

The Role of gp130-mediated Signals in Osteoclast Development: Regulation of Interleukin 11 Production by Osteoblasts and Distribution of Its Receptor in Bone Marrow Cultures

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Summary

Interleukin (IL)-11 is a multifunctional cytokine whose role in osteoclast development has not been fully elucidated. We examined IL-11 production by primary osteoblasts and the effects of rat monoclonal anti-mouse glycoprotein 130 (gp130) antibody on osteoclast formation, using a coculture of mouse osteoblasts and bone marrow cells. IL-1, TNF α , PGE₂, parathyroid hormone (PTH) and 1 α ,25-dihydroxyvitamin D₃ (1 α ,25(OH)₂D₃) similarly induced production of IL-11 by osteoblasts, but IL-6, IL-4, and TGF β did not. Primary osteoblasts constitutively expressed mRNAs for both IL-11 receptor (IL-11R α) and gp130. Osteotropic factors did not modulate IL-11R α mRNA at 24 h, but steady-state gp130 mRNA expression in osteoblasts was upregulated by 1 α ,25(OH)₂D₃, PTH, or IL-1. In cocultures, the formation of multinucleated osteoclast-like cells (OCLs) in response to IL-11, or IL-6 together with its soluble IL-6 receptor was dose-dependently inhibited by rat monoclonal anti-mouse gp130 antibody. Furthermore, adding anti-gp130 antibody abolished OCL formation induced by IL-1, and partially inhibited OCL formation induced by PGE₂, PTH, or 1 α ,25(OH)₂D₃. During osteoclast formation in marrow cultures, a sequential relationship existed between the expression of calcitonin receptor mRNA and IL-11R α mRNA. Osteoblasts as well as OCLs expressed transcripts for IL-11R α , as indicated by RT-PCR analysis and in situ hybridization. These results suggest a central role of gp130-coupled cytokines, especially IL-11, in osteoclast development. Since osteoblasts and mature osteoclasts expressed IL-11R α mRNA, both bone-forming and bone-resorbing cells are potential targets of IL-11.

Interleukin (IL)-11 is a functionally pleiotropic cytokine that was isolated from a bone marrow-derived stromal cell line based on its ability to stimulate the proliferation of IL-6-dependent cells (1). An indication that IL-11 might regulate connective tissue responses was given by its expression in relatively restricted cells of the mesenchymal lineage, such as lung fibroblasts, bone marrow stromal cells, placental stromal cells, articular chondrocytes, and synovio-cytes (1-4).

The IL-11 receptor is a cell surface receptor that consists of two components: a unique ligand-binding 150-kD glycoprotein chain (IL-11R α) (5, 6) and a non-ligand binding, signal transducing 130-kD glycoprotein chain (gp130)¹ (7, 8). Both components are necessary for high affinity binding and signal transduction (6, 8). Signaling occurs after ligand-induced dimerization of gp130 that activates down-

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¹Abbreviations used in this paper: 1 α ,25(OH)₂D₃, 1 α ,25-dihydroxyvitamin D₃; CTR, calcitonin receptor; gp130, glycoprotein 130; IL-11R α , IL-11 receptor; IL-6R α , IL-6 receptor; LIF, leukemia inhibitory factor; OCL, osteoclast-like multinucleated cell; OSM, oncostatin M; PTH, parathyroid hormone; sIL-6R α , soluble IL-6 receptor; TRAP, Tartrate-resistant acid phosphatase.

stream molecules. These include members of the JAK family of non-receptor kinases and a latent transcription factor, acute phase response factor or signal transducer and activator of transcription factor 3 (APRF/STAT 3) (9, 10). The gp130 signal transducer mediates biological effects exerted not only by IL-11, but also IL-6, oncostatin M (OSM), leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), and cardiotrophin-1 (CT-1) (11, 12).

Several lines of evidence suggest that IL-11 is an important osteotropic factor. IL-11 receptor transcripts are present in chondroblastic and osteoblastic progenitor cells during mouse embryogenesis (13). IL-11 itself is produced by human osteosarcoma SaOs-2 cells (14), and Girasole et al. (15) showed that IL-11 dose-dependently stimulated osteoclast-like multinucleated cell (OCL) formation in cocultures of mouse osteoblasts and bone marrow cells. They reported that monoclonal anti-IL-11 antibody inhibited osteoclast formation induced by several osteotropic factors. We also reported that IL-11, IL-6 together with its soluble IL-6 receptor (sIL-6R α), OSM and LIF similarly triggered OCL formation in cocultures (16, 17).

These observations focus interest on the modulation of IL-11 and its receptor during OCL development. In the present study, we measured IL-11 production by primary mouse calvarial cell cultures using a novel bioassay based on the expression of functional IL-11 receptors in Ba/F3 cells (6). We examined the effects of IL-1, TNF α , PGE₂, parathyroid hormone (PTH) and 1 α ,25-dihydroxyvitamin D₃ [1 α ,25(OH)₂D₃], all of which induce OCL formation in mouse cocultures. The role of gp130-mediated signals in OCL formation was determined using a rat monoclonal anti-mouse gp130 antibody. The expression of gp130 and IL-11R α mRNA was studied by Northern analysis and reverse transcription-PCR, respectively. The distribution of IL-11R α transcripts was investigated by *in situ* hybridization.

We found that osteotropic factors induced IL-11 production by osteoblasts and in addition, modulated steady-state levels of osteoblast gp130 mRNA. Interleukin-11 receptor transcripts were detected in osteoblasts and osteoclast-like cells. In the present study we demonstrate that gp130-mediated signals are critical cofactors for osteoclastogenesis induced by a variety of bone resorbing factors.

Materials and Methods

Animals and Cytokines. Newborn and 6–9-wk-old male C57/B16J mice were purchased from Monash University Animal Services Center (Clayton, Australia). Recombinant human IL-11 (specific activity 10⁸ U/mg protein) was obtained from Dr. T. Willson (Walter and Eliza Hall Institute, Melbourne, Australia). Neutralizing monoclonal murine anti-human IL-11 antibody was generously provided by Genetics Institute (Cambridge, MA). Recombinant mIL-11, mIL-6, hOSM, mIL-3, mIL-4, hLIF, mIL-1 α , and mTNF α were purchased from Peprotech (Rocky Hill, NJ). Mouse sIL-6R was prepared from CHO cells as described (16). Recombinant hTGFB1 was the gift of Genentech Inc. (San Francisco, CA). 1 α ,25(OH)₂D₃ was obtained from Wako Pure Chemical

Co. (Osaka, Japan). Bacterial collagenase was obtained from Worthington Biochemical Co. (Freefold, Australia). Other chemicals and reagents were of analytical grade.

