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Bone Morphogenetic Protein-2 Enhances Osteoclast Formation Mediated by Interleukin-1α through Upregulation of Osteoclast Differentiation Factor and Cyclooxygenase-2

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Bone morphogenic protein-2 (BMP-2) is a member of the transforming growth factor β (TGF-β) superfamily. While BMP-2 is capable of inducing bone formation ectopically, little is known about its role on osteoclastogenesis. In this study, we examined the effect of BMP-2 on osteoclast-like multinucleated cell (OCL) formation in cocultures of osteoblast-like cells and hematopoietic cells of bone marrow origin. BMP-2 alone did not stimulate OCL formation in this culture system; however, it strongly enhanced OCL formation in a dose-dependent fashion in the presence of interleukin-1α (IL-1α). Western blot analysis showed that a simultaneous addition of BMP-2 and IL-1α synergistically enhanced cyclooxygenase-2 (COX-2) expression in osteoblast-like cells. Moreover, Northern blot analysis revealed that the level of osteoclast differentiation factor (ODF) mRNA increased by treatment with BMP-2 and IL-1α in osteoblast-like cells. It is noted that BMP-2 alone did cause an increase in the expression of both COX-2 and ODF genes. The stimulatory effect of BMP-2 was abolished by adding nonsteroidal anti-inflammatory drugs, such as indomethacin and a selective COX-2 inhibitor NS-398. Addition of NS-398 inhibited the expression of the ODF gene in osteoblast-like cells treated with BMP-2 and IL-1α. These results indicated that the combination of BMP-2 and IL-1α stimulated osteoblast-like cells to elevate the expression of both COX-2 and ODF genes, resulting in an enhanced OCL formation. Since BMP-2 alone induced the expression of COX-2 and ODF genes in osteoblast-like cells, it appears to be one of the regulating factors of osteoclastogenesis.

Key Words: **osteoclast; osteoblast; BMP-2; ODF; OPGL; COX-2; IL-1α; bone resorption.**

Osteoclasts are unique multinucleated cells responsible for bone resorption that are derived from a hematopoietic stem cell population (1, 2). Osteoclast-like multinucleated cells (OCLs) can be developed *in vitro* from hematopoietic cells by coculturing with osteoblasts or bone marrow stromal cells in the presence of such osteotropic factors as 1α,25-dihydrovitamin D_3 (1α,25$(OH)_2D_3$), prostaglandin E_2 (PGE_2), or interleukin-1 (IL-1) (1, 2). The target cells of these osteotropic factors are mostly osteoblasts or stromal cells (1, 2). Very recently, an essential factor provided by osteoblasts or stromal cells has been identified and named osteoclast differentiation factor (ODF) (3) or osteoprotegerin ligand (OPGL) (4). ODF/OPGL stimulates OCL formation from hematopoietic cells in the presence of macrophage colony stimulating factor (M-CSF) (3, 4). It is noted that *in vitro* osteoclast formation requires only ODF/OPGL and M-CSF (5, 6). Osteoblasts, stromal cells, or the above osteotropic factors such as 1α,25$(OH)_2D_3$ or PGE_2 are not required in this culture system. It is also reported that the expression of the ODF/OPGL gene is strongly upregulated in osteoblasts upon exposure to the osteotropic factors (3). These results strongly suggest that ODF/OPGL expression by osteoblasts or stromal cells is essential for osteotropic factor-mediated osteoclast formation.

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Abbreviations used: ODF, osteoclast differentiation factor; OPGL, osteoprotegerin ligand; OPG, osteoprotegerin; OCIF, osteolcastogenesis inhibitory factor; COX-2, cylooxygenase-2; PGE_2, prostaglandin E_2; IL-1α, interleukin-1α; M-CSF, macrophage colony stimulating factor; TGF-β, transforming growth factor beta; OCL, osteoclast-like multinucleated cell.

