Activation of Peroxisome Proliferator-activated Receptor-γ Pathway Inhibits Osteoclast Differentiation*

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Gabriel Mbalaviele§§, Yousef Abu-Amer¶, Alice Meng‡, Rama Jaiswal‡, Steve Beck¶, Mark F. Pittenger‡, Mark A. Thiede‡, and Daniel R. Marshak¶¶

From §Osiris Therapeutics, Inc., Baltimore, Maryland 21231, the ¶Barnes-Jewish Hospital at Washington University School of Medicine, St. Louis, Missouri 63110, and The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

The nuclear receptor and transcription factor, peroxisome proliferator-activated receptor-γ (PPAR-γ), regulates the activity of other transcription factors in the adipogenic differentiation and inflammatory response pathways. We examined the possible function of the PPAR-γ pathway in osteoclast (Ocl) formation from CD34+ hematopoietic stem cells (CD34+ HSCs), using a co-culture system comprised of human mesenchymal stem cells (hMSCs) and CD34+ HSCs, both derived from bone marrow. Ocl formation in this co-culture system is enhanced by the addition of exogenous osteoprotegerin ligand (OPGL), an essential Ocl differentiation factor, and macrophage-colony stimulating factor (M-CSF). The data indicate that soluble OPGL (sOPGL) and M-CSF stimulate Ocl formation in the co-cultures up to 4-fold compared with CD34+ HSCs alone treated with sOPGL and M-CSF. CD34+ HSCs, but not hMSCs, express PPAR-γ and 15-deoxy-Δ12,14-prostaglandin-J2 (15d-PG-J2), a PPAR-γ agonist, completely blocked the effects of sOPGL and M-CSF on Ocl formation and activity. The inhibitory effect of 15d-PG-J2 is specific to the Ocl lineage in both human and mouse models of osteoclastogenesis. Accordingly, parallel experiments demonstrate that sOPGL activates the NF-κB pathway within mouse Ocl progenitors, and this effect was abolished by 15d-PG-J2. These data establish a link between PPAR-γ and OPGL signaling within Ocl progenitors, and support a role for PPAR-γ pathway in the modulation of osteoclastogenesis.

Osteoclasts (Ocls) arise from precursor cells of the monocytic/macrophage lineage and are the primary cells responsible for physiological and pathological bone resorption. These cells are targets for therapies designed to retard or abolish metabolic bone loss, such as osteoporosis. Abnormal levels of cytokines and growth factors in the bone marrow microenvironment contribute to pathological resorption of bone by the Ocls (1). Accordingly, transgenic mice expressing high levels of G-CSF (2) or OPGL (3) become osteoporotic, while mice harboring a mutation in the M-CSF gene (4) or deletion of the OPGL gene (5) are osteopetrotic. On the other hand, mice overexpressing soluble TNF-α receptor (6) or carrying deletions in the IL-6 gene (7) are protected against bone loss caused by estrogen deficiency. Because the Ocls and their progenitors are sensitive to cytokines, uncovering the regulatory mechanisms of the actions of key cytokines is important to identifying new drug targets to prevent excessive bone resorption.

One candidate in regulating cytokine effects is the peroxisome proliferator-activated receptor-γ (PPAR-γ), a member of the nuclear receptor superfamily of transcription factors expressed in adipose tissue, adrenal gland, spleen, endothelium, and hematopoietic tissue (8–11). Treatment of monocytes or HL-60 monocytic leukemia cells with PPAR-γ agonists results in the conversion of these into foam cells (12, 13). Treatment of activated monocytes/macrophages with PPAR-γ agonists reduces the expression of inflammatory molecules (e.g. inducible nitric oxide synthase, gelatinase B, scavenger receptor A, interleukin (IL)-6, IL-1β, and tumor necrosis factor α (TNF-α)) in response to phorbol ester stimulation (9, 10). Thus, PPAR-γ appears to regulate several functions of lineage-committed hematopoietic cells.

