Cross-Talk between the Interleukin-6 and Prostaglandin E₂ Signaling Systems Results in Enhancement of Osteoclastogenesis through Effects on the Osteoprotegerin/Receptor Activator of Nuclear Factor-κB (RANK) Ligand/RANK System

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The osteoprotegerin (OPG)/receptor activator of nuclear factor-κB ligand (RANKL)/receptor activator of nuclear factor-κB (RANK) system is the dominant and final mediator of osteoclastogenesis. Abnormalities of this system have been implicated in the pathogenesis of many skeletal diseases. Cyclooxygenase (COX)-2 and prostaglandin (PG)E₂, a major eicosanoid product of the COX-2-catalyzed pathway, play key roles in normal bone tissue remodeling. PGE₂ exerts its actions by binding and activating the E series of prostaglandin (EP) receptor. Activation of EP₂ and EP₄ receptors is associated with PGE₂-induced osteoclast differentiation. IL-6, a major proinflammatory cytokine, has also been reported to induce osteoclast differentiation. Although interactions between the COX-2/PGE₂ and IL-6 systems have been described in bone cells, the mechanisms underlying these cooperative signaling pathways and the possible involvement of the OPG/RANKL/RANK system have not been fully elucidated. We demonstrate that COX-2, PGE₂, and IL-6 stimulate osteoblast growth and osteoclast differentiation. Effects on osteoclast differentiation, particularly with IL-6, were most marked when osteoclast precursor cells were grown in coculture with osteoblasts, indicating a possible role of the RANK/RANKL/OPG system. COX-2 and PGE₂ stimulated osteoclastogenesis through inhibition of OPG secretion, stimulation of RANKL production by osteoblasts, and up-regulation of RANK expression in osteoclasts. PGE₂ stimulated IL-6 secretion by bone cells, whereas COX-2 inhibitors decreased this same parameter. IL-6, in turn, increased PGE₂ secretion, COX-2, and EP receptor subtype expression in bone cells. Finally, IL-6 was the mediator of PGE₂-induced suppression of OPG production by osteoblasts. These findings provide evidence for cross-talk between the PGE₂ and IL-6 signaling enhance osteoclast differentiation via effects on the OPG/RANKL/RANK system in bone cells. (Endocrinology 146: 1991–1998, 2005)

Bone Tissue Remodeling results from the coordinated activities of osteoblasts and osteoclasts. Osteoclasts are highly specialized multinucleated cells that are uniquely capable of lacunar bone resorption. Osteoblasts are bone-forming cells that are progressively transformed into osteocytes, imprisoned in their own secretory products, which form lacunae after mineralization (1). Imbalances in the bone remodeling process result in metabolic bone diseases characterized by either enhanced osteoclast activity and increased bone resorption (i.e. osteoporosis and osteolytic bone lesions in cancer) or increased osteoblastic bone formation (i.e. osteopetrosis and prostate cancer-induced osteoblastic metastases) (2, 3).

Although both osteogenic cells (osteoblasts and osteoclasts) contribute individually to bone remodeling, their cellular interactions determine the type and extent of bone remodeling. These interactions can be established through cell-cell contact (4) and are mediated by receptor activator of nuclear factor κB ligand (RANKL) on the osteoblasts and receptor activator of nuclear factor κB (RANK) on the osteoclast surface (5, 6). Bone marrow stromal cells (osteoblast precursor cells) also produce a soluble glycoprotein called osteoprotegerin (OPG), a decoy receptor for RANKL that prevents osteoclast activation (5, 7). Osteoblast function is intimately tied to osteoclast activity via osteoblast production of those essential cytokines. Hormones, growth factors, cytokines, and prostaglandins (PGs) regulate these processes (3, 8), mainly via direct effects on osteoblasts and the OPG/RANKL/RANK system.

