Leukemia inhibitory factor and oncostatin M stimulate collagenase-3 expression in osteoblasts

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Leukemia inhibitory factor and oncostatin M stimulate collagenase-3 expression in osteoblasts. Am. J. Physiol. 276 (Endocrinol. Metab. 39): E456–E471, 1999.—Leukemia inhibitory factor (LIF) and oncostatin M (OSM) have multiple effects on skeletal remodeling. Although these cytokines modestly regulate collagen synthesis in osteoblasts, their effects on collagenase expression and collagen degradation are not known. We tested whether LIF and OSM regulate the expression of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) in osteoblast-enriched cells isolated from fetal rat calvariae. LIF and OSM increased collagenase-3 (MMP-13) mRNA and immunoreactive protein levels in a time- and dose-dependent manner. LIF and OSM enhanced the rate of collagenase gene and stabilized collagenase mRNA in transcriptionally arrested cells. LIF and OSM failed to regulate the expression of gelatinase A (MMP-2) and B (MMP-9). LIF and OSM modestly stimulated the expression of TIMP-1 but did not alter the expression of TIMP-2 and -3. In conclusion, LIF and OSM stimulate collagenase-3 and TIMP-1 expression in osteoblasts, and these effects may be involved in mediating the bone remodeling actions of these cytokines.

Cytokines; collagen degradation; matrix metalloproteinases; tissue inhibitors of metalloproteinases; gelatinases

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Leukemia inhibitory factor (LIF) and oncostatin M (OSM) are members of the IL-6 family of cytokines that share a common signal transducing receptor component, glycoprotein 130 (gp130). LIF binds LIF receptors (LIFR), whereas OSM can bind a specific OSM receptor (OSMR) as well as LIFR (34). The cellular actions of LIF and OSM are elicited by the heterodimerization of LIFR or OSMR with gp130 to generate intracellular signals for the JAK/STAT signal transduction pathway.

LIF and OSM are primarily produced by cells of hematopoietic lineage, such as activated monocytes and T lymphocytes. LIF is also synthesized by parathyroid hormone (PTH)-stimulated osteoblasts (8). LIF and OSM are modest mitogens for osteoblastic cells, and they regulate the expression of important osteoblastic markers such as type I collagen and alkaline phosphatase (10, 29, 36). The actions of LIF and OSM on bone resorption are somewhat controversial, and depending on the culture model stimulatory and inhibitory effects have been reported (10, 19, 29, 36). Based on the actions of LIF and OSM in bone cultures, these cytokines are regarded as potent bone remodeling agents with pleiotropic effects on bone formation and resorption.

Collagenases are matrix metalloproteinases (MMPs) that degrade components of extracellular matrix (25). Three mammalian collagenases, collagenase-1, -2 and -3 (MMP-1, -8, and -13), are known, and these proteinases can degrade fibrillar collagen at neutral pH. Collagenase-3 was originally identified in human breast carcinoma and was found to be expressed in normallignant human cells, including chondrocytes and osteoblasts (33). Collagenase-3 expression is stimulated in rodent osteoblasts by several bone remodeling agents, including PTH, IL-1, and IL-6 (7, 13, 26). Collagenase is synthesized and secreted as a proenzyme and is activated by other proteases in the extracellular matrix by proteolytic cleavage. Bone cells also synthesize gelatinases A (72-kDa gelatinase or MMP-2) and B (92-kDa gelatinase or MMP-9), which can complement collagenase activity by the activation of procollagenase and by further degradation of collagen fragments that are generated by the initial cleavage of intact collagen fibrils by collagenase (18). The biological activity of collagenase is suppressed by tissue inhibitors of metalloproteinases (TIMPs), which are also expressed by osteoblastic cells (7).

Because OSM and LIF have important effects on bone remodeling, we postulated that they may regulate the synthesis of proteases that regulate collagen turnover. In this study, we examined the regulation of collagenase-3, gelatinases A and B, and TIMP-1, -2, and -3 by LIF and OSM in primary cultures of osteoblast-enriched (Ob) cells isolated from fetal rat calvariae.

