Interleukin-6 (IL-6) is a multifunctional cytokine produced by various cells to regulate hematopoiesis, inflammation, immune responses, and bone homeostasis. IL-6 is also known to modulate the differentiation of osteoblasts and osteoclasts. IL-6 is believed to play a positive regulatory role in osteoclast differentiation by inducing the expression of receptor activator of NF-κB ligand (RANKL) on the surface of osteoblasts: RANKL then interacts with RANK expressed on osteoclast progenitors, inducing osteoclast differentiation via the RANK signaling pathway, which involves NF-κB, JNK, and p38. In this report, we demonstrate that IL-6 can also directly act on osteoclast progenitors to suppress their differentiation via an inhibition of RANK signaling pathways. IL-6 specifically suppressed RANK-mediated IκB degradation and JNK activation. Microarray analysis revealed that costimulation with IL-6 and RANKL up-regulates the transcription of MKP1 and MKP7, which encode enzymes that dephosphorylate JNK, and down-regulates the transcription of Semp2 and Cul4A, which are related to the ubiquitin pathway. Thus, IL-6 directly acts on osteoclast progenitors and suppresses their differentiation by regulating the transcription of specific genes related to MAPK phosphatases and the ubiquitin pathway.

Bone tissue is continuously remodeled under physiological conditions (1), but dysregulation of the balance between bone formation and resorption induces pathological conditions, including osteoporosis and osteosclerosis. Bone remodeling is maintained by two key cell populations, osteoblasts and osteoclasts, which are regulated by cytokines, hormones, and growth factors (2, 3). Among the cytokines, the interleukin-6 (IL-6) family cytokines are known to modulate both osteoblast and osteoclast differentiation (3–5).

IL-6 is a multifunctional cytokine produced by various cell types that regulates hematopoiesis, acute phase reactions, immune responses, and bone homeostasis (6–8). The receptor for IL-6 consists of a ligand-binding subunit and a common signal-transducing subunit, gp130 (9). gp130 contains a number of tyrosine residues in its cytoplasmic region, as well as four copies of the YXXQ motif, which is required for the tyrosine phosphorylation of STAT3. Activated STAT3 dimerizes, enters the nucleus, and regulates the transcription of various genes that regulate cell survival, proliferation, and differentiation in a cell-specific manner (6, 8). Tyr-759 of gp130 is required for the tyrosine phosphorylation of Src homology 2 domain-containing tyrosine phosphatase (SHP)-2, which activates the MAPK ERK via a complex comprising SHP2, Gab1/2, and phosphotidylinositol 3-kinase p85 (10–12).

It has been reported that IL-6 together with soluble IL-6 receptor acts to induce the expression of receptor activator of NF-κB ligand (RANKL) on the surface of osteoblasts. RANKL interacts with RANK, which is expressed on the surface of osteoclast progenitors. Coculture experiments have demonstrated that this interaction is indispensable for the differentiation of osteoclast progenitors into mature osteoclasts (13). Induction of RANKL expression on the surface of an osteoblastic cell line via the IL-6-gp130-STAT3 signaling pathway was demonstrated in vitro (14). Although these in vitro data suggested that IL-6-gp130-STAT3 signaling stimulates osteoblasts and supports osteoclastogenesis, IL-6 transgenic mice showed decreased osteoclast numbers and bone resorption (15). One critical difference in vivo might be the lack of soluble IL-6 receptor under physiological steady-state conditions. The complexity of the in vivo situation is further underscored by a report showing that gp130 signaling has opposing effects in terms of bone formation in osteoblasts versus osteoclasts; gp130 signaling up-regulates the expression of RANKL on osteoblasts but down-regulates RANK expression on osteoclast progenitors (16). These results have made it difficult to interpret the phenotypes of mutant mouse lines in which genes expressed in both osteoblasts and osteoclasts are manipulated and suggest that the in vivo roles of IL-6-gp130-STAT3 signaling in osteoclast phosphatase; CTR, calcitonin receptor; TRAP, tartrate-resistant acid phosphatase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; STAT3, signal transducers and activators of transcription 3; SHP2, Src homology 2 domain-containing tyrosine phosphatase; JNK, c-Jun NH₂-terminal kinase; M-CSF, macrophage-colony stimulating factor; ERK2, extracellular signal-regulated kinase 2; HPRT, hypoxanthine phosphoribosyltransferase; sRANKL, soluble RANKL.
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blasts and osteoclasts should be examined separately. To this end, we disrupted the STAT3 gene in an osteoblast-specific manner. The resulting osteoporotic phenotype clearly demonstrated that STAT3 signaling in osteoblasts in vivo has a positive regulatory role in the differentiation of this cell type, leading to bone formation but not osteoclastogenesis (8). On the other hand, other groups have reported that hematopoietic cell-specific STAT3-deficient mice also display an osteoporotic phenotype but due to the augmentation of osteoclast differentiation (17). The latter phenotype, which is reciprocally compatible with the phenotype observed in IL-6 transgenic mice, implies that IL-6/STAT3 plays a negative regulatory role in the differentiation of osteoclast progenitors. The mechanism underlying this negative regulatory role, however, has not yet been clarified, prompting us to reevaluate the in vitro effects of IL-6 on osteoclast progenitors.

