenuates CIA-induced OSM expression and cartilage erosion in vivo

To further confirm the role of OPN in vivo, we first assessed the effect of shRNA-mediated OPN knockdown in the amelioration of mice with CIA. For local delivery of Lenti-shOPN or control-shRNA into the joint cavity, hind paws of mice with CIA were injected with 7.1 × 10⁶ PFU Lenti-shOPN or control-shRNA once weekly for 4 wk after second immunization. The incidence of arthritis in mice with CIA was very high within 4 wk, and 95% of the mice developed severe arthritis. The CIA group injected with Lenti-shOPN had significantly attenuation in paw swelling compared with the control group (Fig. 6A) as reflected by the hind paw volume data (Fig. 6B). In addition, micro-CT imaging of hind paws showed that mice with CIA had significant reduction of BMD at the ankle joint (Fig. 6C, 6D), as well as a statistically significant increase in the ratio between BS and BV, parameter quantifying surface density (Fig. 6E). In contrast, the Lenti-shOPN infected CIA mice showed higher BMD and had a lower BS:BV ratio at the ankle joints, as compared with the control group (Fig. 6D, 6E). Concomitantly, the level of intra-articular cartilage erosion was assayed by staining with H&E and Safranin O. IHC staining of the ankle joints revealed that the numbers of OSM-immunopositive cells were significantly increased in mice with CIA but markedly diminished in CIA mice treated with Lenti-shOPN (Fig. 6F). Taken together, our results confirmed that Lenti-shOPN prevents cartilage erosion and OSM expression in vivo, suggesting OPN as a potential therapeutic target for the treatment of RA.

Discussion

Recent studies have focused on the impact of inflammation in osteoblasts, which exhibit important functions within the arthritic bone microenvironment and thus in RA pathogenesis (16, 34); whereas, during the past decade, RA studies had focused on the effects of inflammation on osteoclast-mediated bone resorption.
These studies highlight that the influence of inflammation on bone is specific to the site of inflammation and dependent on the cytokines present within the local bone microenvironment. It has also been shown that OPN has implications for inflammation involving osteoclasts, monocytes, lymphocytes, and neutrophils (37), and in fact, cumulative evidence suggests that OPN is involved in the pathogenesis of RA because it is important in both joint inflammation and destruction (38). The physiologic concentration of...
OPN is 20–35 ng/ml (39, 40); however, high concentrations of OPN have been detected in RASFs and have even been known to induce chondrosarcoma cell migration (41, 42). Therefore, in our current study, we treated osteoblasts with 25–200 ng/ml OPN. In addition to OSM being expressed in osteoblasts, we also found that other types of proinflammatory cytokines, such as IL-6, IL-1β, and TNF-α, as well as other proteases like matrix metalloproteinases, were induced by OPN (Supplemental Figs. 1, 2). To further investigate OSM is not

FIGURE 6. Administration of Lenti-shOPN attenuates bone erosion and OSM expression in a collagen-induced arthritis (CIA) model. (A) Group of 10 mice with CIA were intra-articularly injected with 7.1 x 10^6 PFU Lenti-shOPN or control-shRNA on day 14 and were sacrificed on day 42. The swelling of hind paws was photographed. Original magnification 1 cm. (B) A digital plethysmometer was used to measure hind paw volumes of mice once weekly until sacrifice. (C, upper panels) Representative micro-CT images of the hind paws on day 42 (original magnification 1 mm). Bottom panels, The severest area of hind paws in the indicated group. (D and E) Quantification of bone mineral density (D) and ratio between bone surface and bone volume (E). Values were analyzed using micro-CT Skyscan Software. (F, upper panels) Histologic sections of ankle joints were stained with H&E or Safranin O in indicated groups. Lower panels, Histologic sections of ankle joints were immunostained with IgG (5 ng/ml) or OSM (2 μg/ml). Results are representative of 10 mice per experimental group. Bars in (B), (D), and (E) show the mean ± SEM. *p < 0.05 versus corresponding day 0 value (B) or control group (D and E), #p < 0.05 versus mice with CIA.
only a molecular signaling protein but also a significant proinflammatory indicator of RA, we demonstrated OSM mAb reduced OPN-increased proinflammatory cytokines expression, including IL-6, IL-1β, and TNF-α, in osteoblasts and RASF (Supplemental Figs. 1, 3), resulting that OSM is a functional effector in vitro. Otherwise, a previous in vivo study also reported the significance of OSM in arthritis pathology using an OSM neutralizing Ab in CIA model (43).

Above in vitro and in vivo results both displayed OSM as a functional mediator of RA pathogenesis. Consequently, because OSM expression is dependent on OPN, OPN could be developed as a useful target in osteoblasts for RA treatment; however, the function and the mechanism of action of OPN in RASF remain to be elucidated.

