Activation of the JAK-STAT Signal Transduction Pathway by Oncostatin-M in Cultured Human and Mouse Osteoblastic Cells*

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ABSTRACT

Oncostatin M (OSM) is one member of the leukemia inhibitory factor/interleukin-6 family of cytokines that has been shown to be a growth regulatory molecule. In osteoblastic cultures, OSM causes marked phenotypic changes and the enhanced secretion of interleukin-6. In this study, we have shown that stimulation of murine and human osteoblastic cultures and a human osteosarcoma cell line with OSM resulted in the tyrosine phosphorylation of a number of cellular proteins including members of both the Janus kinase (JAK) and signal transducer and activator of transcription (STAT) family of signaling proteins. The JAKs, a family of intracellular kinases, and the STATs, a family of transcription factors, have both previously

been shown to be tyrosine phosphorylated and activated in response to various cytokines, interferons, and growth factors in cells of non-skeletal origin. Using three different sources of cells of the osteoblast lineage, we demonstrate that OSM induces a rapid but transient tyrosine phosphorylation of the three JAK family members tested, JAK1, JAK2, and Tyk2. In addition, two members of the STAT family, Stat1α and Stat3, are tyrosine phosphorylated in osteoblastic cells in culture in response to OSM. OSM activation of this pathway in cells of the osteoblast lineage will result in the transcription of specific genes that ultimately may be associated with osteoblast function. (*Endocrinology* **137**: 1159–1165, 1996)

C YTOKINES are soluble factors that mediate communication between cells and play important roles in biological processes including hematopoiesis, neuropoiesis, embryonic development, cachexia, inflammation, muscle proliferation, and bone remodeling (1–3). The majority of cytokines bind to their cell surface receptors and initiate intracellular signaling events by inducing the rapid tyrosine phosphorylation of specific proteins. Structurally many cytokine receptors do not possess intrinsic tyrosine kinase activity, but rather associate with and activate members of the Janus (JAK) family of protein tyrosine kinases (reviewed in Refs. 4–6). Ligand binding to receptor leads to activation of specific members of the JAK family, which results in the tyrosine phosphorylation of the kinase itself, subunits of the cytokine receptor, and several cytoplasmic proteins. One major group of cytoplasmic proteins that becomes tyrosine phosphorylated has been named STATs (signal transducers and activators of transcription). When activated by tyrosine phosphorylation, the STATs translocate to the nucleus and bind to specific DNA sequences to stimulate transcription (7–14). The identification of the JAK-STAT signaling path-

way used by cytokines has provided an important link between events elicited by tyrosine kinases at the cell surface to downstream events activated in the nucleus.

With respect to the cellular process of bone remodeling, there is increasing evidence that cytokines are critically important. Cells in the osteoblast lineage, which are responsible for new bone formation during the process of remodeling, respond to a variety of stimuli and secrete a specific set of cytokines including macrophage-colony stimulating factor and granulocyte-macrophage colony stimulating factor (15, 16). In addition, several members of the leukemia inhibitory factor (LIF)/interleukin (IL)-6 subfamily of cytokines have been shown to have demonstrable bone regulatory activity. This family including LIF, oncostatin-M (OSM), IL-6, IL-11, ciliary neurotrophic factor, and a new member, cardiotrophin-1, are related by protein structure, overlapping function, and share a common signal transducing receptor component, gp130 (17–22). It has been suggested that IL-6, the prototypic member of this family, plays an important role in osteoclastogenesis, particularly in the development of postmenopausal osteoporosis (23, 24). Furthermore, treatment of cocultures of osteoblasts and bone marrow cells, as a source of osteoclast precursors, with IL-11, LIF, and OSM also induce osteoclast formation (23, 25). Some of the other LIF/IL-6 family members have been shown to exert differential effects on osteoblast and/or osteoclast function. For example, osteoblast-like cells are able to secrete and respond to IL-11, LIF, and IL-6 (26–32). Moreover, transgenic mice that overexpress the gene encoding for OSM under the control of the

metallothionein promotor develop osteopetrosis (33). There-fore, understanding the complex circuitry by which the members of the IL-6 family regulate osteoblast-osteoclast interactions and activity is critical in light of the fact that these factors appear to play important roles in the regulation of normal and pathologic bone remodeling.

