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Soluble interleukin-6 receptor triggers osteoclast formation by interleukin 6

(bone resorption/gp130/interleukin-11/oncostatin M/leukemia inhibitory factor)

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ABSTRACT **It has been reported that soluble interleukin (IL)-6 receptor (sIL-6R) is detected in the serum of healthy individuals and its level is increased in patients with multiple myeloma and human immunodeficiency virus infection. Although several reports have suggested that sIL-6R potentiates IL-6 action, its physiological role remains unclear. In this study, we examined the role of sIL-6R on osteoclast formation by IL-6, using a coculture of mouse osteoblasts and bone marrow cells. Neither recombinant mouse IL-6 (mIL-6) nor mouse sIL-6R (smIL-6R) induced osteoclast-like multinucleated cell (MNC) formation when they were added separately. In contrast, simultaneous treatment with mIL-6 and smIL-6R strikingly induced MNC formation. These MNCs satisfied major criteria of authentic osteoclasts, such as tartrate-resistant acid phosphatase (TRAP) activity, calcitonin receptors, and pit formation on dentine slices. The MNC formation induced by mIL-6 and smIL-6R was dose-dependently inhibited by adding monoclonal anti-mouse IL-6R antibody (MR16-1). It is likely that osteoblasts and osteoclast progenitors are capable of transducing a signal from a complex of IL-6 and sIL-6R through gp130, even though they may have no or a very small number of IL-6Rs. Factors such as IL-11, oncostatin M, and leukemia inhibitory factor, which are known to exert their functions through gp130 (the signal-transducing chain of IL-6R), also induced MNC formation in our coculture system. These results suggest that increased circulating or locally produced sIL-6R induces osteoclast formation in the presence of IL-6 mediated by a mechanism involving gp130. This may play an important physiological or pathological role in conditions associated with increased osteoclastic bone resorption.**

Interleukin (IL)-6 is a multifunctional cytokine that regulates pleiotropic functions of cells and tissues (1). Several lines of evidence have suggested that IL-6 is an osteotropic factor as well. Löwik *et al.* (2) and we (3) independently reported that IL-6 stimulated *in vitro* bone resorption in fetal mouse metacarpals and calvaria, respectively. IL-6 is produced by both osteoblastic MC3T3-E1 cells and primary osteoblasts in response to IL-1, tumor necrosis factor α, and lipopolysaccharides (3). Littlewood *et al.* (4) also showed that human osteoblasts produced IL-6 in response to several external stimuli. Besides these *in vitro* studies, IL-6 has been reported to stimulate osteoclast formation and bone resorption *in vivo* as well. Yoneda *et al.* (5) showed that transplantation of human tumor (MH-85) cells into *nude* mice, in which these cells produce a high level of human IL-6, caused hypercalcemia and that administration of neutralizing anti-IL-6 antibody to these tumor-bearing mice lowered the serum calcium to a normal level. Similar hypercalcemic action of IL-6 was reported by Black *et al.* (6), who used Chinese hamster ovarian (CHO) cells transfected with the IL-6 gene. More recently, Jilka *et al.* (7) reported that IL-6 may contribute to bone loss caused by estrogen deficiency. They showed that estrogen inhibited IL-6 production by osteoblasts *in vitro* (8) and that anti-IL-6 antibody prevented osteoclast development caused by estrogen deficiency *in vivo* (7). Taken together, these data suggest that IL-6 plays an important role in stimulating bone resorption in some physiological and/or pathological conditions. However, the mechanism of action of IL-6 responsible for osteoclast recruitment and bone resorption *in vitro* has not been established.

We have developed a coculture system of mouse primary osteoblastic cells and bone marrow cells (or spleen cells) to assess the effects of hormones and cytokines on osteoclast formation (9, 10). In this system, osteoclast-like multinucleated cells (MNCs), which satisfied major criteria of authentic osteoclasts, were formed in response to several osteotropic factors, such as 1α,25-dihydroxyvitamin D_3 [$1\alpha,25(OH)_2D_3$] (9, 10), parathyroid hormone (9, 11) and parathyroid hormone-related peptides (12), prostaglandins (13), and IL-1 (14). To date, however, we have failed to demonstrate stimulative effects of IL-6 on MNC formation in our coculture system. This led us to speculate that increased production of IL-6 may not be enough to induce osteoclast formation.

