IL-6, Leukemia Inhibitory Factor, and Oncostatin M Stimulate Bone Resorption and Regulate the Expression of Receptor Activator of NF-κB Ligand, Osteoprotegerin, and Receptor Activator of NF-κB in Mouse Calvariae

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*J Immunol* 2002; 169:3353-3362; doi: 10.4049/jimmunol.169.6.3353
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IL-6, Leukemia Inhibitory Factor, and Oncostatin M Stimulate Bone Resorption and Regulate the Expression of Receptor Activator of NF-κB Ligand, Osteoprotegerin, and Receptor Activator of NF-κB in Mouse Calvariae

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IL-6, leukemia inhibitory factor (LIF), and oncostatin M (OSM) are IL-6-type cytokines that stimulate osteoclast formation and function. In the present study, the resorptive effects of these agents and their regulation of receptor activator of NF-κB ligand (RANKL), RANK, and osteoprotegerin (OPG) were studied in neonatal mouse calvaria. When tested separately, neither human (h) IL-6 nor the human soluble IL-6R (shIL-6R) stimulated bone resorption, but when hIL-6 and the shIL-6R were combined, significant stimulation of both mineral and matrix release from bone explants was noted. Semiquantitative RT-PCR showed that hIL-6 plus shIL-6R enhanced the expression of RANKL and OPG in calvarial bones, but decreased RANK expression. Human LIF, hOSM, and mouse OSM (mOSM) also stimulated 45Ca release and enhanced the mRNA expression of RANKL and OPG in mouse calvaria, but had no effect on the expression of RANK. In agreement with the RT-PCR analyses, ELISA measurements showed that both hIL-6 plus shIL-6R and mOSM increased RANKL and OPG proteins. 1,25-Dihydroxyvitamin D3 (D3) also increased the RANKL protein level, but decreased the protein level of OPG. OPG inhibited 45Ca release stimulated by RANKL, hIL-6 plus shIL-6R, hLIF, hOSM, mOSM, and D3. An Ab neutralizing mouse gp130 inhibited 45Ca release induced by hIL-6 plus shIL-6R. These experiments demonstrated stimulation of calvarial bone resorption and regulation of mRNA and protein expression of RANKL and OPG by D3 and IL-6 family cytokines as well as regulation of RANK expression in preosteoclasts/osteoclasts of mouse calvaria by D3 and hIL-6 plus shIL-6R. The Journal of Immunology, 2002, 169: 3353–3362.

osteoclasts arise from hemopoietic precursor cells of the monocyte/macrophage family (1). The formation of fully differentiated, bone-resorbing osteoclasts from progenitor cells is mediated by stromal cells/osteoblasts (1). M-CSF, receptor activator of NF-κB ligand (RANKL), and osteoprotegerin (OPG) are key factors regulating osteoclastogenesis (1–4). M-CSF is a secreted product of stromal cells/osteoblasts that enhances macrophage and osteoclast survival. RANKL is a transmembrane protein produced in stromal cells/osteoblasts. RANKL plays an essential role in osteoclast differentiation and function by binding to receptor activator of NF-κB (RANK) on osteoclast progenitor cells. The interaction between RANK and RANKL can be blocked by OPG, a soluble protein released from stromal cells/osteoblasts that acts as a decoy receptor for RANKL. Mice that do not express RANKL or RANK, or are deficient in M-CSF, do not have functional osteoclasts and develop osteopetrosis (5–8). In contrast, mice with a targeted deletion of opg develop multiple fractures and have decreased trabecular bone volume and numerous osteoclasts (9).

IL-6-type cytokines are a family of cytokines composed of IL-6, IL-11, leukemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor, cardiotrophin-1, and novel neutrophin-1/B cell stimulatory factor-3 (10, 11). These agents use the same receptor subunit, gp130, for signaling (12) and often have similar and overlapping functions. Signaling by the IL-6-type cytokines involves binding to specific receptors and activation of Janus kinases and transcription factors of the STAT family (10). The IL-6R, gp80, can exist either as a membrane-bound or a soluble protein (sIL-6R) (13). Binding of IL-6 to either the membrane-bound or soluble form of IL-6R promotes homodimerization of gp130 molecules, whereas the membrane receptors for LIF and OSM heterodimerize with gp130. Recent studies with dominant negative STAT3 and gp130 cells have shown that IL-6-type cytokines can stimulate RANKL expression in stromal cells/osteoblasts by mechanisms involving gp130 and increased STAT transcriptional activity (14).

Although few data currently exist defining the roles of LIF and OSM in skeletal turnover in the human, IL-6 has been suggested to be an important mediator of pathological bone loss (15–22). Estrogen decreases the expression of IL-6 in stromal cells/osteoblasts (23), and the loss of bone caused by ovarectomy in mice can be prevented by infusion of Abs neutralizing IL-6 (24) or by IL-6...
gene disruption (25). These observations have strongly implicated IL-6 in the decrease in bone mass that occurs in postmenopausal osteoporosis (15). In addition, enhanced IL-6 levels have been noted in a number of other conditions characterized by excessive loss of bone, such as periodontal disease (17, 18), Paget’s disease (19), multiple myeloma (20), rheumatoid arthritis (21), and hyperparathyroidism (22). However, IL-6 has not been noted to be an effective stimulator of osteoclast formation in mouse bone marrow cultures unless combined with sIL-6R (26). Furthermore, when sIL-6R and IL-6 are not combined in commonly employed in vitro bone cell culture assays, such as neonatal mouse calvarial bones, bone resorption does not occur (27, 28).

