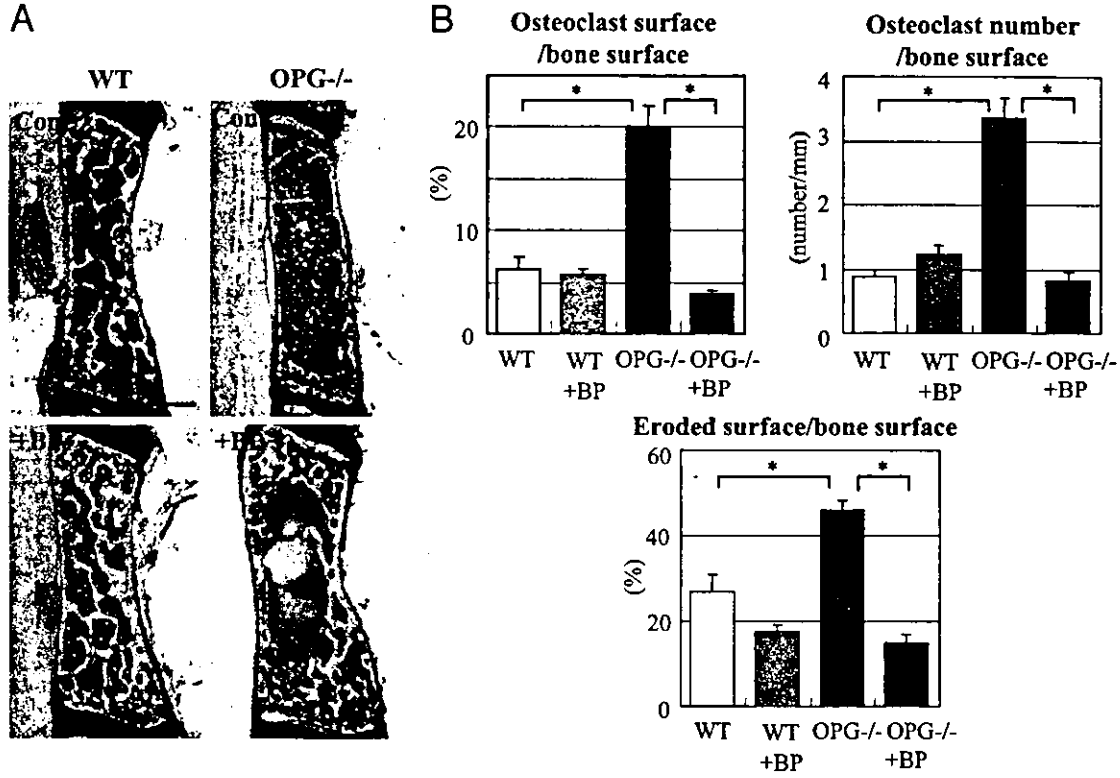

FIG. 2. Effects of risedronate treatment on bone resorption in vertebrae of OPG$^{-/-}$ and WT mice. OPG$^{-/-}$ and WT mice were injected daily with (+BP) or without (Cont) risedronate (0.01 mg/kg body weight·d) for 30 d. Mice were given interval doses of tetracycline and calceine on d 26 and 28, respectively. Vertebrae were removed on d 30 for histological and histomorphometric analyses. A, Vertical sections of vertebrae. *Bar*, 500 μm. B, Bone resorption-related parameters in bone histomorphometric analysis of vertebrae. Data are expressed as the mean ± SEM of four to six animals. Statistical significance was analyzed by *t* test: *, $P < 0.0001$.

Ectopic bone formation

We finally examined whether ectopic bone formation induced by rhBMP-2 is stimulated in OPG$^{-/-}$ mice (Fig. 6A). rhBMP-2-containing collagen disks were implanted into the left dorsal muscular pouches of OPG$^{-/-}$ and WT mice and then recovered after 3, 6, 9, and 12 wk. Soft x-ray images reveal that in collagen disks recovered after 3 wk the areas of calcified mass in the OPG$^{-/-}$ mice were similar in size to those in WT mice, whereas in disks recovered at 6 wk the areas were apparently less in the OPG$^{-/-}$ mice than in the WT mice (Fig. 6A). BMD of the implants recovered after 3 wk from OPG$^{-/-}$ mice was similar to that from WT mice, but BMD after 6 wk was significantly lower in OPG$^{-/-}$ mice than in WT mice (Fig. 6B). There was no significant difference in bone mineral content between OPG$^{-/-}$ and WT mice at 3 wk (data not shown). In addition, histological examination revealed that rhBMP-2 similarly stimulated trabecular bone formation in OPG$^{-/-}$ and WT mice at 3 wk (data not shown). These results suggest that ectopic bone formation induced by rhBMP-2 was not accelerated even with a high turnover rate of bone in OPG$^{-/-}$ mice.

Discussion

Although bone formation is thought to be coupled with bone resorption, the characteristics of the coupling phenomena have not been determined systematically *in vivo* and *in vitro* (1). OPG$^{-/-}$ mice exhibited a high turnover rate of bone (6, 7). Recent studies on juvenile Paget's disease revealed that homozygous deletion of the gene encoding OPG results in Paget's disease with rapidly remodeling woven bone (8). It was also shown that autosomal recessive idiopathic hyperphosphatasia with increased bone formation arises from inactivation of OPG protein (10). These results suggest that loss of OPG function induces activation of osteoblastic bone formation through unknown mechanisms. Is stimulation of osteoclastic bone resorption responsible for the activation of osteoblasts in the loss of OPG function? To answer this question, we studied a possible coupling between bone formation and bone resorption in OPG$^{-/-}$ mice.

Our histomorphometric analysis clearly showed that the elevated osteoblast function in OPG$^{-/-}$ mice was sharply decreased after the suppression of bone resorption by daily injection of risedronate. OPG$^{-/-}$ mice also showed high serum levels of ALP activity and osteocalcin, both of which were decreased to levels lower than those in WT mice by bisphosphonate administration. Thus, the osteocalcin concentration as well as ALP activity in serum sharply reflected the status of osteoblast function. Treatment of primary osteoblasts with risedronate at 10^{-7} M showed no inhibitory effect on ALP activity (data not shown), whereas the same concentration of risedronate significantly inhibited pit-forming activity of osteoclasts placed on dentine slices (24). Neither RANKL (1–300 ng/ml) nor OPG (1–1000 ng/ml) showed inhibitory or stimulatory effects on the proliferation