Cyclooxygenase (COX)-2 is the highly inducible form of COX that catalyzes the conversion of arachidonic acid to PGs. COX-2 acts as a stress response gene and is responsible for the high levels of PG production observed in cancer and inflammation. The roles of COX-2 and PGE₂, a major eicosanoid product of the COX-2-catalyzed reaction, in bone remodeling have been described in studies with mice that are genetically deficient in COX-2. Mice lacking COX-2 expression display reduced bone resorption in response to PTH or 1,25-dihydroxy vitamin D₃ (9). Systemic or local injection of PGE₂ stimulates bone formation in response to mechanical strain (10, 11), and this effect is also mediated by the COX-
2-catalyzed pathway (12). More recently COX-2 has been demonstrated to be a critical regulator of mesenchymal cell differentiation into osteoblasts and an essential element in bone repair (13). It has also been reported that COX-2 and PGE2 play important roles in osteoclast formation in vitro (14) and are required for debris-induced osteoclastogenesis and osteolysis in an in vivo mouse calvaria model (15). These data indicate that COX-2 and PGE2 are involved in both cytokine-mediated osteoclast activation and osteoblastic bone formation. Moreover, it has been demonstrated that the actions of PGE2 in bone homeostasis are mediated via the cAMP signaling pathway, activated by PGE2 binding to the E series of prostaglandin (EP) receptors, particularly the EP2 and EP4 receptor subtypes (16–19).

IL-6, originally identified as a B cell-differentiated factor, is a multifunctional cytokine that, apart from its immunomodulatory effects, enhances osteoclastic bone resorption and has thus been implicated in postmenopausal osteoporosis, Paget's disease, and rheumatoid arthritis (20). IL-6 is produced by both immune and nonimmune cells, including bone stromal cells (osteoblasts and synoviocytes) (21). IL-6 has been proposed to increase osteoclast recruitment by acting on early hematopoietic cells from the granulocyte-macrophage lineage that contain the progenitors of the osteoclastic lineage (22). In multiple myeloma, IL-6 is one of the key cytokines that promotes bone destruction (23). The mechanisms underlying these effects, however, are not well delineated. IL-6 exerts its actions by binding to either the cognate IL-6 receptor or, in most cases, the soluble IL-6 receptor leading to dimerization of gp130 (24, 25). These events result in the simultaneous activation of signal transducer and activator of transcription (STAT)-1/3 and MAPK signaling and the induction of target proteins (26, 27). It has been demonstrated that IL-6 induction of osteoclast differentiation is dependent on IL-6 receptors expressed in osteoblast cells (28).

Several lines of evidence support the notion that IL-6 interacts with PGE2 in different systems. EP4 receptor knockout mice have reduced circulating levels of IL-6 and significantly less IL-6 production by liver and macrophages (29). COX-2 promotion of human oropharyngeal carcinoma growth has been reported to be mediated by IL-6 (30). Moreover, we observed that PGE2 stimulation of human prostatic intraepithelial neoplasia cell growth is through activation of the IL-6/gp130/STAT-3 signaling pathway (31). Stromal osteoblast cells support osteoclast differentiation by their ability to secrete IL-6 and RANKL, in response to PTH, 1,25-dihydroxy vitamin D3, and PGE2 (26, 32, 33). These data provide mechanistic evidence of cross-talk between the COX-2/PGE2 and IL-6 signaling systems.

In the present study, we demonstrate that COX-2 and PGE2 stimulate osteoclast formation through inhibition of OPG secretion and enhancement of RANKL production by osteoblasts and stimulation of RANK expression in osteoclasts. We also demonstrate that, although IL-6 has little direct effect on the regulation of OPG/RANKL/RANK system, IL-6 enhances PGE2 production and PGE2 receptor expression, particularly the EP4 subtype, in both osteoblast and osteoclast cells. These findings provide evidence that the COX-2/PGE2 and IL-6 systems have interactive effects on osteoclast activation that are mediated by the OPG/RANKL/RANK system.

### Materials and Methods

#### Cell culture and reagents

The osteoblastic MC3T3 cell line and murine monocyte/macrophage-like cell line, RAW 264.7, commonly used as an osteoclast precursor cell phenotype, were obtained from American Type Culture Collection (Manassas, VA). Cells were cultured in a humidified 5% CO2 incubator at 37°C with DMEM containing 10% fetal bovine serum (FBS). For coculture experiments, MC3T3 cells were seeded in 12-well cluster culture plates, and RAW264.7 cells were seeded in cell-culture inserts with 0.4 μm pores. NS398, PGE2, PGE2 alcohol, Butaprost, and PGE2 ELISA detection kits were purchased from Cayman Chemical Co. (Ann Arbor, MI); Meloxicam was purchased from BioMol Inc. (Plymouth Meeting, PA); IL-6 (mouse origin) was obtained from Calbiochem Co. (San Diego, CA). IL-6 neutralizing antibodies and ELISA kits for OPG and IL-6 detection were purchased from R&D Systems (Minneapolis, MN). Antibodies against COX-2 were purchased from Oxford Biomed Inc. (Oxford, MI), and anti-RANK, RANKL, and actin antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). All antibodies react with mouse tissues.

