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Bone morphogenetic protein 2 enhances PGE₂ stimulated osteoclast formation in murine bone marrow cultures

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Abstract

Bone morphogenetic protein 2 (BMP-2) is used clinically to stimulate bone formation and accelerate fracture repair. Adding prostaglandin (PG) E₂ or PGE₂ receptor agonists to BMP-2 has been proposed to improve BMP-2 efficacy. However, this may enhance bone resorption, since PGE₂ can increase receptor activator of NF-κB ligand (RANKL) expression and decrease osteoprotegerin (OPG) expression in osteoblasts, and the RANKL: OPG ratio is critical for osteoclast formation. We used bone marrow (BM) cultures and BM macrophage (BMM) cultures from outbred CD-1 mice to examine effects on osteoclast formation of BMP-2 and PGE₂. In BM cultures, which contain both osteoblastic and osteoclastic lineage cells, BMP-2 (100 ng/ml) alone did not increase osteoclast formation but enhanced the peak response to PGE₂ by 1.6- to 9.6-fold. In BMM cultures, which must be treated with RANKL because they do not contain osteoblastic cells, BMP-2 did not increase osteoclast formation, with or without PGE₂. Our results suggest that BMP-2 can increase osteoclast formation in response to PGE₂ by increasing the RANKL:OPG ratio in osteoblasts, which may have therapeutic implications for the use of BMP-2.

Keywords

prostaglandin; cyclooxygenase 2 (COX-2); bone morphogenetic proteins; osteoclasts; mice; receptor activator of NF-kappa B ligand (RANKL); osteoprotegerin (OPG); macrophage colony-stimulating factor (M-CSF); bone marrow macrophage (BMM)

Introduction

Bone morphogenetic protein 2 (BMP-2) increases bone formation both *in vitro* and *in vivo*¹,². BMP-2 is used clinically as an alternative to bone grafting³. We have previously shown *in vitro* that BMP-2 requires cyclooxygenase (COX)-2 dependent prostaglandins (PGs) for full activity in stimulating osteoblastic differentiation². Furthermore, combining BMP-2 with a

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PGE₂ receptor agonist increases ectopic mineralization *in vivo*^{4–6}. These data suggest that PGE₂ can enhance BMP-2 anabolic activity in bone.

Changes in bone mass are due to inequalities in formation of bone by osteoblasts and resorption of bone by osteoclasts. BMP-2 and PGE₂ increase bone formation by increasing the differentiation of osteoblasts. PGE₂ also increases osteoclast formation by stimulating osteoblasts to increase expression of receptor activator of nuclear factor κ B ligand (RANKL), which, with macrophage-colony stimulating factor (M-CSF), is required for the differentiation of monocytic cells into active osteoclasts^{7,8}. In addition, PGE₂ can decrease osteoblastic expression of osteoprotegerin (OPG), a decoy receptor for RANKL, which inhibits osteoclast formation⁹.

A number of reports describe stimulatory effects of BMP-2 on osteoclast formation^{10–15}. On the other hand there are reports that BMP-2 may suppress osteoclast formation, possibly by increasing OPG expression^{16–18}. We previously found that BMP-2 increased osteoclast formation in murine bone marrow (BM) cultures from wild type but not COX-2 knockout mice². Our data also suggested that the combination of BMP-2 with PGE₂ resulted in greater osteoclast formation compared to BMP-2 or PGE₂ alone. The goal of the present study was to determine if the BMP-2 enhancement of PGE₂-stimulated osteoclast formation was due to effects of BMP-2 on RANKL and OPG expression in osteoblastic cells or due to direct effects of BMP-2 on osteoclastic precursors. We found that BMP-2 enhanced PGE₂-stimulated osteoclast formation and RANKL:OPG ratio in BM cultures, but had no stimulatory effect on osteoblast-independent osteoclast formation in BM macrophage (BMM) cultures.

Materials and Methods

Animals

CD1 mice, aged 6–12 weeks, were used in all experiments. Mice were euthanized with carbon dioxide followed by cervical dislocation. All protocols were approved by the animal care committee of the University of Connecticut Health Center.