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Bone morphogenetic proteins (BMPs) are members of the transforming growth factor β (TGF-β) superfamily and are known to induce ectopic bone formation when implanted into muscular tissues (7). BMPs are implicated in osteoblast differentiation during skeletal development (8, 9). Furthermore, several lines of evidence indicate that the role of BMPs is extended to diverse developmental events in animals (8). Since BMPs are involved in the differentiation of osteoblasts, it might be assumed that BMPs are involved in the process of osteoclastogenesis as well. In fact, BMP-2 and BMP-7/OP-1 (osteogenic protein-1) are reported to stimulate *in vitro* osteoclast formation (10, 11). However, the mechanisms of the stimulatory effect of these BMPs has not been elucidated.

In the present study, we report that BMP-2 promotes osteoclast formation in the presence of IL-1α. We also found that upregulation of cyclooxygenase-2 (COX-2) and ODF in osteoblasts is involved in this osteotropic effect of BMP-2. Our observations suggested that BMPs may play an important role in the production of osteoclastogenic factors in osteoblasts and stromal cells during the process of osteoclastogenesis.

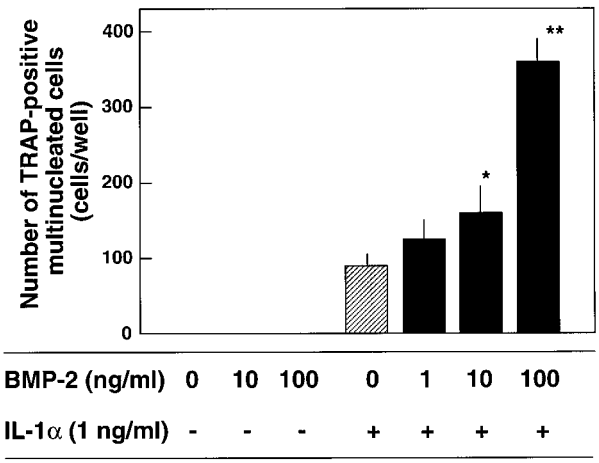

FIG. 1. Effect of BMP-2 on IL-1α-mediated OCL formation in cocultures of mouse bone marrow cells and osteoblast-like cells. Bone marrow cells (1×10^6 cells per well) and osteoblasts (5×10^3 cells per well) were cocultured in the presence or absence of IL-1α and BMP-2 in 24-well culture plates. After being cultured for 8 days, the TRAP-positive multinucleated cells were counted. Data are expressed as the means $\pm$ SD of quadruplicate cultures. Significantly different from the culture of IL-1α alone; *$p < 0.05$ and **$p < 0.01$.

MATERIALS AND METHODS

Mice and reagents. Female mice, ddY strain, were obtained from Japan SLC (Hamamatsu, Japan). PGE_2 and indomethacin were purchased from Sigma Chemicals (St. Louis, MO, USA). 1α,25$(OH)_2D_3$ was purchased from Wako Pure Chemicals (Osaka, Japan). NS-398 was purchased from Calbiochem-Novabiochem International (San Diego, CA, USA). Recombinant IL-1α and TGF-β1 were purchased from R&D Systems (Minneapolis, MN, USA). Recombinant human BMP-2 and activin A were kindly supplied by Yamanouchi Pharmaceutical Co. (Tokyo, Japan), and Ajinomoto Co. (Tokyo, Japan), respectively.

Mouse bone marrow cultures. Bone marrow cells and primary osteoblast-like cells were isolated from ddY mice as described previously (12-14). The cells were suspended in alpha minimum essential medium (α-MEM; GIBCO BRL, Grand Island, NY, USA) containing 10% fetal calf serum (FCS; GIBCO BRL). Marrow cells (1×10^6 cells per well) and osteoblast-like cells (5×10^3 cells per well) were seeded into 24-well flat-bottomed culture plates. Cultures were fed every 3 days by replacing 0.5 ml of old medium with fresh medium containing stimulants. After being cultured for 8 days, the adherent cells were then subjected to staining for tartrate-resistant acid phosphatase (TRAP) as the marker enzyme of osteoclasts, using an acid phosphatase kit (Sigma) (14). TRAP-positive cells containing 3 or more nuclei were counted as OCLs (3–5, 12, 14). The results were expressed as the mean $\pm$ SD of 4 cultures. Statistical differences were analyzed by a Student's *t*-test.