We now report that IL-6 acts directly on osteoclast progenitors to suppress their differentiation. Furthermore, we found that the suppressive effect of IL-6 on osteoclastic cells is mediated through a reduction in both JNK activation and IκB degradation. Microarray analyses revealed reduced expression of Senp2 and Cull4A, which are involved in the ubiquitination pathway, and increased expression of MKP1 and MKP7, which encode MAPK phosphatases. We thus demonstrate that IL-6 inhibits osteoclast differentiation by specifically suppressing the RANK signaling pathways.

EXPERIMENTAL PROCEDURES

Osteoclast Cultures—Bone marrow cells obtained from the femurs of ddY mice (4–6-week-old adults) were cultured for 16 h in α-minimal essential medium supplemented with 10% fetal bovine serum and 10 ng/ml M-CSF. Nonadherent hematopoietic cells were harvested and further cultured with 10 ng/ml M-CSF for 2 days. Adherent cells were used as bone marrow macrophages (BMMs) and were incubated for 24 h with 100 ng/ml IL-6 in the presence of 10 ng/ml M-CSF and 100 ng/ml RANKL. After 24 h, the adherent cells were fixed and stained for tartrate-resistant acid phosphatase (TRAP), and the number of TRAP-positive multinucleated cells was counted.

Resorption Pit Assay—BMMs were plated onto dentine slices and cultured with or without 100 ng/ml IL-6 in the presence of 10 ng/ml M-CSF and 100 ng/ml RANKL for 7 or 14 days. The culture medium was replaced with fresh medium containing these reagents every 3 days. At culture termination, the slices were rinsed with phosphate-buffered saline (−) and left overnight in 1 M ammonium hydroxide to remove all attached cells. The slices were then washed in phosphate-buffered saline (−) and stained with Mayer’s hematoxylin (Sigma-Aldrich).

MTT Assay—Cell proliferation was measured using the MTT assay. The BMMs (1 × 10⁶ cells) were cultured in 100 μl of medium containing fetal calf serum and M-CSF (10 ng/ml) with or without IL-6 (100 ng/ml). For the last 4 h of culture, 10 μl of MTT (5 mg/ml diluted in phosphate-buffered saline) was added. Finally, 100 μl of 0.04 N HCl in isopropanol was added and mixed by pipetting, and the absorbance at 570 nm was measured using a plate reader.

Western Blotting—After the osteoclast progenitors were stimulated with IL-6 family cytokines, cell lysates were prepared in lysis buffer (20 mM Tris HCl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 500 μM sodium vanadate, 1 mM dithiothreitol, 5 μg/ml aprotinin, 5 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride). Proteins were diluted with 20 μl of 3× Laemmli SDS loading buffer, separated on a 4–20% gradient polyacrylamide gel (Dai-ichi Kagaku, Tokyo), and transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore, Bedford, MA). The membranes were blocked with TBST (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.1% Tween 20) containing 1% gelatin and incubated with rabbit anti-phospho-p38, anti-phospho-JNK (Cell Signaling, Beverly, MA), anti-p38, anti-JNK, anti-IκBα (Santa Cruz Biotechnology, Santa Cruz, CA), or anti-ubiquitin (Sigma-Aldrich) antibodies for 1 h at room temperature. The membranes were then washed three times with TBST for 10 min each and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG diluted 1:10,000 (Zymed Laboratories Inc., South San Francisco, CA). The membranes were then washed three times with TBST. Immune complexes were visualized using a chemiluminescence system (Renaissance, PerkinElmer Life Sciences).

