Osteoclastogenesis in the Nonadherent Cell Population of Human Bone Marrow Is Inhibited by rhBMP-2 Alone or Together with rhVEGF

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ABSTRACT: During bone development and repair, angiogenesis, osteogenesis, and bone remodeling are closely associated processes that share some common mediators. In the present study nonadherent human bone marrow mononuclear cells under the induction of sRANKL and M-CSF, differentiated into osteoclasts with TRAP-positive staining, VNR expression, and Ca-P resorptive activity. The effects of various combinations of rhBMP-2 (0, 3, 30, and 300 ng/mL) and rhVEGF (0 and 25 ng/mL) on osteoclastogenesis potentials were examined in this experimental system. The percentages of TRAP-positive multiple nucleated cells represent osteoclast differentiation potential, and the percentages of resorptive areas in the Ca-P coated plates resemble osteoclast resorption capability. The presence of rhBMP-2 at 30 and 300 ng/mL showed inhibitory effects on osteoclast differentiation and their resorptive capability in the human osteoclast culture system. rhVEGF (25 ng/mL) enhanced the resorptive function of osteoclast whenever it was used alone or combined with 3 ng/mL rhBMP-2. However, rhVEGF-induced resorptive function was inhibited by 30 ng/mL and 300 ng/mL rhBMP-2 in a dose-dependent manner. Statistical analysis demonstrated that an interactive effect exists between rhBMP-2 and rhVEGF on human osteoclastogenesis. These findings suggested that an interactive regulation may exist between BMPs and VEGF signaling pathways during osteoclastogenesis; exact mechanisms are yet to be elucidated. © 2005 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 24:29–36, 2006

Keywords: BMP-2; VEGF; osteoclastogenesis; human bone marrow; nonadherent cell

INTRODUCTION

During bone development and repair, angiogenesis, osteogenesis, and bone resorption are closely associated processes that share common mediators. Bone morphogenic proteins (BMPs), vascular endothelial growth factor (VEGF), and other cytokines released from bone matrices during bone resorption are candidates for these common mediators. It is well established that both receptor activator of nuclear factor-κB ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) are two essential factors produced by osteoblasts that promote osteoclast differentiation, and the soluble decoy receptor for RANKL, osteoprotegerin (OPG), produced by osteoblasts inhibits osteoclast differentiation.[1–8] Recently, BMP-2, a potent osteogenic factor, and VEGF, the most important mediator of angiogenesis, were shown to have involved in the regulation of osteoclastogenesis.[9–11] Itoh et al.[9] reported that BMP-2 directly enhanced osteoclastic differentiation in mouse bone marrow macrophage cultures treated with RANKL and M-CSF. It was also shown that rhVEGF caused a dose- and time-dependent increase in bone resorption in vitro and in vivo.[10,11] Two distinct VEGF receptors, KDR/Flk-1 and Flt-1, were detectable in osteoclasts at the gene and protein levels, and VEGF induced tyrosine phosphorylation of proteins in osteoclasts.[10] Thus, mediator for osteogenesis such as

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BMP-2 and for angiogenesis such as VEGF together with RANKL, M-CSF, and OPG, may regulate osteoclastogenesis.[1,5–7,12,13] We hypothesize that BMP-2 and VEGF may play a regulatory role in human osteoclastogeneis.

The aims of this study were, therefore, to investigate the effects of recombinant human BMP-2 (rhBMP-2) and recombinant human VEGF (rhVEGF) alone or in combination on human osteoclast differentiation and bone resorptive function in vitro. In this study, human nonadherent bone marrow mononuclear cells were employed as a source of osteoclast progenitors; the effects of various concentrations of rhBMP-2 and rhVEGF in combination on osteoclastic differentiation and bone resorption potentials were examined and compared.