Immunoblot analysis. The cells were dissolved in 50 mM Tris-HCl containing 0.2% SDS (pH 6.8) and then centrifuged. The protein concentration of the supernatants was determined using a Bio-Rad protein assay reagent (Bio-Rad Laboratories, Richmond, CA, USA), and 50 μg of extracted proteins were separated in 7.5% SDS polyacrylamide gels and then electroblotted on polyvinyldine fluoride membranes. Goat anti-COX-2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used for the immunodetection of the COX-2 protein. Detection was performed using an ECL Western blotting detection system (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK) according to the manufacturer's instruction.

Northern blot analysis. Primary osteoblast-like cells were cultured for 18 h in α-MEM containing 10% FCS with or without stimulants. Poly $(A)^+$ RNA was isolated from the osteoblast-like cells using a QuickPrep mRNA Purification Kit (Amersham Pharmacia Biotech). For Northern blotting, 2 μg of poly $(A)^+$ RNA were resolved by electrophoresis in formaldehyde gels and transferred onto nylon membranes (Hybond N^+; Amersham Pharmacia Biotech), then hybridized with α ^{32}P-labeled cDNA probes. Mouse ODF/OPGL cDNA probe was cloned by reverse transcription-polymerase chain reaction (3, 4). Mouse COX-2 cDNA was purchased from Oxford Biomedical Research, Oxford, MI, USA. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was used as a control probe.

RESULTS

Effect of BMP-2 on osteoclast formation mediated by IL-1α. In cocultures of bone marrow cells and osteoblast-like cells, BMP-2 alone did not produce OCLs (Fig. 1). However, in the presence of 1 ng/ml IL-1α, BMP-2 enhanced the formation of OCLs in a dose-dependent manner (Fig. 1). The maximal effect was seen at 100 ng/ml of BMP-2 which increased the number of OCLs by 3 to 4-fold as compared with the control cultures (IL-1α alone). A preliminary study indicated that the stimulating effect of BMP-2 was similarly observed in the cultures containing higher concentrations of IL-1α (up to 10 ng/ml of IL-1α; data not shown). BMP-2 also increased the number of OCLs in a dose-dependent manner in the presence of 10 nM 1,25α $(OH)_2D_3$ (data not shown). Activin A, a member of the TGF-β superfamily, also enhanced the formation of OCLs in the presence of IL-1α (Fig. 2A), but the effect of TGF-β on OCL formation was biphasic; it enhanced