Studies from our laboratory demonstrate that treatment of osteoblastic cells in culture with OSM causes marked phe-notypic changes including a modest increase in cell prolif-eration, increased collagen synthesis, decreased alkaline phosphatase activity, and a marked increase in IL-6 secretion (34). OSM elicits these biological effects in osteoblast-like cells through the utilization of specific signaling pathway(s). The work presented here is the first to elucidate a signal transduction cascade induced in OSM-treated primary mu-rine and human osteoblastic cells in culture.

Materials and Methods

Reagents and antibodies

Recombinant human OSM (bacterial derived, purified by sequential chromatography with no detectable endotoxin) was the gift of Dr. A. Gregory Bruce, Bristol-Myers Squibb (Seattle, WA). CLS-2 bacterial col-lagenase was obtained from Worthington Biomedical Corp. (Freehold, NJ). New born calf serum (NBCS) was purchased from Irvine Scientific (Santa Ana, CA), and trypsin was supplied by GIBCO Laboratories (Grand Island, NY). The Micro BCA protein assay kit was supplied by Pierce (Rockford, IL). Protein A-Sepharose was obtained from Sigma Chemical Co. (St. Louis, MO). Nitrocellulose filter paper was purchased from Schleicher and Schuell (Keene, NH). The enhanced chemilumi-nescence (ECL) system was obtained from Amersham (Arlington Heights, IL).

Antibodies against the tyrosine kinases JAK1, JAK2, and Tyk 2 were purchased from Upstate Biotechnology (Lake Placid, NY) and used according to the conditions recommended by the supplier. A mixture of antiphosphotyrosine monoclonal antibodies 4G10 (Upstate Biotechnol-ogy) and PY20 (ICN Biomedicals, Costa Mesa, CA) each at a final concentration of 0.5 μg/ml was used for Western blotting. Character-ization of the N- and C-terminal antibodies against Stat1α has been described (35). Anti-Stat3 antibodies were generated against a bacterial fusion protein expressing amino acids 717–769 of murine Stat3 (14). Peroxidase-conjugated secondary antibodies were obtained from Bio-Rad (Hercules, CA).

Cells

Neonatal murine calvarial derived osteoblastic cells were prepared as previously described (36). Calvariae from 3- to 5-day-old C57BL/6J mice were removed and pretreated with 4 mM EDTA in PBS [10 mM sodium phosphate (pH 7.4), 137 mM NaCl] twice (10 min each). The calvariae were then subjected to sequential enzymatic digestion using CLS-2 bac-terial collagenase at 200 U/ml in PBS over a 65-min period. The first two incubations of cells with collagenase each lasted 10 min and were fol-lowed by three 15-min digestions. Cells released after each digestion were collected, and fractions 3–5 were pooled. When examined imme-diately after isolation, the released cells in this pool were highly enriched for cells in the osteoblast lineage (37). The cells were washed twice in MEM with 10% NBCS, counted, plated at a low density of approximately 1–2 × 10^4 cells per ml (1000 cells/cm^2), and grown to confluence (5–7 days) before stimulation. Seeding at this density selects for proliferating cells consistent with osteoblast precursors and osteoblasts (38).

All animals were acquired and maintained in accordance with the NIH Guide for the Use of Laboratory Animals, and the tissue used in this study was obtained using protocols approved by Yale's Animal Care and Use Committee.

Normal human osteoblastic cells were obtained using techniques previously described (27). Sterile normal human bone was obtained

from patients (>60 years old) who were undergoing surgery after ac-cidental injury or hip replacement. The bone was cleared of soft and connective tissue, and the trabecular surfaces were exposed. The tra-becular bone was processed into small chips by mechanical reaming, and the chips were washed in serum-free medium and further minced with a straight microdissecting scissors in sterile glass vials with conical bottoms. The minced bone chips were washed extensively to remove blood components and incubated in medium containing 250 U/ml of CLS-2 bacterial collagenase twice for 40 min at 37 C in a shaking water bath. The chips were then washed three times in DMEM, resuspended in α-MEM with 10% NBCS, placed in 100-mm tissue culture dishes and incubated at 37 C. After this procedure, adherent cells could not be observed on any chip surfaces as determined by scanning electron mi-croscopy. After approximately 1 week of incubation, cells were noted to migrate out of the chips. Confluent cultures were obtained after 3–4 weeks. To separate the cells from the chips, the cultures were treated with 0.02% trypsin for 5 min at 37 C. The released cells were then washed and plated at low density (1–2 × 10^4/ml; 1000/cm^2) in α-MEM with 10% NBCS and grown to confluence (7–10 days). This population of cells was enriched for osteoblast markers including the expression of alkaline phosphatase as determined by FACS analysis, the constitutive secretion of the osteoblast-specific protein, osteocalcin, and the enhanced secre-tion of osteocalcin in response to 1,25-(OH)$_2$D$_3$ (Gundberg, C., and M. Horowitz, unpublished observations). For all experiments, monolayer cultures (murine and human) were used within 36 h of attaining con-fluence. Cells at confluence stop proliferating, display a low rate of DNA synthesis (39), but have not yet entered the phase of mineralization (40).