MATERIALS AND METHODS

Reagents and Materials

Recombinant human BMP-2, VEGF, M-CSF, and soluble RANKL were purchased from PeproTech EC Ltd (Pepro Tech, London, UK). The basal culture medium was prepared with alpha minimal essential medium (α-MEM) (Gibco, UK), containing 15% fetal bovine serum (Gibco, UK), L-glutamine (2 mM), penicillin 100 IU/mL, and streptomycin 100 mg/mL. α-MEM alone or phosphate-buffered solution (PBS) were used for cell isolation. Other reagents and chemicals used were of analytical grade. Twenty-four-well calcium phosphate (Ca-P)-coated plates were purchased from OCT USA, Inc. (Invine, CA). Monoclonal mouse antihuman CD51 was purchased from Novocastra Laboratories Ltd (Newcastle upon Tyne, UK), and monoclonal mouse antihuman CD14 was from DakoCytomation (DakoCytomation Denmark A/S, Glostrup, Denmark).

Induction of Human Osteoclasts from Nonadherent Bone Marrow Mononuclear Cells

Mononuclear cells were isolated by LymphoPrep density gradient centrifugation from bone marrow washouts in bone samples from patients who underwent total hip replacement. The sample harvest was approved by the institute regulation on organ and tissue collection, and consented by the patients. Mononuclear cells were plated at a density of $1 \times 10^6/cm^2$ in T-75 flasks with basal culture media. The first media change was made at days 5–7, when the nonadherent cells were collected from the withdrawn media by centrifugation, and replated in separate flasks. The floating cells in the replated flasks were harvested and replated again 24 h later in a similar fashion. The nonadherent mononuclear cells were further cultured in 24-well plates with or without Ca-P coating, with osteoclast-inducing media (OC media) containing sRANKL 30 ng/mL and M-CSF 30 ng/mL, the media were changed every 4 days thereafter.

Identification of Osteoclastic Cells

Osteoclastic cell phenotype was defined by cytochemical staining for osteoclast-associated enzyme tartrate resistant acid phosphatase (TRAP) activity as previously described[14] with a leukocyte acid phosphatase kit (Sigma Diagnostics Inc, Poole, UK). TRAP-positive multiple nucleated cells (nuclei $\geq$3) were considered as osteoclasts. Osteoclasts were also confirmed by positive immunocytochemical staining for CD51, the vitronectin receptor (VNR), and negative immunostaining for CD14 (marker for human monocytes and micropphages) using indirect two-step immunostaining methods.[15,16]

Osteoclast Resorption Assay

The resorptive pit/area assay is employed as the functional assay of osteoclast bone resorption capability. In brief, cells were cultured under various media in 24-well Ca-P coated plates for 18 days; the plates were then washed in PBS and fixed in 10% formalin (pH 7.2), stained for TRAP. Six digital photographs (×200 magnification) of randomly chosen fields of each well were then taken under light microscopy and saved in a TIFF file. The six randomly selected fields covered approximately 1% of the total Ca-P coated area in each well. Numbers of total TRAP-positive cells and TRAP-positive multiple nuclei cells (nuclei $\geq$3) (MNCs) were counted in the six randomly chosen fields of each well, and the percentage of TRAP-positive MNCs was expressed as the number of TRAP-positive MNCs divided by the total number of TRAP-positive cells. The resorptive areas on the Ca-P–coated plates were easily recognized under microscopic photographs; the resorptive areas (pixel density) and the total Ca-P coated area per field in the six randomly selected fields were measured using the Sicon Image (Sicon Image, NIH), and the percentage of the resorptive areas of the total Ca-P coated area in the six randomly selected fields was calculated as pixel densities of the resorptive areas divided by the total pixel densities of the selected fields. Our preliminary experiments had confirmed that the data generated by the measurement methods in this study were comparable and reproducible by three independent observers.