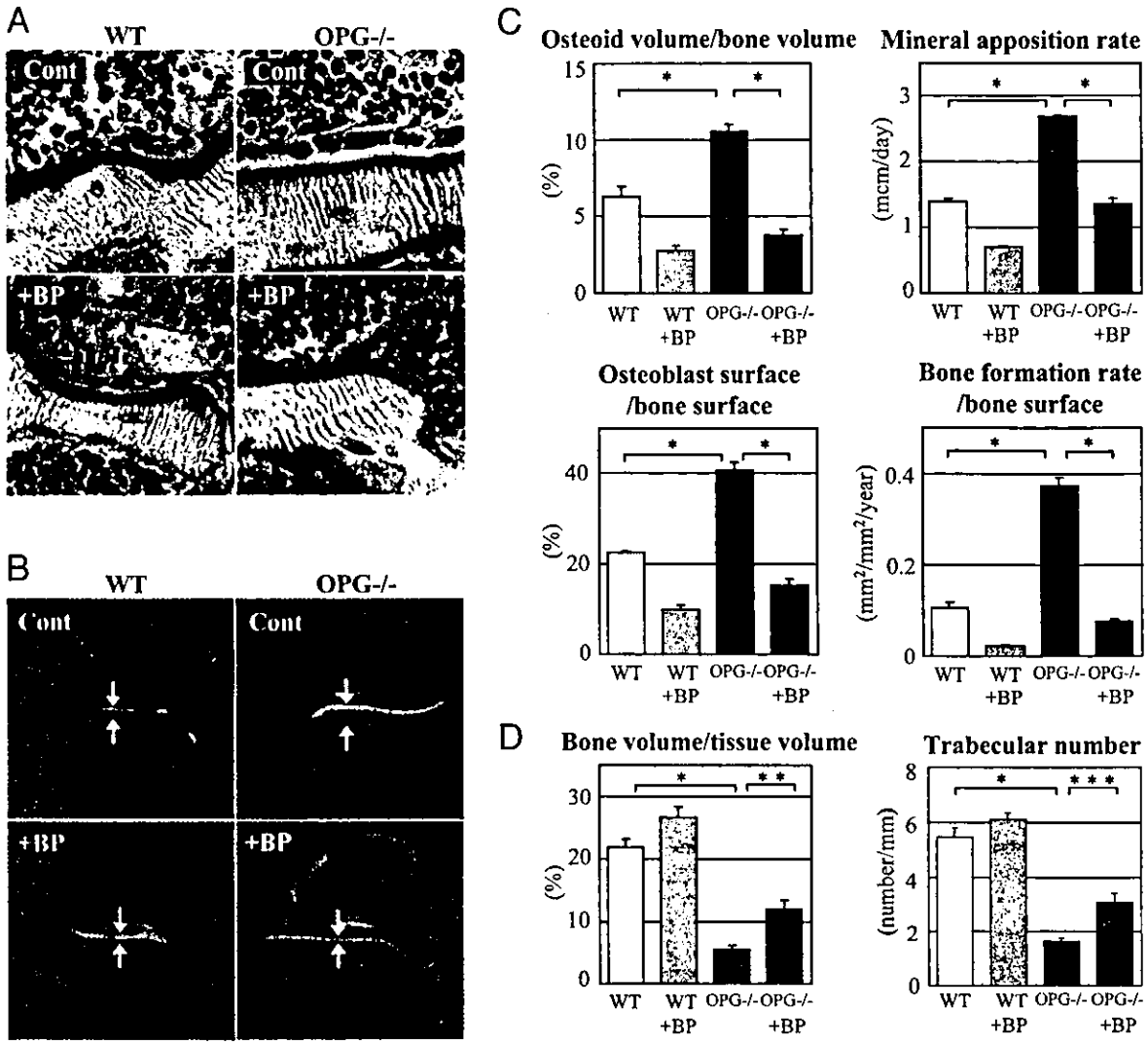

FIG. 3. Effects of risedronate treatment on bone formation in vertebrae of $OPG^{-/-}$ and WT mice. $OPG^{-/-}$ and WT mice were injected daily with (+BP) or without (Cont) risedronate (0.01 mg/kg body weight·d) for 30 d. Mice were given interval doses of tetracycline and calceine on d 23 and 28, respectively. Vertebrae were removed on d 30 for histological and histomorphometric analyses. A, Histology of trabecular bone in vertebrae. *Arrows* indicate osteoblasts along the bone surface. Osteoblasts of $OPG^{-/-}$ mice not treated with risedronate appear more cuboidal in shape than those of WT mice or those of $OPG^{-/-}$ mice treated with risedronate. *Bar*, 25 μm. B, Fluorescent micrographs show double-labeled mineralization in the fronts of trabecular bone in vertebrae. *Bar*, 100 μm. C, Bone formation-related parameters in bone histomorphometric analysis of vertebrae. Data are expressed as the mean ± SEM of four to six animals. Statistical significance was analyzed by *t* test: *, $P < 0.0001$; **, $P < 0.002$; ***, $P < 0.0005$ (WT *vs.* $OPG^{-/-}$ mice).

and ALP activity in the culture of primary osteoblasts derived from $OPG^{-/-}$ mice and WT mice (data not shown). These results suggest that bone formation is tightly coupled with bone resorption in $OPG^{-/-}$ mice.

To date, many studies have shown that induction of bone resorption by bone-resorbing factors (*e.g.* PTH, 1,25-dihydroxyvitamin D_3, and prostaglandin E_2) resulted in the stimulation of bone formation (25–27). However, these factors act on osteoblasts to induce RANKL expression (5). Therefore, it is difficult to determine whether the bone formation induced by bone-resorbing factors is the result of bone resorption or is a phenomenon independent of bone resorption. Our results showed that inhibition of osteoclast activity in $OPG^{-/-}$ mice by a bisphosphonate results in suppression of osteoblast function without any change in the RANKL-RANK interaction. High serum concentrations of RANKL in $OPG^{-/-}$ mice remained unchanged even after suppression of bone resorption by bisphosphonate. These results further support the hypothesis that osteoclastic bone resorption directly activates osteoblast function.

Clarifying the mechanism of coupling between bone resorption and formation is important for understanding the regulation of bone metabolism. Activated osteoblasts with cuboidal shape were often observed near sites where osteoclasts were actively resorbing bones. Such osteoblasts were observed not only in trabecular bones, but also in cortical bones in $OPG^{-/-}$ mice. Wide double lines of calceine and tetracycline, which indicate accelerated bone formation, were localized at sites where osteoclastic bone resorption appeared to have taken place. These results suggest that a

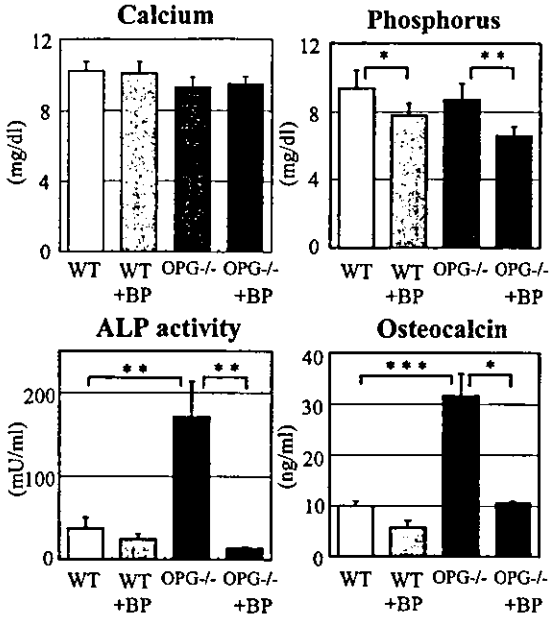

FIG. 4. Serum concentrations of calcium, phosphorus, and osteocalcin and serum ALP activity in $OPG^{-/-}$ and WT mice. $OPG^{-/-}$ and WT mice were injected daily with (+BP) or without risedronate (0.01 mg/kg body weight·d) for 30 d. Serum was collected on d 30 for determination of calcium, phosphorus, and osteocalcin concentrations and ALP activity. Data are expressed as the mean ± SEM of four to six animals. Statistical significance was analyzed by *t* test: *, $P < 0.05$; *, $P < 0.005$; ***, $P < 0.01$.

coupling factor is released during osteoclastic bone resorption as a local factor. In our preliminary experiments osteoclasts produced a factor that induced differentiation of immature mesenchymal cells into ALP-expressing cells (28). However, the width of the double labeling in the periosteal surface of cortical bones was also increased in $OPG^{-/-}$ mice. Bone resorption in the periosteal surface was not as clearly evident as resorption in the endosteal surface. Osteocyte-mediated signals that relate to bone strain induced by bone resorption may stimulate osteoblast function (29). This would explain the increase in periosteal bone formation when bone resorption was not increased.

Although increases in the width of the double labeling were observed in the periosteal surface of cortical bones in $OPG^{-/-}$ mice, cortical bone parameters (cortical area and cortical thickness) were not significantly increased in the mutant mice (data not shown). These results suggest that turnover in cortical bone and that in trabecular bone in growing mice are regulated differently. The coupling itself or the coupling factor might not be directly involved in the determination of the size of cortical bone. However, it should be noted that enlarged porous areas of the cortical bone were observed only in $OPG^{-/-}$ mice, and risedronate treatment significantly reduced the porous areas. These results suggest that OPG plays an important role in turnover in cortical bone.

Whyte *et al.* (8) reported that serum levels of RANKL were markedly elevated in a patient with juvenile Paget's disease. We confirmed that serum RANKL was similarly elevated in $OPG^{-/-}$ mice. Risedronate treatment of $OPG^{-/-}$, $OPG^{+/-}$, and WT mice showed no effect on the circulating levels of RANKL and OPG in both types of mice. We examined

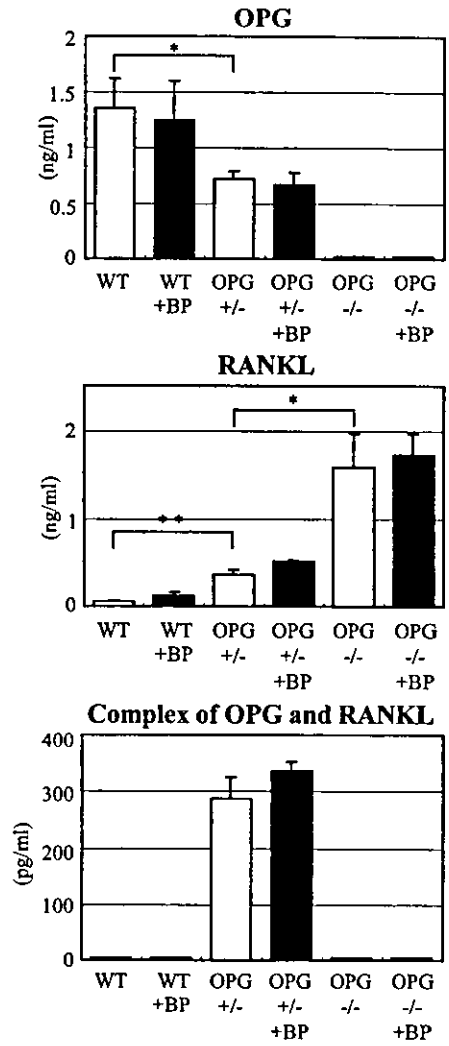

FIG. 5. Serum concentrations of OPG, RANKL, and complex of OPG and RANKL in $OPG^{-/-}$, $OPG^{+/-}$, and WT mice. $OPG^{-/-}$, $OPG^{+/-}$, and WT mice were injected daily with (+BP) or without risedronate (0.01 mg/kg body weight·d) for 30 d. Serum was collected on d 30 for determination of OPG, RANKL, and OPG-RANKL complex concentrations. Data are expressed as the mean ± SEM of four to six animals. Statistical significance was analyzed by *t* test: *, $P < 0.05$; **, $P < 0.005$. No significant differences were found between the value of control mice and that of mice treated with risedronate (+BP).