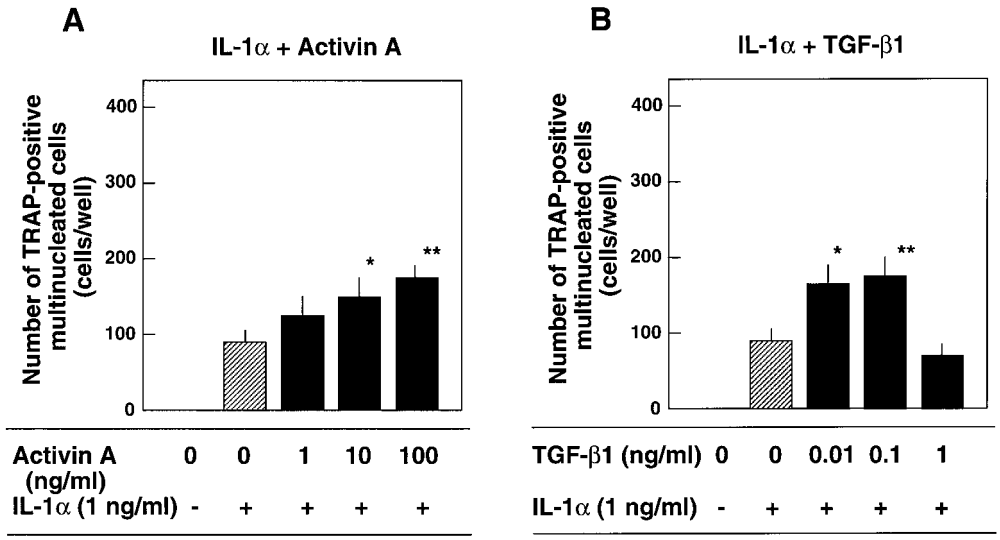

FIG. 2. Effect of activin A (A) or TGF-β1 (B) on IL-1α-mediated OCL formation in cocultures of mouse bone marrow cells and osteoblast-like cells. Bone marrow cells (1×10^6 cells per well) and osteoblasts (5×10^3 cells per well) were cocultured in 24-well culture plates. After being cultured for 8 days, the TRAP-positive multinucleated cells were counted. Data are expressed as the means $\pm$ SD of quadruplicate cultures. Significantly different from the culture of IL-1α alone; *$p < 0.05$ and **$p < 0.01$.

the OCL formation at concentrations of 0.01 and 0.1 ng/ml whereas it suppressed the OCL formation at a concentration of 1 ng/ml (Fig. 2B).

Involvement of PGE_2 in the osteoclast formation mediated by BMP-2 and IL-1α. A radioimmunoassay for PGE_2 in the culture supernatants of the coculture of bone marrow cells and osteoblast-like cells revealed that the concentration of PGE_2 increased in cultures stimulated with the combination of BMP-2 and IL-1α (data not shown). COX-2 is known as a rate limiting enzyme for the conversion of arachidonic acid to prostanoids. Thus, we examined the expression of COX-2 in osteoblast-like cells by Western blot analysis, and found a synergistical increase of COX-2 level in osteoblast-like cells treated with BMP-2 and IL-1α (Fig. 3A). The increase of COX-2 levels was transient, and the levels were reduced at 3 days after the treatments. An increase in COX-2 mRNA level was observed by Northern blot analysis as well (Fig. 3B). Treatment with BMP-2 alone caused an increase of the COX-2 expression (Fig. 3).

Indomethacin and NS-398 are selective inhibitors of COX-2, and abolish the production of PGE_2. These COX-2 inhibitors supressed the IL-1α-mediated OCL formation in the coculture system (Fig. 4). Moreover, we found that they both effectively inhibited the OCL formation mediated by the combination of BMP-2 and IL-1α (Fig. 4).

Northern blot analysis of ODF expression. It is known that ODF is the key molecule in osteoclastogenesis. Therefore, we examined the expression of the ODF gene in osteoblast-like cells treated with BMP-2, IL-1α, or BMP-2 and IL-1α by Northern blot analysis.

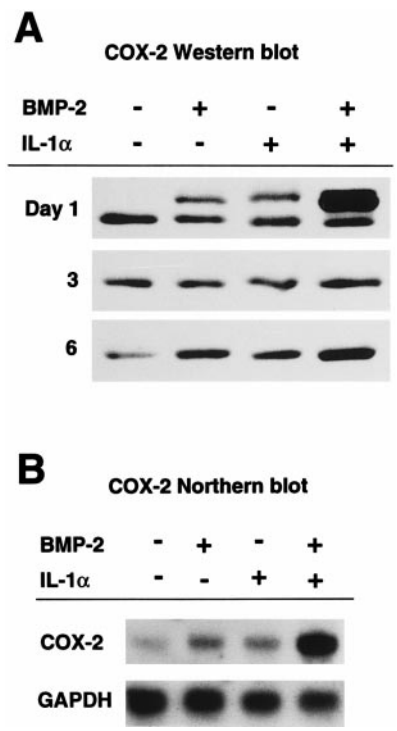

FIG. 3. Western (A) and Northern (B) blot analysis of the effect of BMP-2, IL-1α or BMP-2 and IL-1α on the expression of COX-2 in mouse osteoblast-like cells. (A) Western blot. Cells were cultured in the presence or absence of BMP-2 or IL-1α for 1, 3, or 6 days. COX-2 in cell lysates was detected by using the polyclonal anti-COX-2 antibody. (B) Northern blot. Poly(A)$^+$ RNA (2 μg) from osteoblasts cultured for 18 h in the presence of BMP-2, IL-1α, or BMP-2 and IL-1α as indicated were blotted onto the nylon membranes. Blots were probed with COX-2 or GAPDH cDNA.