IL-6 exerts its activity via a cell surface receptor which consists of two components: a ligand-binding 80-kDa glycoprotein chain (IL-6R) and a non-ligand-binding but signal-transducing 130-kDa glycoprotein chain (gp130) (15). When IL-6R is occupied by IL-6, IL-6R is associated with gp130, then mediates the IL-6 functions. More recently, it was reported that the genetically engineered human and mouse soluble IL-6R (sIL-6R), which lacks transmembrane and cytoplasmic regions, could also mediate the IL-6 signal through gp130 (15–18). Native sIL-6R has been detected in the urine (19) and sera of healthy subjects (20), and its level is increased in patients with multiple myeloma (21) and human immunodeficiency virus infection (20). Suzuki *et al.* (22) reported that serum sIL-6R levels in MRL/*lpr* mice, an animal model of autoimmune diseases, were increased with age.

In this study, we examined the role of mouse sIL-6R (smIL-6R) in MNC formation in the presence of mouse IL-6

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Abbreviations: IL, interleukin; IL-6R, IL-6 receptor; sIL-6R, soluble IL-6R; smIL-6R, mouse sIL-6R; mIL-6, mouse IL-6; mIL-6R, mIL-6 receptor; $1\alpha,25(OH)_2D_3$ or $1,25D_3$, 1α,25-dihydroxyvitamin D_3; MNC, multinucleated cell; CT, calcitonin; OSM, oncostatin M; LIF, leukemia inhibitory factor; FBS, fetal bovine serum; TRAP, tartrate-resistant acid phosphatase.

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(mIL-6) in our coculture system of mouse osteoblastic cells and bone marrow cells. The results presented here indicate that smIL-6R triggers MNC formation by mIL-6, suggesting that locally or systemically produced sIL-6R is involved in IL-6-mediated osteoclast recruitment and osteoclastic bone resorption.

MATERIALS AND METHODS

Animals and Drugs. Female Wistar rats and BALB/c *nu/nu* mice were purchased from Charles River Japan (Kanagawa). Newborn ddY mice and 6- to 9-week-old male ddY mice were obtained from Shizuoka Laboratories Animal Center (Shizuoka, Japan). $1\alpha,25(OH)_2D_3$ was purchased from Duphar (Weesp, The Netherlands). Recombinant human interleukin 1α (IL-1) was purchased from Genzyme. Synthetic salmon calcitonin (CT) was purchased from Peninsula Laboratories. Recombinant human IL-11, human oncostatin M (OSM), and human leukemia inhibitory factor (LIF) were purchased from Pepro Tech (Rocky Hill, NJ). Collagen gel solutions (Cellmatrix, type I-A) were obtained from Nitta Gelatin Co. (Osaka). Bacterial collagenase was obtained from Wako Pure Chemical (Osaka). Dentine (ivory) was kindly provided by Nishide Inzai Co. (Tokyo). Other chemicals and reagents used in this study were of analytical grade.

Preparation of Recombinant mIL-6. The mIL-6-producing CHO cells were established by transfection of dihydrofolate reductase-deficient CHO cells with an mIL-6 cDNA expression vector. The mIL-6 cDNA was isolated from P388D1 (IL-1) cells by PCR cloning. A high producing clone was selected by amplifying with 50 nM methotrexate. The established clone was cultured in α minimal essential medium (α-MEM) containing 5% fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 5% CO_2 in air. The culture media were collected and used as mIL-6 preparations in the following studies. Concentrations of mIL-6 in the media were determined by a sandwich ELISA using monoclonal anti-mIL-6 antibody 6B4 (kindly provided by J. Van Snick, Ludwig Institute for Cancer Research, Brussels), and rabbit polyclonal anti-mIL-6 antibody.

