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Interleukin-6 with Its Soluble Receptor Enhances the Expression of Insulin-Like Growth Factor-I in Osteoblasts*

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ABSTRACT

Interleukin (IL)-6, a cytokine produced by skeletal cells and known to increase bone resorption, has mitogenic effects for bone cells, possibly by regulating the synthesis of other local factors. We tested the effects of IL-6 and its soluble receptor (IL-6sR) on the expression of insulin-like growth factor (IGF)-I and IGF-II in cultured osteoblast-enriched cells from fetal rat calvariae (Ob cells). IL-6 did not modify IGF-I messenger RNA (mRNA) levels, but when tested in the presence of IL-6sR, IL-6 at 1 to 100 ng/ml increased IGF-I transcripts by up to 3.2-fold after 24 h. IL-6sR caused a small increase in IGF-I mRNA levels when tested alone. IL-6 and IL-6sR increased immunoreactive IGF-I levels by 2.4-fold after 24 h and 6.4-fold after 48 h. Cycloheximide prevented, and indomethacin markedly decreased, the effect of IL-6 and IL-6sR on IGF-I mRNA levels, but hydroxyurea did not. IL-6 and IL-6sR did not alter the decay of IGF-I mRNA in transcriptionally arrested Ob cells, and the half-life of the predominant 6.5-kb IGF-I transcript was about 11 h in control and treated cells. In addition, IL-6 and IL-6sR increased the levels of IGF-I heterogeneous nuclear RNA. IL-11 also increased IGF-I mRNA levels, whereas oncostatin M and leukemia-inhibitory factor did not. In contrast to their effects on IGF-I, IL-6 and IL-6sR caused only a modest increase in IGF- II mRNA and polypeptide levels. In conclusion, IL-6, in the presence of IL-6sR, increases IGF-I synthesis in Ob cells; this effect may lead to a secondary increase in bone formation. (*Endocrinology* **138:** 5248–5255, 1997)

INTERLEUKIN-6 (IL-6), a cytokine produced by cells of the osteoblast and osteoclast lineages, increases the recruitment of osteoclasts (1–5). As a consequence, IL-6 causes an increase in bone resorption and possibly bone remodeling. IL-6 appears to mediate the effects of selected hormones on bone resorption and has important interactions with other cytokines present in the bone microenvironment (2–7). Although IL-6 plays a role in bone resorption, its mechanism of action is not fully understood, and its actions on bone formation are not known. The effect of IL-6 on the recruitment of osteoclast-like cells requires the presence of the IL-6 soluble receptor (IL-6sR) (8). In addition, the IL-6sR is present in the systemic circulation, and it appears relevant to the actions of IL-6 in physiological and pathological conditions (9, 10).

In nonskeletal cells, IL-6 stimulates cell growth, an effect that is mediated by locally produced growth factors (11). IL-6 also stimulates DNA synthesis in UMR-106 osteoblastic cells and in primary cultures of rat osteoblasts, and there is an increase in bone cell replication in transgenic mice overexpressing IL-6 in osteoblasts (Refs. 12 and 13 and N. Franchimont and E. Canalis, unpublished observations). The effect of IL-6 on cell replication may be secondary to the induction of selected growth factors by skeletal cells. Insulin-like growth factor (IGF)-I and IGF-II are among the most abundant growth factors present in bone tissue (14–17). IGFs stimulate bone collagen synthesis and have mitogenic properties for cells of the osteoblastic lineage (18). Systemic hormones, as well as local growth factors, regulate the synthesis of IGF-I and IGF-II in osteoblasts, and IGFs may play a role in the coupling of bone formation to bone resorption (14, 19–21). Consequently, we postulated that IGFs might mediate some of the effects of IL-6 in osteoblasts and play a role in the coupling of bone formation to the IL-6-induced bone resorption. An initial step to test this hypothesis would be the demonstration that IL-6 regulates IGF-I or IGF-II synthesis in skeletal cells.

In the present study, we examined the actions of IL-6, in the presence and absence of the IL-6sR, on IGF-I and IGF-II transcripts and protein levels in cultures of osteoblast-enriched cells from 22-day fetal rat calvariae (Ob cells). The effect of IL-6 was compared with that of related cytokines, including IL-11, oncostatin M, and leukemia inhibitory factor (LIF) (22, 23).

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Materials and Methods

Culture technique

The culture method used was described in detail previously (24). Parietal bones were obtained from 22-day-old fetal rats immediately after the mothers were killed by blunt trauma to the nuchal area. This project was approved by the Institutional Animal Care and Use Committee of Saint Francis Hospital and Medical Center. Cells were obtained

by five sequential digestions of the parietal bone, using bacterial collagenase (CLS II, Worthington Biochemical Corp., Freehold, NJ). Cell populations harvested from the third to the fifth digestions were cultured as a pool and were previously shown to express osteoblastic characteristics (24). Ob cells were plated at a density of 8,000–12,000 cells/cm^2 and cultured in a humidified 5% CO_2 incubator at 37 C, until reaching confluence (about 50,000 cells/cm^2). Cells were cultured in DMEM supplemented with 10% FBS (both from Summit Biotechnology, Fort Collins, CO). At confluence, the cells were transferred to serum-free medium for 20–24 h, after which they were rinsed and exposed to test or control medium in the absence of serum for 2–48 h. In 48-h treated cultures, the medium was replaced after 24 h with fresh control or test solutions. Recombinant human IL-6, IL-6sR, IL-11, and oncostatin M (all from R&D Systems, Inc., Minneapolis, MN) were dissolved in PBS containing 0.1% BSA and diluted in DMEM; an equal amount of PBS and BSA was added to control cultures. LIF (Genzyme, Cambridge, MA) was dissolved in PBS containing 0.02% polyoxyethylene sorbitan monolaurate (Tween-20) and 0.1% BSA and diluted in DMEM containing 0.1% BSA; control cultures contained 0.1% BSA and equal amounts of PBS and Tween-20. Hydroxyurea (Sigma Chemical Co., St. Louis, MO) was added directly to the culture medium. Cycloheximide, indomethacin, and 5,6-dichlorobenzimidazole riboside (DRB) (all from Sigma) were dissolved in ethanol and diluted 1:3000, 1:1000, and 1:200, respectively, in DMEM. Control and test cultures contained equal amounts of ethanol. At the end of the incubation, the medium was harvested in the presence of 0.1% Tween-20 (Pierce, Rockford, IL) and stored at −80 C before protein assay, and the cell layer was extracted for RNA analysis or, following labeling, for DNA synthesis analysis and stored at −80 C.