Following ligand binding, PPAR-γ heterodimerizes with the retinoid X receptor, and serves as a transcriptional regulator of target genes. PPAR-γ cooperates with members of the CAAT/enhancer-binding protein family to promote adipocyte differentiation in certain cell types (14), and it inhibits gene expression in monocytes/macrophages, in part by antagonizing the activity of other transcription factors, including NF-κB and AP-1 (9). The NF-κB family of transcriptional activators regulates the expression of a variety of cytokines involved in Ocl differentiation, including IL-1, TNF-α, IL-6, and granulocyte/macrophage-colony stimulating factor (GM-CSF) (15). Consistent with these findings, mice with a deletion of the gene encoding p50/p52 heterodimer of the NF-κB family exhibited osteopetrosis due to a deficiency in Ocl differentiation (16, 17). However, the relationship between Ocl-regulating factors and PPAR-γ has not been investigated.

Recent studies have demonstrated that OPGL and OPG are key regulators of Ocl differentiation, activity, and survival (3, 5, 18–20). OPGL stimulates Ocl development, while OPG acts as OPGL decoy receptor, and blocks its effects. To test the involvement of PPAR-γ in Ocl formation, we employed a co-culture system in which the promotion of Ocl formation from CD34+ hematopoietic stem cells (CD34+ HSCs) by human mesenchymal stem cells (hMSCs) is enhanced by exogenous cytokines, including OPGL. hMSCs are multipotent, adult stem cells that are isolated from bone marrow and can give rise to...
various structural and connective tissue types, including bone marrow stroma, osteoblasts, chondrocytes, and adipocytes (21). We found that CD34⁺ HSCs, but not hMSCs, expressed PPAR-γ and that activation of PPAR-γ pathway in Ocl precursors inhibited sOPGL-induced NF-κB activation, and consequently, blocked sOPGL-induced Ocl formation and activity.

MATERIALS AND METHODS

Reagents—Ciglitazone, 15-deoxy-Delta12,14-prostaglandin-J2 (15d-PG-J2), and PG-J2 were purchased from Biomol Research Laboratory (Plymouth Meeting, PA). Dexamethasone, indomethacin, and insulin were purchased from Sigma. Collagenase was purchased from Roche Molecular Biochemicals (Indianapolis, IN). M-CSF and dOPG were purchased from PeproTech, Inc. (Rocky Hill, NJ).

Isolation and Differentiation of hMSCs—Human bone marrow aspirates were purchased from Poteickas, a division of BioWhittaker (Gaithersburg, MD). The hMSCs were isolated from fresh bone marrow aspirates and culture expanded as described previously (21). Culture medium for hMSCs consisted of Dulbecco’s modified Eagle’s medium-low glucose (DMEM; Life Technologies) supplemented with 10% (v/v) of selected lots of fetal bovine serum (FBS; HyClone, Salt Lake City, UT) and 1% antibiotic-antimycotic solution (Life Technologies, Grand Island, NY).

Adipogenic differentiation of hMSCs was induced as described previously (21). Briefly, hMSCs were plated at a density of 2 × 10⁴ cells/cm² and cultured in hMSC medium until they reached confluence. Cultures were then maintained for 72 h in adipogenic medium (MDII) containing DMEM-high glucose, 10% (v/v) FBS, 10 μg/ml insulin, 1 μM dexamethasone, 0.1 mM indomethacin, and 0.5 mM isobutylmethylxanthine. The medium was changed and cultures were then incubated for 24 h with adipogenic maintenance medium containing DMEM-high glucose with 10% (v/v) FBS and 10 μg/ml insulin. This sequence was repeated three times, after which the cultures were maintained in adipogenic maintenance medium for 1 week. Medium was changed every 3–4 days. Half of the cultures were then fixed and stained with Oil-Red O (21) to visualize adipocytes. The remaining cultures were selected to be approximately the same order to avoid preferential amplification of any fragment based on this methodology.