Cell Culture and Determination of Osteoclast Characteristics. To isolate osteoblastic cells, 6–10 calvaria obtained from 1-d-old mice were cut into small pieces in 10-cm culture dishes with 5 ml of α -MEM (GIBCO BRL, Gaithersburg, MD) containing 30% FBS (Cytosystems, Castle Hill, NSW, Australia). Type-I collagen gel solution (70%, 5 ml) (Cellmatrix Type I-A; Nitta Gelatin Co., Osaka, Japan) was added in the culture dish according to the manufacturers' instructions. After culture for 4–6 d, osteoblastic cells grown from the calvaria were collected by treating with PBS containing 0.3% collagenase for 20 min at 37°C (17). These freshly isolated osteoblastic cells were cocultured with bone marrow cells as described (16, 17). In short, primary osteoblastic cells (10⁴ per well) and nucleated marrow cells (2 \times 10⁵ per well) were cocultured in 48-well plates (Corning Glass Ins., Corning, NY) with 0.3 ml of α -MEM containing 10% FBS in the presence of test chemicals. Cultures were incubated in quadruplicate and cells were replenished on day 4 with fresh medium. OCL formation was evaluated after culturing for 6 d. For tartrate-resistant acid phosphatase (TRAP) staining, adherent cells were fixed with 4% formaldehyde in PBS for 3 min. After treatment with ethanol-acetone (50/50, vol/vol) for 1 min, the well surface was air dried and incubated for 10 min at room temperature in an acetate buffer (0.1 M sodium acetate, pH 5.0) containing 0.01% naphthol AS-MX phosphate (Sigma Chem. Co., St. Louis, MO) as a substrate and 0.03% red violet LB salt (Sigma) as a stain for the reaction product in the presence of 50 mM sodium tartrate. TRAP positive cells appeared as dark red cells. The expression of calcitonin receptors (CTR) was assessed by autoradiography using [¹²⁵I]-salmon calcitonin as described (16–20).

Mouse bone marrow cell cultures were prepared as described (18, 20). The marrow cells were plated at 1 \times 10⁶ cells/ml and incubated in α -MEM containing 12.5% FBS in 9-cm petri dishes (Nunc, Inc. Naperville, IL). OCL formation was induced by 1 α ,25(OH)₂D₃ (10 nM), added on the first day and every third day thereafter, when half the medium was replaced with fresh medium. In parallel cultures, the cells were cultured on top of 13-mm Thermanox coverslips (Nunc) for enumeration of OCLs by TRAP-staining and [¹²⁵I]-calcitonin binding.

For *in situ* hybridization, after coculturing on collagen gels for 7 d (19), cells were treated with collagenase (0.2%), and subcultured on top of 13-mm diameter Thermanox coverslips (Nunc) in 24-well plates, before processing.

A stock of the mouse bone marrow-derived stromal cell line ST2 (21, 22), was obtained from RIKEN cell bank (Tsukuba, Japan). ST2 cells in the 12th passage were grown to sub-confluence in 75-cm² tissue culture flasks (Nunc), in phenol red-free α -MEM containing 10% FBS. The medium was changed to 2% FBS overnight, and cells were treated with osteotropic factors before extraction of total RNA at the appropriate times.

Biological Assay for IL-11. IL-11 activity was measured using the Ba/F3 cell microproliferation assay (6, 23). Survival and proliferation of Ba/F3 cells are supported by IL-3, and also by IL-11 after transfection with cDNAs for mIL-11R α and gp130. Transfected cells also respond to high concentrations of the complex of mIL-6 and sIL6R α (3 μ g/ml IL-6 and 500 ng/ml sIL-6R α , respectively), but do not proliferate in response to OSM, IL-6, or LIF (6). The cells did not show effects with other cytokines or reagents used in the present study. Ba/F3 clones were grown in DME containing 10% (vol/vol) FBS and 10% (vol/vol) WEHI-3B D-conditioned medium as a source of IL-3 (24). Cells were

maintained by twice weekly passage and used in exponential growth phase. Cell proliferation was measured in Lux-60 microwell HL-A plates (Nunc). For use, Ba/F3 cells were washed three times in DME containing 20% (vol/vol) FBS and resuspended at a concentration of 20,000 cells/ml in the same medium. 10- μ l aliquots of cell suspension were then placed in the culture wells with 5 μ l of serial dilutions of recombinant IL-3, IL-11, or culture supernatants. After 2 d of incubation at 37°C in a fully humidified incubator containing 10% CO₂ in air, viable cells were counted using an inverted microscope. IL-11 concentrations in the supernatants were determined from a standard curve set up with known amounts of recombinant IL-11. The detection limit of this assay was typically 50–100 pg/ml (Fig. 1).

ELISA for sIL6R α . Concentrations of sIL-6R α were determined by a sandwich ELISA using rat monoclonal anti-mIL-6R α antibody and rabbit polyclonal anti-mIL-6R α antibody, as described (25). The detection limit of this assay was 1 ng/ml.

Northern Analysis. Total cellular RNA was extracted using the guanidine thiocyanate-phenol chloroform method (26), fractionated by electrophoresis on 1.5% agarose-formaldehyde gels (20 μ g/lane), and transferred to nylon membranes (Hybond-N; Amersham Corp., Arlington Heights, IL). A 2.7-kb mouse gp130 cDNA (27) was random prime labeled with [³²P]dCTP to a specific activity of 1 \times 10⁹ U/min/mg DNA (Boehringer Mannheim, Mannheim GmbH, Germany). Hybridization and washing were carried out as previously described (28). Specifically bound cDNA was quantified using a PhosphorImager and ImageQuant software (Molecular Dynamics, Sunnydale, CA). Relative mRNA levels were normalized for loading variability by comparison with 18S ribosomal RNA levels in the same membranes, probed with a ³²P-labeled 18S ribosomal RNA oligonucleotide.

PCR Amplification of Reverse-transcribed mRNA. Expression of IL-11R α , IL-6R α , and calcitonin receptor (CTR) mRNA reverse-transcribed from primary osteoblasts or bone marrow cell cultures were determined by PCR and Southern blot analysis. First strand cDNA was synthesized from 2.5 μ g of total RNA by incubation for 1 h at 42°C with 12 U of avian myeloblastosis virus reverse transcriptase (Promega Corp., Madison, WI) after random hexanucleotide priming. The reaction mixture (25 μ l) was made up to 100 μ l with sterile distilled water, and 10 μ l (1/10) were submitted for PCR to amplify the sequences of the mouse IL-11R α , IL-6R α , CTR, and GAPDH mRNAs specified below. The reaction mixture contained 50 pmol of each primer, 0.25 mM dATP, dGTP, dCTP, and dTTP (Pharmacia, Uppsala, Sweden), 2 μ l 10 \times reaction buffer, 1 U of Taq DNA polymerase (Boehringer Mannheim) and sterile distilled water, and was overlaid with 50 μ l of paraffin oil. PCR for CTR, IL-11R α , and IL-6R α was performed in a DNA Thermal Cycler 480 (Perkin-Elmer Cetus Instrs., Norwalk, CT) as described (17). To permit semiquantitation of the PCR products, preliminary experiments were performed to ensure that the number of PCR cycles employed for each product in a given experiment (described in the legends to Figs. 5 and 9) was within the exponential phase of the amplification curve. PCR products were resolved on a 2% wt/vol agarose gel and the specificity of the reaction was confirmed by Southern transfer onto nylon membranes (Hybond-N; Amersham) and hybridization with ³²P-labeled internal oligonucleotide probes.

