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Interleukin-6 and Its Soluble Receptor Regulate the Expression of Insulin-Like Growth Factor Binding Protein-5 in Osteoblast Cultures*

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ABSTRACT

Interleukin-6 (IL-6), a cytokine produced by bone cells, is known to influence bone resorption by stimulating the development of osteoclasts from precursor cells and to have mitogenic actions on osteoblastic cells. Insulin-like growth factors (IGFs) are important local regulators of bone formation, and IGF binding protein (IGFBP)-5 stimulates bone cell growth and enhances the effects of IGF-I. We tested the effects of IL-6 in the presence and absence of its soluble receptor (sIL-6R) on IGFBP-5 expression in cultures of osteoblast-enriched cells from 22-day-old fetal rat calvariae (Ob cells). When tested individually, IL-6 and sIL-6R had a modest stimulatory effect on IGFBP-5 messenger RNA (mRNA) levels. In contrast, when IL-6 and sIL-6R were tested in combination, they caused a considerable increase in IGFBP-5 mRNA levels, and IL-6 at 100 ng/ml and sIL-6R at 125 ng/ml increased IGFBP-5 transcripts by 5- to 7-fold after 24 h. The effect of IL-6 and sIL-6R on IGFBP-5 transcripts was not blocked by indomethacin, but cycloheximide markedly inhibited IGFBP-5 mRNA levels in control and treated cultures. IL-6 and sIL-6R did not modify the decay of IGFBP-5 mRNA in transcriptionally arrested Ob cells, and stimulated the rate of IGFBP-5 transcription as demonstrated by a nuclear run-on assay. IL-6 and sIL-6R did not increase intact IGFBP-5 levels in the extracellular matrix and increased IGFBP-5 fragments in the culture medium. Conditioned medium from Ob cells induced the proteolytic fragmentation of an IGFBP-5 standard, an effect that was accelerated and enhanced by conditioned medium from IL-6/sIL-6R-treated cultures and prevented by metalloprotease inhibitors. In conclusion, IL-6, in the presence of sIL-6R, stimulates IGFBP-5 mRNA expression in Ob cells by transcriptional mechanisms, and accelerates the fragmentation of the protein. (*Endocrinology* **138:** 3380–3386, 1997)

INTERLEUKIN (IL)-6 is a pleiotropic cytokine produced by a wide variety of cells, including cells of the osteoblast and osteoclast lineages (1, 2). IL-6 exerts well-established effects on bone resorption. IL-6 stimulates the recruitment of osteoclasts from precursor cells, and mediates the effects of PTH, 1,25 dihydroxyvitamin D_3, and IL-1 on bone resorption (3–7). In contrast, the effects of IL-6 on bone formation remain controversial. IL-6 stimulates DNA synthesis and inhibits protein synthesis in UMR-106–01 rat osteosarcoma cells, and inhibits the differentiation of cells of the osteoblast lineage from rat calvariae (8, 9). In contrast, IL-6 in the presence of its soluble receptor (sIL-6R), induces the differentiation of uncommitted embryonic fibroblasts toward cells of the osteoblastic lineage (10).

Bone remodeling is affected by hormones and local growth factors. Insulin-like growth factors (IGFs) are autocrine factors that enhance the differentiated function of the osteoblast and have modest mitogenic activity for cells of the osteoblastic lineage (11). Recently, we found that IL-6, in the presence of sIL-6R, stimulates IGF I expression in cultures of osteoblast-enriched cells from fetal rat parietal bone (Ob cells), suggesting that IL-6 could influence bone cell function through the IGF axis (12). Osteoblasts not only express the two IGF genes, but also express six IGF binding proteins (IGFBPs), which can modify the half-life and activity of IGF-I and IGF-II (13–17). Some IGFBPs have specific functions, and IGFBP-5 is unique because it induces cell growth and enhances the actions of IGF I in bone cell cultures (18). Therefore, changes in IGFBP-5 synthesis could be responsible for changes in bone formation.

The synthesis of IGFBP-5 in osteoblasts is induced by prostaglandin E_2 (PGE_2), retinoic acid, and IGF-I, and is inhibited by transforming growth factor-β-1, platelet-derived growth factor-BB, fibroblast growth factor-2, and cortisol (17, 19–21). Agents that modify IGFBP-5 synthesis tend to have a similar effect on IGF-I expression, so that in osteoblasts the synthesis of IGF-I and IGFBP-5 is to an extent coordinated (13, 22). Although there is information about the regulation of IGF-I, IGF-II, and IGFBP-5 by the more classic growth factors and by other agents that modify bone formation, less is known about their regulation by bone resorbing cytokines of the interleukin family, such as IL-6. Both IL-6 and IGFBP-5 have mitogenic properties for cells of the osteoblastic lineage. Furthermore, IL-6 modifies the differentiation of these cells, and the expression of IGFBP-5 depends on the stage of osteoblastic cell differentiation (23). Consequently, we postulated that IL-6 might regulate IGFBP-5 expression in osteoblast cultures.