MATERIALS AND METHODS

Cell culture. Fetuses were removed from 22-day-pregnant Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, MA) and were killed by blunt trauma to the nuchal area, following a protocol approved by the Animal Care and Use Committee of Saint Francis Hospital and the costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Medical Center. Ob cells from parietal bones of fetal rat calvariae were isolated as described previously (24). Briefly, bone was digested with bacterial collagenase (Worthington, Freehold, NJ), and the cells from the third to fifth digestions were pooled and plated at 6,000 to 12,000 cells/cm² in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with nonessential amino acids, 100 µg/ml L-ascorbic acid, 20 mM HEPES (all from Life Technologies, Grand Island, NY), and 10% fetal bovine serum (Summit Biotechnology, Fort Collins, CO) and cultured at 37°C in a humidified CO₂ incubator. At confluence, the medium was replaced with serum-free DMEM for 16–24 h, and cells were exposed to control medium with or without test agents. Recombinant mouse LIF (Genzyme, Cambridge, MA) was dissolved in serum-free DMEM containing 0.1% bovine serum albumin (BSA; Sigma Chemical, St. Louis, MO), and recombinant human OSM (R&D Systems, Minneapolis, MN) was added directly to serum-free DMEM. For determination of mRNA stability, cells were incubated with control medium with or without LIF and OSM for 16 h before the addition of 5,6-dichlorobenzimidazole riboside (DRB; Sigma), which was dissolved in ethanol and diluted 1:200; an equal amount of ethanol was present in control and test cultures. For nuclear run-off assays, cells were grown to subconfluence, trypsinized, replated, grown to confluence, serum-deprived for 16 h, and exposed to control and test solutions. At the end of the culture, the cell layer was extracted for isolation of total RNA for Northern blot analysis, and nuclei were isolated to determine rates of transcription. Medium was collected and stored at −20°C after the addition of polyoxyethylene sorbitan monolaurate (Pierce Chemical, Rockford, IL) to a final concentration of 0.1% for procollagenase determination.

Northern blot analysis. Total RNA was isolated from Ob cells by the method of Chomczynski and Sacchi (4) or by using an RNAeasy kit (Qiagen, Chatsworth, CA) and was fractionated on a 1% agarose (Sigma) formamide gel containing 100 µg/ml ethidium bromide (Sigma) as described (4, 31). After electrophoresis, RNA was transferred onto a Biotrans nylon membrane (ICN Biomedicals, Aurora, OH) by capillary action. The integrity and equal gel loading of RNA and efficiency of transfer were assessed by visualizing 28S and 18S ribosomal RNA bands under ultraviolet (UV) light. RNA was cross-linked to nylon membranes using a CL-1000 UV cross-linker (UVP, San Gabriel, CA). cDNA fragments were isolated by restriction endonuclease (New England Biolabs, Beverly, MA) digestion of plasmid clones containing a 2.6-kilobase (kb) rat collagenase-3 cDNA (kindly provided by Dr. Cheryl Quinn, St. Louis University School of Medicine, St. Louis, MO), a 0.8-kb murine TIMP-1 cDNA, a 0.7-kb murine TIMP-2 cDNA, a 0.75-kb murine TIMP-3 cDNA (all kindly provided by Dr. Dylan Edwards, University of Calgary Health Sciences Center, Calgary, AB, Canada), a 1.1-kb human gelatinase A cDNA (American Type Culture Collection, Rockville, MD), a 0.78-kb murine gelatinase B cDNA (kindly provided by Dr. Ghislain Opdenakker, University of Leuven, Brussels, Belgium), and a 0.8-kb rat glyceroldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (kindly provided by Dr. Ray Wu, Cornell University, Ithaca, NY; see Refs. 14–16, 22, 26, 35). cDNAs were radiolabeled by the random-priming method using [α-32P]dATP and [α-32P]dCTP (3,000 Ci/mmol; DuPont, Wilmington, DE) and DNA polymerase large fragment (New England Biolabs), and the hybridization of the RNA blots was performed for 16–24 h at 42°C with radiolabeled cDNAs in the presence of 50% formamide (Sigma) as described (31). The final wash was performed at 55°C in 0.15 M sodium chloride-0.015 M sodium citrate, pH 7 (1× SSC)-0.1% sodium dodecyl sulfate (SDS; all from Sigma) for the detection of collagenase-3, TIMP-1, -2, and -3, and GAPDH mRNAs. The final wash was performed at 62°C in 0.2× SSC-0.1% SDS for the detection of gelatinase A and B mRNAs. mRNA bands were visualized by autoradiography using Kodak X-AR film (Eastman Kodak, Rochester, NY) in the presence of a DuPont Cronex Lightning Plus intensifying screen. The intensity of RNA bands was quantified by densitometric tracing of the autoradiographs. Changes in collagenase-3, TIMP-1, -2, and -3, and gelatinases A and B were normalized for changes in GAPDH. For each test condition, Northern blot analysis was performed using samples from two or more independent cultures.