Preparation of Recombinant smIL-6R. The smIL-6R-producing CHO cells were prepared as described (17). The cells were cultured in α-MEM containing 5% FBS at 37°C in a humidified atmosphere of 5% CO_2 in air. Conditioned media were collected and used as smIL-6R preparations in the following studies. Concentrations of smIL-6R in the media were determined by a sandwich ELISA using rat monoclonal anti-mIL-6 receptor (mIL-6R) antibody RS15 (17) and rabbit polyclonal anti-mIL-6R antibody.

Preparation of Rat Monoclonal Anti-mIL-6R Antibody MR16-1. smIL-6R was purified from smIL-6R preparations with an affinity column-adsorbed rat monoclonal anti-mIL-6R antibody (RS12) (17). Wistar rats were immunized with 50 μg of purified smIL-6R in Freund's complete adjuvant (s.c.), followed 2 weeks later by four 50-μg boosts of smIL-6R in Freund's incomplete adjuvant once a week (s.c.). One week after the last boost, the rats were injected with 50 μg of smIL-6R in 100 μl of phosphate-buffered saline (PBS) (i.v.). The rats were sacrificed 3 days later, and spleen cells were fused with mouse P3U1 myeloma cells at a ratio of 10:1 with polyethylene glycol 1500 (Boehringer Mannheim). After overnight incubation at 37°C in the wells of a 96-well plate (Falcon 3075) with 100 μl of complete RPMI 1640 medium containing 10% FBS, 100 μl of hypoxanthine/aminopterin/thymidine (HAT) containing human IL-6 (23) was added to each well. Half of the culture medium was replaced with HAT medium daily for 4 days. Seven days later, hybridomas producing anti-smIL-6R were selected by a smIL-6R-binding assay (ELISA). Briefly, culture supernatants of the hybridomas (100 μl) were incubated for 60 min in plates coated with rabbit polyclonal anti-rat IgG antibody (Cappel) at 1 μg/ml. The plates were washed, and incubated with smIL-6R at 100 ng/ml. After washing, rabbit polyclonal anti-mIL-6R antibody was added at 2 μg/ml, and the plates were washed and incubated with alkaline phosphatase-conjugated goat polyclonal anti-rabbit IgG antibody (Tago) for 60 min. Finally, after washing, the plates were incubated with a substrate (Sigma 104; *P*-nitrophenyl phosphate) of alkaline phosphatase for 30 min, and were read by using a plate reader (Tosoh, Tokyo) at 405 nm. Hybridomas recognizing smIL-6R were cloned twice by limiting dilution. For ascites production, BALB/c *nu/nu* mice were injected twice with 0.5 ml of pristane, and 3 days later, 3×10^6 established hybridoma cells were injected (i.p.). Ascites fluid was collected 10–20 days later, and the monoclonal antibody (MR16-1) was purified from the fluid with protein G column (Oncogene Science).

Coculture of Osteoblastic Cells and Bone Marrow Cells. Coculture of osteoblastic cells and bone marrow cells was performed by the method of Akatsu *et al.* (10) using *ddy* mice. In short, primary osteoblast-like cells (1×10^4 per well) obtained from mouse calvaria and nucleated marrow cells (2×10^5 per well) were cocultured in the wells of a 48-well plate (Sumitomo Bakelite, Tokyo) with 0.3 ml of α-MEM containing 10% FBS in the presence of test chemicals. Cultures were performed in quadruplicate and cells were fed on day 4 by replacing 0.25 ml of old medium with fresh medium. To determine bone-resorbing activity of osteoclast-like MNCs formed, we used MNC preparations as described (10). In short, primary osteoblast-like cells (5×10^5 per dish) and nucleated marrow cells (6×10^6 per dish) were cocultured in the presence of mIL-6 at 200 ng/ml and smIL-6R at 500 ng/ml on collagen-gel coated dishes. After culturing for 6–7 days, dishes were treated with 4 ml of 0.2% bacterial collagenase for 20 min. The recovered cell suspensions were gently layered on 35% Percoll solution and centrifuged at $250 \times g$ for 20 min. The cells which accumulated at the interface were collected as MNC preparations, suspended in α-MEM containing 10% FBS, and used for determining the activity of pit formation on dentine slices.