RNA Isolation for Northern Blotting—Cells were maintained in either hMSC medium or adipogenic medium MDII for 1, 2, 3, or 5 days. Total RNA was isolated by guanidinium lysis and separated by density centrifugation using a 40-Ti rotor. Lysates (9.0 ml) were spun at 100,000 × g for 23 h through a 2.5-mL cushion of 5.7 M CsCl and 0.1 M EDTA at pH 7.0. RNA (10 mg) was separated in formaldehyde-agarose gels and transferred onto nylon modified membranes (Hybond). Mouse embryonic stem cell total RNA was isolated from 10 embryonic day 12.5 (E12.5) embryos. The membrane was labeled by random primer method using [α-32P]dATP and [α-32P]dCTP. Membranes were hybridized for 16 h at 42 °C, washed with Tris buffer (50 mM Tris, pH 8.6, 1 mM NaCl, 2 mM EDTA, 1% (v/v) SDS) at 42 °C, then with citrate buffer (30 mM sodium citrate, pH 7.0, 300 mM NaCl, 0.1% (v/v) SDS) at 42 °C, and again with citrate buffer at 60 °C. Membranes were exposed to x-ray films (Kodak BioMax MS film) at −70°C in盒exposing screens for 1 day.

Electrophoretic Mobility Shift Assay—Nuclear extracts were prepared from cultured cells following the method of Abu-Amer et al. (27). In brief, mouse Ocl precursors were plated in 100-mm tissue culture dish (7 × 10⁶/dish) in α-MEM in the presence of 10 ng/ml M-CSF. On day 3, cells were treated with vehicle or 5 μM 15d-PG-J2 for 30 min followed by treatment with 20 ng/ml sOPG for the indicated time points. Controls were treated with ethanol, 5–10 ng/ml TNF-α, or 100 ng/ml lipopolysaccharide for 30 min. Cells were washed twice with ice-cold PBS, and treated with 5 mM EDTA and 5 mM EGTA in PBS for 30 min. Cells were then resuspended in hypotonic lysis buffer B (10 mM HEPES, pH 7.8, 1.5 mM MgCl₂, 0.5 mM dithiothreitol, 0.5 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 5 μg/ml leupeptin) and incubated on ice for 15 min. Nonidet P-40 was added to a final concentration of 0.64% (v/v), and nuclei were pelleted by centrifugation at 16,000 × g. The supernatant was removed, and nuclei were resuspended in nuclear extraction buffer B (20 mM HEPES, pH 7.8, 420 mM NaCl, 1.2 mM MgCl₂, 0.2 mM EDTA, 25% (v/v) glycerol, 0.5 mM dithiothreitol, 0.5 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 5 μg/ml pepstatin A, 5 μg/ml leupeptin) and rotated for 30 min at 4 °C. The extracts were centrifuged at 16,000 × g for 4 min, and the cytoplasmic fractions were collected. The protein concentration was measured using standard BCA kit (Pierce, Rockford, IL). Aliquots (10 μg) of nuclear extracts were incubated with 5'-32P-end-labeled double-stranded oligonucleotide probes. The oligonucleotide sequence (5'-AAACAGGGCCGTTTTCCCTC-3') was derived from the Kappa B3 site of the TNF promoter. The reaction was performed at room temperature for 30–60 min in a total of 20 μl of

Depending on the conditions, a range of cell types can be isolated and cultured. This protocol involves the isolation of hMSCs from fresh bone marrow aspirates and their culture expansion. The cells are then maintained in adipogenic medium for 1 week, during which time adipogenesis is induced. The effectiveness of the adipogenic differentiation is monitored by staining for Oil-Red O to visualize adipocytes. This method allows for the study of the molecular mechanisms underlying adipogenesis and the potential therapeutic applications of hMSCs.
binding buffer (20 mM HEPES, pH 7.8, 100 mM NaCl, 0.5 mM dithiothreitol, 1 μg of poly(dI-dC), and 10% glycerol). Samples (10 μl) were fractionated on a 4–20% gradient TBE gel and the proteins were visualized by autoradiography.

Statistical Analysis—All data were analyzed by a paired t test. Samples were run in triplicates and data represent the mean ± S.E. Independent experiments were performed at least twice.