Molecular Characterization of Osteoclast Maturation Using Reverse Transcription-PCR Analysis—RNA was isolated from BMMs incubated for 24 h with or without 100 ng/ml IL-6 in the presence of 50 ng/ml M-CSF and 100 ng/ml RANKL using Sepasol-RNA I (Nacalai Tesque, Kyoto, Japan), and reverse transcribed. Reverse transcription-PCR analysis was conducted as described previously (11). The primer sequences used were the following: TRAP forward, 5′-AAATCTACCTTCTCAAGACCC-3′; TRAP reverse, 5′-TTATTGAACAGCATGACAG-3′; calcitonin receptor (CTR) forward, 5′-ACCCGGAGAACCGCTAGCC-3′; CTR reverse, 5′-GCCCTACAGCGCTTACGTTAC-3′; HPRT forward, 5′-AGCGATGATGAACCAGGT-3′; and HPRT reverse, 5′-GGCTGTACTCTTAAACCA-3′.

Microarray Analysis—A comparison of GeneChip array data was obtained using the KURABO custom analysis services (KURABO Industries, Osaka, Japan, an authorized service provider for Affymetrix Japan K.K. (Tokyo)). Total RNA was isolated from BMMs incubated for 24 h with or without 100 ng/ml IL-6 in the presence of 10 ng/ml M-CSF and 100 ng/ml RANKL using Sepasol-RNA I. Total RNA was reverse transcribed to cDNA using T7 oligo(dT) primer (Affymetrix). The cDNA products were used in an in vitro transcription reaction containing T7 RNA polymerase and biotinylated nucleotide analogs (pseudouridine base). Next, the labeled cDNA products were fragmented, loaded onto GeneChip(R) Mouse Genome 430 A2.0 arrays (Affymetrix), and hybridized according to the manufacturer’s protocols. Streptavidin-phycocerythrin (Molecular Probes, Eugene, OR) was used as a fluorescent conjugate to detect the hybridized target sequences. Raw intensity data from the GeneChip array were analyzed with GeneChip operating software (Affymetrix).

Real-time PCR—Quantitative real-time PCRs were performed using TaqMan Fast Universal PCR Master Mix (Applied Biosystems, Foster City, CA). TaqMan probes were also purchased from Applied Biosystems. All quantitative reverse transcription-PCRs were performed using an ABI 7500 Fast Real-Time PCR system (Applied Biosystems), and all samples were run in triplicate. The ABI 7500 Fast Sequence detector was programmed with the following PCR amplification con-
ditions: 40 cycles of 95 °C for 3 s and 60 °C for 30 s. All quantitation was normalized to the housekeeping gene HPRT, an endogenous control that accounted for variability in the initial concentrations of RNA and in the conversion efficiency of the reverse transcription reactions. Analysis of the relative quantitation required calculations based on the threshold cycle, i.e. the cycle number at which the amplification plot crosses a fixed threshold above the baseline (Ct). Relative quantitation was performed using the comparative ΔΔCt method according to the manufacturer’s instructions.

RESULTS

IL-6 Has a Suppressive Effect on Osteoclast Differentiation—IL-6 induces the surface expression of RANKL on osteoblasts, which supports osteoclast differentiation via a direct interaction between RANKL and RANK. To examine the direct effect of IL-6 on osteoclasts, a culture system was used in which BMMs differentiate into TRAP-positive multinuclear cells upon stimulation with M-CSF and RANKL. In this culture system, the addition of IL-6 strongly suppressed the differentiation of BMMs, as evaluated by the number of TRAP-positive multinuclear cells (Fig. 1, A–C). To determine the resorption activity of the differentiated osteoclasts, we employed pit assays. Resorption pits were observed on dentin slices when the BMMs were cultured with M-CSF and RANKL (Fig. 1, D and F). When IL-6 was added, however, the resorption activity was almost completely suppressed (Fig. 1, E and G). These results imply that IL-6 has a suppressive effect on the differentiation of BMMs into osteoclasts. Moreover, we confirmed the suppressive effect of IL-6 on osteoclast differentiation by analyzing marker gene expression. BMMs cultured with M-CSF and RANKL showed high expression levels of TRAP mRNA after 24 h and cathepsin K mRNA after 48 h. The expression of CTR mRNA was induced at 24 h, and the level of expression

FIGURE 1. The suppressive effect of IL-6 on osteoclast progenitor cells. A, BMMs were cultured in the presence of M-CSF (10 ng/ml), M-CSF plus sRANKL (100 ng/ml), or M-CSF plus sRANKL plus IL-6 (100 ng/ml). TRAP-positive multinuclear cells were counted, and similar results were obtained in three independent experiments. Data are expressed as means ± S.D. B and C, microphotographs of differentiated TRAP-positive multinuclear cells cultured with M-CSF plus sRANKL (B), or with M-CSF plus sRANKL plus IL-6 (C). D–G, resorption activity of differentiated osteoclasts cultured with M-CSF plus sRANKL (D and F) or with M-CSF plus sRANKL plus IL-6 (E and G) for 7 (D and E) and 14 days (F and G), and similar results were obtained in three independent experiments. Bars = 20 μm (B and C); bars = 100 μm (D–G).