Control PCR reactions were carried out on non-reverse transcribed RNA; in none of these samples were PCR products detected.

Oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer model 381A (Foster City, CA). The oligonucleotides for mouse IL-6R α (17) were: 5'-CCTGTGTGGGGT-

TCCAGAGGAT-3' (3' primer complementary to nucleotides 980–1001) and 5'-CTGCCAGTATTCTCAGCAGCTG-3' (5' primer complementary to nucleotides 488–519). The products were verified by Southern hybridization with the internal oligonucleotide, 5'-CACAAACGAAGCGTTTCACAGCTT-3'. The oligonucleotides for mouse IL-11R α (6, 17) were: 5'-GGAGGCCTCCAGAGGGT-3' (3' primer complementary to nucleotides 661–667) and 5'-GGTCCCTCCAGGGGTCCAGTATGG-3' (5' primer complementary to nucleotides 133–156). The products were verified by Southern hybridization with the internal oligonucleotide, 5'-CTCCTGTACTTGGAGTCCAGG-3'. The oligonucleotides for mouse CTR were: 5'-ACAAACTGGA(T/C)(T/G)CCCAGCAGGGGCAC-3' (3' primer complementary to nucleotides 1663–1689) and 5'-AAGAACATGTT(C/T)CT(C/G/T)ACTTA (5' primer complementary to nucleotides 1058–1079), as we previously reported (20). The CTR PCR products were verified with the internal sense strand oligonucleotide, 5'-ACCAAGATGAGGCAAACC-3', by Southern hybridization.

To ensure equal starting quantities of cDNA for the experiments, and to allow semiquantitation of the PCR products representing mL11-R α or mL-6R α , the reverse-transcribed RNA samples were also amplified using oligonucleotide primers specific for GAPDH (29). A fragment of ~420 bp was amplified using 5'-specific oligonucleotide, (5'-CATGGAGAAGGCTGGGGCTC-3', representing nucleotides 306–325 of rat GAPDH) and 3'-specific oligonucleotide, (5'-AACGGATACATTGGGGGTAG-3', representing nucleotides 701–720). Products were verified with [³²P]-labeled internal oligonucleotide (5'-GCTGTGGGCAAGGT-CATCCC-3', representing nucleotides 640–659). The signals were quantitated using a PhosphorImager and ImageQuant software (Molecular Dynamics).

Preparation of Anti-Mouse gp-130 Antibody. Wistar rats were immunized with 100 μ g of soluble mouse gp130 in Freund's complete adjuvant, followed by seven 50- μ g boosts of soluble mouse gp130 in Freund's incomplete adjuvant once a week. 3 d after the last boost, the rats were sacrificed and spleen cells were fused with P3U1 mouse myeloma cells with polyethylene glycol 1500 (Boehringer Mannheim). Hybridomas were established by conventional hypoxanthine/aminopterin/thymidine selection methods, and hybridomas producing anti-mouse gp130 were selected by flow cytometry using BAF-m130 cells, which were established by transfection of BAF-B03 cells with mouse gp130 cDNA (27).

Briefly, culture supernatants of the hybridomas (100 μ l) were added to BAF-m130 cells (5 \times 10⁵ cells) with 100 μ l of Ca²⁺, Mg²⁺-free phosphate-buffered saline containing 2% heat-inactivated FBS and 0.1% sodium azide. The cells were incubated for 20 min on ice, and then incubated for 20 min with FITC-conjugated mouse anti-rat IgG (2 μ g/ml) on ice. Stained cells were analyzed by flow cytometry using FACScan[®] (Becton Dickinson Immunocytometry Sys., Mountain View, CA). Hybridomas recognizing soluble mouse gp130 were cloned twice by limiting dilution. Established hybridomas were expanded as ascite tumors in BALB/C nude mice, and the antibody was purified from the fluid with protein G-agarose column (Oncogene Science, Inc., Manhasset, NY).

Synthesis of Riboprobe. A riboprobe for mouse IL-11R α was synthesized and labeled with digoxigenin (DIG) by using a RNA labeling kit (Boehringer Mannheim) according to the manufacturer's instructions. A 1350-bp PCR fragment in the BstXI site of the mammalian expression vector pEF-BOS (6) was subcloned into the XbaI site of pBluescript (Stratagene, Inc., La Jolla, CA). The antisense and sense probes were then obtained after linearization of the plasmid with SmaI for T3 and NotI for T7 RNA poly-

merase transcription, respectively. The specificity of the cRNA probe for its target sequence was verified by Northern analysis of total RNA derived from transfected Ba/F3 cells.

In Situ Hybridization. In situ hybridization was performed as described with minor modifications (30). Cocultures established on top of 13-mm coverslips in 24-well plates were rinsed in ice-cold PBS, and fixed in 4% paraformaldehyde (PFA) in PBS for 30 min. After washing in PBS, they were pretreated with 0.2 M HCl for 20 min, washed with sterile distilled water for 10 min, and digested with 5 $\mu\text{g/ml}$ proteinase K (37°C; 30 min) in PBS. After treatment with 2 mg/ml of glycine in PBS to inactivate proteinase K, they were refixed with 4% PFA-PBS for 15 min. Hybridization was carried out with 2–4 ng/ μl of antisense cRNA in a damp chamber in a solution containing 50% formamide, 5 \times SSC, 2% blocking reagent (Boehringer Mannheim), 0.02% sodium dodecyl sulfate, and 0.1% *N*-lauroylsarcosyl, for 18 h at 42°C. After hybridization, coverslips were washed sequentially with 2 \times SSC for 30 min, treated with 20 $\mu\text{g/ml}$ ribonuclease (RNase, DNase free; Boehringer Mannheim) in 2 \times SSC for 30 min to remove excess cRNA, then washed with 1 \times SSC for 30 min, and finally with 0.1 \times SSC for 30 min. Detection of hybridized probe was with the anti-DIG-alkaline phosphatase conjugate, according to the manufacturer's instructions. The coverslips were rehydrated in ethanol, and counterstained with nuclear fast red before mounting.

Cytospin preparations of transfected Ba/F3 cells served as positive controls. Hybridization specificity was verified by elimination of signals after pretreatment with 100 $\mu\text{g/ml}$ of RNase for 1 h before hybridization with antisense riboprobe.

Statistical Analysis. Statistical significance was determined by Student's *t* test.

Results

Regulation of IL-11 Production by Primary Murine Osteoblasts. We first analyzed IL-11 production by primary osteoblasts derived from mouse calvaria. Subconfluent osteoblasts were incubated in α -MEM containing 10% FBS in the presence or absence of osteotropic factors, and IL-11 activity in the culture supernatants was quantitated using the Ba/F3 bioassay. Fig. 1 shows the effect of graded concentrations of rhIL-11 on the viability of transfected Ba/F3 cells expressing the mL-11R α chain and gp130 (*left*), and the neutralization of this response by monoclonal murine anti-human IL-11 antibody (α IL-11 mAb, *right*). Parental Ba/F3 cells did not respond to rhIL-11, whereas both parental and transfected cells proliferated in response to rmIL-3 (data not shown). The specificity of this microassay depended on the parallel response of transfected and parental cells.