RESULTS

OPG Enhances the Promotion of Human Osteoclastogenesis by hMSCs—We showed previously that hMSCs promote osteoclastogenesis while hMSCs treated with substances that promote osteogenic (osteoblast) differentiation failed to support Ocl formation in vitro (24). In the first part of this study, we wanted to validate hMSC-based osteoclastogenesis model by determining the expression of OPG and OPGL, two key regulators of osteoclastogenesis (3, 5, 18–20). Using RT-PCR we found that both OPG and OPGL mRNA were expressed by hMSCs, and that osteogenic differentiation of hMSCs was associated with a decline in OPGL/OPG ratio (Fig. 1, top panel) and a concomitant increase in OPG mRNA levels (Fig. 1, middle panel). Quantitative competitive RT-PCR was used to demonstrate that levels of OPGL mRNA in hMSCs were 10 times higher than those of OPG mRNA (Fig. 1B, −OS), while in osteogenic hMSCs (Fig. 1B, +OS) OPG mRNA levels were 100 times higher than those of OPGL mRNA. These data indicate that in vitro differentiation of hMSCs into the osteogenic lineage is associated with a decrease in OPG/OPG ratio.

To determine further the role of OPGL in hMSC-based osteoclastogenesis, CD34+ HSCs and hMSC co-cultures were treated for 1 week with purified, recombinant sOPGL. Co-cultures treated with sOPGL had ~3 times more Ocls than untreated co-cultures (Fig. 2). M-CSF, another osteoclastogenic factor is also expressed by hMSCs (24, 28); addition of this factor alone to hMSCs and CD34+ HSC co-cultures had no effect on Ocl formation. In contrast, the addition of both sOPGL and M-CSF significantly stimulated Ocl formation over control or M-CSF alone. Addition of both sOPGL and M-CSF to the cultures of CD34+ HSCs without hMSCs induced Ocl formation (102 ± 22 Ocls/5 × 10^4 CD34+ HSCs). However, the numbers of Ocls produced in these cultures were lower than those produced in sOPGL- and M-CSF-treated co-cultures with hMSCs (440 ± 16 versus 102 ± 22, in the presence or absence of hMSCs, respectively). These data suggest that the sOPGL and M-CSF are not sufficient to induce maximal Ocl differentiation from CD34+ HSCs without hMSCs.

CD34+ HSCs, but Not hMSCs Express PPAR-γ—Evidence that PPAR-γ antagonizes NF-κB transcriptional activity (9), and that the signaling pathway of OPGL also known as RANKL/TRANCE involves NF-κB (29) led us to examine the expression of this nuclear receptor by hMSCs and CD34+ HSCs. We used RT-PCR and Northern blotting to demonstrate that CD34+ HSCs expressed PPAR-γ mRNA (Fig. 3A). The hMSCs that were induced to differentiate into adipocytes (≥90%) upon treatment with adipogenic medium MDII also expressed PPAR-γ mRNA (Fig. 3A and B). However, using RT-PCR and Northern techniques, all attempts failed to show the expression of PPAR-γ mRNA in hMSCs not treated with MDII. The sequence of the DNA amplicons exhibited 100% identity to PPAR-γ2 (30).

PPAR-γ Agonists Inhibit Ocl Formation—Our finding that PPAR-γ is expressed by CD34+ HSCs led us to examine the effects of PPAR-γ agonists on the ability of sOPGL to stimulate Ocl formation in the co-cultures. Addition of relatively low concentrations of 15d-PG-J2 (1–5 μM) blocked sOPGL-induced Ocl formation in a dose-dependent manner (Fig. 4, left bottom panel). Our finding that PPAR-γ is expressed by CD34+ HSCs led us to examine the effects of PPAR-γ agonists on the ability of sOPGL to stimulate Ocl formation. Addition of relatively low concentrations of 15d-PG-J2 (1–5 μM) blocked sOPGL-induced Ocl formation in a dose-dependent manner.
The addition of 15d-PG-J2 inhibited the formation of TRAP mononucleated cells (i.e. Ocl precursors) suggesting that this PPAR-γ agonist interfered with early events in Ocl differentiation. Dose-response experiments (Fig. 5) showed that 15d-PG-J2 is at least 5-fold more potent than PG-J2 in inhibiting Ocl formation in CD34+ HSCs and hMSC co-cultures.