In the present study, we examined the effects of IL-6 on IGFBP-5 expression in Ob cells, and determined possible mechanisms involved in this regulation. Because sIL-6R is

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known to enhance the actions of IL-6 in skeletal and nonskeletal cells, we also tested the effects of IL-6 in the presence of its soluble receptor (24, 25).

Materials and Methods

Cell culture

The culture method used was described in detail previously (26). 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 digestion were cultured as a pool. 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, maintaining a pH of 7.5. Cells were cultured in DMEM supplemented with nonessential amino acids and 10% FBS (both from Summit Biotechnology, Fort Collins, CO). For RNA and protein analysis, cells were grown to confluence (~50,000 cells/cm^2) and transferred to serum-free medium for 20–24 h, and then exposed to test agents for 2–24 h as indicated in the text and figure legends.

For the nuclear run-on experiment, subconfluent cultures of Ob cells were trypsinized, subcultured at a 1:10 dilution, and grown to confluence in DMEM supplemented with 10% FBS. Cells were serum-deprived for 24 h, and treated for 2–24 h in serum-free DMEM. Recombinant human IL-6 and sIL-6R (R&D Systems, Minneapolis, MN) were dissolved in PBS containing 0.1% BSA. Cycloheximide (Sigma Chemical Co., St. Louis, MO) was added directly to the culture medium, and 5,6-dichlorobenzimidazole riboside (DRB) and indomethacin (both from Sigma) were dissolved in absolute ethanol and diluted 1:200 and 1:1000, respectively, in DMEM. Control and treated cultures contained equal amounts of vehicle. At the completion of the treatment period, the cell layer was extracted with guanidine thiocyanate for RNA analysis or nuclei were isolated by Dounce homogenization for the nuclear run-on assay.

For protein analysis of IGFBP-5, culture medium was collected in the presence of 0.2% polyoxyethylene sorbitan monolaurate (Pierce, Rockford, IL), and the extracellular matrix was extracted as described (27, 28). Both were stored at −70 C. To assay for IGFBP-5 proteolytic activity, the culture medium was collected and tested at the completion of the cell culture period.

Northern blot analysis

Total cellular RNA was isolated with guanidine thiocyanate, at acid pH, followed by a phenol-chloroform (Sigma) extraction or by RNeasy kit in accordance with manufacturer's instructions (Qiagen, Chatsworth, CA) (29). The RNA recovered was quantitated by spectrometry, and equal amounts of RNA from control or test samples were loaded on a formaldehyde agarose gel following denaturation. The gel was stained with ethidium bromide to visualize ribosomal RNA (rRNA), documenting 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 documented by revisualization of rRNA. A 300 bp *Hin*dIII restriction fragment of a rat IGFBP-5 complementary DNA (cDNA) (kindly provided by Dr. S. Shimasaki, La Jolla, CA) and a 700-bp *Bam*HI-*Sph*I fragment of a mouse 18S rRNA cDNA clone (ATCC, Rockville, MD) were purified by agarose gel electrophoresis (30). IGFBP-5 and 18S rRNA cDNAs were labeled with [α-^{32}P] deoxyadenosine triphosphate (dATP) 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 (31).

Hybridizations were carried out at 42 C for 16–48 h, and posthybridization washes were performed at 65 C in 1× SSC for IGFBP-5 and 0.1× SSC for 18S. The bound radioactive material was visualized by autoradiography on DuPont Reflection Film (DuPont), employing Cronex Lightning Plus intensifying screens (DuPont) (32). Relative hybridization levels were determined by densitometry. Northern analyses are representative of three or more cultures.

Nuclear run-on assay

To examine changes in the rate of transcription, nuclei were isolated by Dounce homogenization in a Tris buffer, pH 7.4, containing 0.5% Nonidet P-40 (32). Nascent transcripts were labeled by incubation of nuclei in a reaction buffer containing 500 μM each adenosine, cytidine, and guanosine triphosphates, 150 U RNasin (Promega, Madison, WI) and 250 μCi [α-^{32}P]uridine triphosphate (UTP) (3000 Ci/mmol, DuPont) (33). RNA was isolated by treatment with DNase I and proteinase K, followed by phenol-chloroform extraction and ethanol precipitation. Linearized plasmid DNA containing approximately 1 μg cDNA was immobilized onto GeneScreen Plus by slot blotting according to manufacturer's directions (DuPont). The plasmid vector pGL2-Basic (Promega) was used as a control for nonspecific hybridization, and a mouse 18S rRNA cDNA clone was used to estimate uniformity of the loading. Equal cpm of [^{32}P]RNA from each sample were hybridized to cDNAs at 42 C for 72 h and washed in 1× SSC at 65 C for 20 min. Hybridized cDNAs were visualized by autoradiography, and IGFBP-5 hybridization levels were determined by densitometry. Nuclear run-on assay was performed in two separate experiments.