RANKL mRNA expression in tibiae obtained from $OPG^{-/-}$ and WT mice using RT-PCR techniques. The level of tibial RANKL mRNA expression in $OPG^{-/-}$ mice was similar to that in WT mice (data not shown). This result was consistent with our previous finding that calvarial osteoblasts prepared from $OPG^{-/-}$, $OPG^{+/-}$, and WT mice constitutively expressed similar levels of RANKL mRNA, which were similarly elevated by the treatment with 1,25-dihydroxyvitamin D_3 (30). We have also shown that RANKL expressed by OPG-deficient osteoblasts functions as a membrane- or matrix-associated form (30). These results suggest that OPG deficiency does not affect local expression of RANKL mRNA, and the serum concentration of RANKL is tightly regulated by circulating OPG at the posttranslational level. RANKL expressed by osteoblasts as a membrane- or matrix-associated form appears to play essential roles in increased oste-

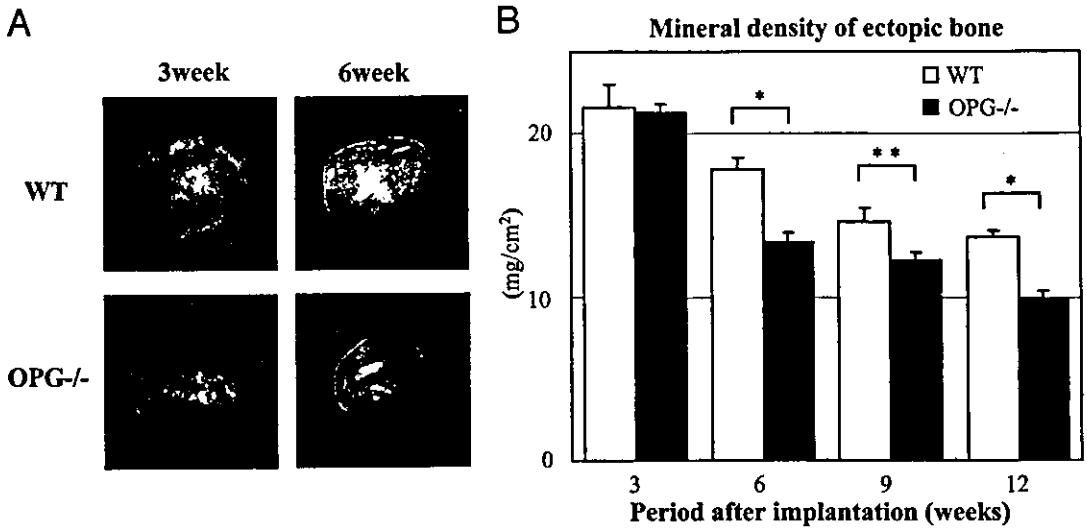

FIG. 6. Ectopic bone formation induced by BMP-2 in $OPG^{-/-}$ mice and WT mice. Ectopic bone formation was induced by implantation of collagen disks impregnated with BMP-2 in $OPG^{-/-}$ mice and WT mice. After implantation for 3, 6, 9, and 12 wk, the implants were recovered, and the BMD of the implants was determined as described in *Materials and Methods*. A, Soft x-ray images of ossicle formed after 3 wk of implantation. B, BMD of the implants recovered after 3, 6, 9, and 12 wk. Data are expressed as the mean ± SEM of four to six animals. Statistical significance was analyzed by *t* test: *, $P < 0.005$; **, $P < 0.05$.

oclast differentiation and function in $OPG^{-/-}$ mice. However, the possibility that circulating RANKL as well as locally expressed RANKL have important roles in OPG deficiency-induced bone resorption cannot be excluded.

Lam *et al.* (11) reported that RANKL increased anabolic bone formation *in vivo* when administered as an amino-terminal glutathione-*S*-transferase fusion protein into mice. However, our results suggest that circulating RANKL does not reflect the status of bone formation in $OPG^{-/-}$ mice. It is therefore unlikely that circulating soluble RANKL is a coupling factor transmitting bone resorption and bone formation in $OPG^{-/-}$ mice. Serum levels of RANKL and OPG in $OPG^{+/-}$ mice were intermediate between those of $OPG^{-/-}$ and WT mice. Interestingly, the complex of OPG and RANKL was detected only in the serum of $OPG^{+/-}$ mice. Serum concentrations of RANKL in $OPG^{+/-}$ mice were similar to those of the RANKL-OPG complex, suggesting that most of RANKL detected in the serum of $OPG^{+/-}$ mice forms the complex with OPG. Although several reports showed that T cells release RANKL as the soluble form (31–33), the origin of serum RANKL remains unknown. The mechanism of action of OPG in the release of RANKL and the origin of soluble RANKL in $OPG^{-/-}$ mice are currently under investigation in our laboratories.

To determine whether the coupling factor is a systemic factor, we examined ectopic bone formation induced by implantation of BMP-2 into $OPG^{-/-}$ and WT mice. BMD of ectopic bone evaluated 3 wk after the implantation showed no significant difference between $OPG^{-/-}$ and WT mice. Thus, BMP-induced ectopic bone formation in $OPG^{-/-}$ mice was not accelerated even in the high turnover state of bone, suggesting that the coupling factor is a local factor. Attenuation of minerals from ectopic bones 6 wk after implantation was clearly more pronounced in $OPG^{-/-}$ mice than in WT mice. The number of osteoclasts appearing in the ectopic bones was higher in $OPG^{-/-}$ mice than in WT mice (data not shown). Thus, the increase in osteoclastic bone resorption in BMP-induced ectopic bone may have masked systemic and anabolic signals for bone formation in $OPG^{-/-}$ mice. Further experiments are needed to determine the role of osteoclastic bone resorption in ectopic bone formation.

Both bone resorption and formation were extremely enhanced in $OPG^{-/-}$ mice, but bone volume in these mice was gradually decreased after birth. This suggests that bone resorption induced by OPG deficiency exceeds bone formation induced by the coupling mechanism. The decrease in bone volume even in the high turnover state of bone is also observed in postmenopausal osteoporosis. Recently, Kawano *et al.* (34) reported that androgen receptor-deficient male mice exhibited high bone turnover with increased bone resorption and formation, but their trabecular and cortical bone masses were significantly reduced. They also found that deficiency in androgen receptors enhanced RANKL expression in osteoblasts that resulted in the stimulation of osteoclastogenesis (34). These results suggest that bone loss is induced even in the high turnover state of bone if bone resorption exceeds bone formation in some bone deceases.

In conclusion, bone formation was tightly coupled with bone resorption in $OPG^{-/-}$ mice. BMP-induced ectopic bone formation was not accelerated even at the high turnover state of bone in $OPG^{-/-}$ mice. Bisphosphonates appear to be first choice medicines for the treatment of diseases that have a high turnover rate of bone, such as Paget's disease (12). Moreover, our results support the usefulness of bisphosphonate treatment in children with OPG deficiency-related idiopathic hyperphosphatasia and suggest that a coupling factor transmitted from bone resorption to bone formation exists at the local sites of bone. Further studies are necessary to elucidate the characteristics of this coupling factor. Such an approach will provide valuable information for the treatment of metabolic bone diseases such as osteoporosis and rheumatoid arthritis.

Acknowledgments

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The Journal of Immunology

Suppression of Osteoprotegerin Expression by Prostaglandin E_2 Is Crucially Involved in Lipopolysaccharide-Induced Osteoclast Formation[1]

Koji Suda,*† Nobuyuki Udagawa,‡ Nobuaki Sato,‡ Masamichi Takami,† Kanami Itoh,† Je-Tae Woo,¶ Naoyuki Takahashi,[2§] and Kazuo Nagai¶

Lipopolysaccharide is a potent stimulator of bone resorption in inflammatory diseases. The mechanism by which LPS induces osteoclastogenesis was studied in cocultures of mouse osteoblasts and bone marrow cells. LPS stimulated osteoclast formation and PGE_2 production in cocultures of mouse osteoblasts and bone marrow cells, and the stimulation was completely inhibited by NS398, a cyclooxygenase-2 inhibitor. Osteoblasts, but not bone marrow cells, produced PGE_2 in response to LPS. LPS-induced osteoclast formation was also inhibited by osteoprotegerin (OPG), a decoy receptor of receptor activator of NF-κB ligand (RANKL), but not by anti-mouse TNFR1 Ab or IL-1 receptor antagonist. LPS induced both stimulation of RANKL mRNA expression and inhibition of OPG mRNA expression in osteoblasts. NS398 blocked LPS-induced down-regulation of OPG mRNA expression, but not LPS-induced up-regulation of RANKL mRNA expression, suggesting that down-regulation of OPG expression by PGE_2 is involved in LPS-induced osteoclast formation in the cocultures. NS398 failed to inhibit LPS-induced osteoclastogenesis in cocultures containing OPG knockout mouse-derived osteoblasts. IL-1 also stimulated PGE_2 production in osteoblasts and osteoclast formation in the cocultures, and the stimulation was inhibited by NS398. As seen with LPS, NS398 failed to inhibit IL-1-induced osteoclast formation in cocultures with OPG-deficient osteoblasts. These results suggest that IL-1 as well as LPS stimulates osteoclastogenesis through two parallel events: direct enhancement of RANKL expression and suppression of OPG expression, which is mediated by PGE_2 production. *The Journal of Immunology*, 2004, 172: 0000–0000.

Osteoclasts are bone-resorbing multinucleated cells that originate from hemopoietic progenitors of the monocyte/macrophage lineage (1–4). Osteoblasts or bone marrow stromal cells are involved in osteoclastogenesis through a mechanism involving cell-to-cell contact with osteoclast progenitors (4, 5). Studies of M-CSF-deficient *op/op* mice have shown that M-CSF produced by osteoblasts is an essential factor for osteoclastogenesis (6, 7). Receptor activator of NF-κB ligand (RANKL)[3] (3) was also identified as another factor essential for osteoclastogenesis (8–11). RANKL is a member of the TNF-ligand family that is expressed by osteoblasts/stromal cells as a membrane-associated factor. Osteoclast precursors express RANK, a receptor of RANKL; recognize RANKL through cell-cell interaction; and differentiate into osteoclasts in the presence of M-CSF (12, 13). Osteoblasts/Stromal cells also produce a soluble decoy receptor for RANKL, osteoprotegerin (OPG), which inhibits osteoclast formation in vivo and in vitro by interrupting the interaction between RANKL and RANK (14, 15).

In mouse cell cocultures, osteoclasts are formed in response to bone-resorbing factors such as 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), parathyroid hormone (PTH), PGE_2, and IL-11 (4). Almost all of the bone-resorbing factors stimulate expression of RANKL in osteoblasts/stromal cells (4). Three independent signals have been proposed to induce RANKL expression in osteoblasts/stromal cells: vitamin D receptor-mediated signals induced by $1,25(OH)_2D_3$, cAMP/protein kinase A (PKA)-mediated signals induced by PTH or PGE_2, and gp130-mediated signals induced by IL-11 (4). Among these signals, vitamin D receptor- and cAMP/PKA-mediated signals suppress OPG expression in osteoblasts/stromal cells. Recently, we reported that compounds that elevate intracellular calcium, such as ionomycin, A23187, cyclopiazonic acid, and thapsigargin, stimulated osteoclast formation in mouse cocultures (16). Treatment of primary osteoblasts with those compounds stimulated the expression of RANKL. Thus, the signal mediated by calcium and protein kinase C (PKC) is proposed to be another (fourth) signal that induces RANKL expression in osteoblasts/stromal cells.