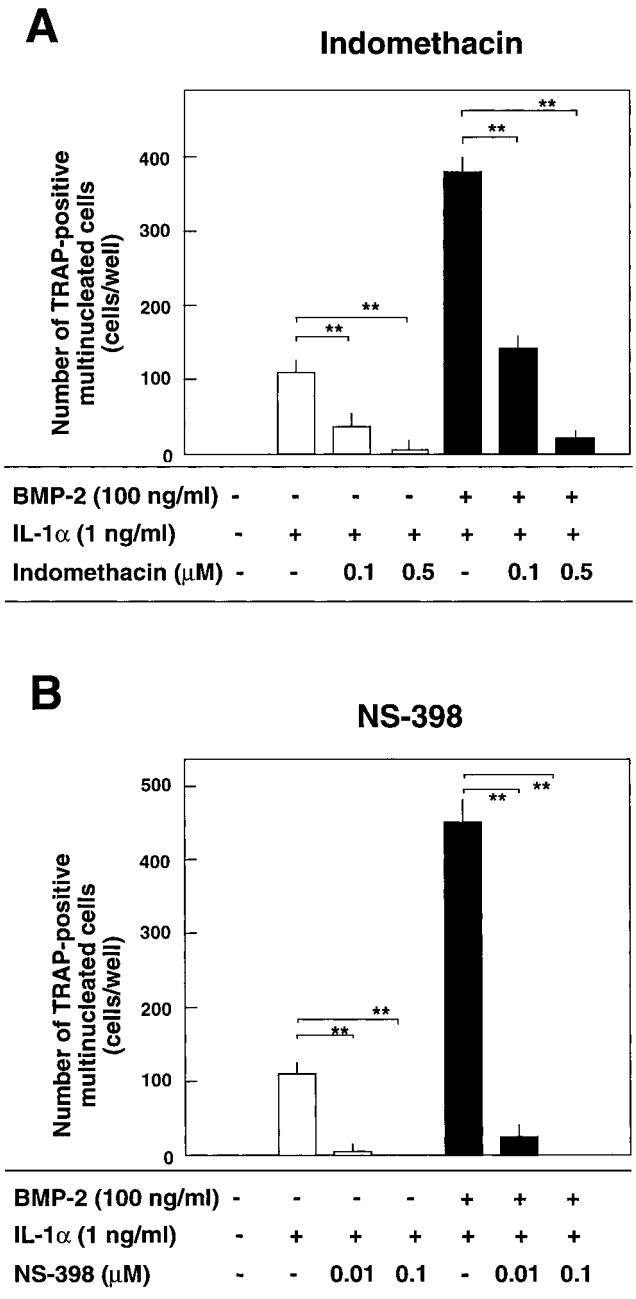

FIG. 4. Effect of indomethacin or NS-398 on OCL formation mediated by BMP-2 and IL-1α. Mouse bone marrow cells (1×10^6 cells per well) and osteoblast-like cells (5×10^3 cells per well) were cocultured with IL-1α and BMP-2 in 24-well culture plates. Indicated concentrations (μM) of indomethacin (A) or NS-398 (B) were added to the cultures. After being cultured for 8 days, the TRAP-positive multinucleated cells were counted. Data are expressed as the means $\pm$ SD of quadruplicate cultures. Significant differences between the values indicated were shown; **$p < 0.01$.

Approximately 2.4 kb of ODF mRNA was detected in osteoblast-like cells treated with BMP-2, IL-1α, or BMP-2 and IL-1α (Fig. 5A). The combination of BMP-2 and IL-1α gave the highest expression of ODF mRNA. NS-398, a COX-2 inhibitor, inhibited the upregulation of ODF mRNA (Fig. 5B).