Northern blot analysis

Total cellular RNA was isolated with guanidine thiocyanate followed by a phenol-chloroform (Sigma) extraction and ethanol precipitation (25) or by RNeasy kit per manufacturer's instructions (Qiagen, Chatsworth, CA). The RNA recovered was quantitated by spectrometry, and equal amounts of RNA from control or test samples were loaded on a formaldehyde agarose gel after denaturation. The gel was stained with ethidium bromide to visualize ribosomal RNA, confirming equal RNA loading of the various experimental samples. RNA was then blotted onto Gene Screen Plus-charged nylon (DuPont, Wilmington, DE), and the uniformity of transfer was confirmed by revisualization of ribosomal RNA. A 500-bp rat prepro-IGF-I complementary DNA (cDNA) (kindly provided by L. S. Murphy, Winnipeg, Manitoba, Canada) and a 1400-bp murine prepro-IGF-II cDNA (kindly provided by G. Bell, Chicago, IL) were purified by agarose gel electrophoresis (26, 27). IGF-I and IGF-II cDNAs were labeled with [α-^{32}P]deoxy-ATP and [α-^{32}P]deoxycytosine triphosphate (dCTP) (50 μCi each at a specific activity of 3,000 Ci/mmol; DuPont) using the random hexanucleotide-primed second strand synthesis method (28). Hybridizations were carried out at 42 C for 16–72 h, and posthybridization washes were performed at 65 C in 1 × saline-sodium citrate. The blots were stripped and rehybridized with a α-^{32}P-labeled 752-bp *Bam*HI-*Sph*I restriction fragment of the murine 18S ribosomal RNA cDNA (American Type Culture Collection, Rockville, MD) under the same conditions, but posthybridization washes were performed in 0.1 × saline-sodium citrate at 65 C. The bound radioactive material was visualized by autoradiography on DuPont Reflection film, employing Cronex Lightning Plus intensifying screens (DuPont). Relative hybridization levels were determined by densitometry. Northern analyses shown are representative of three or more cultures.

Heterogeneous nuclear RNA (hnRNA) analysis

IGF-I hnRNA was analyzed by RT-PCR (29) using specific primers designed to amplify DNA from intron 1 to intron 2 of the rat IGF-I gene, in accordance with published sequences (29–31). A sense intron 1-specific amplimer, 5′-CTTTCAGGACGCAGAAGGAT-3′ and an antisense intron 2-specific amplimer, 5′-AATCCAGAATCCTGGTAGCA-3′, were synthesized commercially. Total RNA from control and test samples was prepared as described for Northern analysis. One microgram of RNA was treated with DNase I, and reverse-transcribed in the presence of the IGF-I intron 2-specific antisense amplimer at 42 C for 30 min with Moloney murine leukemia virus reverse transcriptase (Life Technologies, Grand Island, NY). The newly transcribed cDNA was amplified by 25 PCR cycles of 94 C/1 min, 55 C/1 min, and 72 C/1 min after the addition of the sense intron 1-specific amplimer, *Taq* DNA polymerase and 5 μCi [α-^{32}P]dCTP (3000 Ci/mMol, DuPont) (29, 30). The PCR products were fractionated by electrophoresis on a 6% polyacrylamide denaturing gel, visualized by autoradiography, and quantitated by densitometry. The PCR product increased linearly with increasing amounts of RNA. Ten femtograms of an internal DNA standard were included in the PCR to correct for variations in amplification. The standard was obtained by amplification of SV40 promoter sequences in the pGL2-P plasmid DNA (Promega, Madison, WI) using the composite sense primer 5′-CTTTCAGGACGCAGAAGGAT cgttgctagtaccaaccta-3′ and the composite antisense primer 5′-AATCCAGAATCCTGGTAGCA gtatccagatccacaacctt-3′. The *capital letters* indicate IGF-I sequences, and the *lowercase* letters represent SV-40 sequences in the pGL2-P plasmid. No signal of the hnRNA product was detected when the RT step was omitted before the PCR, eliminating the possibility of DNA contamination. To determine the variability of the procedure, Ob cell RNA was pooled, independent aliquots were reverse-transcribed and amplified by PCR, and hnRNA was quantitated by densitometry, which revealed a coefficient of variation of 11% (n = 13) for the assay. Data on hnRNA are representative of three or more cultures.

IGF-I and IGF-II RIA

IGF-I and IGF-II were measured by RIA in aliquots of the culture medium after separation from IGF-binding proteins (IGFBPs) by acidification (1 M acetic acid final concentration), followed by size-exclusion ultrafiltration using a 10,000 mol wt cut-off filtration device (UFP l LGC, Millipore Corp., Bedford, MA) (15, 20). IGF-I was quantitated using a rabbit polyclonal antibody to human IGF-I (prepared by L. Underwood and J. J. Van Wyk and distributed by the National Hormone and Pituitary Program, Baltimore, MD) as described (15). IGF-II was quantitated using a monoclonal antibody to rat IGF-II (Amano International, Troy, VA) as described (20). Data for IGF-I and IGF-II are expressed in nanomolar concentrations.