Determination of Osteoclast Characteristics. MNCs were fixed and stained for tartrate-resistant acid phosphatase (TRAP), and the number of TRAP-positive MNCs was counted (10). Pit formation was assessed as described (24). Briefly, MNC preparations were put on dentine slices (diameter 4 mm, $\approx$200 μm thick) in the wells of a 96-well plate (Corning 25820). The slices were then incubated in humidified atmosphere at 37°C in α-MEM containing 10% FBS with or without CT. After they had been cultured for 48 h, resorption pits were visualized with Mayer's hematoxylin staining, and the areas of image of Mayer's hematoxylin were measured with a personal image analysis system (LA-525, PIAS Co., Tokyo) linked to a light microscope. For autoradiography using ^{125}I-labeled salmon CT (^{125}I-CT), cocultures were performed on coverslips placed in 24-well plates. Cultures were then incubated with 2×10^{-10} M ^{125}I-CT, stained for TRAP, and processed for autoradiography as described (10).

Statistical Analysis. Data are expressed as mean ± SEM. The statistical significance of the differences between the control and the experimental group was determined by Student's *t* test.

RESULTS

MNC Formation by mIL-6 and smIL-6R. Fig. 1 shows the effects of mIL-6 and smIL-6R on MNC formation when they were separately applied to the cocultures. $1\alpha,25(OH)_2D_3$ (10^{-8} M) was used as a positive control stimulator of MNC formation. Neither mIL-6 (0.2–200 ng/ml) nor smIL-6R (0.5–50 ng/ml) stimulated MNC formation. smIL-6R at 500

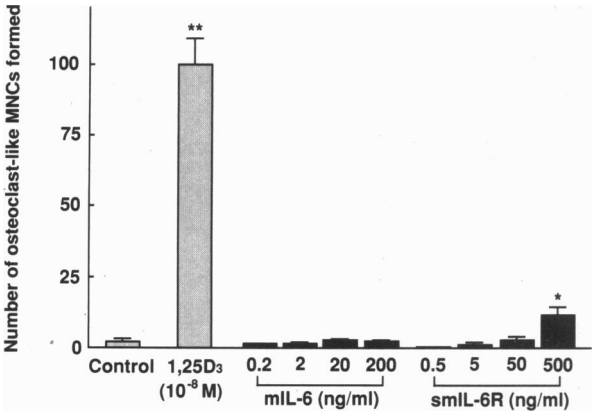

FIG. 1. Effects of mIL-6 or smIL-6R alone on the MNC formation in cocultures of osteoblastic cells and bone marrow cells. Mouse primary osteoblastic cells and bone marrow cells were cocultured with graded concentrations of either mIL-6 or smIL-6R. $1\alpha,25(OH)_2D_3$ ($1,25D_3$, 10^{-8} M) was used as a positive control stimulator of MNC formation. After culture for 7 days, TRAP-positive MNCs formed were counted. Data are expressed as the mean ± SEM of quadruplicate cultures. Significantly different from the control cultures: *, $P < 0.05$; **, $P < 0.01$.

ng/ml induced a small but significant increase in MNC formation. To investigate the possibility that smIL-6R potentiates the mIL-6 action on MNC formation, mIL-6 and smIL-6R were simultaneously added to the cocultures (Fig. 2). mIL-6 and smIL-6R cooperatively stimulated MNC formation, and the potency was increased dose dependently. Minimum concentrations required to induce significant MNC formation were 2 ng/ml of mIL-6 and 62.5 ng/ml of smIL-6R (Fig. 2). These results suggest that coexistence of smIL-6R and mIL-6 is essential for stimulating the MNC formation by IL-6. Since sIL-6R alone fails to transduce the IL-6 signal (15), the MNC formation induced by the maximum dose (500 ng/ml) of smIL-6R alone (Fig. 1) appears to result from the presence of endogenous mIL-6 produced by osteoblastic cells in our cocultures (3).