Severe bone loss due to excessive bone resorption is observed in inflammatory diseases such as periodontitis and osteomyelitis and some types of arthritides (17). LPS, a major constituent of Gram-negative bacteria, is proposed to be a potent stimulator of bone loss in these inflammatory diseases (17–22). Recently, Toll-like receptor 4 (TLR4) was identified as the signal-transducing receptor for LPS (23, 24). The cytoplasmic signaling cascade of TLR4 is sim-

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[3] Abbreviations used in this paper: RANKL, receptor activator of NF-κB ligand; $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; COX2, cyclooxygenase 2; EIA, enzyme

immunoassay; ERK, extracellular signal-regulated kinase; IL-1ra, IL-1 receptor antagonist; OPG, osteoprotegerin; PKA, protein kinase A; PKC, protein kinase C; PTH, parathyroid hormone; RANK, receptor activator of NF-κB; TLR, Toll-like receptor; TRAP, tartrate-resistant acid phosphatase.

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ilar to that of IL-1Rs. Both TLR4 and IL-1Rs use common signaling molecules such as myeloid differentiation factor 88 and TNFR-associated factor 6 (25–29). Macrophages, lymphocytes,

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and osteoblasts/stromal cells express TLR4, and produce PGE_2 and proinflammatory cytokines such as TNF-α and IL-1 in response to LPS (30, 31). These inflammatory factors also stimulate osteoclastogenesis directly or indirectly (17–22, 32).

LPS stimulates PGE_2 production in the target cells through the induction of mitogen-inducible cyclooxygenase 2 (COX2) expression (33). NS398, a nonsteroidal anti-inflammatory agent, specifically inhibits COX2 without affecting COX1 activity (34). Therefore, NS398 has been used as a tool to explore the role of PGE_2 in pathological processes involving COX2 activity. PGE_2 exerts its biological actions through binding to four specific membrane receptors (EP1, EP2, EP3, and EP4) (35). Sakuma et al. (20, 21) reported that induction of osteoclast formation by LPS, TNF-α, and IL-1 was barely observed in cell cultures prepared from EP4 knockout ($EP4^{-/-}$) mice, and that urinary excretion of deoxypyridinoline, a sensitive marker for bone resorption, was not increased in $EP4^{-/-}$ mice injected with LPS. These results suggest that PGE_2 is a key factor in the enhancement of osteoclastogenesis by LPS in vivo and in vitro. However, it is still not known how PGE_2 is involved in the induction of osteoclastogenesis by LPS.

In the present study, we examined the mechanism of the induction of osteoclast formation by LPS in cocultures of mouse osteoblasts and bone marrow cells. We showed that LPS promoted osteoclastogenesis through two parallel events: one was direct enhancement of RANKL expression, and the other was suppression of OPG production mediated by PGE_2 in osteoblasts. In addition, IL-1 stimulated osteoclast formation in the cocultures in a manner similar to LPS.

Materials and Methods

Reagents and mice

LPS (*Escherichia coli* O26:B6) and PGE_2 were purchased from Sigma-Aldrich (St. Louis, MO). NS398 was from Calbiochem (San Diego, CA). Human rOPG and mouse rIL-1β were obtained from PeproTech (London, U.K.). Mouse rTNF-α and mouse rIL-1 receptor antagonist (rIL-1ra) were obtained from R&D Systems (Minneapolis, MN). Anti-mouse TNFR1 Ab was obtained from Genzyme Diagnostics (Cambridge, MA). Six- to 9-wk-old male and newborn ddY mice were obtained from Sankyo Laboratory Animal Center (Tokyo, Japan). C57BL/6 (B6) mice and OPG-deficient ($OPG^{-/-}$) mice (C57BL/6 (B6)) were obtained from Clear Japan Clea (Tokyo, Japan). This study was reviewed and approved by the Showa University Animal Care and Use Committee.

Cell preparation and osteoclast formation assay

Primary osteoblasts were obtained from calvariae of newborn ddY mice, C57BL/6 (B6) mice, and $OPG^{-/-}$ mice by the conventional method using collagenase (36). Bone marrow cells were collected from femora and tibiae of 6- to 9-wk-old male mice. Primary osteoblasts (1×10^4 cells) and bone marrow cells (2×10^5 cells) were cocultured for 5 days in α-MEM containing 10% FCS (CSL, Victoria, Australia) in 96-well tissue culture plates (Corning, Corning, NY) (0.2 ml/well). Cocultures were incubated in the presence of LPS (0.001–10 μg/ml), PGE_2 (1 μM), IL-1β (10 ng/ml), or TNF-α (10 ng/ml) for the final 3 days. Some cocultures were pretreated with NS398 (1 μM), OPG (100 ng/ml), IL-1ra (10 μg/ml), and TNFR1 Ab (10 μg/ml) for 1 h before adding LPS, PGE_2, IL-1β, or TNF-α. Then cocultures were fixed and stained for tartrate-resistant acid phosphatase (TRAP; a marker enzyme of osteoclasts). TRAP-positive cells containing >3 nuclei were counted as osteoclasts. The results obtained from a typical experiment of three independent experiments are expressed as the mean ± SD of four cultures.

Measurement of PGE_2 production

Primary osteoblasts (3×10^4 cells) and bone marrow cells (6×10^5 cells) tios with several eicosanoids: PGE_2, 100%; PGE_2 ethanolamide, 100%: PGE_3, 43%; PGE_1, 18.7%; 6-keto $PGF_1\alpha$, 1%: and 8-*iso* $PGF_2\alpha$, 0.25%.

Northern blot analysis

Primary osteoblasts (1×10^6 cells) were seeded in cell culture dishes (60 mm in diameter; Corning) and cultured in α-MEM containing 10% FCS for 3 days. After incubation in α-MEM containing 0.1% FCS for 3 h, the cells were incubated with LPS (1 μg/ml) or IL-1β (10 ng/ml). In some experiments, osteoblasts were cocultured with bone marrow cells (2×10^7 cells) in the presence of LPS (1 μg/ml) for 3 or 48 h. Some cultures were also treated with NS398 (1 μM) for 1 h before the addition of LPS. Total RNA was isolated from cultures using TRIzol (Life Technologies, Grand Island, NY). Northern blot analysis was performed using denaturing formaldehyde/agarose gels, as described (16). Double-stranded cDNA fragments encoding mouse RANKL OPG and COX2 were kindly provided by H. Yasuda (Snow Brand Milk Products, Tochigi, Japan). cDNA probes (RANKL, OPG, COX2, and β-tubulin) labeled with ^{32}P were synthesized using a cDNA labeling kit (Takara, Tokyo, Japan). The RANKL, OPG, COX2, and β-tubulin probes were hybridized with membranes to which total RNA isolated from osteoblasts had been transferred. The membranes were exposed to Kodak BioMax MS film (Rochester, NY) for 3–48 h. Signals of RANKL, OPG, COX2, and β-tubulin mRNA were quantified using a radioactive image analyzer (BAS2000; Fuji Photo Film, Tokyo, Japan). Signals of RANKL, OPG, and COX2 mRNAs were normalized with the respective β-tubulin mRNA expression levels to calculate the relative intensity.

Results

PGE_2 is required for osteoclast formation induced by LPS

LPS stimulated TRAP-positive osteoclast formation in cocultures of primary osteoblasts and bone marrow cells in a dose-dependent manner (Fig. 1*A*). The maximal number of osteoclasts was observed at 1 μg/ml of LPS. We then examined whether PGE_2 is involved in the induction of osteoclast formation by LPS. LPS (1 μg/ml) as well as PGE_2 (1 μM) induced TRAP-positive osteoclast formation in the cocultures (Fig. 1, *B* and *C*). NS398 (1 μM), a specific inhibitor of COX2, suppressed the induction of osteoclast formation by LPS, but not by PGE_2 in the cocultures (Fig. 1, *B* and *C*). Both LPS- and PGE_2-induced osteoclast formation in the cocultures was strongly inhibited by simultaneous addition of OPG (100 ng/ml) (Fig. 1, *B* and *C*). These results suggest that both PGE_2 production and RANKL-RANK interaction are required for LPS-induced osteoclast formation in the cocultures. We next examined whether LPS induces osteoclastogenesis through IL-1 and TNF-α. Recombinant IL-1β (10 ng/ml) and TNF-α (10 ng/ml) induced osteoclastogenesis in the cocultures. IL-1ra (10 μg/ml) and anti-mouse TNFR1 Ab (10 μg/ml) strongly inhibited the osteoclast formation induced by IL-1β and TNF-α, respectively. However, neither IL-1ra nor TNFR1 Ab affected osteoclastogenesis induced by LPS (Fig. 1*D*). These results suggest that PGE_2 is a critical factor in LPS-induced osteoclastogenesis. F1