Western immunoblot analysis

Extracellular matrix was prepared as described (27, 28). Briefly, Ob cells were rinsed in PBS, and cell membranes were removed with 0.5% Triton X-100 (Sigma), pH 7.4, and nuclei and cytoskeleton were removed by incubation with 2 mM ammonium acetate, pH 9.0. The extracellular matrix was rinsed with PBS and scraped from the culture plates. Conditioned medium was concentrated approximately three times by speed vac centrifugation. Aliquots of extracellular matrix extracts or conditioned medium were mixed with Laemmli sample buffer to give a final concentration of 2% SDS. Samples were fractionated by electrophoresis on a 12% polyacrylamide gel (34). Proteins were transferred to Immobilon P membranes (Millipore, Bedford, MA), blocked with 2% BSA, and exposed to a 1:500 dilution of rabbit antiserum raised against native human IGFBP-5 (UBI, Lake Placid, NY) in 1% BSA overnight. Blots were exposed to goat antirabbit IgG antiserum conjugated to horseradish peroxidase, washed, and developed with a horseradish peroxidase chemiluminescence detection reagent (DuPont). IGFBP-5 was identified by comigration with purified human IGFBP-5 (kindly provided by Dr. D. Clemmons, Chapel Hill, NC). Western blot analyses of the conditioned medium and extracellular matrix are representative of two and eight different cultures, respectively.

To detect IGFBP-5 proteolytic activity in the culture medium of Ob cells, 100 ng of a purified human IGFBP-5 standard were incubated with 100 μl conditioned medium from control or treated cultures in the presence and absence of 1 mM 1,10-phenanthrolene, 10 mM EDTA, or 10 mM phenylmethylsulfonyl fluoride (PMSF). Samples were incubated at 37 C for 0–16 h in a shaking water bath, and proteolytic degradation was terminated by adding 2× Laemmli sample buffer and heating at 100 C for 3 min. Samples were fractionated by electrophoresis and processed for Western immunoblot analysis. Western blot analysis to detect IGFBP-5 proteolytic activity was performed three times in the absence and twice in the presence of protease inhibitors.

Statistical methods

Data are expressed as means ± SEM. Slopes of messenger RNA (mRNA) decay were examined by linear regression, and differences in decay were determined by the method of Sokal and Rolf (35).

Results

Confirming previous observations, Northern blot analysis of Ob cell extracts revealed a predominant IGFBP-5 transcript of 6.0 kilobases (kb) (19, 21). Continuous treatment with IL-6 at 100 ng/ml for 2–6 h did not cause a consistent increase in IGFBP-5 mRNA levels in Ob cells, whereas treatment for 24 h increased IGFBP-5 transcripts by 1.5- to 2-fold (Fig. 1). This effect was magnified when Ob cells were treated with IL-6 in the presence of sIL-6R. IL-6 at 100 ng/ml in the presence of sIL-6R at 125 ng/ml induced a 2- to 3-fold in-

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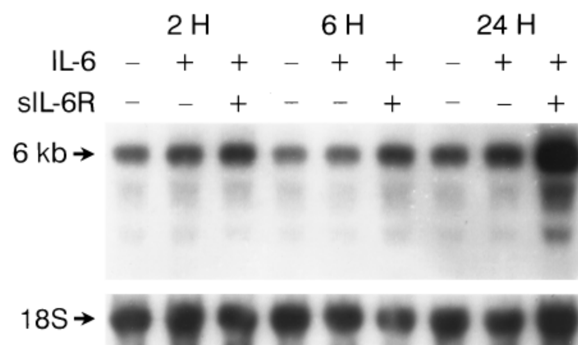

FIG. 1. Effect of IL-6 at 100 ng/ml in presence or absence of sIL-6R at 125 ng/ml on IGFBP-5 mRNA levels in cultures of Ob cells treated for 2, 6, or 24 h. Total RNA from control or IL-6- and sIL-6R-treated cultures was subjected to Northern blot analysis and hybridized with a ^{32}P-labeled rat IGFBP-5 cDNA. Blot was stripped and rehybridized with a ^{32}P-labeled 18S rRNA cDNA. IGFBP-5 mRNA was visualized by autoradiography (*top*); 18S rRNA (*bottom*).