Using this assay, it was clear that unstimulated osteoblasts produced levels of IL-11 that were below the limits of detection in our assays. In contrast, cells that were stimulated with mL-1 α , mTNF α , PGE₂, PTH, and 1 α ,25(OH)₂D₃ produced readily detectable levels of IL-11. As seen in Fig. 2, the time-dependent effects of IL-1, TNF α , or PGE₂ were appreciated only after incubation for 6 h, and were near-maximal after 12-h incubation. The levels of IL-11 present after PTH or 1 α ,25(OH)₂D₃ stimulation were not maximal before 24 h.

Fig. 3 demonstrates the dose-dependent nature of these

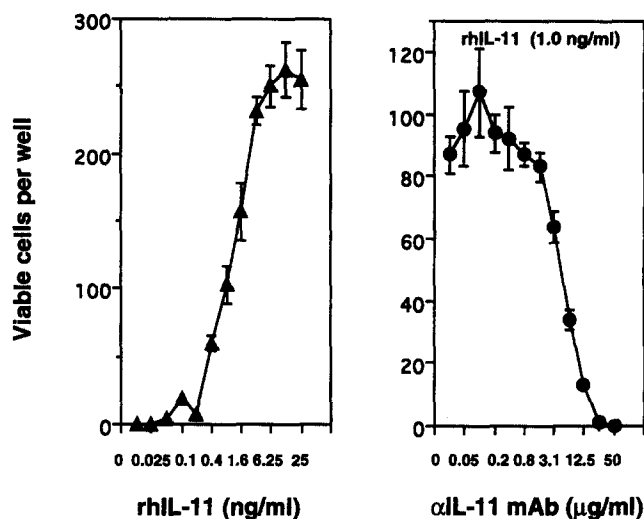


Figure 1. Left panel shows a microassay for Ba/F3 cell proliferation. Transfected Ba/F3 cells expressing the mL-11R α chain and gp130 were incubated at 200 cells per well in a volume of 15 μl with the indicated amounts of rhIL-11. After incubation for 48 h the numbers of viable cells were counted. In parallel incubations, parental Ba/F3 cells were non-viable (not shown). Right panel shows the effect of graded concentrations of monoclonal murine anti-human IL-11 antibody (α IL-11 mAb) on the response of transfected Ba/F3 cells to rhIL-11 (1.0 ng/ml).

responses. The effects of IL-1 or TNF were evident with concentrations as low as 0.1 ng/ml (the lowest concentration tested). Doses as low as 10 pM 1 α ,25(OH)₂D₃ and 25 ng/ml of hPTH (1–34) induced IL-11. Importantly, the ability to induce IL-11 release from osteoblasts was not a general property of all osteotropic cytokines, since mL-6 (20 ng/ml), hOSM (20 ng/ml), hTGF β (1 ng/ml), or mL-4 (10 ng/ml) did not elicit IL-11 release (Fig. 3). The effects of IL-1 and TNF α on IL-11 production were abolished by incubating with the cyclooxygenase inhibitor indomethacin (10⁻⁶ M), but the effects of PGE₂, PTH, or 1 α ,25(OH)₂D₃ were not (data not shown). Thus, prostaglandins seemed to be involved in IL-11 production induced by IL-1 and TNF in these cultures. Indeed PGE₂ itself potently induced IL-11 production by osteoblasts (Figs. 2 and 3).

In view of the possibility that sIL-6R α present in conjunction with IL-6 might also have stimulated the transfected Ba/F3 cells, we assayed the sIL-6R α concentrations by ELISA. In none of the culture supernatants from these experiments was sIL-6R α detectable (detection limit 1 ng/ml; data not shown). In contrast, the growth activity in the supernatants was completely neutralized by monoclonal murine anti-human IL-11 antibody (Table 1). Together, this evidence indicated that the activity in the culture supernatants measured by the microassay was due to the presence of IL-11.

Endogenous IL-11 Production in Cocultures of Mouse Bone Marrow Cells and Primary Osteoblasts. In view of the importance of 1 α ,25(OH)₂D₃ in mediating osteoclastogenesis in the mouse coculture system (31–33), and the apparent ability of monoclonal anti-human IL-11 antibody to abrogate osteoclast formation (15), we measured the effect of 1 α ,25(OH)₂D₃

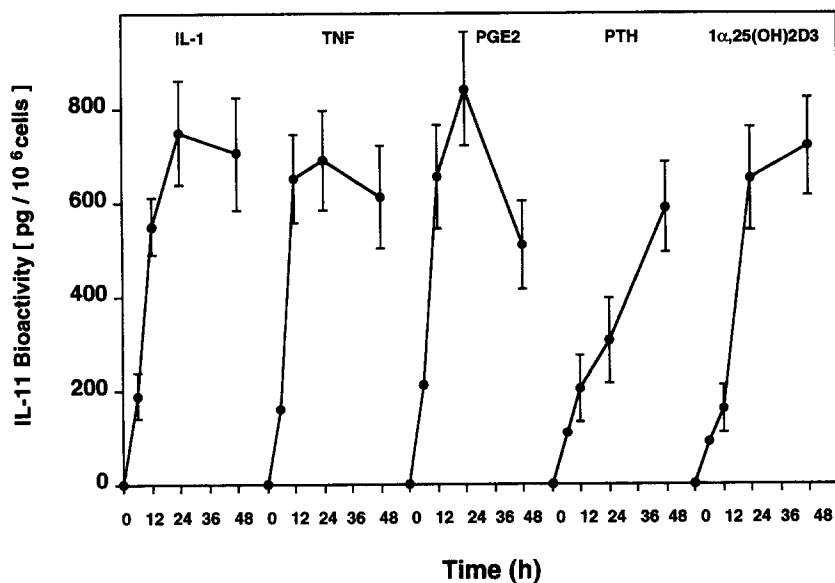


Figure 2. IL-11 production by primary osteoblasts. Subconfluent murine osteoblasts were cultured for up to 48 h in the absence (unstimulated) or presence of mIL-1 α (5 ng/ml), mTNF α (10 ng/ml), PGE $_2$ (10^{-6} M), PTH (200 ng/ml), or 1 α ,25(OH) $_2$ D $_3$ (10 nM). IL-11 released into the supernatants was quantitated at each time point using the Ba/F3 cell bioassay. Each value represents the IL-11 bioactivity (mean \pm SE) of triplicate cultures. Unstimulated osteoblasts did not release detectable IL-11 at any time point (data not shown). These results are representatives of replicate experiments.

on endogenous IL-11 production in cocultures. As shown in Fig. 4, treatment with 10 nM 1 α ,25(OH) $_2$ D $_3$ resulted in striking accumulation of IL-11 in the supernatants, significantly different from untreated cocultures, in which IL-11 was not detected.