Osteoblasts mainly produce PGE_2 in response to LPS

We then measured the PGE_2 concentration in the conditioned medium of cocultures incubated with or without LPS (1 μg/ml) for 6 h (Fig. 2*A*). LPS significantly increased the PGE_2 concentration in the culture medium (Fig. 2*A*). The addition of NS398 (1 μM) to the cocultures completely blocked the induction of PGE_2 production by LPS in the cocultures (Fig. 2*A*). To determine the type of cells that respond to LPS in the cocultures, osteoblasts and bone marrow cells were cultured separately in the presence or absence of LPS for 6 h (Fig. 2*B*). LPS stimulated PGE_2 production in the F2

were cultured separately or in combination with or without LPS (1 μg/ml) or IL-1β (10 ng/ml) in α-MEM containing 10% FCS in 48-well culture plates (Corning). After the cultures were incubated for 6 h, the concentration of PGE_2 in the culture medium was determined using an enzyme immunoassay (EIA; Cayman Chemicals, Ann Arbor, MI). The Ab showed the following cross-reactivity determined by comparing the bond/free ra-

cultures of osteoblasts, but not bone marrow cells. LPS-induced PGE_2 production was strongly inhibited by the addition of NS398 (Fig. 2*B*). Northern blot analysis showed that treatment of osteoblasts with LPS for 3 h stimulated the expression of COX2 mRNA (Fig. 2*C*). These results suggest that osteoblasts in the cocultures

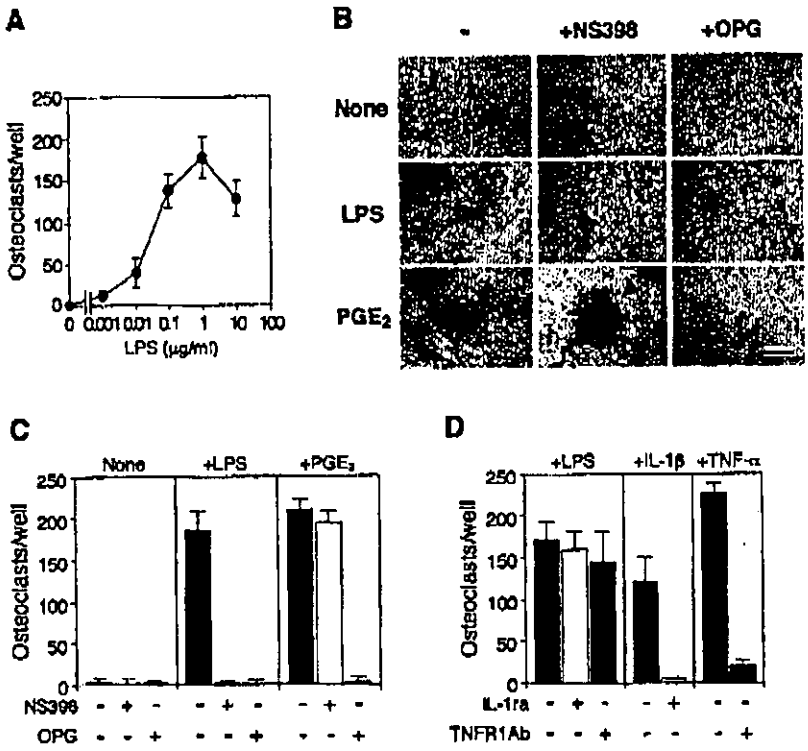

FIGURE 1. Effects of NS398, OPG, IL-1ra, and TNFR1 Ab on osteoclast formation in cocultures treated with LPS. *A*, Mouse primary osteoblasts and bone marrow cells were cocultured for 5 days. LPS (0.001–10 μg/ml) was added to the cocultures for the final 3 days. TRAP-positive multinucleated cells containing >3 nuclei were counted as osteoclasts. Values are expressed as the means ± SD of quadruplicate cultures. *B*, LPS (1 μg/ml) or PGE_2 (1 μM) together with or without NS398 (1 μM) or OPG (100 ng/ml) was added to the cocultures for the final 3 days. The cells were then fixed and stained for TRAP. Arrowheads indicate TRAP-positive multinucleated cells. Bar, 200 μm. *C*, TRAP-positive multinucleated cells containing >3 nuclei were counted as osteoclasts. Values are expressed as the means ± SD of quadruplicate cultures. *D*, LPS (1 μg/ml), IL-1β (10 ng/ml), or TNF-α (10 ng/ml) together with or without IL-1ra (10 μg/ml) and TNFR1 Ab (10 μg/ml) was added to the cocultures for the final 3 days. TRAP-positive multinucleated cells containing >3 nuclei were counted as osteoclasts. Values are expressed as the means ± SD of quadruplicate cultures.

produce PGE_2 in response to LPS via up-regulation of COX2 mRNA expression.

LPS regulates RANKL and OPG gene expression in osteoblasts

We next analyzed the effects of LPS on RANKL and OPG mRNA expression levels in primary osteoblasts by Northern blot analysis (Fig. 3). Treatment of the osteoblasts with LPS increased RANKL mRNA expression with two peaks at 3 and 48 h. The expression of RANKL mRNA after treatment with LPS was increased within 1 h, and was still higher than that of the control cultures even after 72 h (Fig. 3*A*). The expression of OPG mRNA in osteoblasts was also enhanced by the treatment with LPS for 3 h (Fig. 3*A*). However, the expression of OPG mRNA in osteoblasts treated with LPS for 48 or 72 h was decreased to a level lower than that of the control culture (Fig. 3*A*). NS398 (1 μM) had no effect on the level of RANKL mRNA induced by LPS at 3 h (Fig. 3*B*). The LPS-induced up-regulation of RANKL mRNA expression at 48 h was slightly inhibited by the COX2 inhibitor, but the level of the mRNA was much higher than that in the control cultures. In contrast, the LPS-induced down-regulation of OPG mRNA expression in osteoblasts at 48 h after treatment with LPS was completely blocked by the addition of NS398, although the OPG mRNA expression at 3 h was not affected by the COX2 inhibitor (Fig. 3*B*). The expression levels of RANKL and OPG mRNAs in bone marrow cells were lower than those in primary osteoblasts, and were unchanged even after treatment with NS398 for 3 or 48 h (data not shown). These results suggest that PGE_2 produced by osteoblasts plays an important role in the down-regulation of OPG expression, but not the up-regulation of RANKL expression in osteoblasts treated with LPS.

were cocultured with bone marrow cells from wild-type mice in the presence or absence of NS398, OPG, and/or LPS (Fig. 4). In agreement with previously reported findings (37), TRAP-positive osteoclasts were formed in cocultures containing $OPG^{-/-}$ osteoblasts even in the absence of any stimulus (Fig. 4). The number of osteoclasts was further increased in the LPS-treated cocultures containing $OPG^{-/-}$ osteoblasts. NS398 strongly suppressed the spontaneous osteoclast formation in the control cocultures containing $OPG^{-/-}$ osteoblasts. This suggests that endogenous production of PGE_2 plays an important role in the osteoclast formation in cocultures containing $OPG^{-/-}$ osteoblasts. NS398 slightly, but not completely, inhibited LPS-induced osteoclast formation in cocultures containing $OPG^{-/-}$ osteoblasts (Fig. 4). OPG completely suppressed osteoclast formation in the cocultures treated or not treated with LPS (Fig. 4). These results suggest that the down-regulation of OPG expression by PGE_2 is crucially involved in the osteoclast formation induced by LPS in the cocultures.

IL-1 stimulates osteoclast formation in a manner similar to LPS

Because the signaling pathway of IL-1Rs is quite similar to that of TLR4 (26–30), we finally examined whether IL-1 stimulates osteoclastogenesis in the cocultures in a manner similar to LPS. IL-1β (10 ng/ml) induced osteoclast formation in the wild-type cocultures, and the induction was inhibited by NS398 (1 μM) and OPG (100 ng/ml) (Fig. 5*A*). IL-1β (10 ng/ml) also stimulated PGE_2 production in osteoblasts, but not in bone marrow cells after treatment for 6 h (Fig. 5*B*). Northern blot analysis showed that IL-1β up-regulated COX2 mRNA expression in osteoblasts at 3 h (Fig. 5*C*). IL-1β also stimulated RANKL mRNA expression at 3 h

Suppression of OPG expression is involved in induction of osteoclast formation by LPS

We next examined how PGE_2 production is involved in LPS-induced osteoclast formation using osteoblasts from OPG-deficient ($OPG^{-/-}$) mice. Primary osteoblasts prepared from $OPG^{-/-}$ mice (data not shown). IL-1β stimulated osteoclast formation in the cocultures of $OPG^{-/-}$ osteoblasts and wild-type bone marrow cells (the control; 53 ± 11, the mean ± SD of four cultures) (Fig. 5*D*). NS398 (1 μM) did not completely suppress LPS-induced osteoclast formation in the cocultures with $OPG^{-/-}$ osteoblasts, but

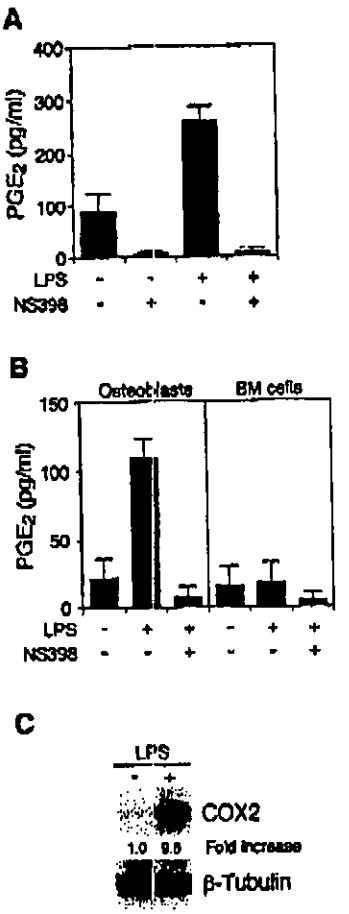

FIGURE 2. LPS induces PGE_2 production and COX2 expression in osteoblasts. *A*, Primary osteoblasts and bone marrow (BM) cells were cocultured with LPS (1 μg/ml) in the presence or absence of NS398 (1 μM). After the cultures were incubated for 6 h, the concentration of PGE_2 in the culture supernatant was determined using EIA. Values are expressed as the means ± SD of quadruplicate cultures. *B*, Primary osteoblasts and bone marrow cells were cultured separately with LPS (1 μg/ml) in the presence or absence of NS398 (1 μM) for 6 h. The PGE_2 concentration in the culture supernatant was determined using EIA. Values are expressed as the means ± SD of quadruplicate cultures. *C*, Primary osteoblasts were treated with LPS (1 μg/ml) for 3 h. Total RNA was isolated from the osteoblasts, and COX2 and β-tubulin mRNA expression was analyzed by Northern blotting. Figures below the signals represent the intensity of the COX2 mRNA signals relative to the β-tubulin mRNA signals.

