Shedding of the Interleukin-6 (IL-6) Receptor (gp80) Determines the Ability of IL-6 to Induce gp130 Phosphorylation in Human Osteoblasts*

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Human osteoblasts produce interleukin-6 (IL-6) and respond to IL-6 in the presence of soluble IL-6 receptor (sIL-6R), but the cell surface expression of IL-6R and the mechanism of sIL-6R production are largely unknown. Three different human osteoblast-like cell lines (MG-63, HOS, and SaO8-2) and bone marrow-derived primary human osteoblasts expressed both IL-6R and gp130 as determined by flow cytometry and immunoprecipitation. However, the membrane-bound IL-6R was non-functional, as significant tyrosine phosphorylation of gp130 did not occur in the presence of IL-6. Phorbol myristate acetate induced a dramatic increase of both IL-6R shedding (i.e. the production of sIL-6R) and IL-6 release in osteoblast cultures, but the cell surface expression of gp130 remained unchanged. IL-6 complexed with sIL-6R, either exogenously introduced or derived from the nonfunctional cell surface form by shedding, induced rapid tyrosine phosphorylation of gp130. This effect was inhibited by neutralizing antibodies to either sIL-6R or gp130, indicating that the gp130 activation was induced by IL-6/sIL-6R/gp130 interaction. Protein kinase C inhibitors blocked phorbol myristate acetate-induced and spontaneous shedding of IL-6R resulting in the absence of sIL-6R in the culture medium, which in turn also prevented the activation of gp130. In conclusion, human osteoblasts express cell surface IL-6R, which is unable to transmit IL-6-induced signals until it is shed into its soluble form. This unique mechanism provides the flexibility for osteoblasts to control their own responsiveness to IL-6 via the activation of an IL-6R sheddase, resulting in an immediate production of functionally active osteoblast-derived sIL-6R.

The balance between bone formation and bone resorption is controlled at least in part by different osteotropic hormones and soluble mediators such as various pro- and anti-inflammatory cytokines. Recently, interleukin-6 (IL-6)1 has attracted special attention because a strong correlation has been found between serum and/or local levels of IL-6 and bone resorption in various diseases (1–5). IL-6 is a pleiotropic cytokine with multiple and diverse effects on various cell types (2, 4, 6, 7). In bone, IL-6 has different effects depending on which type of bone cell is targeted. For example, IL-6 is capable of promoting osteoblast differentiation (4, 8–10) and osteoclast activation (1, 4, 11), processes that depend on the activation of IL-6-induced signaling mechanisms in osteoblasts (8, 9, 12, 13).

IL-6 exerts its effect through the IL-6 receptor complex, which is composed of a ligand binding domain (IL-6 receptor or gp80) and the signal-transducing molecule glycoprotein 130 (gp130). IL-6 binds to its cognate receptor, and the IL-6/IL-6R forms a complex with a gp130 homodimer. This receptor-ligand interaction activates Janus kinases (JAKs). JAKs phosphorylate the tyrosine residues of the cytoplasmic tail of gp130, which then activates various members of the signal transducer and activator of transcription (STAT) family and also the mitogen-activated protein kinase (MAPK) pathway (2, 6–8, 14–17). Both IL-6R and gp130 have soluble forms. Although soluble gp130 inhibits the effect of IL-6 (3, 7, 18), the soluble IL-6R (sIL-6R), unlike other soluble cytokine receptors, promotes the effect of IL-6 (3, 5, 7, 19). Therefore, it is believed that the limiting factor for the IL-6 effect is either the expression of functional cell surface IL-6R or the generation of functionally active sIL-6R.