Expression of Transcripts for IL-11R α Chain and gp130 by Primary Osteoblasts. Since functional responses to IL-11 are dependent not only on local production of the cytokine but also expression of its specific receptors, we next determined expression of IL-11 receptor mRNAs by primary osteoblasts. As shown in Fig. 5, these cells constitutively expressed transcripts for IL-11R α and gp130. By the semi-quantitative RT-PCR procedure, no modulation of IL-11R α mRNA relative to GAPDH mRNA was apparent after 24 h stimulation with 1 α ,25(OH) $_2$ D $_3$, PTH, or IL-1. However, Northern analysis revealed that steady-state gp130 mRNA levels were upregulated. Thus, after 24 h 1 α ,25(OH) $_2$ D $_3$ (10 nM) and PTH (200 ng/ml) elevated gp130 mRNA levels 3.1- and 1.7-fold above control, respectively. Simi-

larly, IL-1 (10 ng/ml) upregulated gp130 mRNA levels 2.4-fold above unstimulated osteoblasts. 1 α ,25(OH) $_2$ D $_3$ also upregulated gp130 mRNA in ST2 cells, a clonal mouse stromal cell line that is capable of supporting osteoclast formation (21, 22). In contrast, other steroid hormones (β -Estradiol or Dexamethasone) did not affect steady-state gp130 mRNA in ST2 cells (Fig. 6).

Effects of Murine Monoclonal Anti-gp130 Antibody on Osteoclast Formation. To establish that gp130-mediated signal transduction is required for osteoclast formation by IL-6 or IL-11, we prepared a rat monoclonal anti-mouse gp130 antibody. The antibody was used to define the role of gp130-mediated signals in osteoclast formation. As shown in Fig. 7, graded concentrations of gp130 antibody added to murine cocultures dose-dependently inhibited osteoclast formation induced by mIL-6 (20 ng/ml) in combination with smIL-6R α (500 ng/ml), or IL-11 (10 ng/ml). We established that 10 μ g/ml of the antibody completely neutralized the osteoclastogenic activity of each cytokine. More importantly,

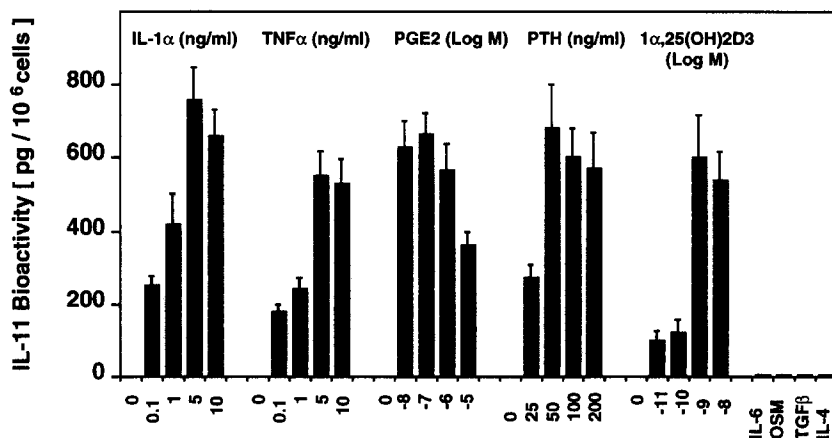


Figure 3. IL-11 production by primary osteoblasts. Subconfluent osteoblasts were cultured for 48 h in the absence (unstimulated) or presence of the indicated concentrations of mIL-1 α , mTNF α , PGE $_2$, PTH, or 1 α ,25(OH) $_2$ D $_3$. IL-11 released into the supernatants was quantitated using the Ba/F3 cell bioassay. Each value represents the IL-11 bioactivity (mean \pm SE) of triplicate cultures. Neither rmIL-6 (20 ng/ml), rhOSM (20 ng/ml), rhTGF β 1 (1 ng/ml), or rmIL-4 (10 ng/ml) induced IL-11 release. The results are representatives of replicate experiments.

Table 1. Specificity of the Ba/F3 Microassay for IL-11 in Osteoblast Culture Supernatants

Culture supernatant (48 h)	α IL-11 mAb added (20 μ g/ml)	
	-	+
No stimulation	3 \pm 1	0
IL-1 (5 ng/ml)	94 \pm 9	6 \pm 2*
TNF α (10 ng/ml)	90 \pm 14	7 \pm 3*
PGE ₂ (10 ⁻⁶ M)	115 \pm 16	9 \pm 2*
hPTH (200 ng/ml)	82 \pm 5	6 \pm 2*
1 α ,25(OH) ₂ D ₃ (10 nM)	63 \pm 6	4 \pm 2*

Transfected Ba/F3 cells were maintained for 48 h in the presence of culture supernatants (diluted 1:2) derived from primary osteoblastic cells treated for 48 h, without or with monoclonal murine anti-human IL-11 antibody (20 μ g/ml). Values shown represent the mean \pm SE number of viable Ba/F3 cells per well from four replicate wells.

**P* < 0.005 vs cells cultured in the absence of monoclonal murine anti-human IL-11 antibody (α IL-11 mAb).

the anti-gp130 antibody abolished osteoclast formation induced by IL-1 α (1 ng/ml) and inhibited, by between 60–75%, osteoclast formation induced by PGE₂ (10⁻⁶ M), 1 α ,25(OH)₂D₃ (10 nM), or hPTH (1–34) (400 ng/ml) (Fig. 8) suggesting a common role of gp130 signals for osteoclastogenesis induced by several bone-resorbing factors. In these experiments, an equivalent concentration of non-immune IgG did not inhibit osteoclast formation (data not shown).

IL-11R α Transcript Expression in Cells of the Osteoclast Lineage. To investigate the possibility that OCLs as well as bone marrow progenitors also express mRNA for IL-11R α , the temporal relationship of the expression of IL-11R α to CTR mRNA was studied during osteoclast formation in bone marrow cell cultures. As shown in Fig. 9, fresh bone marrow cells (day 0) constitutively expressed IL-11R α tran-

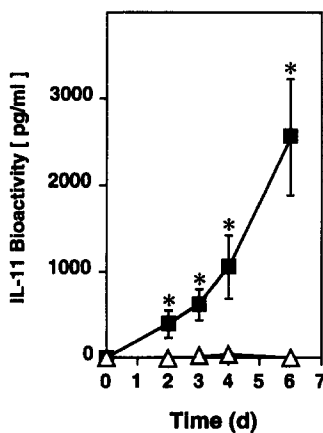


Figure 4. Effect of 1 α ,25(OH)₂D₃ on IL-11 production in cocultures of primary osteoblasts and bone marrow cells. Cocultures were maintained for 6 d in the absence (unstimulated, Δ) or presence (\blacksquare) of 1 α ,25(OH)₂D₃ (10 nM), with a complete medium change after incubation for 3 d. At the times indicated, 50 μ l of the culture medium was collected for quantitation of IL-11 using the Ba/F3 cell bioassay. The amount of IL-11 present 4 and 6 d after initiation of the culture was calculated by adding the IL-11 level obtained at day 3 to the absolute

amounts determined at those times. Each point represents the mean cumulative IL-11 bioactivity of triplicate cultures. **P* < 0.005 vs unstimulated cultures.