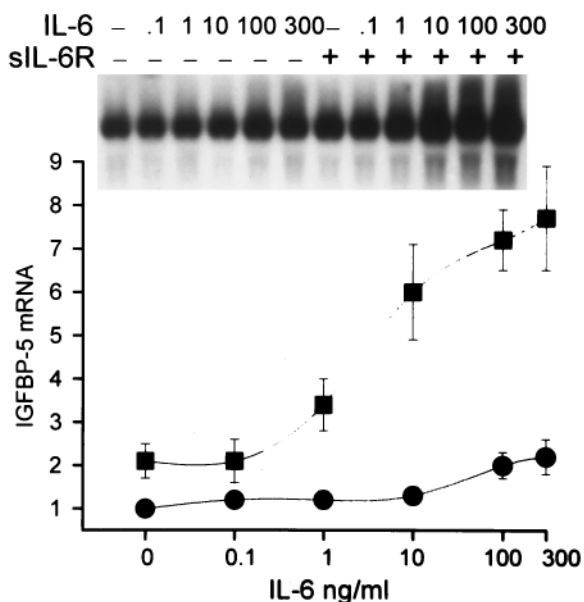

FIG. 2. Effect of IL-6 at 0.1–300 ng/ml in presence or absence of sIL-6R at 125 ng/ml on IGFBP-5 mRNA levels in cultures of Ob cells treated for 24 h. Total RNA from control or IL-6- and sIL-6R-treated cultures was subjected to Northern blot analysis and hybridized with a ^{32}P-labeled rat IGFBP-5 cDNA. IGFBP-5 mRNA was visualized by autoradiography and effect of IL-6 with (■) or without sIL-6R (●) relative to untreated controls was quantitated by densitometry. Symbols represent means ± SEM for three cultures. Inset, Representative Northern blot.

crease in IGFBP-5 mRNA levels after 6 h and a 5- to 7-fold increase after 24 h, as determined by densitometry (Fig. 1). IL-6, when tested by itself, was effective at doses of 100 and 300 ng/ml, which increased IGFBP-5 mRNA levels by 1.5- to 2-fold after 24 h (Fig. 2). When tested in the presence of sIL-6R at 125 ng/ml, IL-6 was modestly active at a dose of 1 ng/ml, and the effect was maximal at 10–300 ng/ml; at these doses IL-6 increased IGFBP-5 transcripts by 6- to 8-fold after 24 h (Fig. 2). In the absence of IL-6, sIL-6R caused a small increase in IGFBP-5 mRNA levels, and at 125–250 ng/ml it increased IGFBP-5 transcripts by approximately 1.5-fold (Fig. 3). In the same experiment, when sIL-6R was tested in the presence of

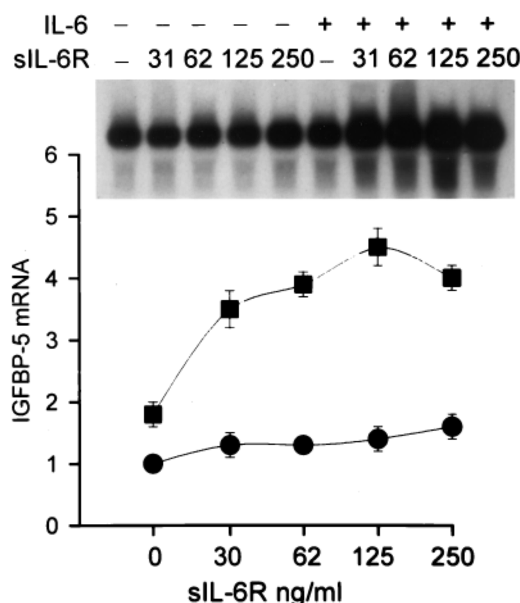

FIG. 3. Effect of sIL-6R at 31–250 ng/ml in presence or absence of IL-6 at 100 ng/ml on IGFBP-5 mRNA levels in cultures of Ob cells treated for 24 h. Total RNA from control or IL-6- and sIL-6R-treated cultures was subjected to Northern blot analysis and hybridized with a ^{32}P-labeled rat IGFBP-5 cDNA. IGFBP-5 mRNA was visualized by autoradiography and effect of sIL-6R with (■) or without IL-6 (●) relative to untreated controls was quantitated by densitometry. Symbols represent means ± SEM for three cultures. Inset, Representative Northern blot.

IL-6 at 100 ng/ml, it was effective at 31–250 ng/ml, increasing IGFBP-5 by 3.5- to 4.5-fold after 24 h. In other experiments, sIL-6R at 125 ng/ml with IL-6 at 100 ng/ml for 24 h increased IGFBP-5 mRNA by 5- to 7-fold (Figs. 1 and 2).