DNA synthesis

DNA synthesis was studied by measuring effects on the incorporation of [methyl-^{3}H]thymidine (5 μCi/ml, specific activity 80 Ci/mmol, DuPont) into acid-insoluble extracts during the last 2 h of culture, as previously described (18, 32). Data are expressed as disintegrations per min per 0.32-cm^2 culture well.

Statistical methods

Data are expressed as means ± SEM. Differences were calculated by ANOVA and *post hoc* examination by Ryan-Einot-Gabriel-Welsh and Dunnett's. Slopes of the decay of IGF-I mRNA from control and treated Ob cells after transcriptional arrest were examined by the method of Sokal and Rohlf (33).

Results

As previously reported, Ob cells revealed four major classes of IGF-I transcripts of 0.9, l.7, 4.1, and 6.5 kb (18, 19) (Fig. 1). Continuous treatment of Ob cells with IL-6 did not cause a significant increase in IGF-I steady state transcripts, but in the presence of its soluble receptor, IL-6 caused a time- and dose-dependent increase in IGF-I mRNA levels in Ob cells. IL-6 in the presence of IL-6sR was not effective after 2 or 6 h, but IL-6 at 100 ng/ml and IL-6sR at 125 ng/ml increased IGF-I transcripts by (mean ± SEM; n = 8) 3.2 ± 0.3 fold ($P < 0.01$) after 24 h, and the effect was sustained for 48 h (Fig. 1). The effect was of similar magnitude in all four mRNA species. After 24 h, IL-6 at 100 ng/ml increased IGF-I transcripts only by 1.2 ± 0.1 fold (n = 9, $P > 0.05$), and the effect was of comparable magnitude at 300 ng/ml (Fig. 2). However, in the presence of IL-6sR, IL-6 stimulated IGF-I expression at concentrations as low as 1 ng/ml, and its activity was

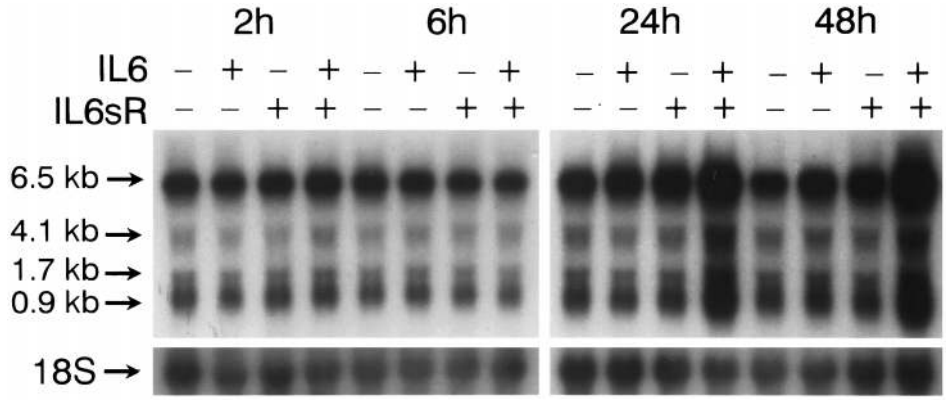

FIG. 1. Effect of IL-6 at 100 ng/ml and of IL-6sR at 125 ng/ml, singly and in combination, on IGF-I mRNA levels in cultures of Ob cells treated for 2, 6, 24, or 48 h. Total RNA from control or treated cultures was subjected to Northern blot analysis and hybridized with a ^{32}P-labeled IGF-I cDNA. The blot was stripped and rehybridized with a ^{32}P-labeled 18S cDNA. IGF-I mRNA was visualized by autoradiography and is shown in the *upper panel* while 18S ribosomal RNA is shown *below*.

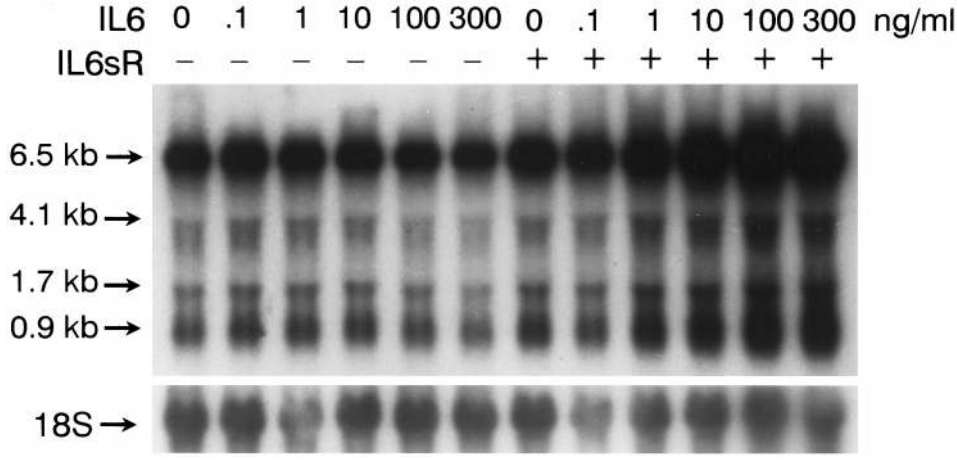

FIG. 2. Effect of IL-6 at 0.1 to 300 ng/ml, in the presence and absence of IL-6sR at 125 ng/ml, on IGF-I mRNA levels in cultures of Ob cells treated for 24 h. Total RNA from control or treated cultures was subjected to Northern blot analysis and hybridized with a ^{32}P-labeled IGF-I cDNA. The blot was stripped and rehybridized with a ^{32}P-labeled 18S cDNA. IGF-I mRNA was visualized by autoradiography and is shown in the *upper panel* while 18S ribosomal RNA is shown *below*.