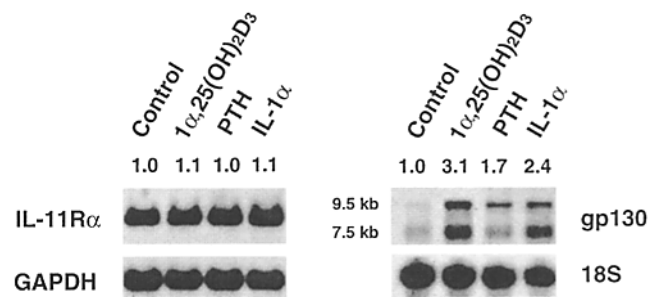


Figure 5. Expression of IL-11R α and gp130 mRNAs by primary osteoblasts. Osteoblasts were cultured without additions (control) or with 1 α ,25(OH)₂D₃ (10 nM), hPTH (200 ng/ml) or IL-1 α (10 ng/ml) for 24 h. Total RNA was reverse transcribed and amplified by 30 cycles for IL-11R α and 20 cycles for mouse GAPDH mRNA using the specific primers described in Methods. PCR products were transferred to a nylon membrane and hybridized with ³²P-labeled internal oligonucleotides specific for mIL-11R α and GAPDH sequences respectively. For Northern analysis, total RNA (20 μ g/lane) was hybridized to ³²P-labeled gp130 cDNA. Hybridization with ³²P-labeled oligonucleotide specific to 18S sequences was used to control for equivalence of loading. The number above each lane is the treated/control ratio of the intensity of the band(s) normalized to that of GAPDH or 18S RNA, measured by densitometry.

scripts. When cultures were stimulated by 10 nM 1 α ,25(OH)₂D₃, a time-dependent increase in CTR mRNA (28 cycles of PCR amplification), relative to GAPDH mRNA (20 cycles of PCR amplification) occurred, reflecting differentiation of bone marrow cell progenitors, as mature osteoclasts were plentiful in the cultures after 5 d. More importantly, after 10 d, we consistently detected a parallel sevenfold rise in abundance of IL-11R α transcripts (30 cycles of PCR amplification) relative to GAPDH mRNA, beginning at day 4 to 5. This phenomenon was relatively specific for IL-11R α mRNA, as evidenced by the lack of appreciable change in abundance of IL-6R α mRNA (25 cycles of PCR amplification) relative to GAPDH mRNA (Fig. 9). This sequential relationship between CTR mRNA and IL-11R α mRNA expression suggested that expression of IL-11R α mRNA was upregulated during OCL differentiation. To test this hypothesis, and to verify IL-11R α gene expression by osteoblasts, we carried out in situ hybridization.

Cocultures grown on collagen gels were treated with 1 α ,25(OH)₂D₃ for 7 d, transferred to coverslips (18) and

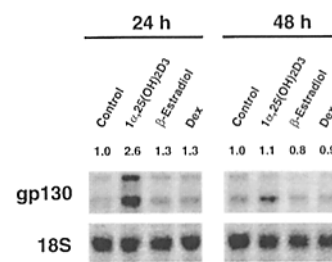


Figure 6. Effects of steroid hormones on gp130 mRNA expression by ST-2 cells. Subconfluent cells were cultured for 24 or 48 h under 2% serum conditions in phenol red free α -MEM without additions (control) or with 1 α ,25(OH)₂D₃ (10 nM), β -Estradiol (10 nM) or Dexamethasone (10⁻⁷ M, Dex). 20 μ g/lane of fractionated total RNA was hybridized with ³²P-labeled gp130 cDNA, as described in Materials and Methods. The number in each lane is the treated/control ratio of the intensity of the bands normalized to that of 18S RNA, measured by densitometry.

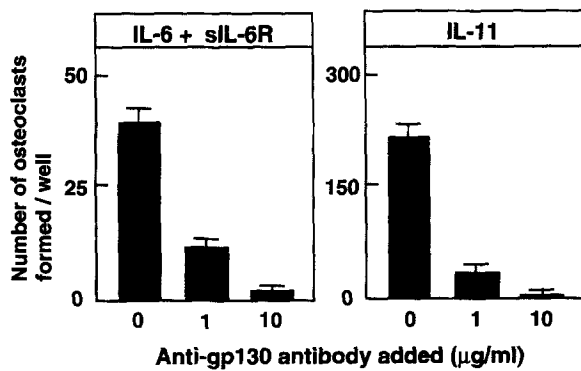


Figure 7. Effect of monoclonal anti-mouse gp130 antibody on OCL formation induced by mIL-6-sIL-6R α , or mIL-11. Cocultures of bone marrow cells and primary osteoblasts were maintained in the presence of mIL-6 (20 ng/ml) in combination with sIL-6R α (500 ng/ml), or mIL-11 (10 ng/ml). Simultaneously, graded concentrations of anti-gp130 antibody were added, as indicated. After culturing for 7 d, TRAP-positive cells were counted. Data are expressed as the mean \pm SE total number of TRAP-positive cells (OCLs) per well of quadruplicate cultures. Pretreatment with non-immune IgG had no effect on osteoclast formation (data not shown). These results are representatives of four separate experiments.

then processed for in situ hybridization. As illustrated in Fig. 10 A, these cocultures contained IL-11R α transcripts detectable by in situ hybridization. Indeed, both osteoblasts and OCLs expressed IL-11R α mRNA. IL-11R α transcripts were located predominantly in OCLs, which were identified by morphology and TRAP-staining (data not shown). The specificity of this result was verified by elimination of hybridization signals after pre-treatment with 100 μ g/ml of ribonuclease for 1 h (Fig. 10 B).

Discussion

The present study focused on the regulation of IL-11 synthesis by primary osteoblasts and furthers our under-

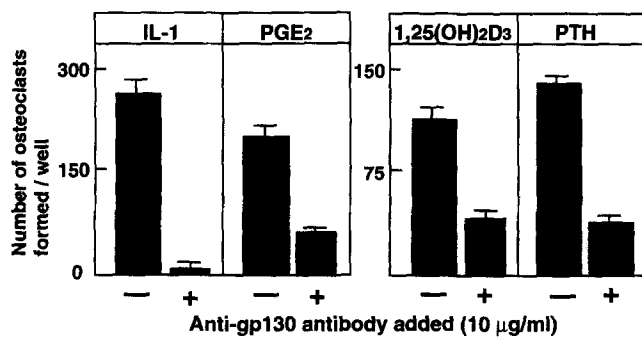


Figure 8. Effect of monoclonal anti-mouse gp130 antibody on osteoclast formation stimulated by IL-1, PGE₂, PTH, or 1 α ,25(OH)₂D₃. Cocultures of bone marrow cells and primary osteoblasts were maintained in the presence of IL-1 α (1 ng/ml), PGE₂ (10⁻⁶ M), 1 α ,25(OH)₂D₃ (10 nM), or hPTH [1-34] (400 ng/ml), each without or with the indicated amounts of anti-gp130 antibody added simultaneously at the beginning of the culture. After culturing for 7 d, TRAP-positive cells were counted. Data are expressed as the mean \pm SE total number of TRAP positive cells (OCLs) per well of quadruplicate cultures. Pretreatment with nonimmune IgG did not influence osteoclastogenesis (data not shown). These results are representatives of five separate experiments.