IL-6 stimulates the synthesis of PGE_2 in osteoblastic cells and PGE_2 induces IGFBP-5 transcripts in Ob cells (8, 17). Therefore, we considered that the stimulation of IGFBP-5 mRNA levels by IL-6 could be mediated through prostaglandin synthesis. To test this possibility, we examined the effects of IL-6 at 100 ng/ml and sIL-6R at 50 ng/ml in the presence of the prostaglandin synthesis inhibitor, indomethacin, at 10 μM. Indomethacin did not inhibit IGFBP-5 transcripts in control or treated cultures, and IL-6 and sIL-6R increased IGFBP-5 mRNA levels by 4.5- ± 0.2-fold in the absence and by 4.1- ± 0.2-fold (both n = 3) in the presence of indomethacin (Fig. 4). To determine whether the effect of IL-6 and sIL-6R was protein synthesis dependent, Ob cells were treated with IL-6 at 100 ng/ml and sIL-6R at 50 ng/ml in the presence or absence of cycloheximide at 3.6 μM. Doses of cycloheximide of 2 μM and higher were shown previously to inhibit protein synthesis in Ob cell cultures by 80–85% (36). Cycloheximide caused a marked decrease in IGFBP-5 mRNA levels, and the effects of IL-6 and sIL-6R on IGFBP-5 transcripts were virtually undetectable in its presence (Fig. 5).

To study the actions of IL-6 and sIL-6R on IGFBP-5 mRNA stability, Ob cells were exposed to DMEM or IL-6 and sIL-6R for 6 h, and then treated with the RNA polymerase II inhibitor DRB at 75 μM in the absence or presence of IL-6 at 100 ng/ml and sIL-6R at 50 ng/ml for 6, 18, or 24 h (37). The

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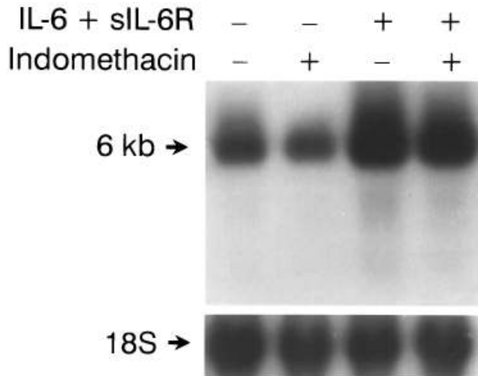

FIG. 4. Effect of IL-6 at 100 ng/ml and sIL-6R at 50 ng/ml in presence or absence of indomethacin at 10 μM on IGFBP-5 mRNA levels in cultures of Ob cells treated for 24 h. Total RNA from control, indomethacin, or IL-6- and sIL-6R-treated cultures was subjected to Northern blot analysis and hybridized with a ^{32}P-labeled rat IGFBP-5 cDNA. Blot was stripped and rehybridized with a ^{32}P-labeled 18S rRNA cDNA. IGFBP-5 mRNA was visualized by autoradiography (*top*); 18S rRNA (*bottom*).

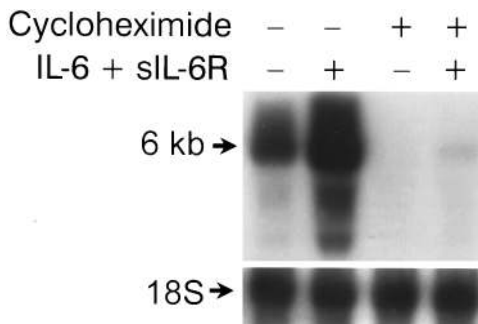

FIG. 5. Effect of IL-6 at 100 ng/ml and sIL-6R at 50 ng/ml in presence or absence of cycloheximide at 3.6 μM on IGFBP-5 mRNA levels in cultures of Ob cells treated for 24 h. Total RNA from control, cycloheximide, or IL-6- and sIL-6R-treated cultures was subjected to Northern blot analysis and hybridized with a ^{32}P-labeled rat IGFBP-5 cDNA. Blot was stripped and rehybridized with a ^{32}P-labeled 18S rRNA cDNA. IGFBP-5 mRNA was visualized by autoradiography (*top*); 18S rRNA (*bottom*).

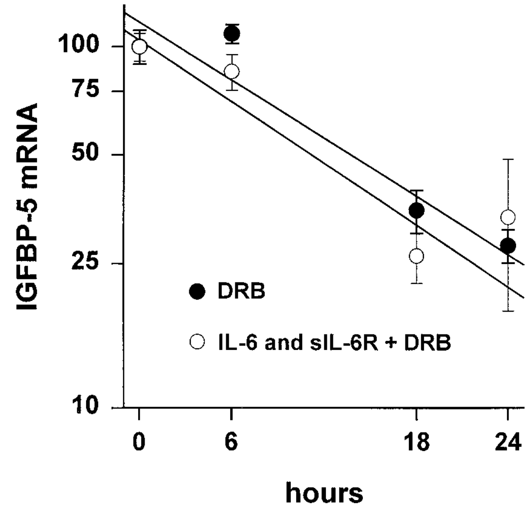

FIG. 6. Effect of IL-6 at 100 ng/ml in presence of sIL-6R at 50 ng/ml on IGFBP-5 mRNA decay in transcriptionally blocked Ob cells. Cultures were exposed to DMEM or treated with IL-6 and sIL-6R 6 h before and 6, 18, and 24 h after addition of DRB. Total RNA from control (●) or IL-6- and sIL-6R-treated cells (○) was subjected to Northern blot analysis and hybridized with a ^{32}P-labeled rat IGFBP-5 cDNA. IGFBP-5 mRNA was visualized by autoradiography, and changes in mRNA levels quantitated by densitometry. Values are means ± SEM for four to six cultures obtained from two experiments. Slopes from control and experimental cultures were not different.