Studies in rodents have indicated that LIF and OSM are involved in the regulation of bone remodeling and bone cell function (reviewed in Refs. 29 and 30). Like IL-6, LIF is produced by osteoblasts in response to parathyroid hormone (PTH) stimulation (31, 32). Both in vivo and in vitro studies have shown that LIF stimulates osteoclastogenesis and bone resorption in mouse calvaria (33, 34). Increased osteoclastogenesis has also been observed in mouse bone marrow cultures following treatment with LIF and OSM (35, 36). Consistent with these observations, transgenic mice overexpressing LIF have been noted to exhibit increased osteoclastic bone resorption (37). However, when mice with targeted deletions of the LIF receptor gene have been studied, they have been found to have increased numbers of osteoclasts and decreased bone volume (38), findings more in line with reports that have indicated small, but significant, inhibition of basal bone resorption in fetal mouse and rat long bones treated with LIF and OSM (39, 40).

While it is known that cytokines in the IL-6 family can stimulate bone resorption, few data defining the effects of IL-6-type cytokines on the expression of RANKL, RANK, and OPG exist. The aims of the present study were to: 1) determine whether IL-6 to–kines on the expression of RANKL, RANK, and OPG exist. The

Materials and Methods

Materials

Recombinant human IL-6 (hIL-6), recombinant mouse IL-6 (mIL-6), recombinant human sIL-6R (shIL-6R), recombinant human LIF (hLIF), recombinant human OSM (hOSM), recombinant mouse OSM (mOSM), recombinant human IL-1β, recombinant mRANKL and mOPG fusion proteins, neutralizing anti-mouse gp130 Ab, and mOPG and mRANKL immunoassay kits were purchased from R&D Systems (Abingdon, U.K.); FBS was obtained from ICN Pharmaceuticals (Costa Mesa, CA); acetazolamide, hydroxyurea, and essentially acid free fetal BSA were purchased from Sigma-Aldrich (St. Louis, MO); oMEM, TRIzol LS reagent, and oligonucleotide primers were obtained from Life Technologies (Paisley, U.K.); First Strand cDNA Synthesis Kit and PCR Core Kit were purchased from Roche (Mannheim, Germany); [3H]CaCl2, RIA kit for PGE2, and l-[3H]proline were obtained from Amersham (Little Chalfont, U.K.); synthetic bovine PTH (PIT-1–34) was obtained from Bachem (Bubendorf, Switzerland); Triton X-100 was purchased from Merck Eurolabs (Darmstadt, Germany); culture dishes and multiwell plates were obtained from Costar (Cambridge, MA). Indomethacin was supplied by Merck, Sharp & Dohme (Haarlem, The Netherlands), flurbiprofen by Boots (Nottingham, U.K.), and 3-amino-1-hydroxy-propylidene-1,1-bisphosphonate (AHPBP) by Henkel (Dusseldorf, Germany). Colchicine was obtained from Sandoz (Basel, Switzerland), and 1,25-dihydroxyvitamin D3 (D3) was supplied by Hoffmann-LaRoche (Basel, Switzerland).

Bone organ culture

Mouse calvarial bone cultures were used as a bone resorption assay and for PGE2 quantifications, PLR analysis and ELISA measurements. Parietal bones from 6- to 7-day-old C57 mice were dissected and cut into four pieces. The bones were preincubated for 18–24 h in αMEM containing 0.1% albumin and 1 μMOL indomethacin. Following preincubation, the bones were extensively washed and subsequently cultured for up to 96 h in multiwell culture dishes containing 1.0 mM indomethacin-free medium with or without test substances (41, 42). The bones were incubated in the presence of 5% CO2 in humidified air at 37°C.

Measurements of mineral release

Mineral mobilization was assessed by analyzing the release of 45Ca from bones prelabeled in vivo. In most experiments 2- to 3-day-old mice were injected with 1.5 μCi [45]CaCl2, and the amount of radioactivity in bone and culture medium was analyzed by liquid scintillation at the end of the culture period. For the time-course experiments, the mice were injected with 12.5 μCi [3H]CaCl2, and radioactivity was analyzed at different time points by withdrawal of small amounts of culture medium. Isotope release was expressed as the percent release of the initial amount of isotope (calculated as the sum of radioactivity in medium and bone after culture) (40). In some experiments the data were recalculated, and the results were expressed as a percentage of the control, which was set at 100%. This allowed for accumulation of data from several experiments.

Measurements of matrix degradation

Bone matrix degradation was assessed by analyzing the release of [3H]H from bones dissected from mice prelabeled with 10 μCi [3H]proline 4 days before dissection. The bones were dissected, preincubated, and cultured in αMEM with or without test substances as described above. The amount of [3H] in the medium at the end of the experiments was analyzed by liquid scintillation. At the end of the culture period, the bones were hydrolyzed, and the radioactivity in the hydrolysates and media was analyzed. The release of [3H]Hproline plus [3H]hydroxyproline parallels the release of [3H]hydroxyproline and thus is a reliable indicator of collagen breakdown.