FIGURE 2. Molecular characterization of osteoclast differentiation. BMMs were cultured with M-CSF (10 ng/ml), M-CSF plus sRANKL (100 ng/ml), or M-CSF plus sRANKL plus IL-6 (100 ng/ml). After 24 and 48 h, RNA was extracted and reverse transcribed in three independent experiments. A, cDNA was amplified using primers specific for TRAP, CTR, cathepsin K, and HPRT. B, samples were subjected to quantitative real-time PCRs using probes specific for TRAP, CTR, cathepsin K, and HPRT. The levels of TRAP, cathepsin K, and CTR mRNA expression were normalized to HPRT expression, and data are expressed as means ± S.D.
increased during the subsequent 24 h (Fig. 2A). The addition of IL-6 strongly suppressed the expression of all these marker genes. The suppressive effects of IL-6 on the expression of these genes were confirmed with quantitative real-time PCR analyses (Fig. 2B). IL-6 suppressed the mRNA expression of TRAP, CTR, and cathepsin K by 71% (at 24 h), 99% (at 48 h), and 99% (at 48 h), respectively. On the other hand, IL-6 augmented the proliferation activity of BMMs (Fig. 3). These results clearly demonstrated that IL-6 acted on the BMMs to strongly suppress osteoclast differentiation.

IκB Degradation and JNK Activation Are Specifically Suppressed by IL-6 Stimulation—Because the RANKL-RANK interaction activates downstream signaling via the TRAF6, NF-κB, JNK, and p38 pathways, we first examined the expression of TRAF6. TRAF6 expression levels in BMMs stimulated by RANKL and IL-6 were similar to those in BMMs stimulated by RANKL alone (Fig. 4). We next analyzed the effect of IL-6 on three other downstream signaling pathways. Although RANKL stimulation alone induced IκB degradation, IκB degradation was not observed in BMMs stimulated by both RANKL and IL-6 (Fig. 5). IL-6 also suppressed the tyrosine phosphorylation of JNK but not of p38 (Fig. 5). These results demonstrated that IL-6 specifically suppresses IκB degradation and the activation of JNK.

Comprehensive Detection of Changes in Gene Expression following RANKL Stimulation or Costimulation with RANKL and IL-6—Of the signaling molecules activated by the RANKL-RANK interaction, IL-6 stimulation specifically suppressed JNK activation and IκB degradation (Fig. 5). To clarify the mechanism underlying the suppressive effects of IL-6 on these two signaling pathways, we compared the gene expression profiles of BMMs stimulated by RANKL with those of BMMs costimulated with both RANKL and IL-6. The identified genes were grouped into categories according to their ascribed functions (Table 1), including signal transduction (19 genes), metabolism (22 genes), transcription (25 genes), protein synthesis (8 genes), transport (11 genes), cell cycle (7 genes), development (6 genes), immune responses (9 genes), apoptosis (1 gene), chemotaxis (4 genes), angiogenesis (1 gene), ESTs (70 genes), and total (183 genes).

**TABLE 1**

| Functional Classification of the Genes with Increased or Decreased Expression Levels in Osteoclast Progenitors |
|---------------------------------------------------------------|
| **Increased** | **Decreased** | **Total** |
| Signal transduction | 16 | 3 | 19 |
| Metabolism | 15 | 7 | 22 |
| Transcription | 16 | 9 | 25 |
| Protein synthesis | 5 | 3 | 8 |
| Transport | 5 | 6 | 11 |
| Cell cycle | 2 | 5 | 7 |
| Development | 4 | 2 | 6 |
| Immune responses | 7 | 2 | 9 |
| Apoptosis | 1 | 0 | 1 |
| Chemotaxis | 4 | 0 | 4 |
| Angiogenesis | 1 | 0 | 1 |
| ESTs | 37 | 33 | 70 |
| Total | 113 | 70 | 183 |
**DISCUSSION**