Extensive research has characterized the central role of the interaction between OPN and the integrins expressed in inflammatory cells derived from activated lymphocytes and macrophages (44, 45). In particular, the interaction of OPN and αvβ3 integrin has been implicated in the pathogenesis of RA by a preclinical study (46). During the process of osteoclastic bone resorption, the interaction between the thrombin cleaved NH2-terminal fragment of OPN containing the RGD sequence and integrin has been well characterized (26, 47); however, in the context of RA osteoblasts, the interaction between OPN and integrin remains to be elucidated. We scanned a variety of cell surface integrins for their involvement in OPN-mediated OSM expression. Only αv and β3 integrins were found to play a role; in fact, treatment with Ab against αvβ3 integrin abolishes the up-regulation of OSM production. Therefore, the axis of OPN/αvβ3 integrin mediated inflammation of human primary osteoblasts is suggested as a functional target in the pathogenesis of RA.

Cumulative evidence suggests that PDGFR signaling plays an important role in the regulation of osteoblasts; in fact, the most recent study has revealed that enhancement of bone formation responds to osteoblasts through transactivation of PDGFRs, in particular PDGFR-α and PDGFR-β (48). In agreement with these findings, our data showed that transactivation of PDGFR-β is involved in OPN-induced OSM expression. In addition, we demonstrated that OPN stimulation of osteoblasts result in PDGFR-β phosphorylation at the Tyr1022 residue in a time-dependent manner, and that AG1296 or PDGFR-β siRNA inhibited OPN-mediated OSM expression. Furthermore, our results provide evidence that MEK/ERK signaling transduction participates in OPN-induced OSM activation in human primary osteoblasts. The fact that the MEK/ERK inhibitors PD98059 and U0126 did not affect OPN-increased PDGFR-β phosphorylation suggests that PDGFR-β transactivation is an upstream event of the MEK/ERK pathway in response to OPN stimulation.

As mentioned earlier, AP-1 is one of the most important binding sites in the 5′ region of the OSM gene (49). AP-1 binds to members of the Jun and Fos families of transcription factors that interact with the AP-1 site to form Jun homodimers or Jun-Fos heterodimers by protein dimerization through their leucine zipper motifs (50). In support of this notion, we showed that OPN-induced OSM production involves the translocation of one of the AP-1 binding complex subunits, c-Fos or c-Jun, into the nucleus of osteoblasts. We also demonstrated that OPN-induced c-Jun phosphorylation and that pretreatment of cells with an AP-1 inhibitor or siRNA abolished OPN-increased OSM expression, indicating that c-Jun activation mediates OPN-induced OSM expression. A ChIP assay further confirmed that OPN increased the binding of c-Jun to the AP-1 element within the OSM promoter. This binding was attenuated by PD98059, PP2, AG1296 and anti-αvβ3 Ab. Therefore, the AP-1 binding site is likely to be the most important one for OSM production in accordance with the previous study (49). In fact, using transient transfection with AP-1 luciferase as an indicator of AP-1 activity, we also observed that OPN induced an increase in AP-1 activity. Similarly, those inhibitors including anti-αvβ3 Ab, PP2, AG1296, PD98059, and U0126, mutant including DN-αv-Src, DN-MEK, and DN-ERK, and siRNA against PDGFR and c-Jun also reduced OPN-induced AP-1 promoter activity.

In mice with CIA are possible to observe the most histopathological characteristics of RA, such as articular cartilage destruction and infiltration of inflammatory cells in subchondral bone (51). IHC staining of the ankle joints showed articular cartilage erosion in mice with CIA, which was ameliorated in Lenti-shOPN mice. In addition, the IHC staining demonstrated the presence of other proinflammatory immunopositive cytokines in the subchondral bone of the CIA mice, which were reduced by Lenti-shOPN (Supplemental Fig. 4), suggesting the effect of OPN in the CIA model might be a response to the expression of other proinflammatory mediators. A recent study demonstrated that OSM is constitutively expressed in the subchondral bone of patients with RA and might be secreted by osteoblasts (15). Of all proinflammatory cytokines, however, OSM-immunopositive cells were significantly suppressed in Lenti-shOPN mice (Fig. 6F, Supplemental Fig. 4), suggesting that OSM expression in subchondral bone is a functional consequence of RA through expression of Lenti-shOPN.

This study, performed on a murine model of RA pathogenesis, provides a direct demonstration that reduced inflammation within the intra-articular microenvironment entails the involvement of subchondral bone and repairs of focal cartilage erosions. Furthermore, it demonstrates that PDGFR-β transactivation in osteoblasts results in OPN-induced OSM expression, which significantly contributes to a signaling pathway that ultimately influences inflammation in the articular microenvironment. We also observed that OPN-induced OSM expression requires an interaction with αvβ3 integrin to trigger c-Src and PDGFR transactivation of MEK and ERK signals, which enhanced binding of c-Jun to the AP-1 site in human osteoblasts. Furthermore, in agreement with our in vitro studies, our in vivo results show that silencing of OPN via Lenti-shOPN injection reduces inflammation and cartilage destruction in mice with CIA. In conclusion, our in vitro and in vivo findings provide a better understanding of the mechanisms involving OPN in RA pathogenesis, thus making it a better therapeutic target against the inflammation of osteoblasts in RA.

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Disclosures
The authors have no financial conflicts of interest.

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