Effects of Monoclonal Anti-mIL-6R Antibody on MNC Formation. To verify that the MNC formation shown in Fig. 2 was due to the formation of a complex of mIL-6 and smIL-6R, we examined the effects of rat monoclonal anti-mIL-6R antibody, MR16-1, on MNC formation. Adding

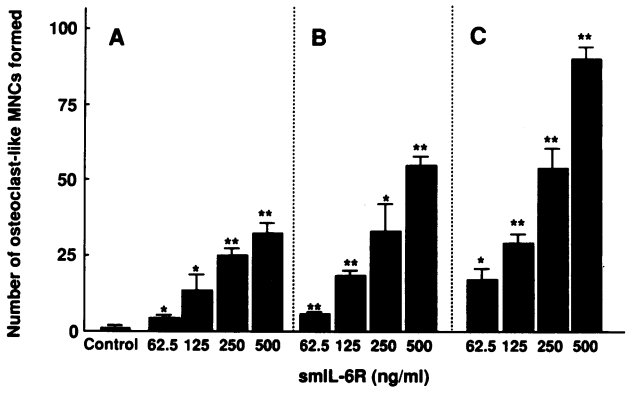

FIG. 2. Cooperative effects of mIL-6 and smIL-6R on the MNC formation in cocultures of osteoblastic cells and bone marrow cells. Mouse primary osteoblastic cells and bone marrow cells were cocultured with mIL-6 at 2 ng/ml (*A*), 20 ng/ml (*B*), or 200 ng/ml (*C*) in the presence of graded concentrations of smIL-6R. After being cultured for 7 days, TRAP-positive MNCs formed were counted. Data are expressed as the mean ± SEM of quadruplicate cultures. Significantly different from the control cultures: *, $P < 0.05$; **, $P < 0.01$.

graded concentrations of MR16-1 dose-dependently decreased MNC formation induced by mIL-6 (20 ng/ml) and smIL-6R (500 ng/ml) (Fig. 3*A*). In contrast, as shown in Fig. 3*B*, MR16-1 at 100 ng/ml had no effect on MNC formation induced by $1\alpha,25(OH)_2D_3$ or IL-1. Control IgG had no inhibitory effect on MNC formation induced by mIL-6 and smIL-6R (data not shown).

Determination of Osteoclast Characteristics of MNCs. To determine the ability to form resorption pits, MNC preparations induced by mIL-6 and smIL-6R were isolated from collagen-gel-coated dishes (10) and cultured for 48 h on dentine slices. MNCs formed many resorption pits on dentine slices (Fig. 4*A*). The resorbing activity of the MNCs expressed by the plan area resorbed per MNC was 4600 ± 400 μm^2, which was comparable to that of $1\alpha,25(OH)_2D_3$-induced MNCs (4100 ± 400 μm^2) (data not shown). When 10^{-11} M salmon CT was added, the ability to form resorption pits of MNCs induced by mIL-6 and smIL-6R was completely inhibited (Fig. 4*B*). Autoradiographic studies using ^{125}I-CT indicated that numerous dense grains due to the ^{125}I-CT binding appeared on more than 95% of the TRAP-positive MNCs (Fig. 4*C*).