#### Proteins isolation and immunoblotting

MC3T3 and RAW264.7 cells, cultured under the indicated conditions, were lysed as described previously (31). Briefly, cells were rinsed twice with ice-cold PBS and scraped with 1.5 ml PBS containing 4 mM iodoacetate. After centrifugation, the pellets were resuspended in [3-(cholamidopropyl)dimethylamino]1-propaneamino sulfonate extraction solution [10 mM [3-(cholamidopropyl)] dimethyl-ammonio]-l-propane-sulfonate, 2 mM EDTA (pH 8.0), and 4 mM iodoacetate in PBS] with protease inhibitors. The samples were then incubated for 30 min on ice and centrifuged at 15,000 × g for 10 min. The supernatants were collected and stored at −70°C. To prepare the protein samples from the culture medium for the detection of soluble RANKL (sRANKL) by Western blotting, conditioned medium was collected, briefly purified by centrifugation, and concentrated 100-fold using a centrifugal filter device (Cen-tricon-10, Amicon Inc., Beverly, MA). For Western blotting, samples were electrophoresed on SDS-PAGE, transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA), and incubated with target antibodies overnight at 4°C. Secondary horseradish peroxidase-linked donkey anti-mouse IgG (Amersham, Arlington Heights, IL) was used. Filters were developed with the enhanced chemiluminescence system (Amersham). Actin was used as the internal control in all Western blot analyses.

#### Osteoclast formation assay

Single-cell suspensions (10^6 cells/well) of RAW264.7 cells were plated in 24-well plates in RPMI 1640 supplemented with 10% FBS overnight. Medium was changed to RPMI 1640 plus 0.5% FBS and treated with or without various reagents. At the end of the experiment, cells were fixed with citrate/formalin/acetone and stained for tartrate-resistant acid phosphatase (TRAP) using a commercially available kit according to the manufacturer’s instructions (Sigma, St. Louis, MO). The number of osteoclast-like cells was determined by counting TRAP-positive multinucleated cells with three or more nuclei.

#### Preparation of conditioned medium for ELISA

Conditioned medium was prepared as described previously (34). Briefly, cells were plated at 1 × 10^5 cells/well in six-well cluster dishes with 2 ml medium containing 10% FBS. After washing with PBS, serum-free medium was replaced. Incubations were continued under the desired conditions. The culture medium was collected, centrifuged at 800 × g for 10 min to remove suspended cells, and stored at −70°C for assays.

#### RT-PCR

Cells were incubated in serum-free medium. Total RNA was extracted with Trizol reagent (Life Technologies, Inc.-BRL, Gaithersburg,
MD). cDNA was prepared by incubating 1 μg total RNA in 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 10 mM dithiothreitol, and RNase inhibitors with 250 U reverse transcriptase, 1 μM of each deoxyribozyme triphosphate and random primers (0.05 μM, Life Technologies, Inc.-BRL) for 60 min at 37°C. The fragment was amplified by PCR using specific primers. A set of specific PCR primers for EP receptor subtypes was prepared as previously reported (35): EP₁ fragment, forward (5′-ACCAGCTGGCAGCCACTGCA-3′; 321–342) and reverse (5′-CGCTGACGGTGTGCACACACG-3′; 750–729); EP₂ fragment, forward (5′-TCAACTGACCTGGCTATGAGTCC-3′; 396–415) and reverse (5′-AGTTATGCAGAAGCTACTGGC-3′; 904–884); and EP₃ fragment forward (5′-GGCTGCCGGCATCAGCTGAC-3′; 456–585) and reverse (5′-CTGCCGGCTCATGAAACTGCCGC-3′; 1050–1030). Primers for β-actin were: forward (5′-GAAGACTAGCAGACTGTCG-3′; 2376–2393) and reverse (5′-TGACCCACATCTCTGTCGA-3′; 2927–2944). PCR was initiated in a thermal cycle programmed at 95°C for 5 min, 94°C for 30 sec, 58°C for 30 sec, and 72°C for 45 sec and amplified with 28 cycles for β-actin and 35 cycles for EP receptor subtypes. The amplified products were visualized on 1.5% agarose gels.