Measurements of prostaglandin biosynthesis

PG formation in the calvarial bones was assessed by analyzing the concentration of PGE2 in the culture medium at the end of the cultures. PGE2 was determined using a commercially available RIA by following the instructions of the manufacturer.

Isolation of mouse calvarial osteoblasts

Bone cells were isolated from calvaria from 2- to 3-day-old mice using the modified time sequential enzyme digestion technique described by Boonekamp et al. (43). Cells from populations 6–10, showing an osteoblastic phenotype as assessed by their cAMP responsiveness to PTH, expression of alkaline phosphatase, osteocalcin and bone sialoprotein expression, as well as the capacity to form mineralized bone nodules (data not shown), were used. The cells were incubated in αMEM/10% FBS at a density of 10 × 103 cells/cm2 in culture dishes and cultured for 48 h before RNA isolation.

RNA isolation and first-strand cDNA synthesis

Calvarial osteoblasts were incubated for 48 h in culture dishes (60 cm2) containing αMEM/10% FBS without test substances, and then RNA was isolated. Calvarial bones, a total of five calvarial halves per group, were incubated in 24-well plates in the absence or the presence of hIL-6 plus sIL-6R, D3, hLIF, hOSM, and mOSM for 24 h before RNA isolation. The five bones belonging to the same group were pooled before total RNA was extracted with TRIzol LS reagent following the manufacturer’s protocol. RNA was quantified spectrophotometrically, and the integrity of the RNA preparations was examined by agarose gel electrophoresis. Only RNA preparations showing intact species were used for subsequent analysis. One microgram of total RNA was reverse transcribed into single-stranded cDNA with a First Strand cDNA Synthesis Kit using oligo(dT)12,18 primers. After incubation at 25°C for 10 min and at 42°C for 60 min, avian myeloblastosis virus reverse transcriptase was denatured at 99°C for 5 min. cDNA was kept at −20°C until used for PCR.

Polymerase chain reactions

The synthesized cDNA was amplified by PCR using a PCR Core Kit and PC-960G Gradient Thermal Cycler (Corbett Research, Sydney, Australia). The PCR analyses for TRAP, GAPDH, RANKL, OPG, RANK, and OSM receptor (OSMR) were performed using PCR Core Kit standard protocol. In the PCR reactions for IL-6, the final MgCl2 concentration was changed from 1.5 to 1 mM, and to 2 mM for gp130 and LIF receptor (LIFR). The conditions for PCR of TRAP, GAPDH, gp130, IL-6R, LIFR, and OSMR were: denaturing at 94°C for 2 min, annealing for 40 s at 58°C for TRAP,
at 57°C for GAPDH, gp130, IL-6R, and OSMR, and at 55°C for LIFR, followed by elongation at 72°C for 60 s; in subsequent cycles denaturation was performed at 94°C for 40 s. Reaction conditions for RANKL, OPG, and LIFR were as follows: denaturation at 94°C for 35 s, annealing at 65°C for 35 s, and elongation at 72°C for 60 s for 10 cycles. In subsequent cycles the primer annealing temperature was decreased stepwise by 5°C every five cycles from 65 to 45°C. The sequence of primers used were: TRAP sense, 5'-AAATCCTCTCTCAGACACCAG-3'; TRAP antisense, 5'-TTATGGAACGAGCAGTAC-3'; GAPDH sense, 5'-AAGCTTGATGATCCCTCCTG-3'; GAPDH antisense, 5'-GGTGGCCGAATTCCTGAATT-3'; RANKL sense, 5'-GGGAGATCTCAGTCTTGTTGCTTG-3'; OPG sense, 5'-TGAGGATCGATAATGTTGCTTG-3'; LIFR sense, 5'-GCTGTCAATTITGCGGTGG-3'; LIFR antisense, 5'-TTCATTTCTCAAGTTTAAGACC; OSMR sense, 5'-GTATGTCACCATGGACCTAGG-3'; and OSMR antisense, 5'-GAGGGACGTTGAG-3'. The GenBank accession numbers and the positions for the 5' ends of the nucleotides for the predicted PCR products are as follows: TRAP, NM 007388, 1072-1384; GAPDH, M32599, 957-1223; RANKL: AF013170, 929-1739; OPG: NM 008764, 428-1147; RANKL: AF019046, 522-921; gp130: X62646, 330-632; IL-6R: X53802, 586-886; LIFR: NM 013584, 2595-2758; and OSMR: NM 011019, 2597-2990. The expression of these factors was compared at the logarithmic phase of the PCR reaction. The PCR products were electrophoretically fractionated in 1.5% agarose gel and visualized using ethidium bromide. The estimated sizes of the PCR products were: TRAP, 313 bp; GAPDH, 267 bp; RANKL, 810 bp; OPG, 720 bp; LIFR, 400 bp; and mOSM, 420 bp. The identity of the PCR products was confirmed using a DYEnamic ET terminator cycle sequencing kit (Amersham Biosciences, Amersham, U.K.) with sequences analyzed on an ABI 377 XL DNA sequencer (PE Applied Biosystems, Foster City, CA).