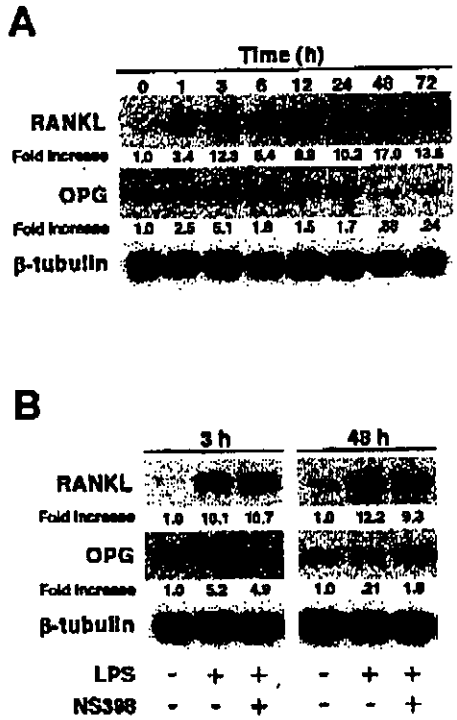

FIGURE 3. LPS regulates the expression of RANKL and OPG mRNAs in osteoblasts. *A*, Primary osteoblasts were treated with LPS (1 μg/ml) for 0–72 h. Total RNA was isolated from the osteoblasts, and the expression of RANKL, OPG, and β-tubulin mRNAs was analyzed by Northern blotting. Figures below the signals represent the intensity of the RANKL and OPG mRNA signals relative to the β-tubulin mRNA signals. *B*, Primary osteoblasts were treated with LPS (1 μg/ml) for 3 or 48 h in the presence of bone marrow cells. NS398 (1 μM) was also added to some cultures. After incubation for the indicated periods, bone marrow cells were removed by pipetting. Total RNA was isolated from osteoblasts, and the expression of RANKL, OPG, and β-tubulin mRNAs was analyzed by Northern blotting. Figures below the signals represent the intensity of the RANKL and OPG mRNA signals relative to the β-tubulin mRNA signals.

OPG did (Fig. 5*D*). These results suggest that IL-1 and LPS stimulate osteoclast formation in the same manner in the cocultures.

Discussion

In vivo and in vitro experiments have shown that PGE_2 is crucially involved in the induction of osteoclastic bone resorption by IL-1, TNF-α, and LPS (20). EP4 subtype-mediated signaling has been shown to be particularly important for the induction of bone resorption by such inflammation-related factors as well as PGE_2 (20). The present study showed that LPS stimulated COX2 expression and PGE_2 production in osteoblasts, and NS398, a specific inhibitor of COX2, strongly blocked the LPS-induced osteoclast formation in cocultures containing wild-type osteoblasts inhibitors did not block this stimulation (21). In agreement with this finding, NS398 failed to inhibit the induction of RANKL expression by LPS in osteoblasts (Fig. 3). These results suggest that LPS induced RANKL expression in a manner that was independent of PGE_2 production in osteoblasts. In contrast, the treatment of osteoblasts in the cocultures with LPS together with NS398 blocked the down-regulation of OPG mRNA expression at 48 h (Fig. 3). This suggests that suppression of OPG by PGE_2 is an important event in osteoclast formation in the cocultures treated with LPS. This notion was further supported by the finding that LPS stimulated osteoclast formation even in the presence of NS398 in cocultures containing osteoblasts derived from $OPG^{-/-}$ mice (Fig. 4). Thus, PGE_2 appears to play an important role as a suppressor of OPG expression rather than an activator of RANKL expression in LPS-induced osteoclast formation (Fig. 6). Recently, Fu et al. (38) reported that the activation of CREB by PTH is required for PTH-induced down-regulation of OPG expression. This suggests that the cAMP-PKA signals play a role in PGE_2-induced suppression of OPG mRNA expression. Further study will elucidate the detail mechanism of the down-regulation of OPG expression by PGE_2.

(Figs. 1 and 2). These results suggest that PGE_2 is somehow involved in LPS-induced osteoclast formation in the cocultures through PGE_2 receptors of EP4 subtype.

It was reported that LPS stimulated the expression of RANKL mRNA in osteoblasts obtained from $EP4^{-/-}$ mice, and that COX

PGE_2 has been shown to induce RANKL mRNA expression in osteoblasts (10). Suzawa et al. (39) reported that PGE_2-induced RANKL expression is mediated through the cAMP signaling pathway. In our experiments, NS398 failed to inhibit RANKL expression in osteoblasts treated with LPS for as long as 48 h (Fig. 3).

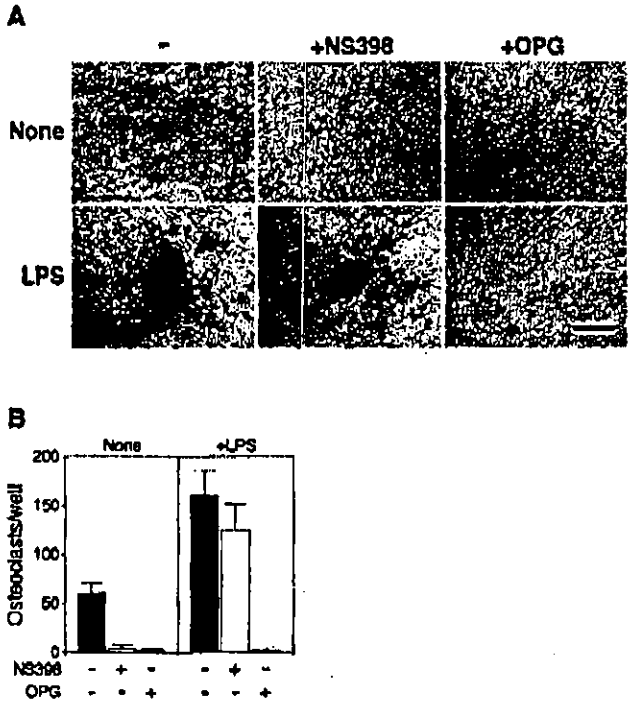

FIGURE 4. Effects of NS398 on LPS-induced osteoclast formation in cocultures containing OPG$^{-/-}$ mouse-derived osteoblasts. *A*, Primary osteoblasts prepared from OPG$^{-/-}$ mice were cocultured with wild-type bone marrow cells for 5 days. LPS (1 μg/ml) was added to the cocultures with or without NS398 (1 μM) or OPG (100 ng/ml) for the final 3 days. The cells were then fixed and stained for TRAP. Arrowheads indicate the TRAP-positive osteoclasts. Bar, 200 μm. *B*, TRAP-positive cells containing >3 nuclei were counted as osteoclasts. Values are expressed as the means ± SD of quadruplicate cultures.

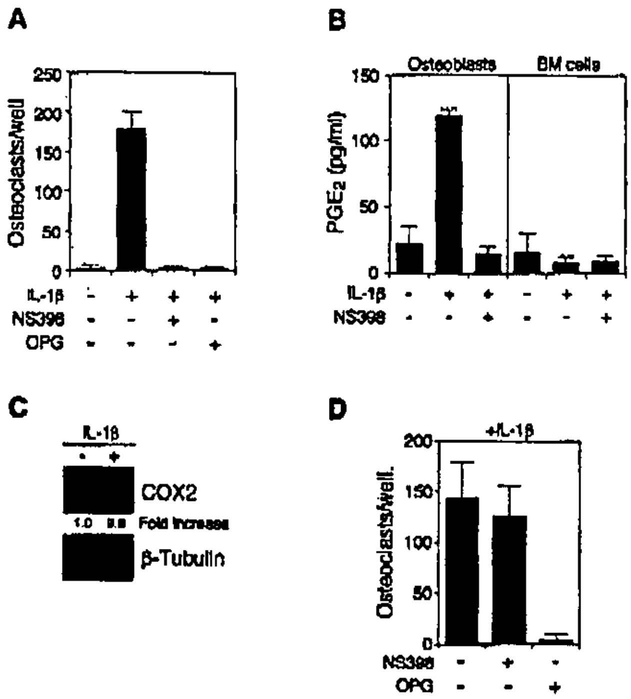

FIGURE 5. IL-1 induces osteoclast formation in the cocultures in a manner similar to LPS. *A*, Mouse primary osteoblasts and bone marrow (BM) cells were cocultured with IL-1β (10 ng/ml) in the presence or absence of NS398 (1 μM) or OPG (100 ng/ml). TRAP-positive cells containing >3 nuclei were counted as osteoclasts. Values are expressed as the means ± SD of quadruplicate cultures. *B*, Primary osteoblasts and bone marrow cells were cultured separately with IL-1β (10 ng/ml) in the presence or absence of NS398 (1 μM) for 6 h. The PGE$_2$ concentration in the culture supernatant was determined using EIA. Values are expressed as the means ± SD of quadruplicate cultures. *C*, Primary osteoblasts were treated with IL-1β (10 ng/ml) for 3 h, and then COX2 and β-tubulin mRNA expression was analyzed by Northern blotting. Figures below the signals represent the intensity of the COX2 mRNA signals relative to the β-tubulin mRNA signals. *D*, Primary osteoblasts prepared from OPG$^{-/-}$ mice and wild-type bone marrow cells were cocultured with IL-1β (10 ng/ml) in the presence or absence of NS398 (1 μM) or OPG (100 ng/ml). TRAP-positive cells containing >3 nuclei were counted as osteoclasts. Values are expressed as the means ± SD of quadruplicate cultures.