Nuclear run-off assay. Nuclei were isolated from Ob cells after Dounce homogenization in Tris buffer containing 0.5% nonionic detergent TGEPC CA630 (Sigma; see Ref. 1). Nascent transcripts were radiolabeled by incubation of nuclei at room temperature for 30 min in a reaction buffer containing 250 µCi (800 Ci/mmol) of [α-32P]UTP (DuPont), 500 µM ATP, CTP, and GTP (all from Life Technologies), and 150 units RNASin (Promega, Madison, WI). [32P]RNA was isolated by treatment with DNase I (Life Technologies) and proteinase K (Boehringer Mannheim, Indianapolis, IN), followed by phenol-chloroform extraction and ethanol precipitation using ammonium acetate. Linearized plasmid DNA containing 1 µg of cDNA for rat collagenase-3 and mouse ribosomal 18S RNA (American Type Culture Collection) or 1 µg of puC18 (Life Technologies) plasmid DNA was immobilized on a Biotrans nylon membrane using a slot-blot apparatus (Life Technologies). Equal counts of [32P]RNA from each sample were hybridized to immobilized cDNAs at conditions identical to Northern hybridization and were washed in 1× SSC-0.1% SDS at 55°C. Hybridization of nascent transcripts was visualized by autoradiography and quantitated by densitometry. Nuclear run-off assay was performed twice for 1 h treatment with LIF and OSM and one time for 4 and 16 h treatment with LIF.

Western immunoblot analysis. Aliquots of equal volume from control and test culture medium were fractionated on a 10% polyacrylamide (Boehringer Mannheim) gel by electrophoresis using denaturing conditions and were transferred onto an Immobilon-P membrane (Millipore, Bedford, MA; see Ref. 1)). After blocking with 2% BSA, the membrane was exposed to a 1:1,000 dilution of rabbit antiserum against rat collagenase (11; kindly provided by Dr. John J. J effrey, Albany Medical College, Albany, NY), followed by the addition of goat anti-rabbit IgG conjugated to horseradish peroxidase (Sigma). The blots were washed and developed with a horseradish peroxidase chemiluminescence detection reagent (DuPont). The chemiluminescent bands were visualized after exposure to DuPont Reflection film employing a reflection intensifying screen. Western blot analysis was performed using samples from two independent cultures.

Statistical methods. Data on collagenase mRNA decay were analyzed by linear regression, and the slopes of the regression lines obtained for control and LIF- or OSM-treated cells were compared for significant differences using the method of Sokal and Rohlf (32).

RESULTS

The effects of LIF and OSM on collagenase-3 transcripts were examined by Northern blot analysis. After treatment of Ob cells with 50 ng/ml of LIF for 2–24 h,
collagenase mRNA levels were increased by 6- to 22-fold (Fig. 1A and Table 1). To determine the dose-dependent changes in collagenase-3 expression, Ob cells were exposed to LIF at doses of 0.1–100 ng/ml for 16 h (Fig. 1B and Table 2). LIF increased collagenase mRNA levels at doses 1 ng/ml and higher. Exposure of Ob cells to OSM at 50 ng/ml for 2–24 h increased collagenase mRNA levels by 5- to 19-fold (Fig. 2A and Table 1). After treatment with OSM at doses of 0.1–100 ng/ml for 16 h, collagenase mRNA levels were increased at doses of 10 ng/ml and higher (Fig. 2B and Table 2).

To investigate whether the collagenase-3 gene was regulated by LIF and OSM at the transcriptional level, we examined the changes in the collagenase gene transcription rate by nuclear run-off assays (Fig. 4). Exposure of Ob cells to LIF at 10 ng/ml for 1, 4, and 16 h increased the synthesis of nascent collagenase transcripts by two-, three-, and sixfold, respectively. OSM at 50 ng/ml caused a fourfold increase in the synthesis of nascent collagenase transcripts after 1 h. Because changes in collagenase mRNA levels may also be secondary to mRNA stabilization, we tested collagenase mRNA decay after transcriptional arrest by adding the RNA polymerase II inhibitor DRB at 75 µM to Ob cells pretreated with or without LIF or OSM at 50 ng/ml for 16 h. The levels of collagenase mRNA from control and LIF-treated cultures were measured before and 3–12 h after transcriptional arrest to determine the rate of collagenase mRNA decay (Fig. 5). The half-life of collagenase mRNA was 4 h in control cultures, and it was estimated to be 24 h in LIF-treated cultures, by extrapolation of values obtained in the first 12 h. The levels of collagenase mRNA from control and OSM-treated cul-