gp130 is ubiquitously expressed in different types of cells. IL-6R expression is also extensive on the surface of various cell types (6, 18, 20–24), but it is assumed to be limited in osteoblasts based on indirect correlations (4, 9, 10, 12, 13, 25–29). Information on the expression and functionality of IL-6R in human osteoblasts is restricted and controversial (8, 9, 25, 27, 30, 31). Although mRNAs coding for IL-6R and gp130 have been identified in murine or human osteoblasts (12, 26, 27, 30), there is no direct evidence (e.g. flow cytometry) demonstrating the presence of IL-6R on the cell surface in human osteoblasts. Although human osteoblasts produce IL-6, and these cells have been shown to respond to IL-6 in the presence of sIL-6R (8, 9, 25, 32, 33), the mechanism of sIL-6R production by human osteoblasts is largely unknown (34).
The sIL-6R can be generated by two distinct mechanisms as described for non-osteoblastic cells: by the shedding of the extracellular domain of IL-6R because of proteolytic cleavage, or by differential splicing (3, 5, 7, 18, 19, 23). In the first case, a highly specific, yet to be identified, enzyme cleaves the extracellular domain of the membrane-anchored IL-6R (3, 5, 19, 35, 36). In the second case, a 94-base pair deletion occurs (exon 2), including the transmembrane coding region, creating a reading frameshift and resulting in the release of a soluble form of IL-6R carrying a unique 10-amino acid sequence at the carboxyl terminus (3, 5, 19, 37–40). Therefore, the two forms of sIL-6R can be distinguished by the characteristic COOH-terminal amino acid sequences.

In this study we examined the synthesis and the cell surface expression of IL-6R and gp130 by human osteoblast-like cell lines (MG-63, HOS, and SaOS-2) and bone marrow-derived primary human osteoblasts. We also investigated the mechanism of sIL-6R generation and analyzed distinct functions of the cell surface IL-6R and the osteoblast-derived sIL-6R by monitoring the IL-6-induced tyrosine phosphorylation of gp130 in human osteoblasts.

**EXPERIMENTAL PROCEDURES**

**Cells and Cell Cultures**—Human osteoblast-like cell lines MG-63, SaOS-2, and HOS and human monocytic cell line THP-1, a positive control for cell surface IL-6R expression (23), were purchased from the American Type Culture Collection (Rockville, MD). The cells were cultured in monolayers in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% fetal bovine serum (FBS; HyClone Laboratories, Inc., Logan, UT) in a standard tissue culture condition (41–43).

Primary osteoblasts were isolated from bone marrow samples of either the iliac crest or vertebral bodies obtained during spine fusion surgeries from patients of both sexes, ranging in age from 25 to 69 years. Bone marrow collection was approved by the Institutional Review Board, and signed consent forms were obtained from each patient. Culture conditions, isolation, and characterization of cells were exactly the same as described earlier (43–45). Briefly, buffy coat-separated nucleated bone marrow cells (2Á10^6/10^7/T75 tissue culture flasks; Corning Inc., Corning, NY) were cultured in α-minimal essential medium (Invitrogen) containing 10% FBS, 10 μM dexamethasone, 50 μg/ml ascorbic acid, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, and 50 μg/ml gentamicin, all purchased from Sigma. The first medium change was performed on day 7, at which time the medium was supplemented with 5 μM β-glycerophosphate (Sigma). Dense colonies of cells were trypsinized and 1Á10^5 cells plated in 10-cm Petri dishes (Corning). Cells were then cultured to obtain a confluent monolayer culture. All experiments with bone marrow-derived osteoblasts were carried out using these first passage cultures. At the time of this

![FIG. 1. Spontaneous cell surface expression of IL-6R by THP-1 (human monocytic cell line), human osteoblast cell lines (MG-63, HOS and SaOS-2), and two independent bone marrow-derived human osteoblasts (POB-1 and POB-2). The expression of IL-6R was analyzed by flow cytometry as described. Closed histograms represent isotype antibody controls, and open histograms show anti-IL-6R antibody-labeled cells on each panel.](image1)

![FIG. 2. The expression of cell surface IL-6R and gp130 in MG-63 cultures after different treatments. Semiconfluent osteoblast cultures were left untreated or treated with PMA or TNF-α for 4 h, and the expression of cell surface IL-6R and gp130 were analyzed by flow cytometry. Closed histograms represent isotype antibody controls, and open histograms show either anti-IL-6R or anti-gp130 antibody-stained cells as indicated. The expression of IL-6R was reduced by both PMA and TNF-α, whereas the level of gp130 was unaffected. The PMA-induced down-regulation of IL-6R expression was essentially abolished by staurosporine (stauro) and partially reversed by Galardin (Gal), whereas the suppressive effect of TNF-α on IL-6R expression was substantially diminished by UO126, a potent inhibitor of MEK1/MEK2.](image2)
First passage, aliquots of cells were also seeded in 24- and 96-well plates (Corning) for viability and cell proliferation tests, and alkaline phosphatase (AP) activity assays. AP activity was measured by Alkaline Phosphatase Colorimetric End point assay (Sigma) in cell lysates of first-passaged osteoblasts. Confluent cultures were stained in situ for AP positivity using Naphthol-AX and Fast Blue reagents (Sigma) following the manufacturer’s instructions. Osteoblast cultures showing higher than 80–85% AP positivity were used in these experiments.