Effects of Combination of rhBMP2 and rhVEGF on Osteoclastogenesis

After 4 days culture in OC media, rhBMP-2 (0, 3, 30, and 300 ng/mL) and rhVEGF (0 and 25 ng/mL) were

added respectively or in combination to the 24-well plastic plates or Ca-P–coated plates, cultured for a further 14 days, with medium changed every 4 days. The combination of various concentrations of rhBMP2 and rhVEGF were divided into eight groups; three wells were assigned in each group: (1) OC media; (2) OC media + rhBMP2 (3 ng/mL); (3) OC media + rhBMP2 (30 ng/mL); (4) OC media + rhBMP2 (300 ng/mL); (5) OC media + rhVEGF (25 ng/mL); (6) OC media + rhBMP2 (3 ng/mL) + rhVEGF (25 ng/mL); (7) OC media + rhBMP2 (30 ng/mL) + rhVEGF (25 ng/mL); (8) OC media + rhBMP2 (300 ng/mL) + rhVEGF (25 ng/mL).

By the end of experiment, the plates were prepared and stained for TRAP, and the percentage of TRAP-positive MNCs as opposed to total TRAP-positive cells and the percentage of resorptive areas as opposed to total Ca-P–coated areas in the six randomly chosen fields of each well, three wells per groups were calculated as described above. All the experiments were repeated three times in triplicate. Two independent observers performed all the measurements. Data were pooled and presented as mean ± SD.

Statistical Analysis

Statistical tests were performed using statistical package SPSS11.0. Because the experiment was a 2×4 factorial design in a randomized order, two-way analysis of variance (ANOVA) was first used and then followed with a one-way ANOVA that included the Student–Newman–Keuls (SNK) test on the eight treatment combinations. The interactive effects between the two factors of rhBMP-2 and rhVEGF were also statistically analyzed. Difference was considered significant at $p < 0.05$.

RESULTS

When cultured in osteoclast-inducing media for 10–14 days, the nonadherent human bone marrow mononuclear cell population gave rise to many TRAP-positive cells containing many multiple nucleated cells (Fig. 1A and B), while rare TRAP-positive multiple nucleated cells were seen under the basal culture media (Fig. 1C).