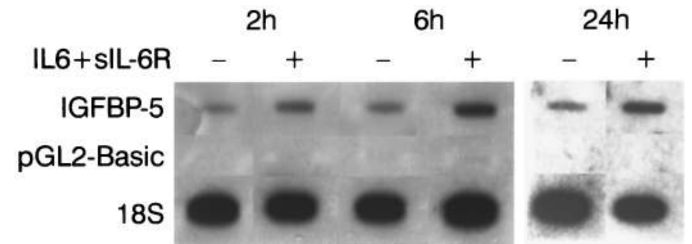

FIG. 7. Effect of IL-6 at 100 ng/ml in presence of sIL-6R at 50 ng/ml on IGFBP-5 transcription rates in cultures of Ob cells treated in Exp A (*left*) for 2 and 6 h and in Exp B (*right*) for 24 h. Nascent transcripts from control (C) or IL-6- and sIL-6R-treated cultures were labeled *in vitro* with [α-^{32}P]UTP, and labeled RNA was hybridized to immobilized cDNA for IGFBP-5. 18S rRNA cDNA was used to demonstrate loading, and pGL2-Basic vector DNA was used as a control for nonspecific hybridization.

half-life of IGFBP-5 mRNA in transcriptionally arrested Ob cells was estimated at approximately 13 h, and it was not modified by treatment with IL-6 and sIL-6R (Fig. 6). To test whether IL-6 and sIL-6R modified the rate of transcription of the IGFBP-5 gene, nuclear run-on assays were performed on nuclei from Ob cells exposed to control medium or to IL-6 at 100 ng/ml and sIL-6R at 50 ng/ml for 2 and 6 h (Fig. 7, Exp A) or 24 h (Fig. 7, Exp B). After 2–24 h, IL-6 in the presence of sIL-6R increased IGFBP-5 transcription rates by 2.5- to 3-fold, demonstrating a transcriptional effect.

Western immunoblots confirmed the presence of a major form of immunoreactive IGFBP-5 in the extracellular matrix of control cultures migrating with a molecular mass of 31 kDa (Fig. 8, *left*). Immunoreactive bands of 45–50 kDa were also observed, but these are not detected by Western ligand blot analysis using ^{125}I-IGF-II as a probe indicating that they are not IGFBPs (20). IL-6 at 100 ng/ml in the presence of sIL-6R at 50–125 ng/ml did not change the levels of the 31-kDa form of IGFBP-5 after 24 or 48 h in the extracellular matrix (Fig. 8, *left*) (n = 8). The 31-kDa form of IGFBP-5, as well as an immunoreactive protein migrating with a molecular mass of approximately 24 kDa, which is the known molecular mass of one of the IGFBP-5 proteolytic fragments, were detected in the culture medium (Fig. 8, *right*) (38, 39). IL-6 in the presence of its soluble receptor decreased the 31-kDa form of IGFBP-5 in the medium and increased the 24-kDa fragment after 24 and 48 h (n = 2). To test for the presence of IGFBP-5 proteolytic activity, purified human IGFBP-5 was incubated with conditioned medium from control and IL-6/sIL-6R-treated cultures for 0–16 h. There was a decrease in the 31-kDa form of IGFBP-5 and the generation of immunoreactive proteins of approximately 17, 20, and 24 kDa (Figs. 9 and 10) (n = 3). The appearance of the proteolytic fragments and the decay of the IGFBP-5 standard were accelerated and enhanced when recombinant IGFBP-5 was incubated in the presence of conditioned medium from IL-6/sIL-6R-treated cultures (Figs. 9 and 10). The generation of proteolytic frag-

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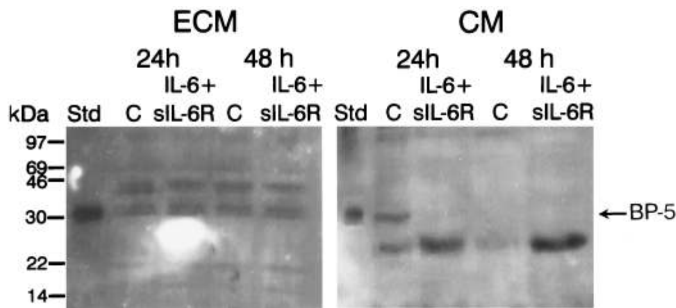

FIG. 8. Effect of IL-6 at 100 ng/ml in presence of sIL-6R at 125 ng/ml on IGFBP-5 polypeptide levels in cultures of Ob cells treated for 24 or 48 h. Extracellular matrix (ECM) or conditioned medium (CM) from control (C) or treated cultures were subjected to Western immunoblot analysis. IGFBP-5 was detected using an anti-IGFBP-5 antibody and identified by comparison with a human IGFBP-5 standard (St), using a chemiluminescence detection system. Molecular mass markers in kDa are indicated on *left*.