U-2OS cells are a moderately differentiated osteosarcoma of the tibia derived from a 15-yr-old girl in 1964 and obtained from American Type Culture Collection (ATCC HTB 96, Rockville, MD). Cells were main-tained by weekly passage in RPMI 1640 medium supplemented with 10% NBCS. To prepare cells for stimulation, osteosarcoma cells were recovered from maintenance culture, washed, and seeded at 10^4 cells/ml into 100-mm tissue culture dishes in growth medium and were grown to 95% confluence (7 days) before stimulation.

Cell stimulation and lysis

Cultures of murine or human osteoblastic cells and U-2OS cells were placed in α-MEM with reduced serum (0.5%) for 16–18 h. The cells were then stimulated with OSM (100 ng/ml) for the time period indicated. At the end of the incubation period, the cells were at least 98% viable as assessed by trypan blue dye exclusion. Cells were immediately rinsed with two changes of ice-cold PBS. After decanting the PBS, the cell monolayers were scraped into an appropriate volume of either lysis buffer A or B. Buffer A contains 20 mM Tris, pH 7.2, 158 mM NaCl, 1.0% Triton X-100, 1.0% sodium deoxycholate, 0.1% SDS, and 5.0 mM EDTA. Buffer B is composed of 50 mM HEPES, pH 7.5, 150 mM NaCl, 1.0% Triton X-100, 10% glycerol, 1.0 mM EGTA, and 1.5 mM MgCl$_2$. Protease and phosphatase inhibitors were added to the PBS and lysis buffers and included 1.0 mM Na$_3$VO$_4$, 1.0 μg/ml pepstatin, 10 μg/ml leupeptin and aprotinin, 1.0 mM phenylmethylsulfonylfluoride, and 50 mM sodium fluoride. Lysates were centrifuged at 12,000 × g for 15 min at 4 C and the supernatants collected. Protein concentration was determined in a Micro BCA protein assay kit using BSA as the standard. Each sample used for immunoprecipitations or Western blotting was adjusted so as to contain equivalent amounts of protein.

Immunoprecipitation and Western blotting

For immunoprecipitation analysis, cell lysates (200–500 μg) were first precleared with Protein A-Sepharose for 1 h at 4 C. After removal of the Protein A-Sepharose, cell supernatants were incubated with the speci-fied antibodies for 2–16 h at 4 C. The antigen-antibody complexes were recovered during a 30- to 60-min incubation using rabbit antimouse immunoglobulin and protein A-Sepharose or protein A-Sepharose alone. The immune complexes were washed 3–5 times with lysis buffer and the immunoprecipitated proteins eluted into SDS-PAGE sample buffer (3% SDS, 60 mM Tris, pH 6.9, 2 mM EDTA, 4% glycerol) by heating the samples to 100 C for 5 min.

Immunoprecipitated material or aliquots of total cell lysates (25–30 μg) were analyzed by 8% SDS-PAGE under reducing conditions fol-

lowed by electrophoretic transfer of the proteins to nitrocellulose. Western blotting procedures were performed essentially according to the procedures in the ECL system specified by Amersham. To reprobe the blots, nitrocellulose filters were stripped of antibodies in a solution containing 62.5 mM Tris, pH 6.7, 2% SDS, and 100 mM 2-mercaptoethanol at 50 C for 30 min.

Results

OSM induces tyrosine phosphorylation of specific cellular proteins in cultured murine and human osteoblastic cells and a human osteosarcoma cell line

In nonskeletal cells, signal transduction by OSM and its related family members has been shown to involve the activation of tyrosine kinases and subsequent phosphorylation of specific cellular proteins (reviewed in Ref. 41). To determine whether OSM induced protein tyrosine phosphorylation in osteoblastic cells, lysates prepared from serum-starved cultures that had been treated with 100 ng/ml OSM over a specified time course were resolved by 8% SDS-PAGE, transferred to nitrocellulose, and blotted with antiphosphotyrosine antibodies. As shown in Fig. 1A, OSM treatment of primary murine osteoblast-like cells induced tyrosine phosphorylation of proteins with mol wt of 150, 125–130, 89, 72, and 45K. The majority of the proteins appeared to be rapidly tyrosine phosphorylated within 1 min after OSM stimulation. Phosphorylation of the proteins reached maximal levels within 5 min after stimulation and was significantly diminished by 30 min.