We have demonstrated here that IL-6 directly suppresses the differentiation and facilitates the proliferation of osteoclast progenitors. Thus, we propose that IL-6 provides a direct inhibitory signal, through up-regulation of RANKL in osteoblasts, for the differentiation of osteoclast progenitors depending on the microenvironmental conditions. This implies that, in the steady-state interactions between osteoblasts and osteoclasts in vivo, IL-6 affects only the osteoclast progenitors to suppress their differentiation. When soluble IL-6 receptor is available, IL-6 additionally acts on osteoblasts to induce their differentiation and to facilitate the osteoclast-inducing activity (7, 13, 18). In other words, IL-6 usually suppresses bone resorption by inhibiting the differentiation of osteoclast progenitors without affecting osteoblasts, whereas under specific conditions, such as inflammation, IL-6 provides discordant signals to osteoclasts and osteoblasts (Fig. 7A) (7, 18). Thus, predicting the net effects of IL-6 on bone homeostasis in vivo is difficult (8, 15, 17–20). Effects of IL-6 on bone formation in vivo are determined by the concentrations and combination of IL-6 family cytokines and soluble IL-6 receptor that are present in the areas around the sites of interaction between osteoblasts and osteoclast progenitors (7, 18). It is possible that the simultaneous inhibition of differentiation and stimulation of osteoclast progenitor proliferation results in a temporary suppression of bone resorption that will begin again after proliferation cases. Although all of the analyses described in this report were performed in vitro, we have clearly demonstrated that IL-6 directly suppresses the differentiation of osteoclast progenitors. Our results also suggest that IL-6 plays roles in preventing excessive bone resorption during steady-state bone remodeling (Fig. 7B).

Analyses of RANK signaling in osteoclast progenitors revealed that the suppressive effect of IL-6 on differentiation was caused by the suppression of IκB degradation and JNK activation. GeneChip analysis of osteoclast progenitors stimulated with RANKL in the presence or absence of IL-6 allowed us to identify four candidate genes related to JNK and NF-κB signaling: MKP1 and MKP7 code for MAP kinase phosphatases, whereas Senp2 and Cul4A are related to the ubiquitin pathway (Fig. 7B).

The MAPK phosphatases are a family of dual-specificity protein phosphatases that can dephosphorylate both phosphothreonine and phosphotyrosine residues, which results in the inactivation of MAP kinase (21, 22). This family includes MKP1 and MKP7, which are known to inhibit JNK activity (23, 24). In our real-time PCR analysis, the expression of MKP1 and MKP7 was reduced by RANKL stimulation, suggesting that the reduced levels of MKP1 and MKP7 efficiently facilitated JNK activation. On the other hand, costimulation with IL-6 and RANKL augmented the expression of MKP1 and MKP7. Thus, MKP1 and MKP7 are likely to be involved in the IL-6-mediated suppression of JNK activation (23–25).

On the other hand, the activation of NF-κB is achieved via the ubiquitination and proteasome-mediated degradation of IκB. When IκB is conjugated with the small ubiquitin-like protein SUMO-1, it becomes resistant to signal-induced degradation (26). Senp2, which belongs to the Senp family, contains a
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In the presence of soluble IL-6Rα, IL-6 evokes discordant responses for bone homeostasis by both inducing bone formation and increasing the osteoclast-supporting activity of osteoblasts (solid line). B, a detailed schematic of the effects of IL-6 on osteoclast progenitors. The RANKL-RANK inter-action activates at least three signaling pathways: p38, NF-κB, and JNK. NF-κB activation and IκB degradation are specifically suppressed in osteoclast progenitors stimulated by IL-6. IL-6 also suppresses the expression levels of Senp2 and Cul4A, which encode JNK phosphatases, thus reducing JNK activity. These factors could explain the suppressive effects of IL-6 on osteoclast differentiation.

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Conserved catalytic domain in the C terminus and SUMO protease activity (27). The reduction in the Senp2 transcription levels might induce the stabilization of IκB-NFκB complexes due to excess IκB sumoylation, leading to diminished NFκB signaling and decreased osteoclast differentiation (25). Cul4A, which belongs to the cullin family, is an ubiquitin liga-plex is associated with IκB ubiquitination, the targets of each cullin family member are now being determined.

Taken together, our data indicate the need for further analysis of the IL-6/gp130/STAT3-mediated suppression of the differentiation of osteoclast progenitors and provide clues as to important pathways and mediators for additional study.