**Statistical analysis**

All results are given as mean ± se. The effects induced by the various treatments were compared with untreated control cells using paired Student’s t test with the Bonferroni adjustment for the comparison of multiple groups. P < 0.05 was considered significant.

**Results**

**Effects of PGE₂ and IL-6 on osteoblast growth and osteoclast formation**

We initially determined the effects of PGE₂ and IL-6 on osteoblast growth and osteoclast differentiation. As shown in Fig. 1A, PGE₂ and IL-6 stimulated MC3T3 osteoblast cell growth by 2- and 1.4-fold over control, respectively, after a 3-d culture period. In contrast, selective COX-2 inhibitors decreased osteoblast cell growth. We tested two distinct COX-2 inhibitors, NS398 and meloxicam. NS398 is a potent selective COX-2 inhibitor that has also been reported to exert COX-2-independent effects via direct inhibition of MAPK activity (36). Meloxicam is another selective COX-2 inhibitor that has a different chemical structure from NS398 and no reported COX-2-independent effects. In this experiment, both inhibitors significantly suppressed MC3T3 osteoblast cell growth by 70 and 77%, respectively, over the control. We next determined whether the effects of the two inhibitors could be reversed by the addition of exogenous PGE₂. As shown in Fig. 1A, the suppression of MC3T3 cell growth by both NS398 and Meloxicam was reversed by PGE₂ addition, suggesting that both compounds were inhibiting osteoblast growth via COX-2-dependent mechanisms (Fig. 1A).

We next determined which subtype of EP receptor mediates the demonstrated effects of PGE₂ on osteoblast proliferation. Butaprost (a selective EP₂ receptor agonist) and PGE₁-alcohol (a selective EP₂ receptor agonist) were added to cultures of MC3T3 cells. Whereas Butaprost had no significant effect on MC3T3 cell growth, PGE₁-alcohol induced a significant increase in osteoblast cell growth (2.4-fold) (Fig. 1A), indicating that PGE₂-stimulated osteoblast cell proliferation is mainly mediated by the EP₄ receptor subtype.

Figure 1B demonstrates the effects of PGE₂, IL-6, and the two distinct COX-2 inhibitors on RAW 264.7 cell osteoclast differentiation, as measured by the TRAP assay. When RAW 264.7 cells were grown alone, the addition of PGE₂ exerted
a minimal stimulatory effect (28% increase) on osteoclast differentiation. Both selective COX-2 inhibitors induced a mild, but statistically significant, decrease in this parameter. IL-6 addition had no effect on osteoclast differentiation when RAW 264.7 cells were grown in the absence of osteoblast cells (Fig. 1B). In contrast, when RAW264.7 cells were grown in coculture with MC3T3 osteoblast cells, both PGE2 and IL-6 significantly increased RAW 264.7 differentiation into osteoclasts and COX-2 inhibitors also had a more dramatic inhibitory effect in the cocultures. The effects of both selective COX-2 inhibitors on osteoclast differentiation were reversed by the addition of exogenous PGE2, again indicating that both NS398 and Meloxicam are acting via COX-2-dependent mechanisms to inhibit osteoclast differentiation (Fig. 1B).

Effects of PGE2 and IL-6 on RANKL production and RANK expression

Osteoblast function is intimately tied to osteoclast activity via osteoblast production of cytokines that either stimulate (RANKL) or inhibit (OPG) osteoclast activation. Osteoblast-derived RANKL binds to either RANK on osteoclasts, resulting in osteoclast activation, or OPG, a decoy receptor for RANKL, inhibiting osteoclast activation (5). Interestingly, the source of both RANKL and its decoy receptor (OPG) is primarily osteoblast cells (37, 38). Our initial studies demonstrated enhanced activity of both PGE2 and IL-6 on osteoclast differentiation when added to cocultures of osteoclast precursor and osteoblast cells. Therefore, we hypothesized that the effects of these factors were mediated by the RANK/RANKL/OPG system.