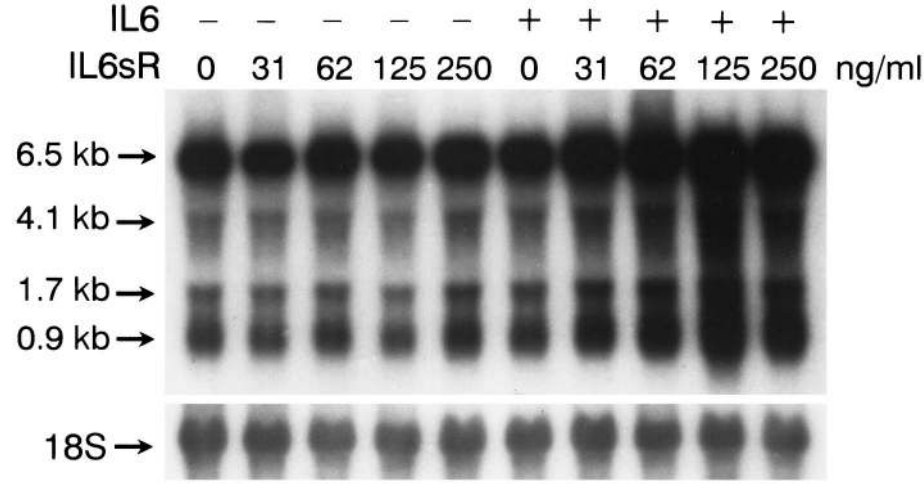

FIG. 3. Effect of IL-6sR at 31 to 250 ng/ml, in the presence or absence of IL-6 at 100 ng/ml, on IGF-I mRNA levels in cultures of Ob cells treated for 24 h. Total RNA from control or treated cultures was subjected to Northern blot analysis and hybridized with a ^{32}P-labeled IGF-I cDNA. The blot was stripped and rehybridized with a ^{32}P-labeled 18S cDNA. IGF-I mRNA was visualized by autoradiography and is shown in the *upper panel* while 18S ribosomal RNA is shown *below*.

maximal at 100 ng/ml, which increased IGF-I mRNA levels by 3-fold after 24 h. IL-6sR alone caused a small, and not statistically significant, increase in IGF-I transcripts and, when tested at 125 ng/ml for 24 h, it increased IGF-I mRNA levels by 1.5 ± 0.2 fold (n = 8, $P > 0.05$) (Figs. 1 and 2). This effect was magnified by IL-6, and IL-6sR at 62 to 250 ng/ml in the presence of IL-6 at 100 ng/ml increased IGF-I mRNA levels by 2.8 ± 0.6 to 3.3 ± 0.5 fold (n = 3 to 4, $P < 0.01$) (Fig. 3). In agreement with its action on IGF-I transcripts, IL-6 by itself had no effect on IGF-I polypeptide levels, but, when tested in the presence of IL-6sR, it increased the levels of immunoreactive IGF-I in the culture medium of Ob cells by 2.4-fold after 24 h and 6.4-fold after 48 h (Table 1).

To determine whether the effect of IL-6 and its soluble receptor on IGF-I mRNA levels was dependent on protein or DNA synthesis, confluent cultures of Ob cells were treated with IL-6 and IL-6sR in the presence and absence of cycloheximide or hydroxyurea at doses known to inhibit protein or DNA synthesis, respectively (18, 32). Cycloheximide at 3.6 μM prevented the stimulatory effect of IL-6 and IL-6sR on IGF-I mRNA levels (Fig. 4). Densitometric analysis indicated that IL-6 at 100 ng/ml and IL-6sR at 50 ng/ml increased IGF-I

TABLE 1. Effect of IL-6 at 100 ng/ml and IL-6sR at 125 ng/ml on IGF-I polypeptide levels in cultures of Ob cells treated for 24 or 48 h

	IGF-I (nM)	
	0 to 24 h	24 to 48 h
Control	3.1 ± 0.2	2.8 ± 0.5
IL-6	2.7 ± 0.3	3.0 ± 0.3
IL-6sR	2.8 ± 0.6	2.5 ± 0.4
IL-6 + IL-6sR	7.4 ± 0.5[a]	18.0 ± 1.7[a]

Values represent the effect of IL-6 and IL-6sR on IGF-I polypeptide levels in Ob cell cultures treated for 24 or 48 h. Culture medium was collected for the first (0–24 h) or the second 24 h (24–48 h) period, and IGF I was determined by immunoassay. Values represent the mean ± SEM for six cultures.

[a] Significantly different from control, $P < 0.05$.

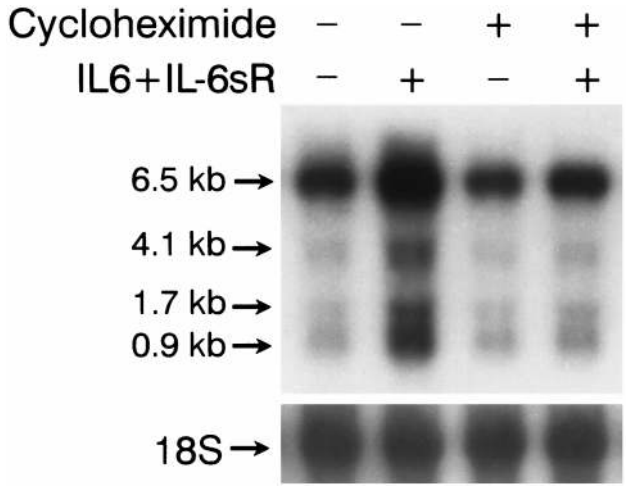

FIG. 4. Effect of IL-6 at 100 ng/ml and IL-6sR at 50 ng/ml, in the presence or absence of cycloheximide at 3.6 μM, on IGF-I mRNA levels in cultures of Ob cells treated for 24 h. Total RNA from control or treated cultures was subjected to Northern blot analysis and hybridized with a ^{32}P-labeled IGF-I cDNA. The blot was stripped and rehybridized with a ^{32}P-labeled 18S cDNA. IGF-I mRNA was visualized by autoradiography and is shown in the *upper panel* while 18S ribosomal RNA is shown *below*.