**Table 1.** Time-dependent changes in steady-state collagenase-3 mRNA levels in LIF- and OSM-treated Ob cells

<table>
<thead>
<tr>
<th>Time, h</th>
<th>LIF</th>
<th>OSM</th>
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<tr>
<td>2</td>
<td>6.2±1.4</td>
<td>4.9±1.9</td>
</tr>
<tr>
<td>4</td>
<td>8.7±2.7</td>
<td>4.6±0.2</td>
</tr>
<tr>
<td>16</td>
<td>20.5±1.6</td>
<td>18.7±6.6</td>
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<tr>
<td>24</td>
<td>21.6±2.5</td>
<td>16.5±9.5</td>
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Changes in steady-state collagenase-3 mRNAs in leukemia inhibitory factor (LIF) and oncostatin M (OSM)-treated cultures relative to control values, after normalizing for changes in GAPDH, are shown as means ± SE; n = 3–6 experiments. Total RNA from control and LIF- or OSM-treated cultures at 0.1–100 ng/ml for 16 h was analyzed for collagenase-3 and GAPDH mRNAs by Northern hybridization followed by densitometry.
atures were measured in a similar manner up to 24 h after transcriptional arrest (Fig. 6). The half-life of collagenase mRNA was ~5 h in control cultures, and it was estimated to be 30 h in OSM-treated cultures, by extrapolation of values observed in the first 24 h.

LIF and OSM at 100 ng/ml for 2–24 h did not alter the expression of gelatinase A or B mRNA levels (data not shown). The regulation of TIMP-1, -2, and -3 transcripts was evaluated after exposure to LIF and OSM at 50 ng/ml for 2–24 h. OSM increased the levels of TIMP-1 mRNA by twofold and LIF by two- to fourfold (Fig. 7 and Table 3). Neither LIF nor OSM changed the levels of TIMP-2 and -3 mRNA (data not shown).

**DISCUSSION**

The present study shows that OSM and LIF stimulate the expression of collagenase-3 in osteoblasts in a time- and dose-dependent manner. The induction of collagenase mRNA by OSM and LIF is similar to that observed for IL-6 in the presence of exogenously added soluble receptor (7, 13). Recently, OSM has been shown to stimulate collagenase-3 in chondrosarcoma cells (5). Unlike IL-6, the receptors for LIF and OSM are ex-
pressed in rodent osteoblastic cells, indicating that these cells are direct targets of these cytokines (2). LIF and OSM regulate collagenase expression at the transcriptional and posttranscriptional level. LIF and OSM are potent mediators of the Jak/STAT signal transduction pathway in osteoblasts (17, 20). Putative DNA elements that can interact with the STAT family of nuclear factors are present within the collagenase gene promoter, and it is possible that LIF and OSM mediate transcriptional regulation of the collagenase gene through these regulatory sequences (27). The activator protein-1 and polyoma viral enhancer PEA3 sites that are present in several MMP promoters, including the collagenase-3, have been shown to be important in the regulation of MMPs by growth factors and cytokines (23, 27). It remains to be established if these regulatory sites within the collagenase-3 promoter influence the transcriptional regulation by LIF and OSM in osteoblasts. Previous studies showed that collagenase-3 expression can be regulated at the posttranscriptional level by altering mRNA stability (37).

LIF and OSM stimulated the secretion of procollagenase. MMPs and proteases that are present in the bone matrix may be responsible for the proteolytic activation of procollagenase in bone. Neither LIF nor OSM altered the expression of gelatinase A and B, possible activators of procollagenase-3 in osteoblastic cells. In contrast, earlier studies indicated that IL-6 can stimulate gelatinase A in osteoblasts (7). The activators of collagenase may also be generated by osteoclasts that are recruited to the bone remodeling site, since osteoclasts synthesize a number of proteolytic enzymes, including MMPs (38). As observed for most stimulators of collagenase-3, LIF and OSM also increase the expression of TIMP-1. Stimulation of TIMP-1 in osteoblasts by LIF and OSM is consistent with the observation that these cytokines increase the expression of TIMP-1 in fibroblasts (30). Expression of TIMP-2 and -3 in osteoblasts was unaffected by these cytokines. TIMPs inhibit MMPs by forming a bimolecular complex with MMPs, thus eliminating their endopeptidase activity. TIMP-1 binds active collagenase, but it does not appear to interact with latent procollagenase. Therefore, the increase in TIMP-1 by LIF and OSM may not affect procollagenase activation but may restrict collagenolytic activity, providing an additional regulatory control for collagen breakdown.