Treatment of Cells—Semiconfluent cultures of cells were subjected to serum starvation (0.3% FBS) for 24 h prior to treatment. Culture media were then replaced with fresh media containing 0.3% FBS and various compounds. Proliferation and viability assays, flow cytometry analysis, and total RNA and protein extractions were carried out on untreated and treated cells. Tissue culture media were collected at various time points, centrifuged, and stored at −80 °C. All experiments were performed in duplicate or triplicate in at least five independent experiments for osteoblast cell lines and in at least three independent experiments for primary osteoblasts.

Reagents listed below were purchased from Calbiochem (La Jolla, CA), R&D Systems (Minneapolis, MN), or Sigma. All concentrations were selected after serial dilutions of each compound tested in either MG-63 or primary osteoblast cell cultures. Only the viable range and the most effective concentrations were used for further experiments. Tumor necrosis factor-α (TNF-α, 20 ng/ml), IL-6R (50 ng/ml), sIL-6R (200 ng/ml), lipopolysaccharide (LPS, O127:B8, 1 mg/ml), phorbol myristate acetate (PMA, 20 ng/ml), actinomycin D (an inhibitor of transcriptional events, 1 μg/ml), cycloheximide (an inhibitor of protein synthesis, 10 μM), brefeldin A (an inhibitor of protein transport from endoplasmic reticulum to Golgi, 1 μM), monensin (an inhibitor of protein transport from Golgi, 10 μM), tunicamycin (an inhibitor of N-glycosylation, 2 μg/ml), wortmannin (an inhibitor of phosphatidylinositol 3-kinase (PI3K), 0.1 μM), SB203580 (an inhibitor of p38 MAPK, 10 μM), U0126 (an inhibitor of MAPK kinase 1 and MAPK kinase 2 (MEK1 and MEK2), 1 μM), staurosporine (0.01 μM), calphostin C (0.1 μM), and bisindolylmaleimide I (1 μM) (all inhibitors of protein kinase C (PKC)), genistein (an inhibitor of protein-tyrosine kinases (PTK), 20 μM), H-89 (a potent inhibitor of protein kinase A (PKA), 30 μM), TAPI-1 (a hydroxamate-based metalloproteinase inhibitor, 50, 100, and 150 μM), and Galardin (GM-6001, a potent metalloproteinase inhibitor, 10, 50, and 100 μM) were added either alone or in different combinations. As calphostin C requires photactivation (46) to inhibit PKC, experiments with calphostin C were carried out in an incubator with a 5-watt light source located 15 cm above culture dishes.

Viability Tests—The trypan blue exclusion test was used to assess the viability of cells. Viability tests were performed in duplicate, and at least 200 cells were counted. Determination of cell viability was used to select the concentrations of each compound in which the cells remained viable (>95%), during the indicated time period.

Measurement of Cytokines and Osteoblast-specific Proteins in Culture Media—Cytokine concentrations in supernatants of osteoblast cultures were measured by sandwich enzyme-linked immunosorbent assays in 96-well microtitration plates following the manufacturer’s instructions.
High sensitivity assay kits for IL-6 (sensitivity range from 3 to 200 pg/ml) and sIL-6R (range from 31 to 2000 pg/ml) were purchased from R&D Systems and BIOSOURCE (Camarillo, CA), respectively.

Detection of Cell Surface Expression of IL-6R and gp130 by Flow Cytometry—Confluent layers of cells were either untreated or treated with different compounds for various times. Cells were harvested with enzyme-free cell dissociating buffer (Intrivogen) and then washed three times in washing buffer (phosphate-buffered saline, pH 7.4, containing 1% bovine serum albumin (Sigma)). Cells were resuspended in 100 μl of washing buffer and incubated with 10 μg/ml anti-human IL-6R monoclonal antibody (IgG1, clone B-R6, BIOSOURCE) or with 50 μg/ml anti-human-gp130 monoclonal antibody (IgG2a, clone B-R3, BIOSOURCE) for 1 h at 4 °C, followed by biotin-labeled polyclonal anti-mouse Ig antibody (10 μg/ml; BD Pharmingen, San Diego, CA). The reaction was developed with streptavidin-phycocerythrin (Intrivogen). Samples were fixed in 2% formalin (Sigma) and then analyzed by FACScan (BD Pharmingen) using Cell Quest software (BD Pharmingen). Isotypic control antibodies corresponding to the primary antibodies were used to determine nonspecific background levels in all experiments. All compounds were tested for autofluorescence. Calphostin C- and bisindolylmaleimide I-treated cells exhibited red fluorescence necessitating the use of streptavidin-fluorescin isothiocyanate (BD Pharmingen) in experiments whenever these two compounds were applied.