transcripts by 2.5 ± 0.4 fold ($P < 0.05$) in the absence, and by 1.3 ± 0.1 fold (NS *vs*. control, $P > 0.05$) in the presence, of cycloheximide (n = 3 to 4). Cycloheximide alone decreased IGF-I mRNA to 0.8 ± 0.1 (NS *vs*. control, $P > 0.05$). After 24 h, IL-6 at 1, 10, and 100 ng/ml increased the incorporation of [^{3}H]thymidine into DNA from (mean ± SEM; n = 6 to 8) 1800 ± 94 dpm/well in control cultures to 3150 ± 380, 3170 ± 400, and 3100 ± 300 dpm/well, respectively (all $P < 0.05$). However, hydroxyurea at 1 mM did not change IGF-I mRNA levels in control or in IL-6- and IL-6sR-treated cultures (Fig. 5). IL-6 at l00 ng/ml and IL-6sR at 50 ng/ml increased IGF-I transcripts by 1.9 ± 0.2 fold in the absence, and by 2.0 ± 0.2 fold (both $P < 0.05$ *vs*. control) in the presence, of hydroxyurea (both n = 3).

To determine whether or not the effect of IL-6 and IL-6sR was dependent on prostaglandin (PG) synthesis, Ob cells were treated with IL-6 and its soluble receptor in the presence and absence of indomethacin at 10 μM for 24 h. The PG synthesis inhibitor decreased the expression of IGF-I transcripts slightly in control and noticeably in treated cultures,

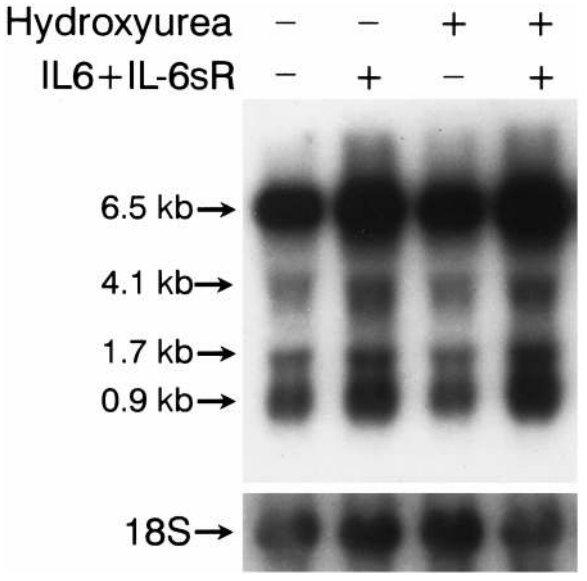

FIG. 5. Effect of IL-6 at 100 ng/ml and IL-6sR at 50 ng/ml, in the presence or absence of hydroxyurea at 1 mM, on IGF-I mRNA levels in cultures of Ob cells treated for 24 h. Total RNA from control or treated cultures was subjected to Northern blot analysis and hybridized with a ^{32}P-labeled IGF-I cDNA. The blot was stripped and rehybridized with a ^{32}P-labeled 18S cDNA. IGF- I mRNA was visualized by autoradiography and is shown in the *upper panel* while 18S ribosomal RNA is shown *below*.

virtually preventing the stimulatory effect of IL-6 and its soluble receptor on IGF-I mRNA levels. Densitometric analysis revealed that IL-6 and IL-6sR increased IGF-I transcripts by 3.7 ± 0.7 fold ($P < 0.05$) in the absence, and by 1.5 ± 0.1 fold (NS *vs*. control, $P > 0.05$) in the presence of indomethacin (both n = 4); indomethacin alone did not change IGF-I mRNA levels, which were 0.9 ± 0.1 of control (Fig. 6).

To determine whether the effect of IL-6 on IGF-I mRNA levels was due to changes in transcript stability, Ob cells were exposed to control or IL-6 and IL-6sR-containing medium for 6 h and then treated with the RNA polymerase II inhibitor DRB at 75 μM, in the presence or absence of IL-6 and IL-6sR, for 6, 18, and 24 h (34). Longer exposure to DRB is not feasible in this culture model because of decreased cell viability beyond 24 h, as determined by trypan blue exclusion (E. Canalis, unpublished observations). The half-life of the 6.5-kb IGF-I transcript in transcriptionally arrested Ob cells was 11 h, and the slopes of the 6.5-kb IGF-I mRNA decay were not different between control and IL-6/IL-6sR treated cultures (Fig. 7). After DRB there was a similar decay in the 1.7- and 4.1-kb transcripts in control and treated cultures, but there was an accumulation of the 0.9-kb transcript in control and IL-6/IL-6sR-treated cultures. To confirm whether or not IL-6 and its soluble receptor modified the transcription of the IGF-I gene, we examined their actions on IGF-I hnRNA levels. The rate of IGF-I transcription in Ob cells is modest, and the number of cells available from primary cultures limited (35). Consequently, it was not possible to obtain results for IGF-I gene transcription using nuclear run-on assays on two occasions (not shown). Furthermore, while changes in hnRNA levels may represent changes in transcription or RNA processing, they correlate well with changes in tran-