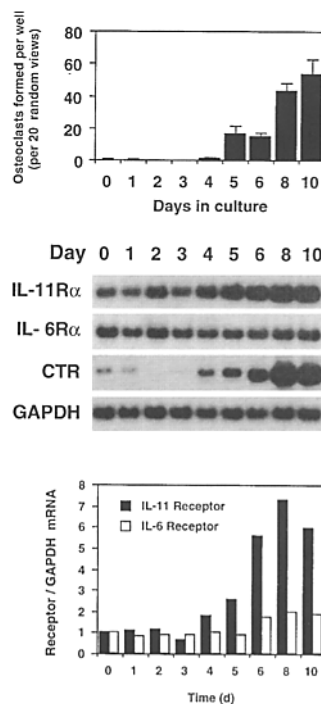


Figure 9. Effect of 1 α ,25(OH)₂D₃ on mIL-11R α , mIL-6R α , and mCTR mRNA expression in bone marrow cell cultures. Bone marrow cells were cultured for 10 d in the presence of 1 α ,25(OH)₂D₃ (10 nM). At each time point, osteoclasts were enumerated as described in Materials and Methods. RNA was reverse transcribed and subjected to 30 cycles of PCR for mIL-11R α amplification, 25 cycles of PCR for mIL-6R α mRNA amplification, 28 cycles of PCR for mCTR mRNA and 20 cycles of PCR for GAPDH mRNA, using specific primers. PCR products were resolved by electrophoresis, transferred to nylon membranes, and hybridized with specific [³²P]-labeled internal oligonucleotides as described in Materials and Methods. Upper panel shows timed appearance of osteoclasts in the cultures. Each value represents the mean \pm SE of osteoclasts (cells exhibiting

both TRAP staining and [¹²⁵I]-CT binding) per well from four replicate cultures. Middle panel shows PCR products for mIL-11R α , mIL-6R α , and CTR, at parallel time points. To allow semiquantitation of the PCR products, reverse transcribed RNA was also amplified using primers complementary to GAPDH sequences. The intensities of autoradiographic signals for mIL-11R α , mIL-6R α , and GAPDH PCR products were quantitated and are shown in the lower panel as the ratio of mIL-11R α /GAPDH and mIL-6R α /GAPDH. This figure is a representative of three separate experiments; similar results were obtained in the other two experiments.

standing of the role of gp130 signal transduction in osteoclast formation. IL-11 receptor transcripts were detected not only in osteoblasts, but in mature osteoclasts, indicating that both bone forming and bone resorbing cells are potential targets of IL-11.

Primary calvarial cell cultures consist of the heterogeneous osteoblastic-stromal cells that are likely to be present in the bone microenvironment in vivo. IL-11 production by primary osteoblasts was induced by IL-1, TNF α , PGE₂, PTH, and 1 α ,25(OH)₂D₃, all of which are potent osteoclastogenic factors in vitro. Unlike osteosarcoma cells (14), primary osteoblasts did not produce IL-11 constitutively. However, our data indicate that appropriately stimulated normal osteoblasts are a source of this cytokine. Although IL-11 was measured with a transfected cell line that is potentially sensitive to growth stimulation by IL-6-sIL-6R α complexes (6), we did not detect sIL-6R α in our experimental systems. We have previously shown that sIL-6R α also is undetectable in murine cocultures despite dexamethasone stimulation, which greatly enhanced expression of osteoblast IL-6R α mRNA and OCL formation (17).

Prostaglandin E₂ was a potent inducer of IL-11 production by osteoblasts, and PGs seemed to mediate IL-11 pro-

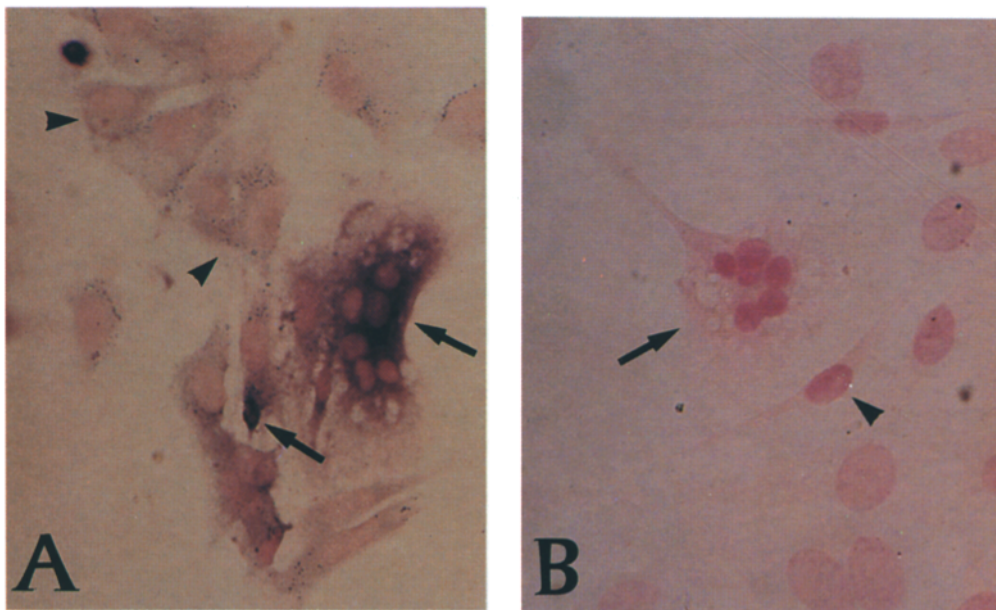


Figure 10. In situ hybridization. Cocultures of bone marrow cells and primary osteoblastic cells were subjected to in situ hybridization as detailed in Materials and Methods. (A) Photomicrograph of cells from the cocultures at day 7. The cells were hybridized with an anti-sense riboprobe specific for mL-11R α . Transcripts for IL-11R α , represented by blue staining, are located in polygonal and spindle-shaped osteoblastic-stromal cells (arrow heads) and OCLs (arrows). (B) Specific hybridization signals were eliminated by pretreatment with RNase. All preparations were counterstained with nuclear fast red. Original magnification, $\times 240$. The results shown in this figure were reproduced in three independent experiments.

duction stimulated by IL-1 and TNF in our primary osteoblast cultures. Prostaglandins are produced in bone by many cells, especially by osteoblasts, and production is stimulated by a variety of cytokines derived from macrophages or hemopoietic cells (34–38). The important role of PGs in IL-1-mediated osteoclast formation and bone resorption has been emphasized (39), and PGs appear to be essential for IL-11 mediated osteoclastogenesis as well (15). An IL-1 response element exists close to the IL-11 gene and, in certain stromal cells IL-1 can stimulate IL-11 by a mechanism independent of prostaglandins (40).