Immunoprecipitation and Western Blot Analysis—Treated and untreated cells were lysed in ice-cold lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% SDS, and 1% Nonidet P-40) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride and 1 unit/ml aprotinin), phosphatase inhibitors (50 mM NaH2PO4, 10 mM sodium pyrophosphate, 50 mM KF, and 1 mM Na3VO4), and 0.01% NaN3, for 1 h at 4 °C. Cell lysates were ultrasonicated (Virtilis, Gardina, NY) for 10 s at 4 °C and cleared by centrifugation, after which 700 μg of cell lysate protein was incubated with either 3 μg of anti-human-IL-6R antibody (rabbit IgG, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or 3 μg of anti-human-gp130 antibody (rabbit IgG, Santa Cruz) for 2 h at 4 °C. Immunocomplexes were collected with Protein G-Sepharose (Amersham Biosciences) after overnight incubation at 4 °C. Protein G-bound complexes were washed in lysis buffer, and then proteins of biotinylated samples were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in reducing condition as described (43). Samples were transferred onto a nitrocellulose membrane (Bio-Rad), and the free binding capacity of the membrane was blocked with 5% skimmed milk in phosphate-buffered saline for 2 h at room temperature. Membranes were immunoblotted with anti-human IL-6R (1 μg/ml), anti-human gp130 (1 μg/ml), or biotinylated anti-phosphotyrosine (clone 4G10, 1 μg/ml; Upstate Biotechnology, Inc., Lake Placid, NY). Antibodies were visualized using an enhanced chemiluminescence (Amersham Biosciences) after using appropriate secondary antibodies conjugated with horseradish peroxidase-labeled reagents (Zymed Laboratories Inc., San Francisco, CA).

Enzymatic Deglycosylation of IL-6R and gp130—N-Glycosidase F (New England Biolabs, Beverly, MA) was used to remove N-linked oligosaccharide chains from both IL-6R and gp130. The immunoprecipitated proteins were digested with N-glycosidase F according to the manufacturer’s protocol and then analyzed using the Western blot method described above.

RNA Isolation and RNase Protection Assay (RPA)—Total RNA samples were isolated from monolayer cultures as described (42, 43). The expressions of cytokine and receptor mRNAs were then analyzed by RPA using a custom-made human multiprobe template set following the manufacturer’s protocol (BD Pharmingen). This multiple template set generated specific RNA probes for IL-6, oncostatin M, leukemia inhibitory factor (LIF), granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-12 p35 subunit (IL-12p35), IL-12p40, IL-6R, gp130, GM-CSF receptor α, and two housekeeping genes (glyceraldehyde-3-phosphate dehydrogenase and L32). Briefly, 32P-labeled riboprobes were synthesized from cDNA templates set using T7 RNA polymerase. The riboprobes were hybridized overnight to the target sample RNA (15 μg), and then the unhybridized (nonprotected) probe and sample RNA were digested by RNase T1 + RNase A. The protected fragments were purified and separated on a sequencing gel. The expression of mRNAs was visualized by autoradiography and quantified using the STORM PhosphorImager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Reverse Transcription of RNA, Polymerase Chain Reaction (PCR), and Sequencing of the PCR Products—The first-strand cDNA was synthesized from 1 μg of total RNA priming by oligo(dT) using SuperScript reverse transcription kit (Invitrogen). The target IL-6R cDNA was amplified by PCR using forward primer (5'-CTAATCTTCTTCCGGTGCCAGC-3') and reverse primer (5'-GGACATTCCAGGTCTTCTTCTTGAG-3') flanking the transmembrane coding region of IL-6R. Templates were initially denatured at 95 °C for 5 min, and then a cycle of 95 °C for 30 s, 60 °C for 40 s, and 72 °C for 40 s was repeated 30 times, followed by a final extension at 72 °C for 5 min. The number of amplification cycles chosen for each reaction was determined to be within the linear range of the assay. To verify semiquantitatively the amounts of mRNA coding for IL-6R, PCR amplification of glyceraldehyde-3-phosphate dehydrogenase housekeeping gene was performed using the same reverse-transcribed cDNA templates and PCR conditions, and a primer pair described earlier (47). PCR products were separated in 1.5% agarose Tris acetate-EDTA gels and stained with ethidium bromide. Representative PCR products were sent to ALF Express II DNA sequencing kit (Amersham Pharmacia Biotech Inc., Valencia, CA) for sequencing using an ABI model 310 genetic analyzer (PerkinElmer, Branenburg, NJ).