Materials

Unless stated otherwise, all reagents were obtained from Sigma-Aldrich (St. Louis, MO). PGE₂ was purchased from Cayman Chemical (Ann Arbor, MI). Recombinant human BMP-2 was produced in *E. coli* using a maltose-binding protein (MBP)-fusion expression vector and the MBP-BMP-2 fusion protein was dimerized and cleaved, as described previously¹⁹. Consistent with our previous study, we found that 100 ng/ml BMP-2 maximally activated the COX-2 promoter in MC3T3-E1 cells². Therefore, for the present study, we selected the same dose of BMP-2 (100 ng/ml) as we had used in the previous study².

Cell culture

Whole BM for culture was obtained as previously described^{2,20,21}. Briefly, femora and tibiae were dissected free of connective tissue. Using aseptic technique, the ends of the bones were cut and BM was flushed with complete cell culture media using a 21-gauge needle. The marrow was resuspended in cell culture media. An aliquot was hemolyzed with 2% acetic acid and counted by hemocytometer. Media for flushing and for all cultures was α MEM with 10% heat-inactivated fetal calf serum (HIFCS), penicillin (100 U/ml), and streptomycin (50 μ g/ml). For BM cultures, marrow was plated at 10⁶ cells/cm² in 12-well dishes. Media and treatments were changed every 3 days. For BMM cultures, 5 $\times$ 10⁶ cells were plated in Petri dishes and expanded with M-CSF (100 ng/ml; R&D Systems, Minneapolis, MN) using the method described by Epple et al.²². When cells reached confluence, they were replated at 5000 cells/well in a 96-

well dish in media with RANKL (30 ng/ml; R&D Systems, Minneapolis, MN), M-CSF (30 ng/ml; R&D Systems) and treatments. Media were changed every 3 days.

Tartrate-resistant acid phosphatase (TRAP) stain

Osteoclasts were defined as TRAP⁺ multi-nucleated cells (MNCs). At the end of culture, cells were fixed with 2.5% glutaraldehyde for 30 minutes at room temperature and stained using a leukocyte acid phosphatase A kit (Sigma-Aldrich). Cells staining for TRAP with 3 or more nuclei were counted as osteoclasts.

Quantitative polymerase chain reaction (qPCR)

Total RNA was extracted with Trizol (Invitrogen, Carlsbad, CA) and reverse transcribed with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). qPCR was performed using TaqMan Gene Expression Assays on the Applied Biosystems ABI Prism 7300 Sequence Detection System instrument utilizing universal thermal cycling parameters. Each sample was run in duplicate and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was the endogenous control, in parallel reactions. Each primer had equal efficiency with GAPDH over a range of target gene concentrations. The $\Delta\Delta CT$ method was used to analyze the relative quantity of target mRNA.

Statistics

Data are presented as means $\pm$ SEM. All experiments were performed with 3–4 replicates. Statistical analysis was performed using two-way analysis of variance, followed by Bonferroni post-hoc test. A p-value of less than 0.05 was considered statistically significant.

Results

BMP-2 enhanced PGE₂-stimulated osteoclast formation in BM cultures

In 8 experiments, the average peak number of osteoclasts formed with BMP-2 (100 ng/ml) alone was 4.7 ± 1.6 osteoclasts/well (range: 0–12), which was slightly greater, but not significantly, than control treated cultures, which formed 1.3 ± 0.4 osteoclasts/well (range: 0–4). Similar to previous reports^{20,23}, PGE₂ (1 μ M) increased osteoclast formation compared with control in 3 of 5 experiments (average peak: 320 ± 183 ; range 12–965).