Protein tyrosine phosphorylation was also induced when cultures of human osteoblastic cells were treated with OSM (Fig. 1B). The majority of proteins that became tyrosine phosphorylated in response to OSM treatment of primary human osteoblast-like cells had similar electrophoretic mobilities to all of the induced tyrosine phosphorylated proteins detected in OSM-stimulated murine osteoblastic cells (designated by the *arrows* in Fig. 1, A and B). However, in human osteoblast-like cells, two additional proteins of mol wt 160K and 50–60K (designated by the single *asterisks* in Fig. 1B) became tyrosine phosphorylated in response to OSM. Tyrosine phosphorylation of all of the proteins was induced within 1–3 min, reached maximal levels at 5 min, and decreased by 30 min after OSM treatment, similar to the time course observed in OSM-stimulated murine osteoblastic cultures.

Osteosarcomas are the most common and malignant forms of bone tumors. They are the transformed counterparts of cells in the osteoblast lineage and are thought to arise from mesenchymal stem cells. Therefore, it was important to determine whether these cells responded to OSM in a fashion similar to that of normal cells. Cell lysates prepared from a human osteosarcoma cell line (U-2OS) cultured in the absence or presence of OSM for various time periods were immunoblotted with antiphosphotyrosine antibodies. Even though the basal level of tyrosine phosphorylation was higher in the U-2OS cells, the pattern of tyrosine phosphorylated proteins induced in the U-2OS cells after treatment with OSM, as shown in Fig. 1C, was similar to that observed in human osteoblastic cultures with the exception of a protein of mol wt 110K, which appeared to be only induced in the U-2OS cells (indicated by the *double asterisk* in Fig. 1C).

OSM stimulates the tyrosine phosphorylation of several JAK family members

The JAK kinases have been shown to be tyrosine phosphorylated and activated in response to cytokine and growth factor stimulation in nonskeletal cells (4–6). Therefore, we determined whether JAK family members became tyrosine phosphorylated upon treatment of osteoblastic cultures with OSM. Lysates from OSM-treated murine osteoblast-like cells were immunoprecipitated using antiserum to JAK1, JAK2, and Tyk2 and analyzed by blotting with antiphosphotyrosine antibodies (αP-Tyr). Data in Fig. 2 (upper) demonstrate that tyrosine phosphorylation of all three JAK family members was evident at times as early as 30 sec. Phosphorylation was transient, being diminished by 30 min after addition of OSM. When the blots were stripped of antiphosphotyrosine antibody and reprobed with antiserum to the three respective kinases (lower), similar levels of protein were observed in all lanes of the time course tested. In addition, tyrosine phosphorylation of JAK1, JAK2, and Tyk2 was observed after a 5-min treatment of human osteoblastic cultures with OSM (Fig. 3) and in OSM-treated U-2OS cells (data not shown). Figure 3 also demonstrates that a major tyrosine phosphorylated protein of mol wt 120–130K induced by OSM-treatment of human osteoblastic cells and observed in the total cell lysate with antiphosphotyrosine antibodies (indicated by the *arrow*) has a similar electrophoretic mobility to phosphoproteins precipitated with αJAK1 and αJAK2 antibody preparations, therefore suggesting that this protein may represent a tyrosine phosphorylated JAK family member.

A. 1° MURINE OB 0 1' 5' 10' 30' kD 210 — 105 — 70 — 45 —

B. 1° HUMAN OB 0 1' 3' 5' 30'

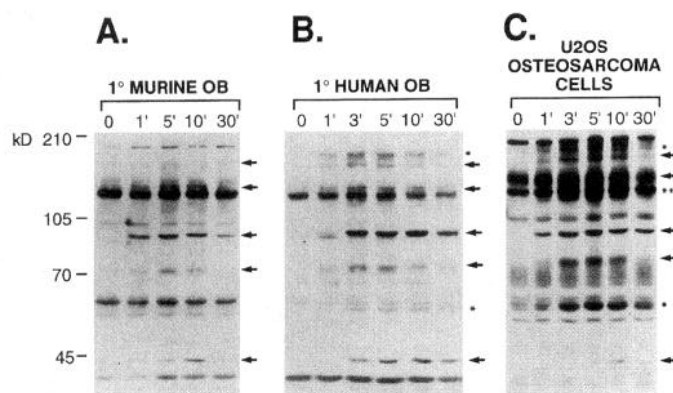**C.** U2OS OSTEOSARCOMA CELLS 0 1' 3' 5' 10' 30'