Statistical Analysis—Descriptive statistics were used to determine group means and standard deviations. Paired Student’s t tests were performed between groups of interest. The level of significance was set at p < 0.05. All statistical analyses were performed using computer-based statistical software (SPSS/PC®, version 4.0.1, SPSS Inc., Chicago, IL).
**RESULTS**

**Osteoblasts Express Membrane-anchored IL-6R and gp130, and Shed the IL-6R into sIL-6R—**First, we determined whether human osteoblasts express IL-6R and gp130 on their cell surface. Human monocyctic cell line THP-1 was used as a positive control (Fig. 1A). All untreated cultures of various osteoblast-like cell lines and bone marrow-derived primary human osteoblasts expressed a large amount of IL-6R and gp130 (Figs. 1 and 2) analyzed by flow cytometry. The expression of IL-6R was reduced in both PMA- and TNF-α-treated cultures (Fig. 2), and the effects were time- and dose-dependent. In contrast to IL-6R, the expression of gp130 was not affected by either PMA or TNF-α (Fig. 2).

The PMA-induced reduction of IL-6R expression (Figs. 2 and 3B) was the result of receptor shedding, as the amount of sIL-6R increased simultaneously in the culture medium as early as 1–2 h (Fig. 3, A and C). Inhibitors of major signaling molecules (PTK, PKA, PI3K, MAPK) or TAPI-1 failed to inhibit IL-6R shedding (not shown). On the other hand, PMA-induced shedding of IL-6R was completely blocked by PKC inhibitors and partially by a potent metalloproteinase inhibitor, Galardin, resulting in both the reversal of IL-6R expression on the cell surface (Fig. 2) and the absence of sIL-6R in the medium (data not shown). These observations suggest that IL-6R shedding is mediated by a PKC-controlled enzyme(s), possibly a membrane-type metalloproteinase(s), in human osteoblasts. Although PKC inhibitors also reduced the spontaneous sIL-6R release, these inhibitors simultaneously slightly up-regulated the cell surface expression of IL-6R in untreated cultures. This might be a consequence of diminished natural IL-6R shedding. This observation further supports the role of a PKC-mediated sheddase in the process of sIL-6R release.

In contrast to the PMA effect, the suppression of IL-6R expression in TNF-α-treated cultures (Fig. 2) was not accompanied by an increased sIL-6R release (Fig. 3A). Instead, sIL-6R release was reduced (Fig. 3A), indicating that the suppression of IL-6R expression by TNF-α was not the result of receptor shedding. TNF-α reduced the total amount of IL-6R in MG-63 (Fig. 4A) and primary human osteoblasts (Fig. 4C) in a time-dependent manner, suggesting either the inhibition of IL-6R synthesis or internalization (degradation) of the receptor. In contrast to the TNF-α effect, PMA had no measurable effect on the total amount of IL-6R (Fig. 4B). TNF-α-induced down-regulation of IL-6R expression (Figs. 2 and 4) was prevented by a MEK1/MEK2 inhibitor U0126 (Figs. 2 and 4D) and by PKC inhibitors (Fig. 4D). These inhibitors also diminished the suppressive effect of TNF-α on sIL-6R production (data not shown). The suppressed cell surface expression of IL-6R and the reduced level of sIL-6R in the conditioned medium of TNF-α-treated osteoblasts were unaffected by inhibitors of PTK, PKA (Fig. 4D), PI3K, or p38 MAPK. Collectively, these results suggest the involvement of the PKC-MAPK pathway (without the participation of p38 MAPK) in TNF-α-induced inhibition of cell surface IL-6R expression.