In the present study, the ability of $1\alpha,25(\text{OH})_2\text{D}_3$ to potently stimulate IL-11 production was shown in osteoblasts and in cocultures of osteoblasts and bone marrow cells. These findings are consistent with a similar effect of $1\alpha,25(\text{OH})_2\text{D}_3$ in bone marrow cell cultures (15). The concentrations of IL-11 present in supernatants of the co-cultures treated with $1\alpha,25(\text{OH})_2\text{D}_3$ exceeded 1 ng/ml after 3 d, which was in accord with our previous estimate of the EC₅₀ of exogenous IL-11 (~ 1.0 ng/ml) necessary for maximal osteoclastogenesis in such cocultures (16).

Cytokines exert their pleiotropic effects by interacting with specific cell surface receptors (11, 12). There is little direct information about the IL-11 receptor in connective tissue cells. During embryogenesis, primitive chondroblastic and osteoblastic progenitor cells seem to express higher levels of a transcript homologous with IL-11R α compared to their more differentiated counterparts (13). Our results indicate that primary osteoblasts not only are a source of IL-11, but express transcripts for the complete IL-11 receptor complex (IL-11R α and gp130). This evidence suggests that IL-11 acts in an autocrine-paracrine fashion. Stimulation of primary osteoblastic cells by $1\alpha,25(\text{OH})_2\text{D}_3$, PTH or IL-1 upregulated gp130 mRNA up to threefold at 24 h, while mRNA levels for IL-11R α were unchanged. Similar

upregulation of gp130 mRNA (and protein) expression by $1\alpha,25(\text{OH})_2\text{D}_3$ and PTH has been reported in murine MC3T3-E1 cells and primary bone marrow cell cultures (41). Indeed, it has been proposed that regulation of gp130 expression by systemic hormones could modulate the sensitivity of osteoblasts to cytokines such as IL-11 and IL-6-sIL-6R α (41, 42). It is not known if gp130 may be rate-limiting for cytokine signal transduction, in the way that low levels of functional IL-6 receptors limit responsiveness of osteoblastic cells to IL-6 under physiological circumstances (16, 17). Expression of gp130 is ubiquitous (26), but it is possible that key target cells, such as bone marrow stromal cells might be relatively deficient in gp130 under certain conditions. Further studies are required to confirm this hypothesis.

We have reported that OCL formation is induced by at least three different mechanisms (32, 33). The first mechanism is the PTH-IL-1-PGE₂ axis, which is mediated by signaling involving cAMP. The second mechanism is $1\alpha,25(\text{OH})_2\text{D}_3$ -induced osteoclast formation, which is mediated by the vitamin D receptor but independent of cAMP. IL-1 induces OCL formation by a mechanism involving PG production (39). The gp130 signal, activated by cytokines such as IL-11, IL-6-sIL-6R α , LIF, or OSM, is clearly an additional and important pathway of OCL formation. We previously showed that anti-IL-6R α antibody inhibited OCL formation by IL-6-sIL-6R α , but not by IL-1 or $1\alpha,25(\text{OH})_2\text{D}_3$ (16). This indicates that IL-6 is not implicated in osteoclastogenesis stimulated by IL-1 or $1\alpha,25(\text{OH})_2\text{D}_3$. In the present study, neutralizing anti-gp130 antibody, abolished osteoclast formation induced by IL-1. Moreover, the antibody partially inhibited the osteoclastogenic effects of PGE₂, PTH, and $1\alpha,25(\text{OH})_2\text{D}_3$. This indicates that gp130 signals, probably evoked by IL-11, are indispensable for IL-1 induced OCL formation. This is consistent with the ability

of IL-1 to induce both PGE₂ and IL-11 production by osteoblasts. Similarly, we speculate that IL-11 contributes, at least in part, to OCL formation induced by PGE₂, PTH, and 1 α ,25(OH)₂D₃. Girasole et al. also reported that a monoclonal anti-IL-11 antibody inhibited PTH-, 1 α ,25(OH)₂D₃-, IL-1-, or TNF α -mediated OCL formation by between 50–100% (15).

Together, these results suggest that the gp130 signal is a pivotal cofactor for osteoclast formation. Targeted disruption of gp130 may clarify the physiological role of IL-11 and related cytokines in osteoclast formation and skeletal development (43).

Osteotropic factors appear to act directly on osteoblastic cells, which in turn produce a factor responsible for osteoclast differentiation (17, 32). This putative factor may be expressed on the cell surface membranes and plays a critical role through a juxtacrine (cell-to-cell contact) mechanism. In support of this concept, we recently established that the ability of IL-6 to induce osteoclast differentiation depends on signal transduction mediated by IL-6 receptors expressed on osteoblastic cells but not on osteoclast progenitors (17). In view of this evidence, it is likely that IL-11 also induces osteoclast formation by activating gp130 signals via IL-11 receptors present on osteoblasts. In bone marrow cell cultures, which are relatively deficient in stromal cells, exogenous IL-11 is insufficient to trigger osteoclastogenesis (15), but augments the effect of 1 α ,25(OH)₂D₃, perhaps because gp130 is upregulated under these conditions.

The demonstration of IL-11R α mRNA expression in mature OCLs suggests an important biological function of IL-11 in osteoclasts, perhaps distinct from its role in osteoclast formation. Ohsaki et al. (44) demonstrated functional IL-6 receptors in human osteoclast-like cells derived from giant cell tumors of bone. In these cells, IL-6 appeared to modulate bone resorbing activity. However, anti-gp130 antibody did not influence pit resorption by OCLs (data not shown). Macrophage colony stimulating factor and IL-1 appear to promote survival of OCLs (45), but we found that IL-11 itself did not support OCL survival (data not shown). Further studies will be required to demonstrate functional IL-11 receptors on OCLs and elucidate the role of IL-11 in mature osteoclasts.

In conclusion, normal osteoblasts are a source of IL-11, the production of which is induced by IL-1, TNF, PGE₂, PTH, and 1 α ,25(OH)₂D₃. Bone resorbing hormones and IL-1 also upregulate gp130 mRNA in these cells and consequently cell responsiveness to IL-11 and related cytokines may be enhanced. Both osteoblasts and mature OCLs express IL-11R α mRNA and thus are potentially able to respond to IL-11. Signals mediated by gp130 may be involved in physiological regulation of osteoclast formation stimulated by osteotropic factors. Since high local concentrations of IL-1, TNF, and PGs occur in rheumatoid arthritis synovium (46), IL-11 could contribute to the excessive osteoclastic bone resorption observed in that disease.

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