FIG. 1. OSM induces tyrosine phosphorylation in cultures of murine and human osteoblastic cells and a human osteosarcoma cell line. Cultures of murine osteoblastic cells (A), human osteoblastic cells (B), and the osteosarcoma cell line, U-2OS (C) were treated with vehicle alone (0') or OSM (100 ng/ml) for the various time periods indicated. Equal amounts of protein solubilized in lysis buffer A (25 µg/time point) were separated on 8% SDS-PAGE, transferred to nitrocellulose and probed with antiphosphotyrosine antibodies. Bound antibody was detected in the ECL reaction. The *arrows* indicate proteins that become tyrosine phosphorylated in response to OSM in both murine and human osteoblastic cells and U-2OS cells. *, Tyrosine phosphorylated proteins induced in OSM-treated human osteoblastic cells and U-2OS cells that are not observed in OSM-stimulated murine cultures. **, Induced protein only observed in U-2OS cells.
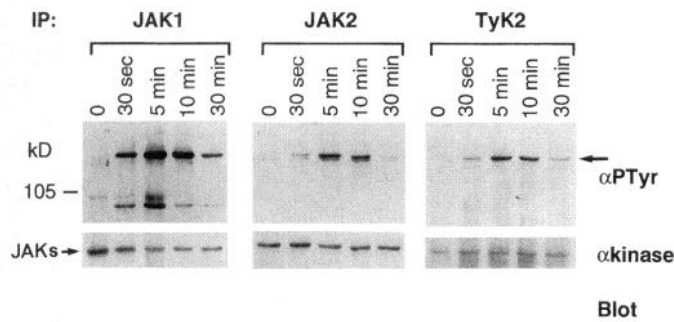
FIG. 2. Multiple JAK family members are transiently tyrosine phosphorylated in OSM-stimulated murine osteoblast-like cells. Murine osteoblastic cells were treated with OSM (100 ng/ml) for the various time periods indicated. At each time point, 500 μg of cell protein prepared in Buffer B was immunoprecipitated with antiserum against JAK1, JAK2, or Tyk2. The immunoprecipitated material was separated on 8% SDS-PAGE, transferred to nitrocellulose, and immunoblotted with antiphosphotyrosine antibody (*upper panels*). The filters in the *upper panel* were stripped of antibody and reprobed in Western blots with antiserum against the appropriate JAK family member that was originally used in the immunoprecipitation. In the JAK1 immunoprecipitation, the proteins migrating at approximately 105 kDa may represent degredation products of JAK1. The *arrow* in the *upper panel* indicates the JAK proteins.
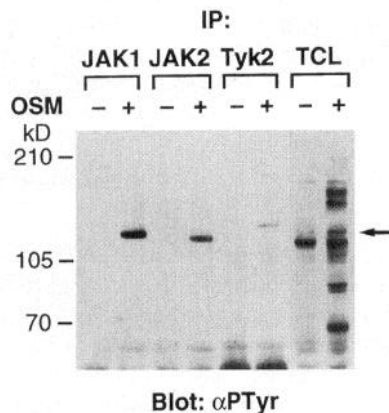

FIG. 3. JAK1, JAK2, and Tyk2 are tyrosine phosphorylated in response to OSM-treatment of human osteoblast-like cells. Protein solubilized in Buffer B (500 μg) prepared from unstimulated or OSM-stimulated (5 min) cultures of human osteoblastic cells were immunoprecipitated with antibodies against JAK1, JAK2, or Tyk2. The immunoprecipitated material was analyzed by antiphosphotyrosine Western blotting. Total cell lysate (TCL; 25 μg) from unstimulated and OSM-stimulated cells was analyzed on the same gel. The *arrow* indicates a tyrosine phosphorylated protein of mol wt 120–130K induced in stimulated cells and observed in total cell lysates.