**Protein analysis**

Measurements of OPG and RANKL protein synthesis were assessed by analyzing the levels of OPG and RANKL in mouse calvarial bones using commercially available ELISAs. Following preincubation, a total of eight calvarial halves per group were individually incubated in 24-well plates in the absence or the presence of hIL-6 plus shIL-6R (both 100 ng/ml), IL-1β (100 ng/ml), and mOSM (10 ng/ml) for 48 h. The bones were then treated with 0.2% Triton X-100 for 24 h at room temperature, and the samples were analyzed by ELISA according to the manufacturer's protocol. The sensitivities for the immunoassay kits were 5 pg/ml. According to the manufacturer, the ELISA for RANKL might be affected by the presence of OPG, and similarly the ELISA for OPG might be affected by RANKL. The interference can be seen at concentrations of RANKL and OPG in the nanogram per milliliter range, which is not likely to be present in our samples (according to the ELISA measurements). D3 was used as a positive control and, as expected, resulted in an increased protein level of RANKL and a decreased protein level of OPG in the calvarial bones. These observations together with the results showing that our protein measurements after treatment with hIL-6 plus shIL-6R and mOSM were in agreement with the RT-PCR analyses suggest that the assessments of protein levels using the ELISAs reflect true changes in RANKL and OPG protein.

**Statistics**

Statistical analysis was performed using the nonparametric Kruskal-Wallis/Mann-Whitney U test.

**Results**

**Effects of hIL-6, in the absence and the presence of shIL-6R, on bone resorption in neonatal mouse calvaria**

Treatment for 96 h with hIL-6, mIL-6 (both 2–200 ng/ml), or shIL-6R (50–500 ng/ml) did not affect either 45Ca or 3H release from neonatal mouse calvarial bones (Fig. 1). However, when the bones were exposed to the combination of hIL-6 (200 ng/ml) and shIL-6R (500 ng/ml), both 45Ca and 3H release were significantly stimulated. The responses were less than those seen with either PTH or D3 at maximally effective concentrations (10 mmol/L), but similar to release caused by a maximum concentration of IL-1β (300 pg/ml).

When hIL-6 was maintained at a constant level (100 ng/ml), the release of 45Ca was dependent on the concentration of shIL-6R (15–500 ng/ml; Fig. 2A). Similarly, when the level of shIL-6R was kept constant (150 ng/ml), the release of 45Ca was related to the concentration of hIL-6 (5–50 ng/ml; Fig. 2B). In time-course experiments, significant stimulation of 45Ca release caused by hIL-6 plus shIL-6R (both 100 ng/ml) was observed at 24 h (the first time point studied; Fig. 2C). This release was similar to release caused by PTH (10 mmol/L) and IL-1β (300 pg/ml), although clearly less pronounced than that produced by PTH.

Stimulation of 3H release by hIL-6 plus shIL-6R (both 100 ng/ml) was significantly inhibited by calcitonin (1 mmol/L), the bisphosphonate AHPrBP (0.1 mmol/L) and acetazolamide (0.1 mmol/L), three inhibitors of osteoclast activity that have different mechanisms of action (Fig. 3A). Semiquantitative RT-PCR analysis showed that the mRNA expression of TRAP, an osteoclast marker, was increased by hIL-6 plus shIL-6R (both 100 ng/ml) and D3 (10 mmol/L; Fig. 3B).

**FIGURE 1.** Effects of hIL-6 and shIL-6R on release of 45Ca (A) and 3H (B) from neonatal mouse calvarial bones cultured for 96 h. Responses are compared with those of PTH (10 nmol/L), D3 (10 nmol/L), and IL-1β (300 pg/ml). Significant increases (p < 0.05) in 45Ca and 3H release were noted following treatment with hIL-6 plus shIL-6R, PTH, D3, and IL-1β, whereas treatment with mIL-6, hIL-6, and shIL-6R separately did not result in any increase in 45Ca release. Values represent the means for six bones, and the SEMs are shown as vertical bars. The Journal of Immunology 3355
100 pg/ml) and PTH (10–100 pmol/L) were not potentiated by addition of shIL-6R (150 ng/ml; Fig. 4).

Importance of PGs in the bone resorption stimulated by hIL-6 and shIL-6R

When calvarial bones exposed to hIL-6 plus shIL-6R (both 100 ng/ml) were treated with either indomethacin (1 μmol/L) or flurbiprofen (1 μmol/L), two structurally different inhibitors of cyclooxygenase activity, a significant (p < 0.01), 40% inhibition of cytokine-stimulated 45Ca release was observed (data not shown). Treatment of mouse calvaria with hIL-6 and shIL-6R (both 100 ng/ml) resulted in a significant (p < 0.05), 4-fold stimulation of PGE2 formation, while treatment with IL-1β (300 pg/ml) and PTH (10 nmol/L) increased PGE2 release 2000- and 40-fold, respectively (data not shown).