As shown in Fig. 2A, Western blot analysis revealed that PGE2 increased the expression of membrane-bound RANKL in MC3T3 osteoblast cells, as identified by the band at the molecular mass of 40 kDa (39). Both selective COX-2 inhibitors decreased RANKL expression in these same cells. We also examined secreted sRANKL levels (in the presence or absence of PGE2) in concentrated culture medium from MC3T3 cells. sRANKL appeared on immunoblots as a band at the molecular mass of 26 kDa, as previously reported (39, 40). Figure 2A also demonstrates that sRANKL expression in osteoblasts was increased by the addition of PGE2 and decreased by addition of either selective COX-2 inhibitor. In contrast, IL-6 showed no detectable effect on the expression of either membrane-bound or sRANKL in osteoblast cells. Osteoclast precursor cells also had some basal expression of RANKL, the levels of which were unchanged by the addition of either PGE2 or IL-6 (data not shown).

We next determined the effects of COX-2, PGE2, and IL-6 on RANK expression in RAW264.7 osteoclast precursor cells. Figure 2B demonstrates that both IL-6 and PGE2 increased the expression of RANK in osteoclasts. The two distinct COX-2 inhibitors, NS398 and Meloxicam, decreased RANK expression in these cells. These data indicate that both PGE2 and IL-6 increase RANK expression on osteoclast precursor cells.

Effects of PGE2 and IL-6 on OPG secretion and expression

OPG was initially cloned and characterized as a soluble decoy receptor belonging to the TNF receptor superfamily and was subsequently found to be a decoy receptor for RANK that inhibits RANKL activation of osteoclasts (7). We next examined the effects of PGE2 and IL-6 on OPG production by MC3T3 osteoblast cells. Figure 3A demonstrates that PGE2 addition decreases OPG secretion by osteoblasts. In contrast, NS398, a selective COX-2 inhibitor, significantly stimulates OPG secretion by MC3T3 cells. Although exogenous IL-6 alone had no detectable effect, IL-6 neutralizing antibodies increased OPG secretion and reversed the observed suppression of OPG secretion by PGE2.

Similar effects of COX-2, PGE2, and IL-6 on OPG protein expression in osteoblast cells as determined by Western blotting were observed (Fig. 3B). MC3T3 cells expressed the full-length OPG protein. Expression levels of OPG were decreased by PGE2 and increased by the COX-2 inhibitor, NS398. IL-6 addition had no demonstrable effect on OPG cellular expression levels. However, neutralizing antibodies to IL-6 increased basal OPG cellular expression and blocked the inhibitory effect of PGE2 addition on this parameter.
Reciprocal interactions between the IL-6 and PGE2 systems

The effects of COX-2 and PGE2 on bone remodeling appear to be mediated, at least in part, by proinflammatory cytokines (13, 14, 42). In the next series of experiments, we investigated the reciprocal interactions between the IL-6 and PGE2 signaling pathways. As shown in Fig. 4A, basal IL-6 secretion was 10-fold higher in RAW264.7 osteoclast precursor cells than in MC3T3 osteoblasts. PGE2 significantly stimulated IL-6 production in both cell lines, although this effect was more marked in the MC3T3 cells than in the RAW264.7 cells (5.7-fold and 2.1-fold increases in IL-6 secretion after PGE2 addition in MC3T3 vs. RAW264.7 cells, respectively).

The two distinct COX-2 inhibitors, NS398 and Meloxicam, significantly inhibited IL-6 secretion in both MC3T3 and RAW264.7 cells. However, NS398, which, as noted, has additional COX-2-independent effects on MAPK inhibition, decreased IL-6 secretion by osteoclast cells more significantly than Meloxicam (Fig. 4A).

The effects of IL-6 addition on COX-2 expression and PGE2 secretion in these cell lines are demonstrated in Fig. 4, B and C, respectively. RAW264.7 osteoclast precursor cells expressed high basal levels of COX-2, whereas COX-2 basal expression in MC3T3 cells was barely detectable as determined by immunoblotting. After IL-6 addition, a significant, time-dependent increase in COX-2 expression was observed in MC3T3 cells. IL-6 also increased COX-2 expression in the RAW264.7 osteoclast cells, although, because of their higher basal COX-2 expression levels, the effect of IL-6 was less dramatic (Fig. 4B).

Figure 4C demonstrates the effect of IL-6 addition on PGE2 secretion in the osteoclast precursor and osteoblast cell lines. PGE2 secretion, both basal and IL-6 induced, paralleled the observed COX-2 expression levels in these cells. Basal PGE2 secretory levels were 2 times higher in RAW264.7 cells than in MC3T3 cells. IL-6 addition had little effect on PGE2 secretion in RAW264.7 cells but significantly increased PGE2 secretion in MC3T3 osteoblast cells (Fig. 4C).