Two distinct STAT proteins are tyrosine phosphorylated in OSM-treated osteoblastic cultures

Signaling initiated by cytokines, interferons, and growth factors induces tyrosine phosphorylation and activation of a new family of transcription factors, the STATs (7–14). One member of this family, Stat1α, has been shown to be tyrosine phosphorylated and activated in response to different members of the LIF/IL-6 family in nonskeletal cells (5, 12). Two antibodies that recognize distinct regions of Stat1α were used in immunoprecipitation analysis to determine whether Stat1α became tyrosine phosphorylated in response to OSM treatment of osteoblastic cells. Immunoprecipitated material

was analyzed by antiphosphotyrosine Western blotting. Data in Fig. 4 demonstrate that antibodies generated against a carboxy-terminal portion of Stat1α recognized a protein that displayed a low level of tyrosine phosphorylation in response to OSM treatment of murine osteoblast-like cells (C-terminal Stat1α; compare lanes 3 and 4). Low levels of tyrosine phosphorylation of Stat1α were also detected using another antibody generated against an amino-terminal fragment of Stat1α (Fig. 4; N-terminal Stat1α; compare lanes 1 and 2). In addition, OSM induced a major tyrosine phosphorylated protein that was only immunoprecipitated with the N-terminal Stat1α antibody and migrated slightly faster than Stat1α at a mol wt corresponding to approximately 88–89K (Fig. 4, lane 2). p88/89 recognized by the N-terminal antibody had a similar electrophoretic mobility to one of the major tyrosine phosphorylated proteins induced by OSM as observed in a total cell lysate by antiphosphotyrosine immunoblotting (lanes 2 and 5; p88 indicated by the *arrowhead*). Because this antibody has been shown to cross-react with Stat3, which migrates slightly faster than Stat1α (5, 42, 43), p88 is likely to represent Stat3 that has become tyrosine phosphorylated in response to OSM.

Stat3 shares 50% homology with Stat1α (12). It is tyrosine phosphorylated in Hep-G2 cells after treatment with IL-6 and related cytokines, as well as in other cell lines stimulated with various growth factors and interferons (5, 12–14). To confirm that the p88/89 tyrosine phosphorylated protein was Stat3, we repeated the studies with a Stat3-specific antibody. A major tyrosine phosphorylated protein induced by OSM treatment of murine (Fig. 5A) and human osteoblastic cells (Fig. 5B) was recognized by this antibody (upper panels; lanes 2). Tyrosine phosphorylated Stat3 had a similar electrophoretic mobility to the protein of mol wt 88–89K that became tyrosine phosphorylated in response to OSM as observed in total cell lysates on antiphosphotyrosine blots (Fig.
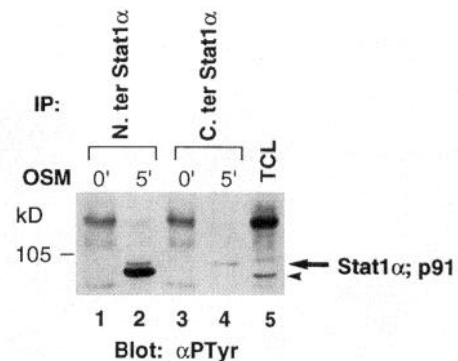

FIG. 4. The transcription factor, Stat1α, and related family members are tyrosine phosphorylated in response to OSM-stimulation of murine osteoblastic cells. Cell lysates prepared in Buffer B from unstimulated (0′) and OSM-stimulated (5′) cultures of murine osteoblast-like cells were immunoprecipitated with two different antisera that recognize Stat1α. One antibody was generated to an N-terminal peptide of Stat1α (N. ter Stat1α; lanes 1 and 2) and the other to a C-terminal peptide (C. ter Stat1α; lanes 3 and 4). The immunoprecipitated material was analyzed by antiphosphotyrosine Western blotting. TCL from OSM-stimulated cells was also included on this gel (lane 5). The position of Stat1α is indicated by the *arrow*. The position of a major tyrosine phosphorylated protein induced by OSM-treatment of osteoblasts and also immunoprecipitated by N-terminal Stat1α antibodies in stimulated cells is indicated by the *arrowhead*.
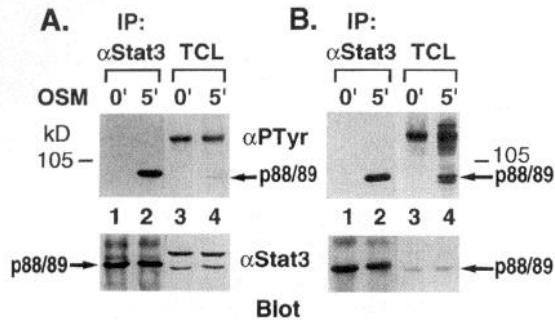
FIG. 5. The STAT family member, Stat3, is tyrosine phosphorylated in response to OSM-stimulation of cultures of murine and human osteoblastic cells. Solubilized protein prepared in Buffer B from unstimulated (0'; lane 1) and OSM-stimulated (5'; lane 2) murine osteoblast-like (A) and human osteoblast-like cells (B) were immunoprecipitated with anti-Stat3 antibodies and then analyzed by antiphosphotyrosine Western blotting (αPTyr; *upper panel*). The filters in the *upper panels* of A and B are stripped of antibody and reprobed with anti-Stat3 antibodies (*lower panels* of A and B). TCL from unstimulated (lane 3) and stimulated (lane 4) cultures of murine osteoblastic (A) and human osteoblastic cells (B) was included on this gel. The designation of Stat3 by p88/89 is indicated.