Effects of hIL-6 and shIL-6R on the expressions of RANKL, RANK, and OPG in neonatal mouse calvaria

Semiquantitative RT-PCR analysis of mRNA expressions of RANKL, OPG, and RANK in bones treated with hIL-6 plus shIL-6R (both 100 ng/ml) for 24 h revealed that the expression of RANKL was increased, but the expression of RANK was decreased (Fig. 5A). The mRNA expression of OPG in bone treated with hIL-6 plus shIL-6R was also increased, but less than that of RANKL. Human IL-6, in the absence of shIL-6R, did not affect the expression of RANKL, RANK, or OPG (data not shown). Treatment of the bones with D3 (10 nmol/L) resulted in enhanced mRNA expression of both RANKL and RANK, but decreased expression of OPG (Fig. 5A). The semiquantitative RT-PCR analyses were normalized with GAPDH, and identical results were obtained in two independent experiments in which RT-PCR analyses were repeated three times. No bands were seen in samples in which the RT reactions were omitted, indicating no amplification of genomic DNA (data not shown).

ELISA measurements revealed that treatment with either hIL-6 plus shIL-6R (both 100 ng/ml) or D3 (10 nmol/L) for 48 h stimulated the synthesis of RANKL protein in calvarial bones (Fig. 5B). The synthesis of OPG protein was also increased by treatment with hIL-6 plus shIL-6R (both 100 ng/ml). In contrast, D3 (10 nmol/L) decreased the synthesis of OPG protein in calvarial bones (Fig. 5C). Similar data were obtained in three independent experiments.

Effects of OPG on bone resorption stimulated by RANKL, D3, and hIL-6 plus shIL-6R

RANKL (1–300 ng/ml) stimulated 45Ca release from mouse calvarial bones in a concentration-dependent manner. Half-maximal stimulation was found at 30 ng/ml, while maximum stimulation (2-fold) was noted at 200 ng/ml (Fig. 6A). OPG (300 ng/ml) abolished 45Ca release induced by RANKL (100 ng/ml; Fig. 6B). Similarly, OPG (3–300 ng/ml) significantly inhibited 45Ca release stimulated by both D3 (10 nmol/L) and hIL-6 plus shIL-6R (both...
100 ng/ml; Fig. 6, C and D). OPG did not affect unstimulated release of $^{45}$Ca in control bones (Fig. 6, B and C).

**Effects of OPG on bone resorption stimulated by hLIF, hOSM, and mOSM**

Human LIF, hOSM, and mOSM (0.1–100 ng/ml) caused concentration-dependent stimulation of $^{45}$Ca release from mouse calvaria (data not shown). Stimulation was seen at and above 0.3 ng/ml, with half-maximal effects at ~0.5–2 ng/ml. Maximum stimulation was observed at 10 ng/ml; 1.5-fold increases were noted with hOSM and hLIF, while a 1.9-fold increase was seen with mOSM.

Treatment of calvarial bones with OPG (300 ng/ml) substantially decreased $^{45}$Ca release stimulated with hLIF, hOSM, and mOSM (all at 10 ng/ml; Fig. 7A). In these experiments OPG also caused a small inhibition of $^{45}$Ca release in the control group.

**Effects of hLIF, hOSM, and mOSM on the expression of RANKL, RANK, and OPG in neonatal mouse calvaria**

Treatment of mouse calvaria with hLIF, hOSM, or mOSM (10 ng/ml, respectively) for 24 h resulted in enhanced mRNA expressions of RANKL and OPG, but neither agent changed the expression of RANK (Fig. 7, B–D). The analyses were normalized with GAPDH and repeated three times using RNA from two experiments. No bands were seen in samples in which the RT reactions were omitted, indicating that genomic DNA was not amplified (data not shown).

ELISA measurements revealed that treatment of calvarial bones with mOSM (10 ng/ml) for 48 h resulted in increased RANKL (Fig. 7E) and OPG (Fig. 7F) protein synthesis. In this experiment D$_3$ also caused increased RANKL and decreased OPG protein levels. Similar data were obtained in three independent experiments.

**Expression of gp130, IL-6R, LIFR, and OSMR in neonatal mouse calvaria and calvarial osteoblasts**

Calvarial bone as well as osteoblasts isolated from these bones expressed mRNA for gp130, IL-6R, LIFR, and OSMR (Fig. 8). Comparison of mRNA expression levels using a semiquantitative RT-PCR method revealed a lower expression level of IL-6R compared with the expression of LIFR and OSMR in both calvaria and isolated osteoblasts. No bands were seen in samples in which the RT reactions were omitted, indicating no amplification of genomic DNA (data not shown).