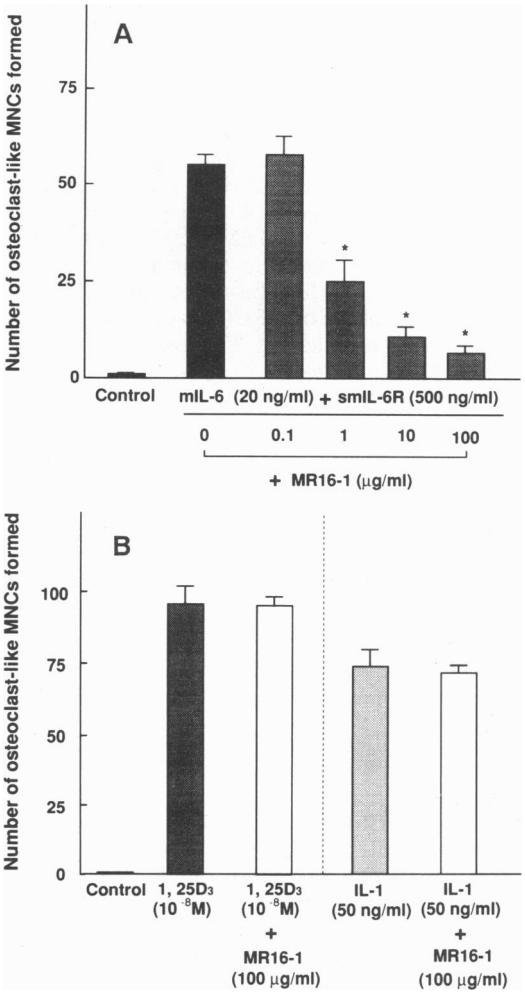

FIG. 3. Effect of monoclonal anti-mIL-6R antibody on MNC formation. Mouse primary osteoblastic cells and bone marrow cells were cocultured with mIL-6 (20 ng/ml) and smIL-6R (500 ng/ml) (*A*), or $1\alpha,25(OH)_2D_3$ (10^{-8} M) or IL-1 (50 ng/ml) (*B*), both with or without rat monoclonal anti-mIL-6R antibody, MR16-1. Graded concentrations (*A*) or 100 μg/ml (*B*) of MR16-1 was added at the beginning of the cocultures. After culture for 7 days, TRAP-positive MNCs formed were counted. Data are expressed as the mean ± SEM of quadruplicate cultures. Significantly different from the cultures treated with mIL-6 and smIL-6R alone: *, $P < 0.01$.

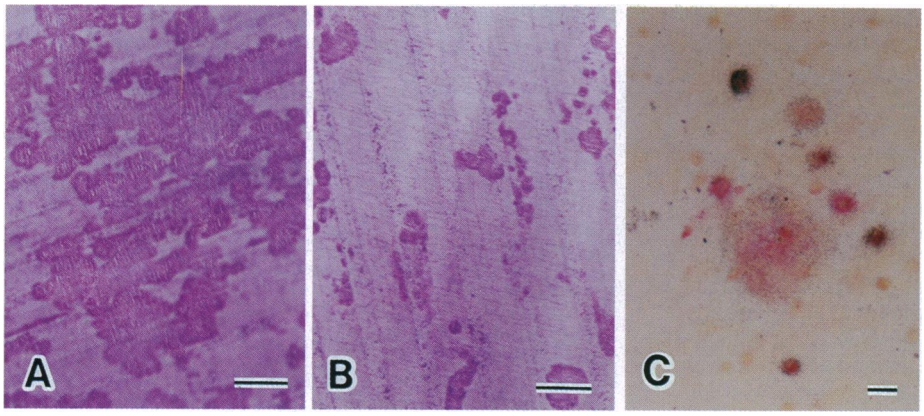

FIG. 4. Osteoclast characteristics of MNCs induced by mIL-6 and smIL-6R. Pit formation on dentine slices by MNCs was examined in the absence (*A*) or presence (*B*) of salmon CT (10^{-11} M) at 37°C for 48 h. Resorption pits were stained with Mayer's hematoxylin and observed under a light microscope. Localization of TRAP activity and binding of ^{125}I-labeled salmon CT in MNCs were assessed as described in the text (*C*). (Bars = 100 μm.)

Effects of Cytokines Which Utilize gp130 as a Common Signal Transducer on MNC Formation. Recent studies have revealed that not only IL-6 but also several cytokines transduce their signals through gp130 (25, 26). To confirm that the signal mediated by gp130 is involved in MNC formation, we examined the effects of IL-11, OSM, and LIF, all of which transduce their signals through gp130, on MNC formation. All the factors tested induced MNC formation dose dependently (Fig. 5). The potency of these factors in inducing MNCs was the highest in OSM, followed by IL-11 and LIF, in that order.