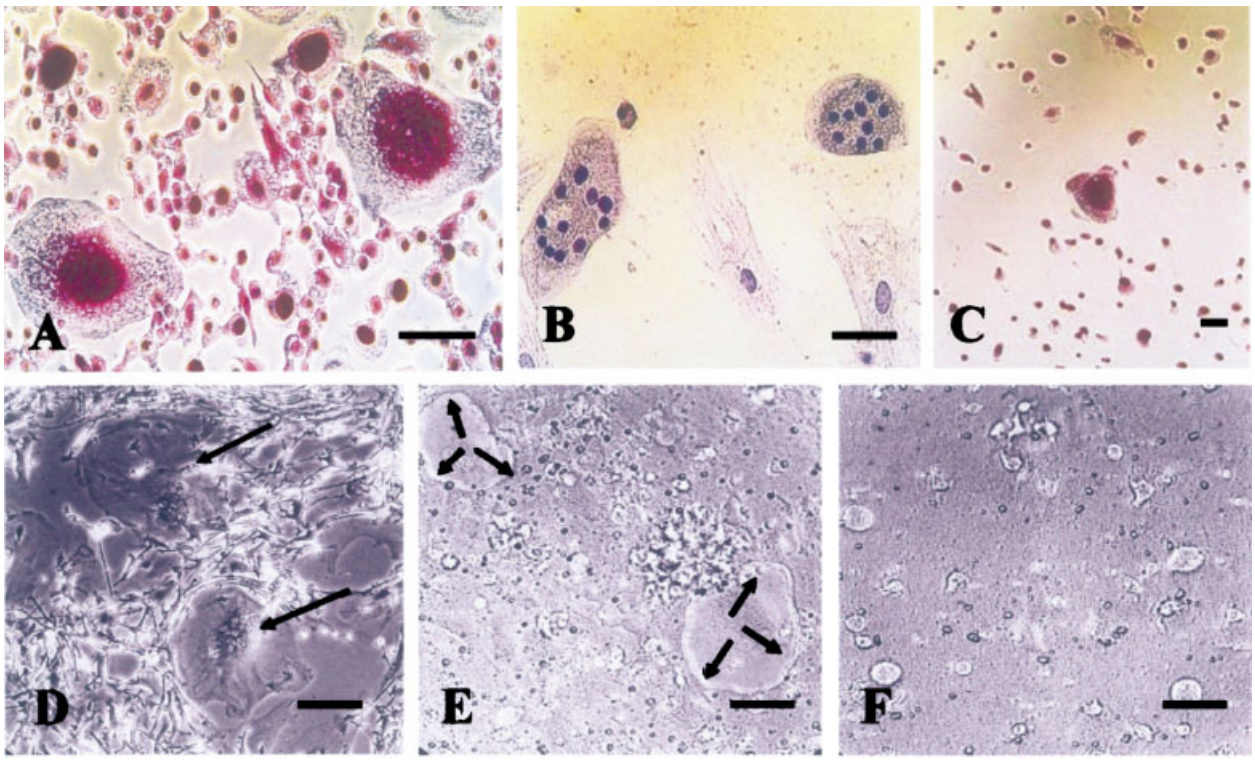

Figure 1. (A, B) TRAP-positive multiple nucleated cells (MNCs) were frequently in culture with OC media. (C) Rare TRAP-positive MNCs were found in culture with basal media. (D) MNCs were seen in close contact with fibloblastic stromal cells in the culture, suggesting that cell–cell interactions are needed for supporting osteoclasts development. (E) Resorptive areas (empty holes highlighted by arrows) on Ca-P coated plates appeared after 12 days culture in the OC media. (F) No or rare resorptive areas were found in the culture with basal media. (A–F) bar = 50 μm. [Color scheme can be viewed in the online issue, which is available at http://www.interscience.wiley.com]

The osteoclastic cells were surrounded by mesenchymal stromal cells in the culture (Fig. 1D), and were positive for VNR staining and negative for CD14 and CD34 (data not shown). Resorptive areas on Ca-P–coated plates appeared after 12 days in culture with the OC media (Fig. 1E), while no or little resorptive areas found in the culture with basal culture media (Fig. 1F).

TRAP-positive multiple nucleated cells (MNCs) were formed in all groups cultured with sRANKL (30 ng/mL) and M-CSF (30 ng/mL), but the percentages of TRAP-positive MNCs and resorptive areas between groups were significantly different. The greatest number of TRAP-positive MNCs and greatest percentage of resorptive areas were both seen in Group 6 (Fig. 2F), followed by Group 5 (Fig. 2E), Group 2 (Fig. 2B), Group 1 (Fig. 2A), and then Group 4 (Fig. 2D) and the lowest were Groups 3 (Fig. 2C), 7 (Fig. 2G) and 8 (Fig. 2H).

Statistical analysis using the SNK procedure for TRAP-positive MNCs percentages resulted in four homogeneous subsets, with the ascendant order as Group 8 < Groups 3, 4, 7 < Groups 1, 2, 5 < Group 6 (Fig. 3A). There was no statistical difference between groups within the same subset, while the percentage of TRAP-positive MNCs in different subset groups was significantly different ($p < 0.05$). The interactive effects of rhBMP-2 and rhVEGF on TRAP-positive MNCs percentage were statistically significant ($p < 0.05$). For resorptive area assay statistical analysis, the SNK procedure also gave four homogeneous subsets, with the ascendant order as Groups 3, 7, 8 < Group 4 < Groups 1, 2 < Groups 5, 6 (Fig. 3B). Again, there was no statistical difference between groups within the same subset, while the resorptive areas in different subset groups was significantly different ($p < 0.05$). The interactive effects of rhBMP-2 and rhVEGF on resorptive area formation was highly statistically significant with