Addition of BMP-2 to PGE₂ increased PGE₂-stimulated osteoclast formation 4.5 ± 0.9 fold in 8 experiments (range: 1.6–9.6 fold; Fig 1A). The average peak osteoclast formation in 8 experiments was 543 ± 236 (range: 34–2047). Osteoclast number peaked on days 5 to 8, and there was no consistent change in the time course of osteoclast formation between PGE₂ alone and PGE₂ plus BMP-2. PGE₂ dose-dependently increased osteoclast formation at 100 nM and 1 μ M (Fig 1B). BMP-2 increased the number of osteoclasts formed at these doses but did not decrease the minimum dose of PGE₂ required to stimulate osteoclastogenesis.

To determine the period of culture during which BMP-2 had its effects, we treated BM cultures with PGE₂ (1 μ M) for 9 days and added BMP-2 (100 ng/ml) for days 3–9, days 6–9, or continuously during cell culture. Osteoclast formation peaked on day 7 for all groups (data not shown). Comparison of groups on day 7 is shown in Fig 2A. BMP-2 added to PGE₂ increased PGE₂-stimulated osteoclast formation similarly when added from day 0–7 or from day 3–7. However, BMP-2 added from day 6–7 had no effect on peak PGE₂-stimulated osteoclast formation.

We also cultured BM for 7 days in basal media before beginning treatment with PGE₂ (1 μ M), BMP-2 (100 ng/ml), or the combination. Using this acute, delayed treatment protocol, we found that PGE₂ stimulated osteoclast formation within 48 hours of treatment (Fig 2B). Treatment

with BMP-2 alone did not stimulate osteoclast formation. There was no significant difference between treatment with PGE₂ alone and BMP-2 and PGE₂. Together, these results suggest that BMP-2 acts early in culture to increase osteoclast formation.

BMP-2 did not increase osteoclast formation in BMM cultures

It is possible that BMP-2 increases osteoclast formation by increasing the number of osteoclast precursors or the ability of the precursors to fully differentiate. To test for effects of BMP-2 on osteoclast precursors in the absence of osteoblasts, we stimulated osteoclast formation in BMM cultures with RANKL (30 ng/ml) and M-CSF (30 ng/ml) and treated with BMP-2 (100 ng/ml), PGE₂ (1 μM) or vehicle for 9 days (Fig 3). There were 2 waves of osteoclast formation. PGE₂ inhibited osteoclast formation during the first wave, but not the second. Treatment with BMP-2 did not increase osteoclast formation, either alone or in the presence of PGE₂, suggesting that BMP-2 is not acting on osteoclast precursors directly to increase osteoclast formation. Similarly, in spleen cell cultures, which also form osteoclasts with RANKL and M-CSF treatment, BMP-2 had no effect on the number of osteoclasts formed²⁴.

Effect of BMP-2 and PGE₂ on regulators of osteoclast formation

BM cultures contain precursors not only for osteoclasts but also for osteoblasts. Cells of the osteoblast lineage can express RANKL, OPG, and M-CSF, all of which regulate osteoclast differentiation. We measured the effects of BMP-2 and PGE₂ on the expression of RANKL and OPG mRNA in BM cultures on days 4–6, prior to peak osteoclast formation, consistent with the early effect of BMP-2 (Fig 4). BMP-2 (100 ng/ml) alone had no effect on RANKL mRNA. Although BMP-2 decreased OPG mRNA 32–54% compared with control, the RANKL:OPG ratio was not significantly increased. Treatment with PGE₂ (1 μM) both increased RANKL mRNA and decreased OPG mRNA, compared with control, resulting in an increased RANKL to OPG ratio. BMP-2 in combination with PGE₂ resulted in small, non-significant increases in RANKL and decreases in OPG, compared with PGE₂ alone. However, the RANKL to OPG ratio was increased by BMP-2 and PGE₂ compared with PGE₂ alone by 64±29% on day 6 in the experiment shown in Fig. 4. In a second experiment, the RANKL to OPG ratio was increased 64±8% on day 5.

BMP-2 (100 ng/ml) had no effect on M-CSF mRNA compared with control (Fig 5). PGE₂ (1 μM) increased M-CSF mRNA. Treatment with the combination of BMP-2 and PGE₂ had no further effect on M-CSF mRNA.