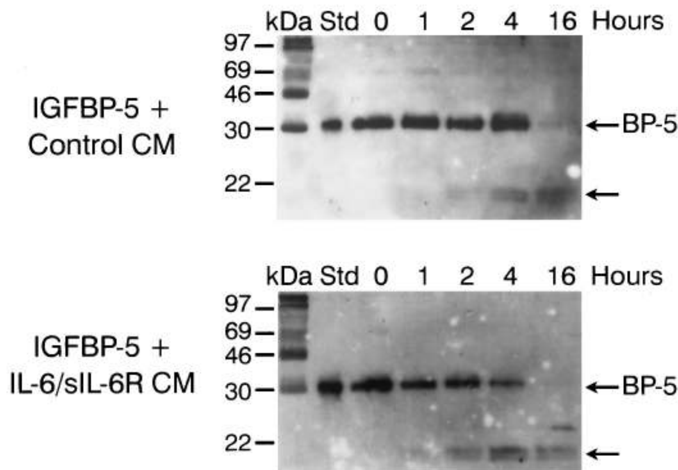

FIG. 9. Effect of IL-6 at 100 ng/ml in presence of sIL-6R at 125 ng/ml on IGFBP-5 proteolytic activity in cultures of Ob cells treated for 24 h. A purified human IGFBP-5 standard (Std) was incubated with conditioned medium (CM) from control (*upper*) or IL-6/sIL-6R-treated (*lower*) Ob cell cultures at 37 C for 0, 1, 2, 4, or 16 h and subjected to Western immunoblot analysis. Intact IGFBP-5 and generated fragments were detected using an anti-IGFBP-5 antibody using a chemiluminescence detection system and are indicated by *arrows* on *right*. Molecular mass markers in kDa are indicated in left margin.

ments was virtually prevented by the metalloprotease inhibitors 1,10 phenanthrolene and EDTA, and only partially prevented by the serine protease inhibitor PMSF (Fig. 10) (40).

Discussion

IL-6 is considered to be an important mediator of bone resorption and stimulates osteoclastogenesis in physiological conditions and in conditions of increased bone resorption, such as estrogen deficiency and multiple myeloma (41–43). Because IL-6 also has mitogenic properties for osteoblastic cells, it may act in the coupling of bone resorption and bone formation. Bone remodeling is modified by local growth factors, which exert their actions by influencing the development, proliferation, and differentiated function of cells of the osteoblast or osteoclast lineage (44). IGFs are among the

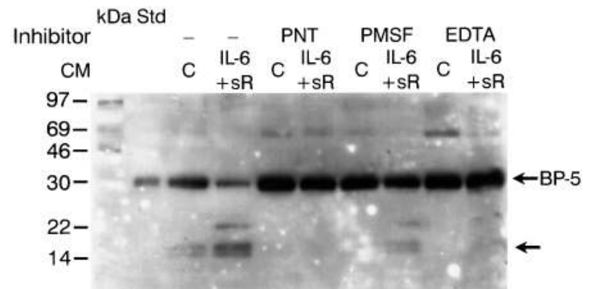

FIG. 10. Effect of IL-6 at 100 ng/ml in presence of sIL-6R at 125 ng/ml on IGFBP-5 proteolytic activity in cultures of Ob cells treated for 24 h. A purified human IGFBP-5 standard (Std) was incubated with conditioned medium (CM) from control (C) or IL-6/sIL-6R-treated Ob cell cultures at 37 C for 4 h in presence and absence of 1,10 phenanthrolene (PNT) at 1 mM, PMSF at 10 mM, or EDTA at 10 mM and subjected to Western immunoblot analysis. Intact IGFBP-5 and generated fragments were detected using an anti IGFBP-5 antibody and a chemiluminescence detection system. Molecular mass markers in kDa are indicated on *left*.

most abundant factors present in bone, and their effects are critical to the formation of new bone and to the maintenance of a normal bone matrix (13). Because the activity of IGFs is regulated by the production and availability of IGFBPs, we examined the effects of IL-6 in the presence or absence of sIL-6R on the synthesis of IGFBP-5 in Ob cells.