DISCUSSION

The present study clearly demonstrates that smIL-6R triggers formation of osteoclast-like MNCs in the presence of mIL-6, but mIL-6 alone does not. MNCs induced by mIL-6 and smIL-6R satisfied major criteria of authentic osteoclasts, such as TRAP activity, functional CT receptors, and pit formation on dentine slices (Fig. 4). This indicates that the MNCs induced by mIL-6 and smIL-6R are indeed genuine osteoclasts. It has been reported that sIL-6R potentiates agonistic effects in the presence of IL-6 (15–18, 21, 22). However, most of the results so far reported have been obtained by using IL-6-dependent cell lines or IL-6R or gp130 gene-transfected cells. We now add MNCs formed in the cocultures of osteoblastic cells and bone marrow cells to the list of cells which require sIL-6R in the action of IL-6. mIL-6 induced MNC formation in the presence of smIL-6R, but either of the two alone did not. Therefore, our coculture system appears to provide useful information to elucidate the role of sIL-6R.

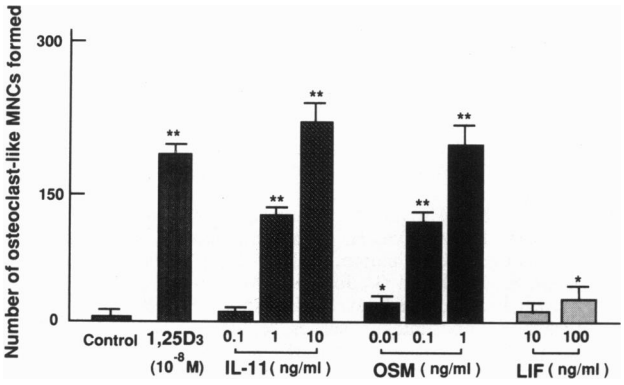

FIG. 5. Effects of several cytokines that utilize gp130 as a common signal transducer on MNC formation. Mouse primary osteoblastic cells and bone marrow cells were cocultured with graded concentrations of IL-11 (0.1–10 ng/ml), OSM (0.01–10 ng/ml), or LIF (10 and 100 ng/ml). 1α,25(OH)$_2$D$_3$ (10^{-8} M) was used as a positive control stimulator of MNC formation. After culture for 7 days, TRAP-positive MNCs formed were counted. Data are expressed as the mean ± SEM of quadruplicate cultures. Significantly different from the control cultures: *, $P < 0.05$; **, $P < 0.01$.

There is very little information on the distribution of membrane-bound IL-6R on osteoblasts and bone marrow cells. Littlewood *et al.* (4) reported that IL-6R mRNA was detected in human osteoblast-like cells, but IL-6 showed no effects on these cells. Both their report and the present study suggest that osteoblastic cells and/or osteoclast progenitor cells present in bone marrow cells have no or a very small number of functional IL-6Rs in physiological conditions. Since the gp130 gene has been reported to be expressed ubiquitously (1), these cells may interact with a complex of mIL-6 and smIL-6R and mediate the IL-6 signal via gp130 even in the absence of IL-6R. Recent studies have revealed that several cytokines mediate their respective signals via gp130 as IL-6 does (25, 26). Passeri *et al.* (27) have reported that IL-11, which also transduces its signal through gp130 (26), stimulates osteoclast formation and osteoclastic bone resorption *in vitro*. In the present study, we examined the effects of three cytokines on MNC formation, OSM, IL-11, and LIF, all of which utilize gp130 as a signal transducer (25, 26). All the cytokines induced MNC formation (Fig. 5). Of the three cytokines tested, OSM and IL-11 were potent MNC inducers. LIF also induced MNC formation, but its potency was much less than that of OSM and IL-11. These cytokines exhibit redundancy (overlapping functions), but each factor also possesses its own specific activities (25). This may be explained by the specific distribution of their respective receptors in the target cells (25). Therefore, the potency of these cytokines to induce MNCs may reflect the receptor number of each cytokine in osteoblastic cells and/or bone marrow cells.