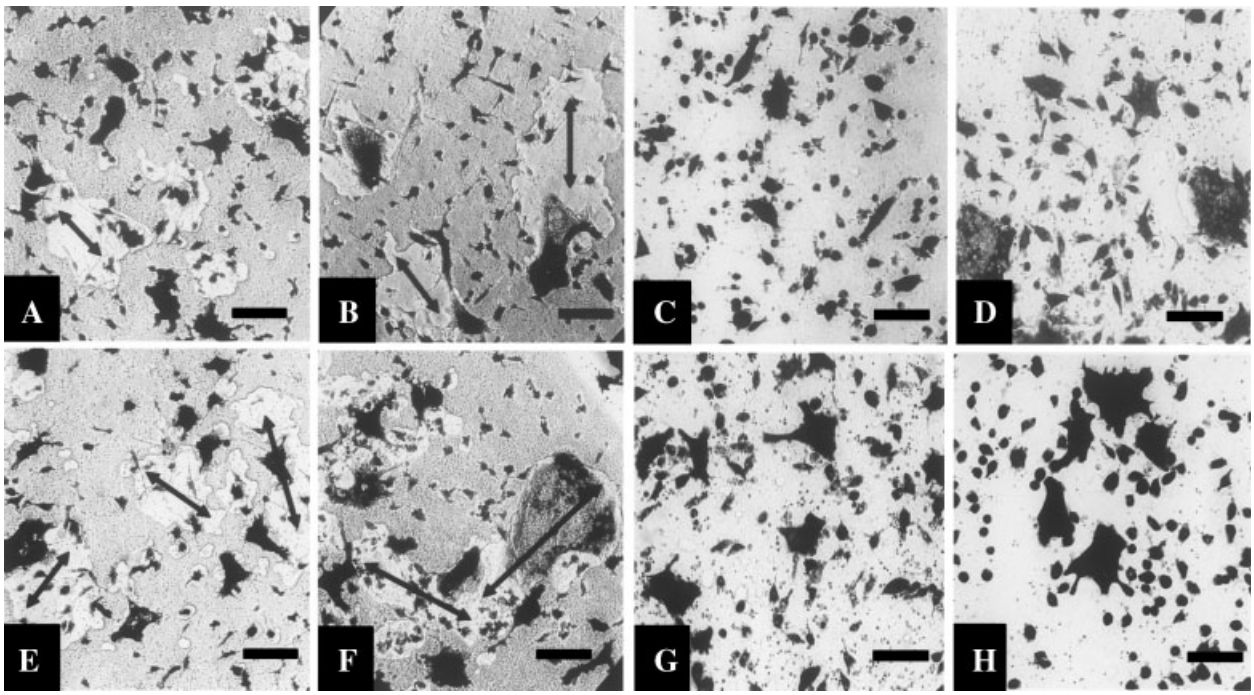

Figure 2. Representative images from each of the group show the TRAP-positive MNCs and the resorptive areas (arrow). The largest resorptive areas percentage and TRAP-positive MNCs were seen in the order of Group(s) $6 \geq 5 > 1$, $2 > 4 \geq 3$, $7 \geq 8$. (A) Group 1, OC media alone. (B) Group 2, OC media + rhBMP-2 (3 ng/mL). (C) Group 3, OC media + rhBMP-2 (30 ng/mL). (D) Group 4, OC media + rhBMP-2 (300 ng/mL). (E) Group 5, OC media + rhVEGF (25 ng/mL). (F) Group 6, OC media + rhBMP-2 (3 ng/mL) + rhVEGF (25 ng/mL). (G) Group 7, OC media + rhBMP-2 (30 ng/mL) + rhVEGF (25 ng/mL). (H) Group 8, OC media + rhBMP-2 (300 ng/mL) + rhVEGF (25 ng/mL). (A–H) bar = 50 μm.