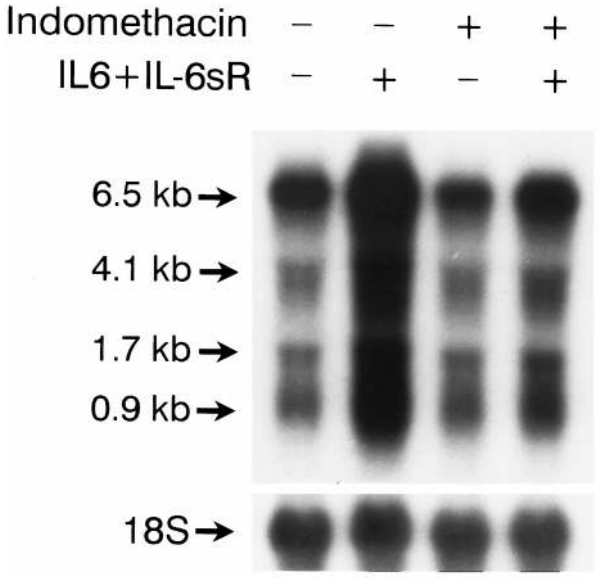

FIG. 6. Effect of IL-6 at 100 ng/ml and IL-6sR at 50 ng/ml, in the presence or absence of indomethacin at 10 μM, on IGF-I mRNA levels in cultures of Ob cells treated for 24 h. Total RNA from control or treated cultures was subjected to Northern blot analysis and hybridized with a ^{32}P-labeled IGF-I cDNA. The blot was stripped and rehybridized with a ^{32}P-labeled 18S cDNA. IGF-I mRNA was visualized by autoradiography and is shown in the *upper panel* while 18S ribosomal RNA is shown *below*.

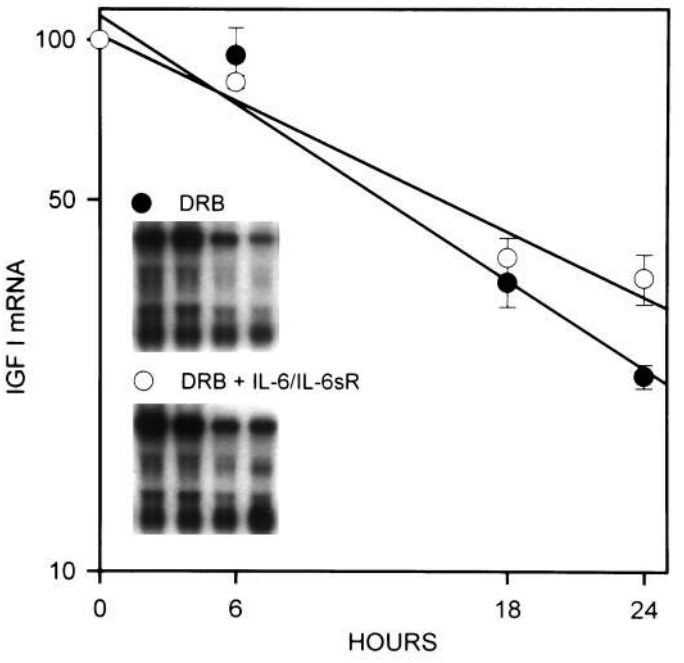

FIG. 7. Effect of IL-6 at 100 ng/ml and IL-6sR at 50 ng/ml on IGF-I mRNA decay in Ob cell cultures. Confluent cultures of Ob cells were serum deprived and exposed to control or IL-6- and IL-6sR-containing medium for 6 h before the addition of DRB at 75 μM. Total RNA, obtained 0–24 h after the addition of DRB alone or in the presence of IL-6 and IL-6sR, was subjected to Northern blot analysis and the 6.5-kb transcript hybridized with a ^{32}P-labeled IGF-I cDNA. IGF-I mRNA was visualized by autoradiography and quantitated by densitometry. Data from control (*closed circles*) and IL-6/IL-6sR-treated (*open circles*) cells are expressed as means ± SEM for three cultures and represent percent levels of the 6.5-kb transcript present before the addition of DRB. Representative Northern blots are shown in the *inset*.

scription obtained by nuclear run-on assays (30). IL-6 at 100 ng/ml and IL-6sR at 50 to 125 ng/ml did not increase IGF-I hnRNA after 2 h and had a modest and variable effect after 6 h. However, after 24 h, IL-6 and its soluble receptor consistently increased IGF-I hnRNA by about 5-fold (Fig. 8).

IL-11, LIF, and oncostatin M, cytokines known to activate similar signal transduction pathways as IL-6, were tested (22, 23). IL-11 at 50 ng/ml after 2 h (not shown) and after 6 h did not modify IGF-I transcripts, but after 24 and 48 h it increased IGF-I mRNA levels by 1.8 ± 0.2 and 3.7 ± 0.1, respectively ($n = 3–4$, $P < 0.01$) (Fig. 9). In contrast, the related cytokines LIF and oncostatin M, each at 100 ng/ml for 2 (not shown), 6, 24, or 48 h, did not modify IGF-I mRNA levels in Ob cells when compared with their respective controls (Fig. 9). Cultures exposed to DMEM-containing Tween-20 and 0.1% BSA, like the LIF-treated cultures, had higher levels of IGF-I mRNA, but LIF did not cause an increase in IGF-I transcripts when compared with this control.

In contrast to its effects on IGF-I gene expression, IL-6 in the presence or absence of its soluble receptor caused only a modest increase in IGF-II mRNA levels in Ob cells. This small effect was time dependent and not observed after 2 or 6 h, whereas IL-6 at 100–300 ng/ml in the presence of IL-6sR at 125 ng/ml increased IGF-II mRNA levels by up to 1.5-fold after 24 h (Fig. 10) or 48 h (not shown). IL-6 at 100 ng/ml in the presence of IL-6sR at 125 ng/ml increased IGF-II polypeptide levels from (mean ± SEM; n = 6) 6.3 ± 0.2 nM in control to 10.0 ± 0.4 nM in treated cultures ($P < 0.01$) after 24 h.