This suggests that LPS induces RANKL expression by the mechanism independent of PGE$_2$ production. Kikuchi et al. (40) reported that LPS induces RANKL through extracellular signal-regulated kinase (ERK) and PKC. We also confirmed that calcium/PKC inhibitors, such as BAPTA-AM (an intracellular calcium chelator) and Ro-32-0432 (a PKC inhibitor), and ERK inhibitor PD98059 inhibited LPS-induced RANKL mRNA expression in osteoblasts (K.S., unpublished observation). PD98059 failed to inhibit the induction of RANKL mRNA expression by PGE$_2$ and the induction of osteoclast formation in cocultures treated with PGE$_2$ (data not shown). These results suggest that LPS directly stimulates RANKL expression through calcium/PKC signals, followed by ERK signals in osteoblasts. It is unlikely that PGE$_2$-induced signals directly cross talk with LPS-induced signals in the induction of RANKL expression in osteoblasts.

The intracellular signaling pathway of TLR4 is quite similar to that of IL-1Rs (25–29). Like LPS, IL-1 stimulated COX2 mRNA expression at 3 h and PGE$_2$ production at 6 h in osteoblast cultures (Fig. 5). IL-1β also induced RANKL mRNA expression in osteoblasts, as previously reported (data not shown) (32). NS398 inhibited IL-1-induced osteoclast formation strongly in cocultures containing wild-type osteoblasts, but only partially in cocultures containing OPG$^{-/-}$ osteoblasts (Fig. 5). These results suggest that PGE$_2$ produced by osteoblasts in response to IL-1 plays a similar of either OPG or NS398 (Fig. 4). These results suggest that RANKL is involved in the spontaneous osteoclast formation, and that PGE$_2$ constitutively produced in the cocultures stimulates RANKL expression in osteoblasts. LPS and IL-1β further enhanced osteoclast formation in cocultures containing OPG$^{-/-}$ osteoblasts (Fig. 4), suggesting that the up-regulation of RANKL expression by LPS and IL-1 enhances the osteoclast formation. The induction of osteoclast formation by LPS and IL-1 in cocultures containing OPG$^{-/-}$ osteoblasts was partially inhibited by the addition of NS398. Therefore, PGE$_2$ induced by LPS and IL-1 appears to be involved in RANKL expression in osteoblasts. Our results indicate that the full inhibition of LPS- and IL-1-induced osteoclast formation by NS398 requires PGE$_2$-dependent suppression of OPG production (Fig. 6).

The previous studies have shown that OPG production by osteoblasts is down-regulated by bone-resorbing factors such as

role to LPS in osteoclast formation through the suppression of OPG expression (Fig. 6).

In cocultures containing $OPG^{-/-}$ osteoblasts, osteoclasts were formed even in the absence of any stimulus (Fig. 4). The spontaneous osteoclast formation was strongly inhibited by the addition of $1,25(OH)_2D_3$, PTH, and PGE_2 (38, 41–44). Our results confirmed the previous finding that osteoclasts spontaneously form in the control cocultures containing $OPG^{-/-}$ osteoblasts. The decrease in OPG production by osteoblasts was a key event for the induction of osteoclastogenesis by LPS and IL-1. $OPG^{-/-}$ mice exhibited

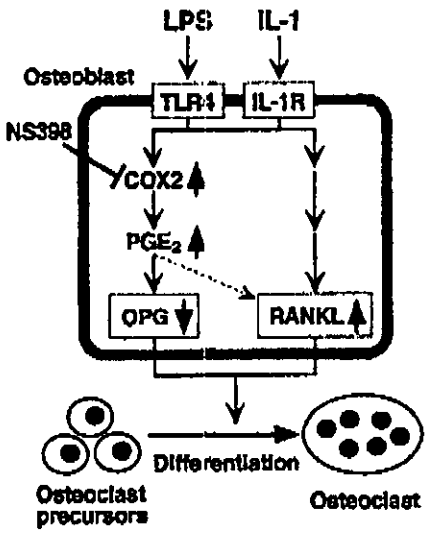

FIGURE 6. A possible mechanism of the induction of osteoclastogenesis by LPS and IL-1. LPS and IL-1 promote the differentiation of osteoclast precursors into osteoclasts through two parallel events in osteoblasts: direct enhancement of RANKL expression, and suppression of OPG production mediated by PGE_2. PGE_2 induced by LPS and IL-1 also stimulates RANKL expression, but the suppression of OPG production in osteoblasts appears to be more important than the induction of RANKL expression in osteoblasts for the stimulation of osteoclastogenesis. See text for details.

severe osteoporosis caused by enhanced osteoclast formation and function (14, 15, 45, 46). These results suggest that OPG is a physiological regulator of bone resorption, and that the balance between RANKL and OPG expressions at bone is particularly important for the regulation of bone resorption in vivo and in vitro.

We previously reported that LPS and IL-1 directly stimulated the survival, fusion, and pit-forming activity of osteoclasts (47). Those results together with the results shown in this study suggest that LPS and IL-1 are involved in the stimulation of osteoclastic bone resorption in several ways: LPS and IL-1 directly stimulate osteoclast function, induce RANKL expression in osteoblasts, and suppress OPG expression through enhancement of PGE_2 production. Further studies will be necessary to elucidate the precise mechanism of the regulation of osteoclastic bone resorption induced by these inflammatory factors.

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Lipopolysaccharide Promotes the Survival of Osteoclasts Via Toll-Like Receptor 4, but Cytokine Production of Osteoclasts in Response to Lipopolysaccharide Is Different from That of Macrophages[1]

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Lipopolysaccharide is a pathogen that causes inflammatory bone loss. Monocytes and macrophages produce proinflammatory cytokines such as IL-1, TNF-α, and IL-6 in response to LPS. We examined the effects of LPS on the function of osteoclasts formed in vitro in comparison with its effect on bone marrow macrophages, osteoclast precursors. Both osteoclasts and bone marrow macrophages expressed mRNA of Toll-like receptor 4 (TLR4) and CD14, components of the LPS receptor system. LPS induced rapid degradation of I-κB in osteoclasts, and stimulated the survival of osteoclasts. LPS failed to support the survival of osteoclasts derived from C3H/HeJ mice, which possess a missense mutation in the TLR4 gene. The LPS-promoted survival of osteoclasts was not mediated by any of the cytokines known to prolong the survival of osteoclasts, such as IL-1β, TNF-α, and receptor activator of NF-κB ligand. LPS stimulated the production of proinflammatory cytokines such as IL-1β, TNF-α, and IL-6 in bone marrow macrophages and peritoneal macrophages, but not in osteoclasts. These results indicate that osteoclasts respond to LPS through TLR4, but the characteristics of osteoclasts are quite different from those of their precursors, macrophages, in terms of proinflammatory cytokine production in response to LPS. *The Journal of Immunology,* **2003, 170: 3688–3695.**

Lipopolysaccharide, the major component of the outer membrane of Gram-negative bacteria, induces the production of proinflammatory cytokines such as IL-1, TNF-α, and IL-6 in macrophages, lymphocytes, and endothelial cells (1–3). These cytokines activate immune systems to defend the host from bacterial infection (4, 5). Recent studies have provided new insights into the receptor system for LPS in the innate immune system (6–9). CD14 is a membrane-anchored glycoprotein that functions as a member of the LPS receptor system (10). Recently, Toll-like receptor 4 (TLR4)[3] was identified as the signal-transducing receptor for LPS (11–13). The binding of LPS to CD14 initiates signal transduction through TLR4, which results in the release of proinflammatory cytokines and the induction of the systemic inflammatory response.

Recent studies have also revealed that the signaling cascade of TLR4 is quite similar to that of IL-1Rs (6–9). Both types of receptors use TNFR-associated factor 6 (TRAF6) as a common signaling molecule (14, 15). LPS and IL-1 induce the degradation of I-κB in the target cells to activate NF-κB (16). Then NF-κB translocates from the cytosol to the nucleus, and regulates the expression of the target genes that regulate immune and inflammatory responses. Recent studies also demonstrated that C3H/HeJ mice, which show extremely low responsiveness to LPS, have a point mutation in the intracellular domain of TLR4 (11–13). These results further support the notion that TLR4 is a signaling receptor responsible for LPS-induced inflammatory responses.

Osteoclasts, the multinucleated giant cells that resorb bone, develop from hemopoietic cells of the monocyte/macrophage lineage (17–20). Osteoblasts or bone marrow stromal cells have been shown to be involved in osteoclastogenesis (20, 21). Studies of M-CSF-deficient *op/op* mice have shown that M-CSF produced by osteoblasts/stromal cells is an essential factor for osteoclastogenesis (22, 23). Recently, the gene for another essential factor for osteoclastogenesis, receptor activator of NF-κB ligand (RANKL), was cloned (24–27). RANKL is a new member of the TNF ligand family, and is expressed by osteoblasts/stromal cells in response to many bone-resorbing factors. Osteoclast precursors express RANK, a TNFR family member, recognize RANKL expressed by osteoblasts/stromal cells, and differentiate into osteoclasts in the presence of M-CSF (28, 29). Osteoprotegerin (OPG), which is produced by many types of cells including osteoblasts/stromal cells, is a soluble decoy receptor for RANKL, and blocks osteoclastogenesis by inhibiting RANKL-RANK interaction (30, 31).