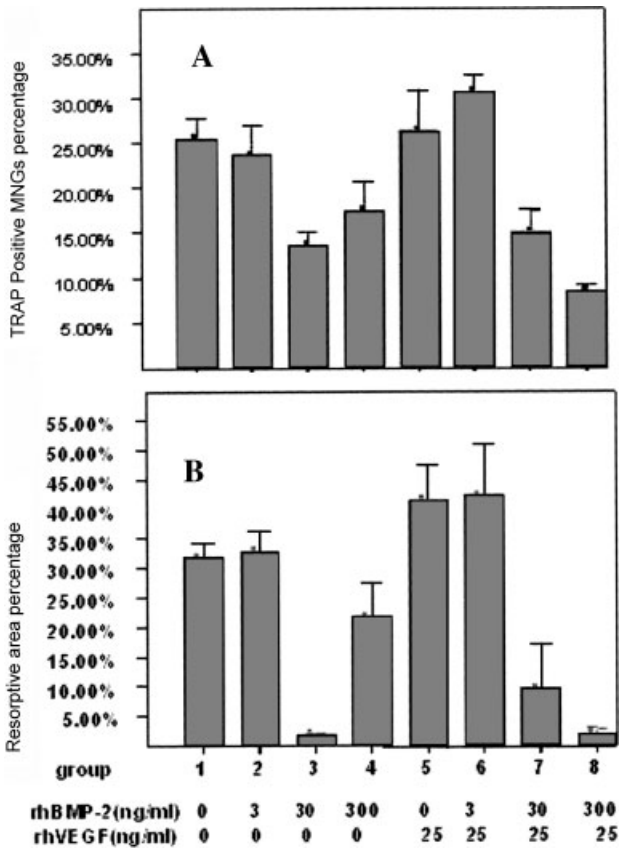

Figure 3. (A) The number of TRAP-positive MNCs in different treatment groups was shown in the order as Group(s) $8 < 3, 4, 7 < 1, 2, 5 < 6$. (B) The percentage of resorptive area on the Ca-P coated plates in different treatment groups was shown in the order as Group(s) $3, 7, 8 < 4 < 1, 2 < 5, 6$. There was no statistical difference between groups within the same subset, while percentage of the TRAP-positive MNCs and the resorptive pit area between each subset was significantly different ($p < 0.05$). All experiments were repeated three times in triplicate and the data were pooled, bars represent mean $\pm$ 1.0 SD of the percentages.

$p = 3.30 \times 10^{-7}$, suggesting the effect of rhBMP-2 on the resorptive area formation at one VEGF level (0 ng/mL) was significantly different from its effect at the other rhVEGF level (25 ng/mL). When rhVEGF (25 ng/mL) was used alone, it had enhancing effects on osteoclastogenesis, with significantly higher resorptive areas in Group 5 compared to Group 1 ($p < 0.05$).

When rhBMP-2 was used alone in the culture system, the number of TRAP-positive MNCs and the resorptive areas percentage in Groups 3 and 4 were lower than those in Groups 1 and 2 ($p < 0.05$), indicating that 30 ng/mL and 300 ng/mL rhBMP-2 had the inhibitory effects on osteoclastic differentiation and their bone resorption activity, but the inhibitory effect of rhBMP-2 on osteoclastogenesis was not dose dependent because 30 ng/mL rhBMP-2 rather than 300 ng/mL caused the stronger inhibitory effect ($p < 0.05$). Interestingly, the low dose of rhBMP-2 (3 ng/mL) in the presence of rhVEGF enhanced osteoclastic differentiation with significantly higher percentages of TRAP-positive MNCs and resorptive areas in Group 6 compared to Group 2 [$p < 0.05$, Fig. 3A and B]. In contrast to the BMP-2-alone groups (Groups 3 and 4), 30 ng/mL (Group 7) and 300 ng/mL (Group 8) rhBMP-2 in the presence of rhVEGF (25 ng/mL) had significant inhibitory effects on osteoclastic differentiation and bone resorptive function ($p < 0.05$) in a dose-dependent manner (Fig. 3A and B).