**Osteoblasts Synthesize IL-6R and gp130 with a High Turnover—**To further confirm that the synthesis of IL-6R can be blocked as rapidly as by TNF-α (Fig. 4A), inhibitors of protein synthesis and intracellular transport were administrated to osteoblast cultures and then the IL-6R release and the expression of both IL-6R and gp130 were investigated. Cycloheximide, brefeldin A, and monensin dramatically reduced the sIL-6R production (data not shown) and the cell surface expression of both IL-6R and gp130 after 4 h (Fig. 5), approximately at the time point when the TNF-α effect became evident (Figs. 2 and 4). Actinomycin D also inhibited the IL-6R release and cell surface expression of both IL-6R and gp130, but only after
12 h (Fig. 5). These results suggest that continuous transcription and translation processes and active intracellular transport mechanisms are necessary for the expression of IL-6R and gp130, and for effective sIL-6R release.

As was shown in Figs. 1 and 2, osteoblasts expressed cell surface IL-6R and gp130. Immunoprecipitation studies confirmed that these proteins were synthesized by osteoblasts with approximate sizes of 88 and 145 kDa (Figs. 4, 6, and 7). The amounts of both IL-6R and gp130 proteins were significantly inhibited by actinomycin D, cycloheximide, and brefeldin A. Unexpectedly, cultures treated with brefeldin A contained the accumulation of the low molecular mass forms of IL-6R (at 80 kDa) or gp130 (130 kDa) shown as var-IL-6R and var-gp130. Note that inhibition of N-glycosylation by tunicamycin or treatment of samples with N-glycosidase F resulted in the 105-kDa band corresponding to gp130 (deglycos-gp130).

Either the inhibition of N-glycosylation by tunicamycin or the removal of sugar components by N-glycosidase F shifted the larger molecular mass to an ~105-kDa band, replacing the 130- and 145-kDa bands (Fig. 6B). As the predicted size of gp130 based on the amino acid sequence is 103.5 kDa (50), this 105-kDa species should represent the nonglycosylated core protein of gp130. Treatment with brefeldin A resulted in a complete shift of the 145-kDa to the 130-kDa form (Fig. 6B), i.e. a partial glycosylation occurred (48).

The Cell Surface IL-6R Is Unable to Transmit IL-6-induced Signals until It Is Shed into Its Soluble Form—Tyrosine phosphorylation of gp130 is an essential step in IL-6-induced signaling mechanisms. Thus, to investigate the functionality of cell surface IL-6R, we tested the tyrosine phosphorylation of gp130 in the presence of IL-6 or IL-6 combined with various compounds. We found a very weak, almost undetectable tyrosine phosphorylation of gp130 in untreated osteoblasts (Fig. 7A). Despite the extensive cell surface expression of IL-6R (Figs. 1 and 2), it was unable to mediate IL-6-induced signals as exogenous IL-6 failed to induce significant tyrosine phosphorylation of gp130 (Fig. 7). In contrast, a co-treatment of osteoblasts with IL-6 and sIL-6R resulted in rapid tyrosine phosphorylation of the 145-kDa gp130, but not in the less glycosylated 130-kDa species (Fig. 7).

The transfer of the conditioned medium from PMA-stimulated cells (which has significant amounts of IL-6 and sIL-6R; Fig. 3A) to an untreated osteoblast culture induced a
strong tyrosine phosphorylation of gp130 after 10 min of treatment. This effect was further increased by adding exogenous IL-6 (Fig. 7A). In contrast, the transfer of conditioned media from untreated cultures did not result in tyrosine phosphorylation of gp130 (Fig. 7A). Likewise, PMA alone failed to induce tyrosine phosphorylation of gp130 after 10 min (data not shown), indicating that PMA could not account for this effect. Tyrosine phosphorylation of gp130, induced by the conditioned medium of PMA-stimulated osteoblasts, was inhibited by neutralizing antibodies to either sIL-6R (Fig. 7A) or gp130 (data not shown), further confirming that the gp130 phosphorylation was indeed induced by the IL-6-sIL-6R gp130 complex and not by another IL-6 type of cytokine. To verify this hypothesis, conditioned medium of PKC-inhibitor-pretreated (blocking IL-6R shedding) and subsequently PMA-treated osteoblasts failed to induce tyrosine phosphorylation of gp130, even in the presence of exogenous IL-6. Taken together, these results suggest that the nonfunctional cell surface IL-6R becomes functionally active after shedding and the (shed) sIL-6R complexed with IL-6 is able to induce gp130-mediated signaling in human osteoblasts.