Our previous experiments suggested that the EP4 is a major subtype of PGE2 receptor that mediates PGE2 effects on bone cells (35). In addition, a recent report demonstrated that homozygous deletion of the EP4 decreased the incidence and severity of rheumatoid arthritis and resulted in lower detectable IL-6 levels in a mouse model (29). The effect of IL-6 on the expression of EP receptor subtypes in the bone cell lines was next assessed. As shown in Fig. 5A, RT-PCR revealed that both MC3T3 and RAW264.7 cells express the EP2 subtype mRNA but do not express detectable levels of EP1, EP2, and EP3 subtype mRNA (data not shown). The expression levels of EP4 mRNA in both cell lines were increased by IL-6. Although MC3T3 osteoblast cells had undetectable basal expression levels of the EP2 receptor subtype, EP2 mRNA expression was induced by IL-6 addition. RAW264.7 cells expressed neither basal nor inducible EP2 mRNA (data not shown). We also examined the effects of IL-6 on EP4 and EP2 protein expression in MC3T3 and RAW264.7 cells. Western blot analysis revealed a similar expression and induction pattern of EP4 and EP2 receptor subtype protein levels by IL-6 as that seen at the mRNA level (Fig. 5B).
Enhanced osteoclastogenesis leading to bone loss is a hallmark of various forms of metabolic bone diseases, including postmenopausal and glucocorticoid-induced osteoporosis. In the bone microenvironment, osteoclast differentiation is regulated by the coordinated synthesis and action of cytokines produced by bone marrow stromal cells and osteoblasts. RANKL and OPG have been identified as members of a ligand-receptor system that directly regulates osteoclast differentiation and bone resorption. Soluble and membranous forms of RANKL, as well as OPG, are preferentially produced by osteoblasts, whereas the specific receptor RANK is expressed on osteoclast progenitors. RANKL binds to either RANK, resulting in osteoclast activation, or OPG, a decoy receptor for RANKL, thereby inhibiting osteoclast activation (5). Several investigators have reported that a variety of hormones and local factors, i.e. PTH, PGs (particularly PGE2), and cytokines such as IL-6, modulate the balance among RANKL, OPG, and RANK in the bone microenvironment (3, 8).

In the present study, we demonstrate that PGE2, derived from the COX-2-catalyzed pathway, increases both osteoblast proliferation and osteoclast differentiation. These results confirm the observation that disruption of COX-2 gene expression results in defective osteoblast secretion of RANKL and impaired osteoclast formation in response to a variety of hormones (9). The COX-2 pathway has also been implicated in osteoclast formation and bone destruction in bone metastases of mammary carcinoma cells (14). Although some effects of PGE2 on the RANK/RANKL/OPG system have been previously reported, those studies generally used isolated cultures of either osteoclasts or osteoblasts (9, 17). We demonstrate that the effects of PGE2 on osteoclasts were more pronounced when they were grown in coculture with osteoblasts. This enhancement of the PGE2 effect on osteoclasts is regulated by the coordinated synthesis and action of cytokines produced by bone marrow stromal cells and osteoblasts. RANKL and OPG have been identified as members of a ligand-receptor system that directly regulates osteoclast differentiation and bone resorption. Soluble and membranous forms of RANKL, as well as OPG, are preferentially produced by osteoblasts, whereas the specific receptor RANK is expressed on osteoclast progenitors. RANKL binds to either RANK, resulting in osteoclast activation, or OPG, a decoy receptor for RANKL, thereby inhibiting osteoclast activation (5). Several investigators have reported that a variety of hormones and local factors, i.e. PTH, PGs (particularly PGE2), and cytokines such as IL-6, modulate the balance among RANKL, OPG, and RANK in the bone microenvironment (3, 8).