On the basis of these results, gp130 appears to be involved in MNC formation induced by IL-6, OSM, IL-11, and LIF. We have reported that MNC formation is induced by at least two different mechanisms; one is parathyroid hormone-, IL-1-, and prostaglandin E$_2$-induced MNC formation, which is mediated by a mechanism involving cAMP production, and the other is 1α,25(OH)$_2$D$_3$-induced MNC formation, the mechanism of which appears to be independent of cAMP production (28). IL-1 induces MNC formation by a mechanism involving prostaglandin production (14). In both mechanisms, osteotropic factors appear to act directly on osteoblastic cells, which in turn produce a factor responsible for osteoclast differentiation (28). Such a factor appears to be expressed on the cell surface membranes and plays a key role through a cell-to-cell contact mechanism (28). In the present study, we showed that anti-mIL-6R antibody inhibited mIL-6- and smIL-6R-induced MNC formation but not IL-1- or 1α,25(OH)$_2$D$_3$-induced MNC formation (Fig. 3). Therefore, IL-6 production does not appear to be involved in the MNC formation by IL-1 or 1α,25(OH)$_2$D$_3$-mediated signals,

respectively. A preliminary experiment showed that, when osteoblastic cells and spleen cells (osteoclast progenitor cells) were cocultured without direct contact, no MNCs were formed in response to smIL-6R and mIL-6, IL-11, or OSM (data not shown). This indicates that a microenvironment provided by osteoblastic cells or cell-to-cell contact between osteoblastic cells and osteoclast progenitor cells is indispensable for gp130-mediated MNC formation. Further studies are necessary to clarify how the gp130-mediated signal stimulates osteoclast formation.

Recent studies have revealed that naturally produced sIL-6R occurs in the urine (19) and sera (20) of healthy subjects, and that its serum levels are increased in patients with multiple myeloma (21), human immunodeficiency virus infection (20). Furthermore, excess IL-6 is produced systemically and locally in patients with multiple myeloma (29, 30). Both IL-6 and sIL-6R levels were also significantly elevated in synovial fluids from patients with rheumatoid arthritis (31, 36). The results of the present study taken together with these results suggest that the elevated levels of both IL-6 and sIL-6R form a complex, which is involved in bone destruction and induces hypercalcemia in these patients. Recently Jilka *et al.* (7) have proposed that IL-6 is responsible for osteoclast recruitment caused by estrogen deficiency. They reported that the increase in osteoclast number after ovariectomy was prevented by the treatment with a neutralizing anti-IL-6 antibody *in vivo* and in *ex vivo* cultures (7). Interestingly, anti-IL-6 antibody had no inhibitory effects on osteoclastogenesis in sham-operated animals (7). Costatini *et al.* (32) reported that IL-6 gene knock-out mice did not lose bone after ovariectomy. This confirms the previous report that IL-6 is involved in bone resorption in estrogen deficiency. We also speculate that estrogen deficiency may cause excess production of not only IL-6 but also (s)IL-6R in bone cells. Further studies are needed to prove these hypotheses.

There have been many reports which suggest that IL-6 plays an important role in osteoclastic bone resorption *in vitro* as well as *in vivo* (33). However, several conflicting results have been reported. Al-Humidan *et al.* (34) and Barton and Mayer (35) failed to demonstrate bone-resorbing activity of IL-6 on neonatal calvariae *in vitro*. Al-Humidan *et al.* (34) also showed that IL-6 might act as a local inhibitor of bone resorption. In addition, Löwik *et al.* (2) reported that IL-6 stimulated bone resorption in metacarpals but not in fetal long bones in organ cultures. The discrepancy may be explained by the differences in experimental conditions or cellular composition of bone tissues used, or species differences, but a clear explanation remains to be found. Our results raise the possibility that the number of IL-6Rs on the cell membrane or the concentration of sIL-6R in assay systems may be important factors controlling IL-6 response in target tissues in physiological and/or pathological conditions.

In conclusion, sIL-6R triggers osteoclast formation in the presence of IL-6. Excess production of IL-6 and (s)IL-6R may contribute to osteoclastic bone resorption in several metabolic bone diseases, such as multiple myeloma, rheumatoid arthritis, and postmenopausal osteoporosis. If this hypothesis is correct, anti-IL-6R antibody will be useful as an inhibitor of sIL-6R and IL-6R for the treatment of metabolic bone diseases.

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