5, A and B, compare lanes 2 and 4). Equal protein loading of Stat3 was verified by reprobing the antiphosphotyrosine blots with antiserum to Stat3 (Fig. 5, A and B; lower panel). Therefore, two related transcription factors, Stat1α and Stat3, are tyrosine phosphorylated in response to OSM treatment of human and mouse osteoblast-like cells.

Discussion

The LIF/IL-6 subfamily of cytokines, which includes OSM, has been shown to induce a signaling cascade that involves the rapid tyrosine phosphorylation of specific cellular proteins ultimately leading to a wide variety of biological changes in nonskeletal cells. However, little is known about how OSM regulates bone cell function. In this report, we show for the first time that OSM induces the tyrosine phosphorylation of specific cellular proteins in biologically responsive murine and human osteoblastic cultures and in a human osteosarcoma cell line. Similar, but not identical, patterns of tyrosine phosphorylated proteins were induced by OSM treatment of Kaposi sarcoma cell lines and in human endothelial cells (44, 45). We demonstrate that a subset of the proteins that are tyrosine phosphorylated in response to OSM-treatment of osteoblasts includes members of the JAK-STAT signaling pathway. More specifically, the protein of mol wt 125–130K that becomes tyrosine phosphorylated in response to OSM is one of the JAK family members whereas the major tyrosine phosphorylated protein at 88–89K is presumably Stat3. In addition, other tyrosine phosphorylated proteins induced by OSM that were observed on antiphosphotyrosine blots of total cell lysates include the protein of mol wt 150K identified to be gp130, of 50–60K identified as one of the isoforms of the adaptor protein Shc, and of 44K as MAP kinase (46). Identification of the remaining tyrosine phosphorylated proteins awaits further investigation.

The receptors for the OSM cytokine family have been shown to use different members of the JAK-Tyk family and

are able to induce distinct patterns of JAK-Tyk tyrosine phosphorylation depending on the cell line examined (5, 6). Stimulation of EW-1 cells with OSM resulted in tyrosine phosphorylation of JAK1 and JAK2 but not Tyk2 (6). In HepG2 cells, OSM stimulated the tyrosine phosphorylation of JAK1 (5), whereas in our studies, OSM treatment of murine and human osteoblastic cells and a human osteosarcoma cell line induce transient tyrosine phosphorylation of all three JAK family members tested, JAK1, JAK2, and Tyk2. This result is not totally surprising in light of the finding in other cell types that stimulation by OSM or related family members leads to tyrosine phosphorylation of multiple JAK proteins (5, 6). Furthermore, in osteoblast-like cells, OSM induced tyrosine phophorylation of at least two related transcription factors, Stat1α and Stat3. Although similar results have been observed in the hepatocyte cell line HepG2, this is the first demonstration of tyrosine phosphorylation of the STAT family members in cultures of osteoblast-like cells in response to OSM (5). In both OSM-treated HepG2 cells and osteoblastic cells, tyrosine phosphorylated Stat3 was much more apparent than the tyrosine phosphorylated Stat1α protein. This could be due to differences in the actual amount of Stat3 and Stat1α present in the cells examined or may actually represent differences in the extent of tyrosine phosphorylation of different STAT family members in response to OSM.