Discussion

We and others have previously reported that BMP-2 alone increased osteoclast formation^{2, 10}. However, in the current study, BMP-2 alone had no significant effect on osteoclast formation in murine BM cultures. It is possible that differences in the effects of BMP-2 on osteoclast formation are due to differences in mouse strains used in the experiments. In this study, we used outbred CD1 mice, whereas in our previous study, the mice were in a mixed C57Bl/6 × 129/sv background. BMP-2 treatment did not increase osteoclast formation in BMM cultures stimulated with RANKL and M-CSF but did increase the RANKL to OPG ratio in PGE₂-treated BM cultures. These results suggest that the major mechanism by which BMP-2 increases osteoclast formation is through an interaction with PGE₂ in cells of the osteoblast lineage.

It is possible that the predominant role of BMP-2 in osteoclast formation is to enhance the effects of other osteoclastogenic stimuli. Similar to our results, BMP-2 has been reported to enhance interleukin 1 (IL-1), 1,25-dihydroxyvitamin D, and parathyroid hormone (PTH) stimulated formation of osteoclasts^{10–12,14}. The BMP-2 enhancement of IL-1 stimulated

osteoclastogenesis was associated with increased COX-2 and inhibited by a COX-2 selective inhibitor, NS-398. Since 1,25-dihydroxyvitamin D and PTH can stimulate COX-2 expression and PG synthesis, it is possible that BMP-2 enhancement of osteoclast formation by these hormones is also mediated in part by PGs²⁵.

Others have found a variable effect of BMP-2 on RANKL-dependent osteoclast formation^{13, 17,26,27}. In our model of osteoclast precursors, BMMs, we found no effect of BMP-2 on RANKL stimulated osteoclast formation. Treatment with RANKL and M-CSF stimulated osteoclast formation with peaks at two different time points. Multiple peaks have been described in other models of osteoclast formation²⁸. PGE₂ inhibited osteoclast formation at the first peak and later had no effect. We have observed similar biphasic effects of PGE₂ on osteoclast formation in spleen cells treated with M-CSF and RANKL²⁹. These results suggest that data obtained at one time point in osteoclast cultures can be misleading and that it is essential to perform a time course.

BMP-2 had small, non-significant effects on RANKL and OPG mRNA in the presence of PGE₂, but these effects combined to increase the RANKL:OPG ratio compared with PGE₂ alone. Koide, et. al., reported that in co-cultures of primary calvarial osteoblasts and BM, BMP-2 and IL-1 individually increased RANKL mRNA and together there was a further increase in RANKL mRNA, which was dependent on endogenous PGs¹¹. BMP-2 has also been reported to increase RANKL expression in C2C12 myoblastic cell and chondrocytes^{30,31}. However, we found no effect of BMP-2 alone on RANKL expression. BMP-2 has been reported to increase expression of M-CSF in C2C12 myoblastic cells^{15,16}. However there was no change in M-CSF mRNA in our BM cultures with BMP-2 treatment. As our effect on osteoclast formation is large and the effect on RANKL and OPG is modest, it is possible that BMP-2 also acts on other osteoblastic factors, such as ITAM-stimulated signaling³².

BMP-2 has been reported to increase OPG expression. Treatment of immortalized human fetal osteoblasts with BMP-2 increased OPG mRNA and protein, but treatment of other human cell lines with BMP-2 did not have any effect on OPG mRNA³³. Additionally, BMP-2 has been found to increase the expression of OPG in the mesenchymal cell line C3H10T1/2 and the myoblastic cell line C2C12^{16,18}. In contrast, we observed a small decrease in OPG mRNA with BMP-2 treatment alone on day 5 and 6, but this effect was not sufficient to alter the RANKL:OPG ratio. It is likely that BMP-2 effects on OPG expression depends the cell type or culture conditions.

BMP-2 could otherwise change the phenotype of osteoblastic cells. This would be consistent with our observation that BMP-2 acts early in culture to enhance PGE₂-stimulated osteoclast formation. This may also account for the variability of published observation of the effect of BMP-2 on OPG expression.