The precise role of collagenase stimulation in bone remodeling mediated by LIF and OSM is unclear. A recent report by Holliday et al. (9) showed that the collagen fragments generated by collagenase can enhance osteoclastic activity. Therefore, it is possible that the increase in collagenase expression may be partly responsible for the stimulation of bone resorption by LIF and possibly by OSM in rodent calvaria. Because collagenase-3 is also present in human skeletal cells, stimulation of collagenase by various cytokines may contribute to the pathogenesis of diseases leading to matrix degradation and osteopenia. Although the physiological levels of cytokines of the IL-6 family may be important in the normal bone remodeling, the increased levels of these cytokines in bone and joints of patients with postmenopausal osteoporosis and rheumatoid arthritis may promote connective tissue degradation and resorption (12, 28). In addition to degrading collagen fibrils, collagenase-3 can process other components of bone matrix. A recent study has demonstrated that collagenase-3 cleaves insulin-like growth factor (IGF) binding protein-5 by which it can generate pep-

Table 3. Time-dependent changes in steady-state TIMP-1 mRNA levels in LIF- and OSM-treated Ob cells

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<thead>
<tr>
<th>Time, h</th>
<th>LIF</th>
<th>OSM</th>
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<tbody>
<tr>
<td>2</td>
<td>1.3±0.2</td>
<td>1.4±0.1</td>
</tr>
<tr>
<td>4</td>
<td>1.7±0.1</td>
<td>1.1±0.1</td>
</tr>
<tr>
<td>16</td>
<td>3.6±0.0</td>
<td>2.2±0.5</td>
</tr>
<tr>
<td>24</td>
<td>4.1±0.5</td>
<td>1.7±0.4</td>
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Changes in steady-state tissue inhibitor of metalloproteinase (TIMP)-1 mRNAs in LIF- and OSM-treated cultures relative to control values, after normalizing for changes in GAPDH, are shown as means ± SD (n=2 experiments) for LIF and means ± SE (n=3 experiments) for OSM treatments. Total RNA from control and LIF- or OSM-treated cultures at 50 ng/ml for 2–24 h was analyzed for TIMP-1 and GAPDH mRNAs by Northern hybridization followed by densitometry.

Fig. 7. Effects of LIF and OSM on tissue inhibitor of metalloproteinase (TIMP)-1 mRNA levels in cultures of Ob cells. Ob cell cultures were treated with LIF (A) or OSM (B) at 50 ng/ml for 2, 4, 16, and 24 h. Total RNA from control (–) or test (−) cultures was analyzed by Northern hybridization using 32P-labeled TIMP-1 and GAPDH cDNAs. Levels of TIMP-1 and GAPDH mRNAs from 1 of 2 (A) or 3 (B) independent cultures are shown.

Collagenase mRNA contains three AREs in the 3' untranslated region and one ARE in the protein-coding region; however, it is unclear if these RNA elements play a role in regulating mRNA stability. It is conceivable that the cytokines, such as LIF and OSM, and other stimulators of collagenase mRNA stability may function by suppressing the normal regulatory function of AREs.
tides that may regulate the actions of IGF-I and II, important local stimulators of bone formation (6). Thus collagenase may coordinate different aspects of bone resorption and formation during the bone remodeling cycle by proteolytic cleavage of different components of bone matrix.

In conclusion, OSM and LIF stimulate expression of collagenase-3 and TIMP-1 in osteoblastic cells. These cytokines do not regulate the expression of gelatinases A and B and TIMP-2 and -3. The stimulation of collagenase-3 and TIMP-1 by LIF and OSM may regulate turnover of collagen and other components of bone matrix that may affect both bone formation and resorption.

We thank Dr. Cheryl Quinn for rat collagenase-3 cDNA, Dr. Dylan Edwards for murine TIMP-1, 2 and 3 cDNAs, Dr. Ghislain Opendaker for murine 92-kDa gelatinase cDNA, Dr. Ray Wu for rat GAPDH cDNA, and Dr. John effrey for rat procollagenase antibody. We also thank Kristine Sasala, Deena Durant, and Susan O’Lone for expert technical assistance.

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