In the present study, we demonstrated that IL-6 alone caused a modest increase in IGFBP-5 mRNA levels in Ob cells, but when tested in the presence of its soluble receptor, the effect was amplified and observed at lower doses of IL-6. This is not surprising because sIL-6R binds IL-6 with similar affinity as the membrane bound receptor, and the sIL-6R/IL-6 complex mediates IL-6 signaling by binding and activating the signal transducing gp 130 (24). By these mechanisms, IL-6R may confer IL-6 responsiveness to cells expressing low levels of IL-6 transmembrane receptor (24). Furthermore, the effect of IL-6 on osteoclast formation also is observed in the presence of its soluble receptor (25). The effect of IL-6 and sIL-6R on IGFBP-5 mRNA was observed at concentrations detected in mouse and human serum, suggesting that it is physiologically relevant (45, 46). sIL-6R alone had a small effect, and its activity is probably due to the presence of significant levels of IL-6 in the bone cell microenvironment (47). Although IL-6 stimulates PGE_2 synthesis in osteoblastic cells, and PGE_2 stimulates the transcription of IGFBP-5 in osteoblasts, the stimulatory effect of IL-6 and sIL-6R on IGFBP-5 is not prostaglandin synthesis dependent, suggesting that IL-6 influences bone cell growth through various mechanisms (8, 48). IL-6 and its soluble receptor also increase IGF-I transcripts and polypeptide levels, and IGF-I increases IGFBP-5 transcription (12, 19). However, IGF-I probably does not mediate the acute effects observed with IL-6 and its soluble receptor on IGFBP-5 synthesis. This is because the effect on IGFBP-5 transcription was detected after 2 h, whereas 24 h of exposure to IL-6 and sIL-6R are needed to detect a stimulation of IGF-I mRNA in Ob cells (N. Franchimont and E. Canalis, unpublished observations). Furthermore, indomethacin decreases the effect of IL-6 and sIL-6R on IGF-I expression without modifying the expression of IGFBP-5. Nevertheless, the increase in IGF-I

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synthesis by IL-6 and sIL-6R may have an important secondary delayed effect not only on the stimulation of IGFBP-5 transcription in osteoblasts, but also on the stabilization of the protein (19, 49).

IL-6 induces the transcription of the IGFBP-5 gene in osteoblasts, and does not stabilize IGFBP-5 mRNA in transcriptionally arrested Ob cells. The gene sequences responsible for the effect have not been determined. In other cells, IL-6 activates the synthesis of the activating protein-1 family of transcription factors, induces nuclear factor-IL-6, a member of the CCAAT enhancer binding protein family, acute-phase response factor, and the octamer binding proteins (50–53). Examination of the IGFBP-5 promoter region reveals potential binding sites for nuclear factor-IL-6 and activating protein-1 in the bp −2700 to +1 region of the gene (54). It is possible that IL-6 acts by inducing or activating transcription factors that bind to one or more of these sequences.

Whereas most of the IGFBPs appear to have inhibitory activities on bone formation, IGFBP-5 has been shown to increase bone cell growth and enhance the actions of IGF-I on this process (14, 18, 55, 56). Although IL-6 and sIL-6R increased IGFBP-5 mRNA, they did not cause a detectable increase in polypeptide levels, suggesting additional effects at the translational or posttranslational level. Our studies demonstrate that IL-6 and sIL-6R enhance the production of IGFBP-5 fragments due to increased proteolytic activity, and this is probably the cause for the lack of a substantial accumulation of intact IGFBP-5 in Ob cells. The osteoblastic MC3T3 cells and fibroblasts secrete serine proteases and matrix metalloproteinases that degrade IGFBP-5 (38, 39, 57, 58). Ob cells seem to secrete similar proteases, because the IGFBP-5 proteolytic activity was inhibited both by the serine protease inhibitor PMSF, and by the metalloproteinase inhibitors EDTA and 1,10 phenanthrolene. Recent studies from our laboratory demonstrated that IL-6 and sIL-6R have stimulatory activity on the synthesis of collagenase 3 or matrix metalloproteinases-13 and of the 72-kDa gelatinase in Ob cell cultures (59), but it is not known whether or not IL-6 also induces serine proteases in these cells. Therefore, the induction of collagenase or other proteases may be responsible for the proteolysis of IGFBP-5 by IL-6 and sIL-6R in Ob cells. It is not clear why IL-6 and sIL-6R induce IGFBP-5 synthesis as well as proteolytic activity for this binding protein. This may be a local mechanism to prevent excessive accumulation of intact IGFBP-5 and binding of IGF-I. The function of the IGFBP-5 fragments is not well established, and it is not known whether they have effects distinct from those of the intact protein. The significance of IGFBP-5 fragmentation is not clear, but it is possible that the fragments have specific actions on bone cell function.

In conclusion, IL-6 in the presence of sIL-6R enhances IGFBP-5 mRNA expression by transcriptional mechanisms in osteoblast cultures, and stimulates the production of IGFBP-5 proteolytic activity and the formation of IGFBP-5 fragments. The effects of IL-6 and sIL-6R on the IGF-IGFBP axis could constitute a pathway for IL-6 effects on bone formation.

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