DISCUSSION

IL-1, a potent mediator of inflammatory reactions, activates osteoclasts to resorb bone, induces bone resorption in organ culture, and induces osteoclast formation *in vitro* (1, 2, 15). Several *in vitro* and *in vivo* studies have established the potent bone-resorbing effect of IL-1, suggesting that IL-1 may be involved in the pathogenesis of bone destruction (15). In this study, we found that BMP-2, a potent bone-inducing cytokine and a member of the TGF-β superfamily, enhanced *in vitro* OCL formation in the presence of IL-1α. We also found that the amount of PGE_2 in supernatants increased in cultures stimulated with the combination of BMP-2 and IL-1α. Northern and Western blot analyses revealed that BMP-2 and IL-1α synergistically increased the COX-2 expression in osteoblast-like cells. Furthermore, OCL formation mediated by BMP-2 and IL-1α was inhibited by the addition of either indomethacin or NS-398. These results suggested that the upregulation of COX-2 levels in osteoblast-like cells may be involved in the stimulatory effect of BMP-2 in IL-1α-mediated OCL formation.

Very recently, an essential factor of osteoclast differentiation was cloned and named ODF/OPGL (3, 4). It has also been just reported that the OCL formation

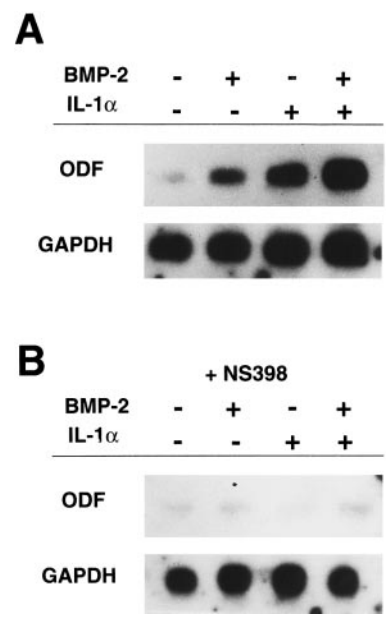

FIG. 5. Expression of ODF mRNA in primary osteoblast-like cells. Poly $(A)^+$ RNA (2 μg) from osteoblasts cultured for 18 h in the presence of BMP-2, IL-1α, or BMP-2 and IL-1α as indicated were blotted onto the nylon membranes. Blots with RNA from cells cultured in the absence (A) or in the presence (B) of NS-398 (0.1 μM) were probed with ODF or GAPDH cDNA.

mediated by PGE_2, $1\alpha,25(OH)_2D_3$, or IL-1 is inhibited by polyclonal anti-ODF antibodies, suggesting that the OCL formation mediated by these factors is through the upregulation of ODF in osteoblasts or stromal cells (16). In the present study, we found that stimulation with BMP-2 and IL-1α enhanced ODF expression in osteoblast-like cells (Fig. 5). IL-1α alone induced the ODF expression as expected, and it is interesting that BMP-2 alone did stimulate it. However, the stimulatory effect of IL-α, BMP-2 and BMP-2 and IL-1α, on the ODF expression seems to depend on the PGE_2 produced by the action of COX-2, because the ODF expression was abolished by the addition of NS-398 (Fig. 5B). In this regard, Akatsu *et al.* (17) reported that indomethacin completely inhibits IL-1-mediated *in vitro* OCL formation from bone marrow cells, suggesting that PGE_2 is involved in the mechanism of IL-1-mediated OCL formation. Our results supported their findings and indicate that the stimulatory effect of BMP-2 on OCL formation is also dependent on the PGE_2 production (Fig. 4).