The cytoplasmic tail of RANK has been shown to interact with TRAF1, TRAF2, TRAF3, TRAF5, and TRAF6 (32–34). Among these TRAFs, TRAF6 appears to play important roles in osteoclast differentiation and function. TRAF6 knockout mice develop severe osteopetrosis (14, 15), and overexpression of TRAF6 in osteoclast

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[3] Abbreviations used in this paper: TLR, Toll-like receptor; ERK, extracellular signal-regulated kinase; IL-1Ra, IL-1R antagonist; MAPK, mitogen-activated protein kinase; MNC, multinucleated cell; OPG, osteoprotegerin; PI-3, phosphatidylinositol-3; RANK, receptor activator of NF-κB; RANKL, RANK ligand; TRAF, TNFR-associated factor; TRAP, tartrate-resistant acid phosphatase.

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progenitors induces their differentiation into osteoclasts (35). We previously reported that IL-1 stimulated the survival, fusion, and bone resorption activity of osteoclasts (36, 37), and that TNF-α directly stimulated the differentiation of osteoclasts (38). The stimulatory effects of TNF-α and IL-1 on osteoclast differentiation and function appear to be independent of the RANKL-RANK interaction, because these TNF-α- and IL-1-induced effects are not inhibited by the simultaneous addition of OPG (38). TNF type I receptor p55 (TNFR1) and type II receptor p75 (TNFR2) have been shown to use TRAF2 as a common signal transducer in the target cells (39, 40). These results suggest that TRAF-mediated signals play central roles in the regulation of osteoclast differentiation and function. Abu-Amer et al. (41) reported that LPS stimulated osteoclast formation in vivo and in vitro, and LPS-induced osteoclastogenesis is mediated by TNFR1.

In the present study, we explored the role of LPS in the function of osteoclasts in comparison with its role in bone marrow macrophages. We found that both osteoclasts and bone marrow macrophages expressed TLR4 and CD14 mRNAs, and induced degradation of I-κB in response to LPS. LPS strongly supported the survival of osteoclasts via TLR4-mediated signals. LPS enhanced the production of proinflammatory cytokines such as IL-1, TNF-α, and IL-6 in bone marrow macrophages and peritoneal macrophages. In contrast, the production of these cytokines in osteoclasts was not stimulated by LPS. Thus, osteoclasts respond to LPS through TLR4, but the responsiveness to LPS is quite different between osteoclasts and macrophages, the precursors of osteoclasts, in terms of proinflammatory cytokine production.

Materials and Methods

Animals and chemicals

Five-week-old male and newborn ddY mice were obtained from Sankyo Laboratory Animal Center (Tokyo, Japan) and Saitama Experimental Animals (Saitama, Japan), respectively. TNFR1 knockout mice (C57BL/6J strain) were obtained from The Jackson Laboratory (Bar Harbor, ME). C3H/HeJ mice that have a point mutation in the TLR4 gene and normal control C3H/HeN mice were obtained from Sankyo Laboratory Animal Center. LPS was purified in our laboratory from *Escherichia coli* strain K235, as described previously (42). Human rM-CSF (Leukoprol) was obtained from Kyowa Hakko Kogyo (Tokyo, Japan). Anti-mouse CD14 goat polyclonal Abs and anti-mouse IL-1β goat polyclonal Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and Genzyme Techne (Minneapolis, MN), respectively. Mouse IL-1R antagonist (IL-1Ra) was obtained from R&D Systems (Minneapolis, MN). ELISA kits for mouse IL-1β and TNF-α were obtained from Genzyme Techne, and that for mouse IL-6 was obtained from Endogen (Woburn, MA). ^{125}I-labeled human calcitonin (sp. act., 74 TBq/mmol) was purchased from Amersham (Buckinghamshire, U.K.). 1,25-Dihydroxyvitamin D_3 was purchased from Wako Pure Chemicals (Osaka, Japan). Recombinant mouse TNF-α, IL-1α, and IL-1β were obtained from Genzyme Techne. Pronase E was obtained from Calbiochem (La Jolla, CA).

Assay of survival of mature osteoclasts

Primary osteoblasts were prepared from calvariae of newborn ddY, C3H/HeJ, or C3H/HeN mice, as described (21, 43). Osteoblasts and freshly prepared bone marrow cells were cocultured in αMEM supplemented with 10% FBS (JRH Biosciences, Lenexa, KS) and 1,25-dihydroxyvitamin D_3 (10^{-8} M) in 100-mm-diameter dishes precoated with collagen gel, as described previously (43). Osteoclasts were formed within 6 days in the cocultures. All the cells in the cocultures were recovered from the dishes by treatment with αMEM containing 0.2% collagenase (43). The purity of osteoclasts in the crude preparation was ~5%. To purify osteoclasts, the crude osteoclast preparation was plated in culture dishes (24-well dishes). After cells were cultured for 4 h, osteoblasts were removed by treatment with PBS containing 0.001% pronase E. Some cultures were then fixed and stained for tartrate-resistant acid phosphatase (TRAP, a marker enzyme of osteoclasts). The purity of osteoclasts in this preparation was ~95% (36, 43). Purified osteoclasts were further incubated for the indicated periods in the presence or absence of IL-1, RANKL, TNF-α, or LPS, and stained for TRAP. TRAP-positive multinucleated cells (MNCs) with more than three nuclei were counted as living osteoclasts.

Mouse bone marrow macrophage and peritoneal macrophage cultures

Bone marrow cells obtained from tibiae of 5- to 8-wk-old ddY mice were suspended in αMEM containing 10% FBS and cultured in 48-well plates (1.5×10^5 cells/0.3 ml/well) in the presence of M-CSF (50 ng/ml) (38). After cells were cultured for 4 days, nonadherent cells were completely removed from the cultures by pipetting. Almost all of the adherent cells expressed macrophage-specific Ags such as Mac-1, Moma-2, and F4/80 (38). These macrophages were further cultured overnight with vehicle (control) or LPS (1 μg/ml). Peritoneal macrophages were obtained from peritoneal exudate cells by peritoneal lavage with cold αMEM medium 4 days after i.p. injection of thioglycolate (2 ml; Remel, Lenexa, KS). Peritoneal exudate cells were seeded at 1.5×10^5 cells in 300 μl αMEM containing 10% FBS/well of 48-well plates, and were allowed to adhere 3 h at 37°C in 5% CO_2. Nonadherent cells were removed by washing in medium to provide cultures routinely comprising >95% adherent macrophages. Peritoneal macrophages were activated with 1 μg/ml LPS, as indicated below. Some cultures were subjected to immunostaining with anti-mouse IL-1β Abs. The concentrations of IL-1β, TNF-α, and IL-6 in the conditioned medium were determined using the respective ELISA kits.

PCR amplification of reverse-transcribed mRNA

For semiquantitative RT-PCR analysis, total cellular RNA was extracted from bone marrow macrophages and purified osteoclasts using TRIzol solution (Life Technologies, Grand Island, NY). First strand cDNA was synthesized from the total RNA with random primers and subjected to PCR amplification with EX *Taq* polymerase (Takara Biochemicals, Shiga, Japan) using the following specific PCR primers: mouse TLR2, 5′-TCGCTTTTTCCCAATCTCAC-3′ (forward, nt 743–762) and 5′-TGTA ACGCAACAGCTTCAGG-3′ (reverse, nt 1123–1142); mouse TLR4, 5′-AATTCCTGCAGTGGGTCAAG-3′ (forward, nt 1178–1197) and 5′-AGGCGATACAATTCCACCTG-3′ (reverse, nt 2359–2378); mouse CD14, 5′-ACATCTTGAACCTCCGCAAC-3′ (forward, nt 454–473) and 5′-AGGGTTCCTATCCAGCCTGT-3′ (reverse, nt 934–953); mouse GAPDH, 5′-ACCACAGTCCATGCCATCAC-3′ (forward, nt 566–585) and 5′-TCCACCACCCTGTTGCTGTA-3′ (reverse, nt 998-1017). The PCR products were separated by electrophoresis on a 2% agarose gel, and visualized by ethidium bromide staining and UV light illumination. The sizes of PCR products for mouse TLR2, TLR4, CD14, and GAPDH are 400, 601, 500, and 452 bp, respectively.

Cytochemical and immunochemical staining

For TRAP staining, cultures were fixed with PBS containing 10% Formalin and treated with methanol-acetone (50:50, v/v) for 1 min (43). Cells were then incubated in an acetate buffer (0.1 M sodium acetate, pH 5.0) containing naphthol AS-MX phosphate as a substrate and red violet LB as a stain in the presence of 50 mM sodium tartrate, as described (43). TRAP-positive cells appeared as dark-red cells. For immunohistochemical staining, cells were washed twice with PBS, fixed with cold methanol-acetone (50:50, v/v) for 10 min, and incubated for 90 min with anti-mouse CD14 Ab and anti-mouse IL-1β goat polyclonal Abs as the first Abs. The bound Abs were visualized using biotinylated second Abs, avidin-biotin-conjugated peroxidase, and an 3-amino-9-ethylcarbazole substrate kit (Histofine, Nichirei, Tokyo, Japan) (44). The positive cells appeared as dark-brown cells.

Autoradiography of ^{125}I-labeled human calcitonin

Osteoclast preparations obtained from mouse cocultures were plated on coverslips in 24-well plates, and incubated with 0.2 nM ^{125}I-labeled human calcitonin in αMEM containing 0.1% BSA for 1 h at room temperature (43, 45). The cells were then washed twice with PBS and fixed for 5 min in 0.1 M sodium cacodylate buffer (pH 7.4) containing 1% formaldehyde and 1% glutaraldehyde. The specimens were then subjected to immunocytochemistry for IL-1β. Thereafter, the coverslips were mounted on glass slides and dipped in NR-M2 emulsion (Konica Photo Film, Tokyo, Japan). They were then stored for 14 days in the dark and developed in Rendol (Fuji Photo Film, Tokyo, Japan). The expression of calcitonin receptors was detected by microscopic examination as a dense accumulation of grains due to ^{125}I-labeled calcitonin binding.

Western blot analysis

Bone marrow macrophages and purified osteoclasts were incubated for various periods in the presence of LPS (1 μg/ml). Cells were washed twice