In conclusion, we found that while BMP-2 alone had minimal effects on osteoclast formation, it enhanced osteoclast formation that was stimulated by PGE₂. Thus, endogenous and exogenous PGs likely increase both osteoblast and osteoclast differentiation in response to BMP-2. Modulating PGs may have important implications in BMP-2 based therapies. For example, in spinal fusion, resorption would likely be deleterious and co-administration of BMP-2 with a COX inhibitor might improve the outcome^{34–36}. On the other hand, treatment with PGE₂ agonists might enhance the response to or decrease the dose of BMP-2 required for conditions in which both bone formation and resorption are critical, such as fracture repair or distraction osteogenesis. *In vivo* inhibition of PGs may have deleterious effects, as we have found that BMP-2 has a diminished capacity to mineralize collagen pellets implanted in COX-2 knockout mice². Future *in vivo* studies of the modulatory effect of PGs on BMP-2 induced bone formation and resorption could help to tailor BMP-2 therapy for improved outcomes.

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Figure 1.

Effect of PGE₂ or the combination of BMP-2 and PGE₂ on osteoclast formation in BM cultures. A) Cultures treated with PGE₂ (1 μ M), or PGE₂ (1 μ M) and BMP-2 (100 ng/ml). Osteoclasts per well were counted on the indicated days. B) Osteoclast numbers in cultures treated with increasing doses of PGE₂ alone or in combination with BMP-2 (100 ng/ml) for 5 days. Data are presented as mean $\pm$ SEM for 3 wells/group. ^aSignificantly different from PGE₂ alone, $p < 0.05$.

Figure 2.

Timing of BMP-2 treatment on enhancement of osteoclast formation stimulated by PGE₂. A) Osteoclast number on day 7 of culture. Cells were treated throughout with PGE₂ (1 μ M). BMP-2 (100 ng/ml) was added on day 0, 3, or 6 of the culture period. B) Acute effects of BMP-2. BM was cultured for 7 days before adding BMP-2 (100 ng/ml), PGE₂ (1 μ M), or the combination. Osteoclasts were counted 48 hours after treatment. Data are presented as mean $\pm$ SEM for 3 wells/group. ^aSignificantly different from PGE₂ alone, $p < 0.05$.

Figure 3.

Effect of BMP-2 on RANKL and M-CSF stimulated osteoclast formation in BMMs. BMM cultures were stimulated to form osteoclasts with RANKL (30 ng/ml) and M-CSF (30 ng/ml), in the presence of BMP-2 (100 ng/ml), PGE₂ (1 μ M), or the combination. Osteoclasts per well were counted on the indicated days. Data are presented as mean $\pm$ SEM for 4 wells/group.

Figure 4.

Effect of BMP-2, PGE₂, or the combination on RANKL and OPG mRNA expression. BM cultures were treated with BMP-2 (100 ng/ml), PGE₂ (1 μM), or the combination. On the indicated days, total RNA was extracted and the mRNA was reverse transcribed. mRNA levels of A) RANKL and B) OPG measured by qPCR. C) RANKL:OPG ratios calculated from the mRNA expression data. Data are presented as mean ± SEM for 3 samples/group. ^aSignificantly different from control, *p* < 0.05. ^bSignificantly different from BMP-2 alone, *p* < 0.05. ^cSignificantly different from PGE₂ alone, *p* < 0.05.

Figure 5.

Effect of BMP-2, PGE₂, or the combination on M-CSF mRNA expression. BM cultures were treated with BMP-2 (100 ng/ml), PGE₂ (1 μ M), or the combination. On the indicated days, total RNA was extracted and the mRNA was reverse transcribed. mRNA levels of M-CSF were measured by qPCR. Data are presented as mean $\pm$ SEM for 3 samples/group. ^aSignificantly different from control, $p < 0.05$. ^bSignificantly different from BMP-2 alone, $p < 0.05$.