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Discussion

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ocal interactions with the COX-2/PGE2 system. Specifically, an increase in RANK expression by osteoclasts and via reciprocation of osteoclast differentiation in cocultures appeared to be due to an secretion by osteoblasts in one study (39), we found little mediation of both these elements of bone remodeling are only partially understood. Although there is convincing evidence that IL-6 alone has no direct effect on osteoclast-like cells in vitro (46, 47) or in vivo (48), neutralizing anti-IL-6 antibodies and anti-IL-6 receptor antibodies have been reported to inhibit the stimulatory effects of 1,25-dihydroxy vitamin D3 on osteoclastogenesis (47, 49). Similarly, PTH-induced bone resorption in cocultures of osteoblasts and osteoclasts has been shown to be partially dependent upon IL-6 receptor activation (50).

We demonstrate that IL-6 increases osteoblast proliferation. IL-6 did not significantly stimulate osteoclast differentiation, unless the osteoclasts were grown in coculture with osteoblasts. Although IL-6 was reported to increase RANKL secretion by osteoblasts in one study (39), we found little effect on this parameter. Instead, IL-6 stimulation of osteoclast differentiation in cocultures appeared to be due to an increase in RANK expression by osteoclasts and via reciprocal interactions with the COX-2/PGE2 system. Specifically, neutralizing antibodies to IL-6 increased basal OPG secretion and blocked the inhibitory effect of PGE2 on this parameter, indicating that endogenous IL-6 mediates PGE2 effects on osteoblast OPG secretion. IL-6 also increased COX-2 expression and PGE2 production in osteoblasts. IL-6 has previously been reported to increase COX-2 expression in osteoblast cultures, although the effect noted in that report was modest and only seen when IL-6 was combined with the soluble IL-6 receptor (41). The osteoblast cultures used in that study differed from the osteoblast cell line employed in the current study, and that may account for the higher degree of COX-2 induction by IL-6 alone in our report. Finally, we demonstrate that IL-6 increases the expression of both the EP4 and EP2 receptor subtypes in bone cells.

PGE2 exerts its actions by binding and activating EP receptors. Among the four subtypes of EP receptor, EP4 is the only subtype expressed in both osteoblasts and osteoclasts (51). EP4 receptor is critical in mediating the effects of PGE2 on RANKL-induced osteoclastogenesis (17, 18, 52) and osteoblastic development (51, 53). Homozygous deletion of the EP4 receptor subtype was shown to decrease the incidence and severity of rheumatoid arthritis in a mouse model (29). Although EP2 is not constitutively expressed in bone cells, some involvement of EP2 in osteoclastogenesis has been reported (17, 19). There is evidence that IL-6 interacts with the EP receptor system in inflammatory carcinogenesis and rheumatoid arthritis (30, 54), through activation of the gp130/STAT-3 signaling pathway (31, 55). In this study, we provide evidence that IL-6 up-regulates the expression of the EP2 receptor subtype in both osteoblasts and osteoclast precursors. In addition, IL-6 induces EP2 receptor subtype expression in osteoblasts. The induction of EP4 and EP2 by IL-6 in osteoblasts, in turn, amplifies the effects of PGE2 on the inhibition of OPG production and stimulation of membrane-bound RANKL expression and sRANKL release. All of these events, coordinately, tip the balance of the RANKL/OPG/RANK system in favor of increased osteoclastogenesis and enhanced bone resorption.

In conclusion, we provide in vitro evidence of cross-talk between the COX-2/PGE2 and IL-6 systems that results in increased osteoclast differentiation via effects on the RANK/ RANKL/OPG system in bone cells. Both PGE2 and IL-6 have demonstrated effects on bone remodeling. Osteoblast-derived IL-6 and PGE2 are critically involved in postmenopausal osteoporosis as well as rheumatoid arthritis (43, 45, 54). These findings provide a rationale for the treatment of these disorders with selective COX-2 inhibitors because they would serve to decrease osteoclast activation due to either PGE2 or IL-6 stimulation.

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References

10. Weinreb M, Suponitzky I, Keila S 1997 Systemic administration of an anabolic...

25. Legrand-Poels S, Schoonbroodt S, Piette J

26. Kishimoto T


1998 Interaction of triiodothyronine with 1α,25-dihydroxyvitamin D₃ on interleukin-6-dependent osteoclast-like cell formation in mouse bone marrow cell culture. Bone 22:237–242


1996 Inducible cyclooxygenase-2 mediates the induction of bone loss in young rats increase the osteogenic capacity of bone marrow. Bone 20:521–526


1999 Inhibition of constitutively activated Stat-3 signaling pathway suppresses growth of prostate cancer cells. Cancer Res 60:1225–1228

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