**Effects of an Ab neutralizing gp130**

Addition of anti-mouse gp130 (2 μg/ml) to calvarial bones inhibited $^{45}$Ca release stimulated by hIL-6 plus shIL-6R (50 ng/ml and...
FIGURE 6. Effects of OPG on 45Ca release stimulated by RANKL, D3, and hIL-6 plus shIL-6R. Bones were cultured for 96 h. A, RANKL dose-dependently stimulated bone resorption. Significant (p < 0.01) increases in 45Ca release were noted at 3–300 ng/ml. The statistically significant (p < 0.001) stimulatory effects on 45Ca release induced by RANKL (100 ng/ml; B), D3 (10 nmol/L; C), and hIL-6 plus shIL-6R (both 100 ng/ml; D) were studied in the absence and the presence of OPG. OPG caused significant (p < 0.001) inhibition of 45Ca release stimulated by RANKL, D3 (with both 90 and 300 ng/ml OPG), and hIL-6 plus shIL-6R (p < 0.01 at and above 90 ng/ml). Values represent the means for 12–24 bones, and SEMs are shown as vertical bars when larger than the height of the symbol. The values for release of 45Ca (percentage of initial) in unstimulated (control) bones in the experiments shown in A–D were 13.01 ± 0.55 (n = 17; A), 12.41 ± 0.36 (n = 24; B), 12.85 ± 0.44 (n = 23; C), and 12.63 ± 0.50 (n = 12; D).

Discussion

There is evidence suggesting that IL-6 can play an important role as a regulator of osteoclastogenesis in a number of disease states characterized by excessive resorption of bone (15–22). However, study of the cellular and molecular mechanisms responsible for the actions of IL-6 have been hampered by the lack of sensitivity to the cytokine displayed by various cell and organ culture systems (26–28). In agreement with two previous reports (27, 28), we found that periosteal/endoosteal resorption characteristic of neonatal mouse calvarial bones could not be stimulated by a wide range of IL-6 concentrations. Soluble receptor proteins can be inhibitors of ligand function, but that is not the case for sIL-6R. In the present study, we found that IL-6 was active in neonatal mouse calvaria only when added to bone cultures with sIL-6R. This is in agreement with investigations in mouse bone marrow cultures, where it has been shown that IL-6 does not stimulate osteoclast formation unless sIL-6R is present (26). Mineral and matrix breakdown were stimulated by hIL-6 plus sIL-6R in neonatal mouse calvarial bones. In addition, resorption stimulated by hIL-6 plus sIL-6R was blocked by three different osteoclast inhibitors: calcitonin, acetazolamide, and AHPBP. Moreover, mRNA expression of the osteoclast marker, TRAP, was increased. The function of TRAP in osteoclasts is not fully understood, but mice lacking TRAP display disrupted ossification and mild osteopetrosis, whereas transgenic mice overexpressing TRAP develop mild osteopetrosis and decreased trabecular density (44, 45). Taken together, our data indicate that resorption caused by hIL-6 plus sIL-6R in neonatal mouse calvaria was mediated by osteoclasts.

Osteoclast formation stimulated by hIL-6 plus sIL-6R in mouse bone marrow cultures can be abolished by exposure to the cyclooxygenase inhibitor indomethacin (46). In neonatal mouse calvaria treated with hIL-6 plus sIL-6R, inhibition of resorption by indomethacin was 40%, and stimulation with hIL-6 plus sIL-6R caused only a small increase in PGE2, suggesting that the major portion of the resorptive effect of hIL-6 plus sIL-6R in calvarial explants was PG independent.

Members of the IL-6 family of cytokines, most notably IL-11 and IL-6, have been suggested to play important roles as mediators of other calcitrophic agents (47–49). In primary hyperparathyroidism patients have been shown to have increased serum levels of IL-6 that correlate with biochemical markers of bone resorption (22). IL-6 release from cultured calvarial bones was stimulated by PTH and IL-1β in the present study. However, resorption stimulated by these agents was not increased by addition of sIL-6R, suggesting that IL-6 is not a mediator of either PTH or IL-1β in neonatal mouse calvaria.

Similar to other IL-6-type cytokines, such as IL-11 and IL-6 (plus sIL-6R), both LIF and OSM were found to be stimulators of 45Ca release in neonatal mouse calvarial bones. Human LIF, hOSM, and mOSM were found to be equipotent, with half-maximal stimulation occurring at 0.5–2 ng/ml. This is the first report of OSM stimulating bone resorption in mouse calvaria. Our results showing
that IL-6-type cytokines are potent resorptive agents in calvaria are in agreement with previous observations that have shown stimulation of osteoclast formation by IL-6 plus sIL-6R, IL-11, LIF, and OSM (14, 26, 35, 47). In the case of LIF the present study agrees with an investigation by Reid et al. (34) in which both human and mouse LIF were found to be in vitro stimulators of mouse calvarial bone resorption. In contrast and for unknown reasons, both LIF and OSM have also been found to exert small inhibitory effects on unstimulated (control) bone resorption in mouse and rat long bones without affecting PTH-stimulated resorption (39, 40).