Osteoblasts Constitutively Express IL-6R and gp130 Genes—To explore whether transcriptional mechanisms are involved in the regulation of IL-6R expression and sIL-6R production, various mRNA levels were analyzed. Human osteoblasts constitutively expressed mRNAs for both IL-6R and gp130, which were not modified by treatments with PMA, LPS, TNF-α, IL-6, or the combination of IL-6 and sIL-6R. Upregulation of IL-6, LIF, and GM-CSF mRNA occurred in TNF-α- or LPS-treated cultures after 24 h (Fig. 8A). PCR analysis of IL-6R showed two specific products with a 94-base pair difference in size (Fig. 8B). TNF-α treatment induced the suppression of the smaller species at 24 h (Fig. 8B). Sequencing analysis of the two PCR products verified that both were specific for IL-6R mRNA, but the smaller product lacked the 94 base pairs (exon 2) including the coding region of the transmembrane domain of the IL-6R (Fig. 8C). This mecha-

![Figure 8](image-url)
nism is identical to those described earlier for human monocytic and tumor cell lines (5, 18, 37–40).

**DISCUSSION**

Although the expression of gp130 on human osteoblast cells is well confirmed, the cell surface expression of IL-6R remains unclear. Herein, we demonstrated the expression of IL-6R on the surface of human osteoblasts using flow cytometry, and also the total amount of IL-6R by immunoprecipitation. In the view of these results, a number of apparent contradictions are resolved, such as those related to the functionality of the IL-6R complex in osteoblasts. For example, it was believed that MG-63 cells lacked IL-6R on the cell surface because IL-6 was unable to induce tyrosine phosphorylation of gp130 (9), although no direct evidence was presented to confirm this hypothesis. In contrast, it was also proposed that MG-63 cells should express functional IL-6R because a weak tyrosine phosphorylation of gp130 could be detected in the presence of exogenous IL-6 (25); however, the presence of IL-6R on osteoblasts was not demonstrated. These contradictory conclusions can be explained, at least in part, by the use of variable antibodies with the differential ability to recognize gp130 and/or phospho-gp130.

In this study we have detected relatively high IL-6R expression on the cell surface of human osteoblast-like cell lines and on bone marrow-derived primary human osteoblasts. We have also identified very weak tyrosine phosphorylation of gp130 in either nontreated or IL-6-treated cultures. The functionality of sIL-6R present in the culture medium was confirmed by the use of a neutralizing antibody to sIL-6R, which completely blocked the tyrosine phosphorylation of gp130. As IL-6 was unable to induce significant tyrosine phosphorylation of gp130, either the cell surface-expressed IL-6R is nonfunctional or an upstream event of IL-6-induced signaling is impaired. Clearly, further studies are necessary to understand why the osteoblast-expressed IL-6R/gp130 complex fails to transmit signals in the presence of IL-6. On the other hand, as described earlier (9, 25), osteoblasts exhibit a strong tyrosine phosphorylation of gp130 in the presence of both IL-6 and sIL-6R (Fig. 7).

Our results indicate that PMA-induced loss of IL-6R from the cell surface is caused by shedding, because the amount of sIL-6R increased simultaneously in the conditioned medium of PMA-treated cultures. As PKC inhibitors eliminated the PMA-induced release of sIL-6R, the activation of the IL-6R shedding is most likely controlled by a member(s) of the PKC family in human osteoblasts similar to that described for human myeloma, monocytic cell lines, and human neutrophils (3, 5, 19, 35, 51). Likewise, the presence of sIL-6R in untreated osteoblast cultures is probably a result of shedding, because PKC inhibitors almost completely blocked the release of sIL-6R and simultaneously increased the expression of IL-6R on the cell surface. Consistent with these findings, inhibitors of protein synthesis and transport rapidly reduced the cell surface expression of IL-6R and the production of sIL-6R. This suggests that the increased sIL-6R release was the consequence of impaired shedding and reduced IL-6R expression. Collectively, the constitutive and PMA-induced IL-6R shedding most likely require the same membrane proteinase, which is controlled by similar signaling mechanisms. The partial effect of Galardin is evidence for the involvement of a metalloproteinase(s) in the shedding process of IL-6R in human osteoblasts. However, as the expression of IL-6R on the cell surface and the sIL-6R production were unchanged in the presence of metalloproteinase inhibitor TAPI-1, IL-6R shedding in osteoblasts is likely executed via an enzyme(s) different from that described for human multiple myeloma and monocytic cells (20, 22, 23).