To demonstrate clearly that PPAR-γ agonists act directly on Ocl progenitors, CD34+ HSC cultures without hMSCs or mouse Ocl precursors were treated with sOPGL and M-CSF in the absence or presence of 15d-PG-J2. The results show that induction of Ocl formation by sOPGL (Fig. 4, right top panel) was inhibited by 15d-PG-J2 in cultures of mouse Ocl precursors (Fig. 4, right bottom panel) and CD34+ HSC cultures (data not shown). We also found that 15d-PG-J2 inhibited Ocl formation induced by the addition of IL-1, IL-3, and GM-CSF to CD34+ HSC cultures in the absence of hMSCs (25.5 ± 1.5 versus 3 ± 2.5, in the absence or presence of 5 μM 15d-PG-J2, respectively).

Taken together, these data establish that PPAR-γ-mediated inhibition of Ocl formation occurs through the Ocl progenitors.

PPAR-γ Agonists Inhibit Ocl Activity—To determine if 15d-PG-J2 affects the ability of pre-formed Ocls to resorb bone, Ocls were isolated from co-cultures of CD34+ HSCs and hMSCs treated for 1 week with sOPGL (40 ng/ml) and M-CSF (25 ng/ml). The purified Ocls were then plated onto elephant tusk slices with hMSCs. Treatment with 15d-PG-J2 for 24 h did not affect the numbers of Ocls (108 ± 18 versus 107 ± 6, in the absence or presence of 15d-PG-J2, respectively), demonstrating that Ocl attachment to bone matrix was not affected by the treatment and that 15d-PG-J2 was not cytotoxic to the cells.

However, we noted that the Ocls in 15d-PG-J2-treated co-cultures were retracted and exhibited high TRAP activity. In contrast, we found that the treatment of cultures with 15d-PG-J2 decreased bone resorption by Ocls stimulated by sOPGL and M-CSF (21 ± 5 versus 9.5 ± 0.5, in the absence or presence of 15d-PG-J2, respectively). These data indicate that 15d-PG-J2 not only blocks Ocl differentiation, but is also a potent inhibitor of Ocl function.

PPAR-γ Agonists Inhibit OPGL Signaling through NF-κB—PPAR-γ antagonizes the transcriptional activity of NF-κB (9), which consists of various combinations of Rel family transcription factors. To delineate the possible mechanism by which PPAR-γ blocked osteoclastogenesis, we determined the effect of PPAR-γ on NF-κB activity. Electrophoretic mobility shift assay was performed on nuclear extracts using the B3 response element of the TNF promoter (27). As shown in Fig. 6, treatment of mouse Ocl precursors with sOPGL increased the DNA binding activity of NF-κB, as did the lipopolysaccharide and TNF-α used as positive controls. Electrophoretic mobility shift assay performed on nuclear extracts from cells treated with sOPGL and 15d-PG-J2 showed that 15d-PG-J2 markedly inhibited sOPGL-induced NF-κB DNA binding activity. Consistent with this observation, IL-6 production which is known to be regulated by NF-κB pathway (22), was stimulated by sOPGL and inhibited by 15d-PG-J2 in mouse Ocl precursors (control, 226 ± 52 ng/mg of proteins; 15d-PG-J2, 173 ± 64 ng/mg of proteins;
PPAR-γ Pathway in Osteoclastogenesis

In this study we found that hMSCs express both OPG and OPGL, two members of the TNF family of key regulators of osteoclastogenesis (24). In contrast, hMSCs that become osteogenic (osteoblasts) following treatment with appropriate stimuli, expressed lower levels of osteoclastogenic cytokines, such as IL-6, IL-11, and leukemia inhibitory factor and the osteogenic hMSCs failed to support osteoclastogenesis (24). Blocking the activities of these cytokines with specific neutralizing antibodies also partially inhibited osteoclastogenesis (24). In this study we found that hMSCs express both OPG and OPGL, two members of the TNF family of key regulators of osteoclastogenesis. Furthermore, osteogenic differentiation of hMSCs is associated with a decline in the ratio of OPGL/OPG, which in turn may reduce the ability of osteogenic hMSCs to support Ocl formation. Addition of sOPGL to the CD34+ HSCs and hMSC co-cultures greatly enhanced Ocl formation. Thus, in light of evidence that cytokines are involved in all aspects of bone metabolism, this co-culture system provides a model useful to characterize downstream factors that regulate cytokine-mediated bone resorption.