Stimulation of biological activity by the IL-6 family of cytokines is dependent on the expression of biologically active cytokine binding proteins and functional gp130 protein. RT-PCR analysis demonstrated the expression of gp130 mRNA in both intact calvaria and calvarial osteoblasts. Since LIF and OSM as well as IL-11 stimulate bone resorption in mouse calvaria, a deficiency in functional gp130 cannot be the reason why IL-6 alone does not stimulate resorption. Other explanations for the depressed sensitivity to IL-6 include such possibilities as low expression of IL-6R or expression of a nonfunctional IL-6R (50). In the present study the former possibility seems to be the more plausible explanation, for RT-PCR analysis revealed only weak expression of IL-6R in whole calvaria and calvarial osteoblasts compared with mRNA

![Figure 7](image_url)

**FIGURE 7.** The roles of RANKL, RANK, and OPG in the bone-resorptive effects of hLIF, hOSM, and mOSM. A, OPG significantly (*p* < 0.001) inhibited ^4^Ca release stimulated by hLIF, hOSM, and mOSM (all at 10 ng/ml). The stimulatory effects of the three cytokines were statistically different (*p* < 0.001) from the control release of ^4^Ca. In these experiments the inhibitory effect of OPG on control release of ^4^Ca was statistically significant (*p* < 0.001). Values represent the means for 18–36 bones, and SEMs are shown as vertical bars. The release of ^4^Ca (percentage of initial) in unstimulated (control) bones was 14.09 ± 0.32 (n = 36). B–D, Study of mRNA expression for RANKL, OPG, and RANK in neonatal mouse calvaria cultured in the absence (control) and the presence of hLIF (B), hOSM (C), and mOSM (D; 10 ng/ml, respectively) for 24 h. The results show increased mRNA expression for RANKL and OPG following treatment with all three cytokines, whereas the expression of RANK was unaffected. The PCR reactions are normalized with GAPDH, and the numbers in the figures indicate the number of PCR cycles. E and F, Protein analysis using ELISA for RANKL and OPG in mouse calvarial bones cultured in the absence or the presence of mOSM (10 ng/ml) or D_3_ (10 nmol/L) for 48 h. E, RANKL levels were significantly (*p* < 0.001) increased in bones stimulated by mOSM and D_3_. F, OPG levels were significantly increased (*p* < 0.05) in bones stimulated by mOSM and were significantly decreased in bones stimulated by D_3_. Values represent the means for eight bones, and SEMs are shown as vertical bars.

![Figure 8](image_url)

**FIGURE 8.** Comparison of the mRNA expression levels of gp130, IL-6R, LIFR, and OSMR in neonatal mouse calvaria and calvarial osteoblasts using a semiquantitative RT-PCR approach. PCRs were performed at different cycles representing the logarithmic phase of the PCR reactions, and the results obtained after 30 cycles are shown.
expression of the receptors for LIF and OSM. This marginal expression of IL-6R, together with the inability of IL-6 to stimulate resorption without added IL-6R, or to alter mRNA expression of RANKL, RANK, or OPG in calvarial explants suggest that a sufficient quantity of IL-6R is not present in mouse calvaria. Similar results have been noted in human osteoblasts (51, 52) and gingival fibroblasts (53), where low levels of mRNA expression for IL-6R have been associated with the inability of the fibroblasts to bind IL-6 and of the osteoblasts to respond to IL-6.

Recent in vitro studies have indicated that hOSM does not activate the mOSMR, but probably functions by binding to the mLIFR. Thus, mLIFR recognizes not only mLIF, but hLIF and

**FIGURE 9.** Effects of an Ab neutralizing gp130 (2 μg/ml) on 45Ca release significantly (p < 0.01) stimulated by hIL-6 plus shIL-6R (50 and 100 ng/ml, respectively), hLIF, hOSM (both 50 ng/ml), mOSM (10 ng/ml; A), PTH, and D3 (both 0.1 nmol/L; B). A significant (p < 0.001) inhibition of the 45Ca release stimulated by hIL-6 plus shIL-6R was noted. Values represent the means for 18 bones, and SEMs are shown as vertical bars. The values for release of 45Ca (percentage of initial) in unstimulated (control) bones were 13.07 ± 0.64 (A) and 12.96 ± 0.67 (B); n = 18.

**FIGURE 10.** Schematic drawing of the effects of major calcium-regulating hormones and IL-6 family cytokines on osteoclast formation mediated by RANKL, RANK, and OPG. IL-6 plus sIL-6R, LIF, and OSM, functioning via gp130, induce increased expression of RANKL and OPG in osteoblasts (left panel). Also shown in the left panel are two major calcium regulatory hormones, PTH and D3, functioning via a membrane-bound PTH receptor subtype 1 and a cytosolic vitamin D receptor, respectively. PTH and D3 increase the expression of RANKL and decrease the expression of OPG. An excess (over OPG) of RANKL stimulates the receptor RANK, expressed on preosteoclasts. This leads to differentiation and fusion of preosteoclasts to multinucleated, bone-resorbing osteoclasts. An osteoblast plasma membrane equipped with receptors for IL-6, IL-11, LIF, and OSM as well as the signal transducing protein gp130 is depicted in the right panel. Binding of IL-6 or IL-11 to their respective receptors promotes homodimerization of gp130 molecules. The LIF and OSM receptors heterodimerize with gp130. Activation of the Janus kinase/STAT pathway by the IL-6 family of cytokines increases the expression of both RANKL and OPG. In contrast, PTH and D3 increase RANKL expression, but decrease that of OPG. This is probably the explanation for why the bone-resorptive responses to PTH and D3 are larger than those caused by the IL-6-type cytokines.
probably hOSM as well (54–56). This multifunctional ability of mLIFR may provide an explanation for the similarities in the actions of hOSM and hLIF on $^{45}\text{Ca}$ release and the mRNA expression of RANKL and OPG in the present study.