As human osteoblasts generate an alternatively spliced variant of IL-6R mRNA lacking the transmembrane coding region (Fig. 8), we cannot rule out the possibility that this sIL-6R isoform also contributes to the total amount of sIL-6R measured in the culture medium. However, it is very unlikely that PKC inhibitors could so rapidly and selectively decrease the production of a splice variant in untreated cultures and that PMA could increase so dramatically the transcription (Fig. 8) and then the synthesis of this splice variant within 2 h (Fig. 3). Thus, the majority of sIL-6R is probably generated by a proteolytic cleavage controlled by a PKC-mediated pathway in human osteoblasts.

Based on the results discussed above, why osteoblasts continuously express nonfunctional IL-6R on the cell surface and why a high level of receptor synthesis and intracellular trafficking is maintained seem to be obvious questions. One possible answer is that the generation of sIL-6R by shedding from a nonfunctional cell surface form is the primary mechanism by which osteoblasts are able to rapidly control their own sensitivity toward IL-6. In other words, human osteoblasts, which can produce a large amount of IL-6, are “resistant” to the autocrine effects of IL-6 in normal conditions. However, they are ready to respond rapidly to IL-6 by shedding of the nonfunctional cell surface IL-6R. This mechanism seems to be more rapid and flexible than either controlling the synthesis and the cell surface expression of a functional IL-6R or regulating the secretion of a functionally active sIL-6R splice variant. In addition, the osteoblast-derived sIL-6R/IL-6 complex may have remote effects on various gp130-expressing cells in a paracrine fashion, especially on osteoclasts.

Another very intriguing finding of this study is that, whereas TNF-α increases IL-6 secretion in osteoblast cultures, this proinflammatory cytokine simultaneously inhibits the expression of IL-6R on the cell surface (Fig. 2), i.e. a fewer number of receptors is available to be shed and to be functionally active. This observation suggests that, although osteoblasts can produce more IL-6 as a response to the proinflammatory cytokine TNF-α (Fig. 3), they simultaneously become less sensitive to IL-6 because of the lack of functionally active IL-6R/sIL-6R complex. This seems to be a particularly important mechanism providing instant “anergy” of the osteoblast to IL-6 under inflammatory conditions. The TNF-α-induced down-regulation of IL-6R is PKC-mediated involving MAPKs (but not p38 MAPK), whereas the PMA-induced shedding is insensitive to inhibitors of MEK1/MEK2. Again, a partially shared regulatory mechanism within the PKC pathway may provide more flexibility for osteoblasts to respond rapidly to the change in the microenvironment, while being protected from the autocrine affects of their own proinflammatory mediator (IL-6).

Taken together, we have shown in this study that human osteoblasts express cell surface IL-6R, which becomes functionally active after shedding, and the sIL-6R mediates the effect of IL-6. Under the control of this unique mechanism, osteoblasts are able to rapidly regulate their own sensitivity to IL-6 by activating an IL-6R shedding. Because the turnover of IL-6R synthesis is high in osteoblasts, the replacement of the shed receptor on the cell surface could be immediately provided if needed. Importantly, osteoblast-derived functional sIL-6R, complexed with IL-6, can mediate autocrine effects on osteoblasts through the gp130-JAK-STAT pathway (8, 9, 25), which then may up-regulate the expression of osteoclast-activating molecules (e.g. receptor activator of nuclear factor-κB ligand), leading subsequently to the activation of osteoclasts (12, 13, 26, 29). Osteoblast-derived sIL-6R complexed with IL-6 may also have a direct paracrine effect on osteoclasts (11). Therefore, in pathological conditions, where the disease is associated with substantial bone loss and IL-6 is present (e.g. rheumatoid ar-
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thritis, multiple myeloma, osteoporosis, periprosthetic osteo-
sis, etc.), the inhibition/blockade of the generation of osteoblast-
derived sIL-6R may be a crucial event to prevent or treat IL-6-mediated bone loss.

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