An important modulator of cytokine effects is the PPAR-γ nuclear transcription factor, which is involved in a variety of biological processes including inflammation (9, 10), atherosclerosis (12, 13, 31), adipogenesis (14), and angiogenesis (11). We postulated that PPAR-γ might regulate Ocl differentiation because PPAR-γ is expressed by cells of the monocyte/macrophage lineage from which the Ocls arise. We found that Ocl formation induced by sOPGL and M-CSF or by IL-1, IL-3, and GM-CSF in both human and rodents, was inhibited by the addition of PPAR-γ agonists, and that these agonists also inhibited Ocl bone resorbing activity. These agonists might inhibit Ocl formation by stimulating the expression of OPG by hMSCs acting as a decoy receptor that blocks OPGL effects (18, 20), thus altering the OPGL/OPG ratio or down-regulating the expression of receptor activator of NF-κB (RANK), the receptor that mediates OPGL signals (32). Our findings that hMSCs did not express PPAR-γ mRNA, and that PPAR-γ agonists inhibited Ocl formation from mouse Ocl precursors or CD34+ HSCs in the absence of hMSCs strongly suggest that Ocl precursors were the main target cells of PPAR-γ agonists. Furthermore, this hypothesis on up-regulation of OPG expression was ruled out since treatment of hMSCs with 15d-PG-J2 failed to induce PPAR-γ expression in these cells, and the OPGL/OPG ratio was not changed (data not shown). The latter hypothesis on down-regulation of RANK expression may be possible, but the establishment of any negative regulation of RANK expression by 15d-PG-J2 should be secondary to an immediate intracellular event induced by 15d-PG-J2 inside the Ocl precursors. We investigated that possibility.

Based on our data that sOPGL plays a key role in Ocl differentiation, we postulated that PPAR-γ might inhibit Ocl formation induced by sOPGL by inhibiting the activity of NF-κB, shown to be downstream of RANK in certain cell types (29). We found that sOPGL activated NF-κB in Ocl precursors and that this effect was inhibited by 15d-PG-J2. This provides a possible mechanism by which 15d-PG-J2 blocks sOPGL-induced Ocl formation and activity. Our data contrast with a recent report showing that OPGL failed to activate NF-κB in the macrophage RAW cell line (32), but the reason of this discrepancy is not clear. To our knowledge, ours is the first demonstration that PPAR-γ negatively regulates Ocl differentiation induced by OPGL through modulation of NF-κB.

PPAR-γ functions as a receptor for synthetic and naturally occurring substances, including nonsteroidal anti-inflammatory drugs (NSAIDs) (10), antidiabetic thiazolidinediones (33), polyunsatured fatty acids, and 15d-PG-J2 (8). Here we report that 15d-PG-J2 inhibits both Ocl formation and activity normally stimulated by osteoclastogenic cytokines, such as sOPGL. Interestingly, one of the current treatments of pathological bone resorption are NSAIDs (34). Based on this new finding, it is possible to speculate that the inhibition of bone resorption by NSAIDs might have two components: inhibition of prostaglandins (PGs) synthesis and stimulation of PPAR-γ activity. It is also noteworthy that IL-4, a potent inhibitor of Ocl differentiation (35, 36) stimulates the expression of PPAR-γ and the synthesis of PPAR-γ agonists (37). This finding raises the possibility that normal modulation of PPAR-γ expression and/or activity in the Ocls occurs through specific surface re-
This possibility can be exploited in drug targeting the Ocls, thus, avoiding pleiotropic effects of some non-physiological PPAR-γ agonists. Together with a recent report on the anti-angiogenic effects of PPAR-γ agonists (14), we propose that the PPAR-γ pathway might be exploited for the prevention of excessive resorption in diseases such as metastatic bone disease, Paget’s disease, rheumatoid arthritis, and periodontal bone disease.

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