Unlike hOSM, mOSM does not bind to mLIFR. In mouse cells, mOSM is thought to form a low affinity complex with gp130, which then binds to the OSMR type II to form a high affinity signaling complex (56, 57). Because of the different receptors activated by human and mouse OSM, we studied the responses of both human and mouse OSM in the calvarial system. The actions of mOSM were indistinguishable from those of hOSM and hLIF, suggesting that activation of mouse OSM and LIF receptors results in similar effects on resorption.

An Ab neutralizing gp130 significantly inhibited $^{45}\text{Ca}$ release stimulated by hLIF-6 plus shIL-6R, but failed to block resorption caused by hLIF, hOSM, and mOSM. The possibility that this failure to suppress hLIF, hOSM, and mOSM was the result of too low a concentration of Ab is not likely, for inhibition did not occur when the concentration of Ab was increased. However, it is possible that the activity of the anti-gp130 Ab is altered depending on whether gp130 homodimerization or heterodimerization occurs. In a recent study by Ahlén et al. (58) the bone-resorptive effect of IL-11 was significantly decreased by addition of the anti-gp130 Ab. Perhaps the ability of the anti-gp130 Ab to interfere with both IL-6 and IL-11 signaling, but not with LIF or OSM signaling, might be due to IL-6 and IL-11 binding to their respective receptors before forming a signaling complex with a gp130 homodimer, whereas LIF and OSM bind to heterodimers consisting of their respective receptor and gp130 (see Fig. 10). Still another possibility may be related to the recent observations by O’Brien et al. (36). These investigators showed that increasing the expression of gp130 in a stromal/osteoblastic cell line resulted in increased STAT3-dependent promoter activity and enhanced osteoclast formation in response to IL-6 plus sIL-6R; decreased STAT3 activation in stromal osteoblastic cells is required for induction of the osteoclast specific gene expression (14). Furthermore, the osteoclastogenic responses to PTH and D$_3$ are not affected by modulating gp130 expression levels (36).

Recent studies have shown that IL-11 stimulates RANKL and OPG mRNA expression in osteoblasts and mouse calvaria (58, 59) without affecting the mRNA expression of RANK in the calvarial bones (58). Exogenous addition of OPG will also inhibit the calvarial bone-resorptive response of IL-11 (58). In the present study, three additional members of the IL-6 family of cytokines, IL-6 (plus sIL-6R), LIF, and OSM, were found to be good stimulators of resorption in neonatal mouse calvarial bones. These cytokines as well as D$_3$ also enhanced the mRNA expression of RANKL. Moreover, IL-6 plus sIL-6R, OSM, and D$_3$ were all shown to stimulate RANKL protein synthesis (see Fig. 10). The importance of increased RANKL expression in the resorptive responses of D$_3$ and the IL-6 family was demonstrated by showing that calvarial bone resorption stimulated by D$_3$, hIL-6 plus shIL-6R, hLIF, hOSM, and mOSM as well as that stimulated by exogenous RANKL could be blocked by OPG.

In addition to increasing the mRNA expression of RANKL, D$_3$ decreased the expression of OPG and increased the expression of RANK. This alteration in the profile of RANKL, OPG, and RANK offers an explanation for why D$_3$ caused greater stimulation of neonatal mouse calvarial bone resorption than the IL-6-type cytokines. In the case of LIF and OSM, these agents increased mRNA expression of the decoy receptor, OPG and had no effect on RANK. Enhanced stimulation of mRNA expression for OPG was additionally noted with hIL-6 plus shIL-6R, but hIL-6 plus shIL-6R decreased the expression of mRNA for RANK. In agreement with the RT-PCR analysis, hIL-6 plus shIL-6R and mOSM were found to increase OPG protein levels, whereas D$_3$ caused a decrease. Although there has been a great deal of study of the regulation of RANKL and OPG in stromal cells/osteoblasts, there are very few data currently available on the regulation of RANK in preosteoclasts and osteoclasts. In preliminary studies we have observed that the mouse preosteoclastic cell line, Raw 264.7, expresses mRNA for both D$_3$ receptors and gp130 (E. Persson and U. H. Lerner, unpublished observations). This suggests that the regulation of RANK by D$_3$ and hIL-6 plus shIL-6R in neonatal mouse calvarial bones may be due to a direct effect of these agents on osteoclasts.

In summary, IL-6 was found to be a good stimulator of osteoclastic bone resorption when combined in calvarial bones with sIL-6R. LIF and OSM were also observed to be IL-6-type cytokines capable of stimulating bone resorption in calvaria. In addition, neonatal mouse calvarial bones proved to be a good model for comparing the resorptive effects of D$_3$ and the IL-6 family of cytokines with the mRNA expression of RANK and mRNA expression and protein synthesis of RANKL and OPG. The roles of RANKL, RANK, and OPG in osteoclast formation and bone resorption stimulated by major calcium regulatory hormones and the IL-6-type cytokines and their receptors are summarized in Fig. 10.

Acknowledgments

We thank Birgit Andertun for skilful technical assistance and Dr. Pernilla Lundberg for valuable advice.

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