It has been reported that TGF-β shows both stimulating and inhibiting effects on bone resorption in organ cultures and *in vitro* osteoclast formation (18). In this study, we found that a lower concentration of TGF-β enhanced *in vitro* OCL formation in the presence of IL-1α, while it inhibited the OCL formation in higher concentrations (Fig. 2B). Very recently, TGF-β is reported to stimulate the production of osteoclastogenesis inhibitory factor (OCIF) (or osteoprotegerin [OPG]) (19, 20) by mouse primary osteoblasts, the stromal-like cell line, ST-2, and the osteoblast-like cell line, MC3T3-E1 (21, 22). Therefore, the decrease in OCL formation in higher concentrations of TGF-β (Fig. 2B) may be due to the production of OCIF/OPG, a decoy receptor of ODF/OPGL (16, 19, 20).

Another member of the TGF-β superfamily, activin A, is reported to enhance the formation of OCLs in a dose-dependent manner either in the presence or absence of $1\alpha,25(OH)_2D_3$ or parathyroid hormone in mouse bone marrow cultures (23). Similarly, BMP-2 and BMP-7/OP-1 increase the capacity of $1\alpha,25(OH)_2D_3$ to induce OCL formation (10, 11). We also found that both BMP-2 and activin A enhanced *in vitro* OCL formation in the presence of $1\alpha,25(OH)_2D_3$ (data not shown). In these cases, the upregulation of COX-2 and enhanced production of PGE_2 may be involved in the stimulatory effect of BMP-2 or activin A on the OCL formation in the presence of $1\alpha,25(OH)_2D_3$. In fact, it is known that simultaneous stimulation with $1\alpha,25(OH)_2D_3$ and PGE_2 enhances OCL formation in bone marrow cultures (1, 2, 12).

In this study, we found that although BMP-2 alone stimulated both ODF and COX-2 expression in osteoblast-like cells, it did not induce OCL formation *in vitro*. It is difficult to explain this fact, since previous studies (10, 11) have reported that both BMP-2 alone and BMP-7/OP-1 alone are capable of inducing OCL formation from mouse hemopoietic blast cells and rat bone marrow cells, respectively (10, 11). It is possible that in the previous studies a trace amount of osteotropic factors such as IL-1 may be spontaneously produced by the bone marrow cells or hematopoietic blast cells, triggering the OCL differentiation. Moreover, further explanations may be possible: 1) In our study, the stimulatory effect of BMP-2 on osteoblasts was insufficient for OCL formation because its effect was only transient (Fig. 3A). 2) BMP-2 induces OCIF/OPG expression in osteoblasts, which should inhibit the OCL formation. It is noted that IL-1 is reported to downregulate OCIF expression (22). Thus, it would be interesting to investigate whether BMP-2 stimulates the production of OCIF/OPG in osteoblasts or stromal cells in the presence or absence of IL-1α.

Since it is reported that the osteoclast formation required not only ODF/OPGL but also M-CSF (5, 6), BMP-2 or IL-1α may also stimulate M-CSF production from osteoblasts or stromal cells. However, a Northern blot study suggested that the expression of M-CSF in osteoblast-like cells was not influenced by the addition of BMP-2 nor IL-1α (data not shown).

Although it must be noted that the osteoblast-like cells used in this study may be composed of not only osteoblastic cells at various stages of differentiation but also fibroblasts and chondrocytes (13), our preliminary study revealed that ST-2, a cell line of osteoblastic and stromal cell characters, showed essentially the same responses against BMP-2 and IL-1α as the osteoblast-like cells used in this study. ST-2 cells will be a useful cell line to elucidate the molecular mechanisms by which BMP-2 regulates the expression of ODF or COX-2 in the complicated network of various cell types in the bone microenvironment. The precise mechanism of regulation mediated by BMP-2 is now being examined in our laboratory using ST-2 cells.

In conclusion, BMP-2 stimulates osteoclast formation in the presence of IL-1α by a mechanism involving not only PGE_2 but also ODF in mouse marrow cultures. Since BMPs are potent bone inducing cytokines, they are finding clinical applications in orthopedics, dentistry, periodontology, and plastic surgery (9). However, our findings indicated that BMP-2 may enhance bone resorption instead of bone formation in inflammatory environments, suggesting that the control of inflammatory reaction in the target tissues may be one of the critical steps in clinical application of BMPs in orthopedics.

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