DISCUSSION

During bone development and repair, osteogenesis and osteoclastogenesis are closely coupled events. It is well documented that many cytokines or growth factors including BMPs, transforming growth factor-beta (TGF-β), VEGF, fibroblast growth factor (FGF), platelet-derived growth factor (PDGF),[17,18] and interleukins such as IL-1, IL-4, IL-6, and IL-11[19,20] are released from bone cells or matrices during bone formation or resorption, some of which regulate osteobast and osteoclast functions. It is believed that bone-resorbing osteoclasts are derived from hematopoietic progenitors of the monocyte-macrophage lineage. Soluble RANKL, together with M-CSF, induced osteoclast differentiation from mouse hematopoietic cells and human peripheral blood mononuclear cells in the absence of osteoblasts/stromal cells.[2–4,8,21,23] In the present study, the nonadherent human marrow mononuclear cell population was replated and induced to differentiate into osteoclastic cells under osteoclast-inducing media containing sRANKL (30 ng/mL) and M-CSF (30 ng/mL), which may be a useful alternative of harvesting osteoclastic progenitors from the peripheral blood mononuclear cells. By day 12, multiple nuclei cells were found in all the experimental groups, with positive TRAP staining, VNR expression, and Ca-P resorptive activity. When the different combinations of rhBMP-2 and rhVEGF on human osteoclastogenesis were examined, the presence of rhBMP-2 at 30 and 300 ng/mL had inhibitory effects on human osteoclast differentiation and their resorptive

capability with or without rhVEGF (25 ng/mL). An interactive effect existed between rhBMP-2 and rhVEGF on human osteoclastogenesis, suggesting that a crosscommunication exists between VEGF and BMPs signaling pathways in regulating osteoclastogenesis.

BMPs were first identified as potent bone-forming factors that induce ectopic bone formation in vivo.[22] The effects of BMPs on bone formation have been well documented; however, their effects on osteoclastogenesis, which is controlled by osteoblasts via the OPG-RANKL-RANK system,[13,23] have not been extensively investigated. In vitro, a serial of osteoclast culture systems have been established to investigate the effects of cytokins and other factors on osteoclast differentiation, and a combination of osteoclast differentiation factor and M-CSF is sufficient to stimulate osteoclast formation from human peripheral blood monocytes and mouse spleen cells.[10] Recently, Itoh et al.[9] reported that BMP-2 directly enhanced mice osteoclastic differentiation of the progenitor cells in bone marrow macrophage cultures treated with sRANKL and M-CSF, and both bone marrow macrophages and purified mature osteoclasts expressed BMPR-IA mRNA. Osteoclast formation induced by sRANKL and BMP-2 was suppressed by the addition of soluble BMPR-IA. Osteoclast formation was also inhibited by soluble BMPR-IA even in the absence of exogenous BMP-2. Kanatani et al.[24] first demonstrated that BMPR-IA mRNA was expressed in hematopoietic blast cells supported by GM-CSF. Kaneko et al.[25] reported that mature rabbit osteoclasts expressed BMP receptors, and BMP-2 directly stimulated their pit-forming activity even in the absence of exogenous RANKL. Koide et al.[26] reported that the combination of BMP-2 and IL-1α improve osteoclast differentiation by upregulating the expression of both cyclooxygenase-2 and RANKL mRNAs in osteoblasts. Abe et al.[27] showed that BMP-2 increased osteoclast formation by four to sixfold in PTH-treated bone marrow cultures that contained both hematopoietic and osteoblastic stromal cells. These findings suggested that BMP-mediated signals probably via their receptors were involved in osteoblastic bone formation as well as in osteoclastic bone resorption, although the exact mechanisms remain unclear. A recent study revealed BMP-2 inhibited the activation of NF-κB induced by RANKL in purified osteoclasts,[9] suggesting that BMPs-mediated signals cross-communicate with RANKL-mediated ones in osteoclastogenesis. In the present human osteoclast culture system, the presence of rhBMP-2 at 30 and 300 ng/mL but not 3 ng/mL had inhibitory effects on osteoclast differentiation and their resorptive capability, supporting the fact that BMP-2 is mainly an anabolic agent that promotes osteogenesis and inhibits bone resorption. The difference between our findings (human osteoclast) and previous findings (mouse osteoclast) may be due to the use of different osteoclast culture systems, the dose difference of BMP2 and the different species, as BMP-2 has a difference in dose response in different species. In addition, the cell culture system used in this study was a system with mixed cells; cells responding to VEGF and BMP-2 will include mesenchymal stromal/stem cells (MSCs), as demonstrated in Figure 1(D), so that effects noted on osteoclast differentiation may include secondary differentiation through cell–cell contact between OC progenitors and MSCs and secondary cytokines/growth factors released by MSCs in response to rhVEGF and rhBMP-2 stimuli. However, these effects are not possible to rule out or single out in the current experimental system. Despite these differences, these findings all suggest that BMP/VEGF-mediated signals, probably through their receptors, involved in regulating osteoclastogenesis.