The combination of JAK and STAT proteins that become phosphorylated is probably dependent on the cell line analyzed and the type of stimulation used. However, one possible explanation for our results in osteoblast-like cells is that the primary cultures contain a heterogeneous population of cells, each of which responds differently to OSM, leading to the tyrosine phosphorylation of multiple JAK and STAT family members. Extensive characterization of the murine osteoblastic cells reveals that the cells examined display markers of the osteoblast phenotype and the cultures are not contaminated with osteoclasts, macrophages, fibroblasts, or endothelial cells (36). Therefore, the great majority of these cells (greater than 95%) appear to represent osteoblastic cells or osteoblast-precursors, suggesting that cell heterogeneity cannot account for tyrosine phosphorylation of multiple JAK and STAT family members. We do recognize that heterogeneity can still exist in the primary cell cultures with respect to various stages of differentiated osteoblast-like cells present in the population. However, the analysis of OSM treatment of the clonal osteosarcoma cell line, derived from the osteoblast lineage, which demonstrated induction of tyrosine phosphorylation of all three JAK family members (data not shown) strongly suggests that OSM induces the tyrosine phosphorylation of multiple JAK family members in cells of the osteoblast lineage. Tyrosine phosphorylation of p88/89 in the OSM-treated osteosarcoma cell line as observed on antiphosphotyrosine immunoblotting further suggests that Stat3 is tyrosine phosphorylated in the clonal cell line as well as in the primary cultures. We therefore feel that our interpretation that multiple JAK members and at least Stat3 are tyrosine phosphorylated in response to OSM treatment of cells of the osteoblast lineage is reasonable based on similar findings in the clonal osteosarcoma cell line.

Treatment of cultures of murine osteoblastic cells with LIF, another IL-6 family member, leads to an enhanced secretion of IL-6 with a profile of induced tyrosine phosphorylated proteins similar to that observed with OSM-stimulated cells (28). Induction of an identical pattern of tyrosine phosphorylated proteins may be attributed to the utilization of the same receptor components by LIF and OSM, which has been reported in other cell types (reviewed in Ref. 41). In contrast, LIF was not very effective in inducing IL-6 secretion when added to cultures of human osteoblastic cells and failed to induce tyrosine phosphorylated proteins in these cells. This difference in responsiveness to LIF may be due to changes in the expression of the shared receptor components between murine and human osteoblast-like cells. Osteoblastic cells generally responded to other members of the LIF/IL-6 family with respect to tyrosine phosphorylation and IL-6 secretion to a much lesser degree than the response observed with OSM (28). Ciliary neurotrophic factor was weak at inducing tyrosine phosphorylation when added to cultures of murine osteoblastic cells and was also a poor inducer of IL-6 secretion. On the other hand, IL-6 only in combination with its soluble receptor component (sIL-6R), could induce tyrosine phosphorylation in human osteoblastic cells, which is consistent with the finding that IL-6 requires the sIL-6R to stimulate osteoclastogenesis in the murine coculture system (23). Finally, IL-11 was able to induce both tyrosine phosphorylation in murine osteoblast-like cells and IL-6 secretion (28). In contrast to our results with OSM on the phosphorylation of the different JAK family members, it has been reported that IL-11 induced phosphorylation of JAK2, with no effect on JAK1 in osteoblasts (47). Based on this latter comparison, the various members of the LIF/IL6 family when added to osteoblastic cells may phosphorylate different combinations of JAK proteins leading to differences in the profiles of tyrosine phosphorylated proteins observed.

Identification of the genes that become transcriptionally activated in osteoblastic cultures in response to OSM is critical in understanding the final biological consequences observed in the treated cells. In light of our findings, implicating the involvement of the JAK-STAT pathway in OSM-treated osteoblast-like cells, we anticipate that the promoters of several genes activated in osteoblastic cells will possess transcriptional elements recognized by various STAT family members. However, we cannot exclude the possibility that OSM induces other signaling pathways that would lead to the activation of transcription factors distinct from the STAT family. For example, in Kaposi cells OSM induces the tyrosine phosphorylation and activation of MAP kinase, which presumably would lead to the activation of different classes of transcription factors (44). Similarly, we recently identified the protein of mol wt 44K that is tyrosine phosphorylated in murine and human osteoblastic cultures in response to OSM as MAP kinase (46). Ultimately the links between the signaling pathways and the genes that become activated will provide a clearer understanding of the biological effects of OSM and the other members of the LIF/IL-6 subfamily of cytokines in osteoblast function.

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