Discussion

The present investigation was undertaken to determine whether IL-6 regulates IGF-I and IGF-II synthesis in cultures of rat osteoblasts. IL-6 by itself did not alter the levels of IGF-I transcripts, but in the presence of its soluble receptor, IL-6 caused a significant time- and dose-dependent stimulation of IGF-I mRNA and protein levels. The effect of IL-6 and IL-6sR on IGF-I mRNA levels was not acute and was observed after 24 h probably because it was dependent on the synthesis of new proteins since it was blocked by cycloheximide. In the present study, we confirmed that IL-6 causes a modest increase in DNA synthesis in cells of the osteoblastic lineage (12). However, the effect of IL-6 on IGF-I expression was not related to its mitogenic properties since it was not blocked by hydroxyurea. This does not preclude a role of IGF-I ultimately mediating the mitogenic actions of IL-6 in bone cells, since the experiments conducted determined only immediate effects. Inhibition of PG synthesis decreased the expression of IGF-I and virtually prevented the effect of IL-6 and IL-6sR. Since IL-6 induces PG synthesis in osteoblasts and PGE_2 induces IGF-I expression, it is likely that IL-6 regulates IGF-I synthesis primarily by PG-dependent mechanisms (12, 36). Although IL-6 and its soluble receptor did not modify the decay of IGF-I transcript in transcriptionally arrested cells, there was accumulation of 0.9- kb transcripts in control and

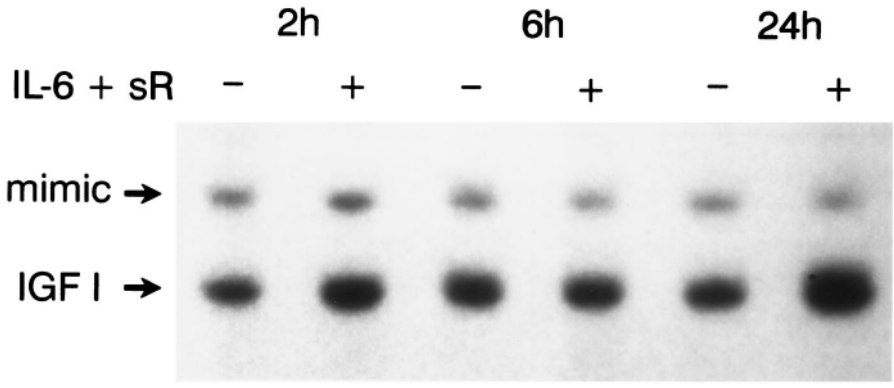

FIG. 8. Effect of IL-6 at 100 ng/ml and IL-6sR at 50 ng/ml on IGF-I hnRNA levels in cultures of Ob cells treated for 2–24 h. Total RNA from control and IL-6/IL-6sR-treated cultures was reverse transcribed and amplified by PCR in the presence of 5 μCi [α-^{32}P]dCTP using IGF-I exon 1- and intron 1- specific primers to generate a 263-bp product. An exogenous DNA standard (mimic), designed to use the same primers, was coamplified with each reaction to assess PCR efficiency. PCR products were visualized by autoradiography.

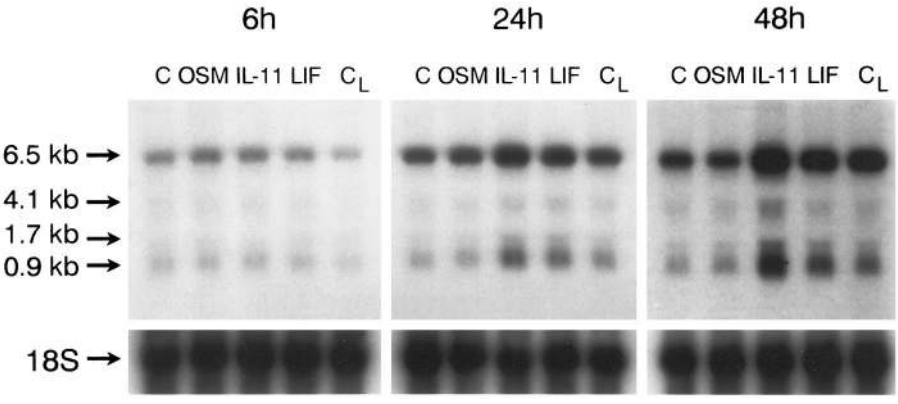

FIG. 9. Effect of LIF, oncostatin M (OSM), both at 100 ng/ml, and IL-11 at 50 ng/ml on IGF-I mRNA levels in cultures of Ob cells treated for 6, 24, or 48 h. Control cultures for IL-11 and OSM contained DMEM (C), and for LIF contained DMEM with Tween-20 and 0.1% BSA (C_L). Total RNA from control or treated cultures was subjected to Northern blot analysis and hybridized with a ^{32}P-labeled IGF-I cDNA. The blot was stripped and rehybridized with a ^{32}P-labeled 18S cDNA. IGF-I mRNA was visualized by autoradiography and is shown in the *upper panel* while 18S ribosomal RNA is shown *below*.

treated cultures, suggesting changes in processing under conditions of transcript arrest. IL-6 and IL-6sR increased IGF-I hnRNA levels. Although changes in hnRNA may reflect alterations in transcription or processing, they correlate well with changes obtained by nuclear run-on assays (29, 30), suggesting that IL-6 stimulates IGF-I expression by transcriptional mechanisms. In contrast to its effects on IGF-I expression, IL-6 and its soluble receptor caused only a modest increase in IGF-II mRNA and protein levels. This suggests that this effect is not biologically important.