VEGF is a specific mitogen for endothelial cells from vascular, and the most important mediator of angiogenesis.[28] It is well known that vascular invasion plays a key role in bone formation during bone development and repair. Although VEGF is secreted by many cell types, among which osteoblasts have been reported to produce VEGF and its receptors,[29,30] the expression of VEGF was upregulated by 1,25-dihydroxyvitamin D_3 and prostaglandin E_2, both of which are potent stimulators of osteoclastic resorption.[31,32] Prior to their removal by chondroclasts, the hypertrophic chondrocytes express VEGF and VEGF receptors.[30,33–35] Inhibition of VEGF activity in the growth plate resulted in nearly complete suppression of blood vessel invasion, concomitant with impaired trabecular bone formation, inhibited chondroclastic resorption, and expansion of the hypertrophic chondrocyte zone. Our data also showed that rhVEGF (25 ng/mL) alone promotes osteoclastognesis and bone resorption capability. Taken together, VEGF promotes chondroclastogenesis and osteoclastogenesis and plays an important role in skeletal development and bone remodeling. Recently, Niida et al.[11] demonstrated that together with sRANKL, VEGF induced osteoclastogenesis, suggesting that VEGF could substitute

the function of M-CSF in osteoclastogenesis to a certain extent. Using a high purity (>95%) rabbit osteoclast culture system, Nakagawa et al.[10] demonstrated that VEGF caused a dose- and time-dependent increase in the area of bone resorption pits, partially by enhancing the survival of osteoclasts. In this study, rhVEGF (25 ng/mL) showed an enhancing effect on osteoclast bone resorptive function whenever it was used alone or combined with 3 ng/mL rhBMP-2. However, rhVEGF-induced osteoclastic resorptive function was inhibited by 30 ng/mL and 300 ng/mL rhBMP-2. It is not known if at a higher concentration rhVEGF would be able to revise the inhibitory effects of rhBMP-2 as seen in the present study. As the data presented, an interactive effect exists between rhBMP2 and rhVEGF on human osteoclastogenesis, the treatment of 30 ng/mL rhBMP-2, 30 ng/mL rhBMP-2 + 25 ng/mL rhVEGF, or 300 ng/mL rhBMP-2 + 25 ng/mL rhVEGF all had inhibitory effects on human osteoclast differentiation and their bone resorptive function in vitro. A crosscommunication may therefore exist between VEGF and BMPs signaling pathways, probably through their receptors, during their regulations of osteoclastogenesis. The underline cellular and molecular mechanisms need further careful investigation.

The present study has documented the inhibitory effects of rhBMP-2 (at 30 and 300 ng/mL) on osteoclastogenesis in a human osteoclast culture system alone or together with rhVEGF (at 25 ng/mL). rhVEGF alone (at 25 ng/mL) promotes osteoclastogenesis in the culture system, but the activation was revised into inhibition by additional rhBMP-2 (at 30 and 300 ng/mL). There was an interactive effect between rhVEGF and rhBMP-2 on human osteoclastogenesis in vitro. Further investigation into the mechanisms of these phenomena may lead to better understanding of the roles of BMP-2 and VEGF in mediating osteoclastogenesis, bone remodeling, and perhaps novel strategies for the treatment of osteolysis and osteoporosis conditions.

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