The experiments reported demonstrate an effect of IL-6 and IL-6sR in osteoblasts; however, IL-6 in the presence of its soluble receptor will activate any cell that expresses gp130, the signaling protein for IL-6 type cytokines (6, 9, 22). Therefore, IL-6 and IL-6sR may also induce IGF-I synthesis in nonskeletal cells. The effect of other cytokines known to activate gp130 in skeletal cells was variable (22, 23). IL-11 induced IGF-I transcripts, whereas oncostatin M and LIF tested at doses known to be mitogenic in rat osteoblastic cells did not cause a consistent increase in IGF-I mRNA levels in Ob cells (23). The reason for the differences in the effects of these related cytokines is not immediately apparent although IL-6 and IL-11 initially bind to α-receptor subunits, and this results in activation and homodimerization of gp130, whereas LIF and oncostatin M bind to β-subunits that form heterodimers with gp130 (22). This could suggest that homodimerization of gp130 is needed for IGF-I induction. Another possibility is that cells responsive to LIF and oncostatin with an increase in DNA synthesis in osteoblastic cultures do not express IGF-I or do not respond to gp130 activation.

IL-6 stimulates bone resorption by increasing osteoclast recruitment and differentiation, and in selected culture systems this effect is observed only in the presence of IL-6sR (8). The effect of IL-6 on osteoclast recruitment requires the presence of osteoblasts and depends on IL-6 receptors expressed by osteoblastic cells (37). Therefore, it is not surprising that IL-6 regulates IGF expression in osteoblasts and that its effect depends on the presence of IL-6sR. The effect of IL-6 and its soluble receptor on IGF-I expression was observed at doses similar to those detected in human serum, suggesting its relevance to physiological or pathological conditions (10, 39). Serum concentrations of IL-6sR are elevated in conditions of increased bone remodeling, and it is possible that IGF-I is important in the coupling of the IL-6-induced bone resorption with bone formation. However, it is important to note that systemic IL-6 and IL-6sR are complexed with other circulating proteins, including soluble gp130, and as such they may be biologically inactive (38). Transgenic mice overexpressing IL-6 have decreased serum concentrations of IGF-I and impaired growth (40). The discrepancy between these results and ours is not surprising since the mechanisms and hormones regulating systemic and local IGF-I differ (14, 41). Furthermore, transgenic mice overexpressing IL-6 in osteoblasts display increased localized cell growth (13). The circulating levels of IGF-I are GH dependent and are derived from liver cells, which express the leader exons 1 and 2 of the IGF-I gene (31, 41). In contrast, osteoblasts express primarily IGF-I exon 1-dependent transcripts, and GH has limited effects on IGF-I synthesis in these cells (36, 42).

IGF-I has been shown to increase IL-6 production in UMR-106 osteoblastic cells, suggesting the existence of a positive feedback mechanism between IGF-I and IL-6 in osteoblasts

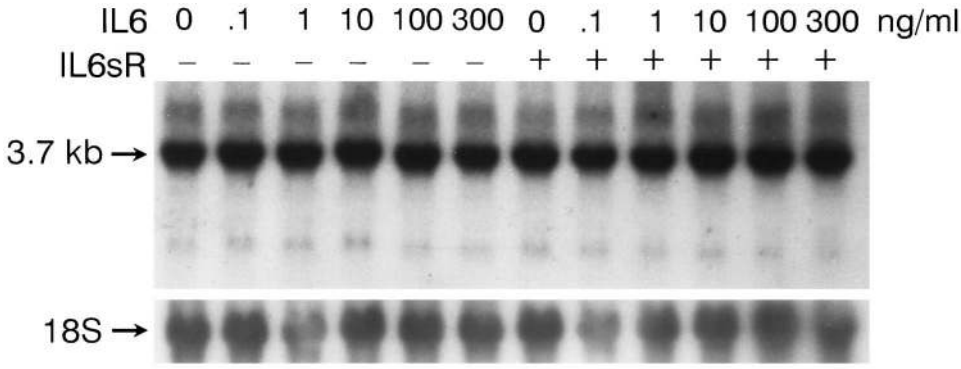

FIG. 10. Effect of IL-6 at 0.1 to 300 ng/ml, in the presence and absence of IL-6sR at 125 ng/ml, on IGF-II mRNA levels in cultures of Ob cells treated for 24 h. Total RNA from control or treated cultures was subjected to Northern blot analysis and hybridized with a ^{32}P-labeled IGF-II cDNA. The blot was stripped and rehybridized with a ^{32}P-labeled 18S cDNA. IGF-II mRNA was visualized by autoradiography and is shown in the *upper panel* while 18S ribosomal RNA is shown *below*.

(43). In addition, GH increases IL-6 mRNA and protein levels in human osteoblasts (44). Although IL-6 and IGF-I and IGF-II enhance DNA synthesis in cells of the osteoblastic lineage, their effects on the differentiated function of the osteoblast differ. IGF-I and IGF-II increase collagen synthesis, matrix apposition rates, and other aspects of the differentiated function of the osteoblast, whereas IL-6 has been reported to have no effect or to inhibit the function of the differentiated osteoblast and the differentiation of cells of the osteoblastic lineage (14, 18, 45–48). This would suggest that the induction of IGF-I does not mediate the acute effects of IL-6 on osteoblastic function, but is consistent with a role in the coupling of bone formation to bone resorption, since the bone-forming response in coupling is not immediate.

The activity of IGF-I and IGF-II is modified by IGFBP, and osteoblasts are known to express IGFBPs 1 to 6 (49–51). Although the exact function of the IGFBPs in bone tissue is not fully understood, only one of the binding proteins, IGFBP-5, stimulates bone cell growth and enhances the effects of IGF-I on this process (52). Recently, we found that IL-6 and its soluble receptor enhance the expression of IGFBP-5 mRNA by the osteoblast (53). This may result in the potentiation of the effect of IGF-I on bone formation after its induction by IL-6.

In conclusion, the present studies demonstrate that IL-6 and its soluble receptor increase IGF-I expression in osteoblasts and cause a modest stimulation of IGF-II synthesis. The induction of IGF-I may ultimately play a role in bone formation and its coupling to bone resorption.

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