Vascular Endothelial Growth Factor Can Substitute for Macrophage Colony-stimulating Factor in the Support of Osteoclastic Bone Resorption

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Summary

We demonstrated previously that a single injection of recombinant human macrophage colony-stimulating factor (rhM-CSF) is sufficient for osteoclast recruitment and survival in osteopetrotic (*op/op*) mice with a deficiency in osteoclasts resulting from a mutation in M-CSF gene. In this study, we show that a single injection of recombinant human vascular endothelial growth factor (rhVEGF) can similarly induce osteoclast recruitment in *op/op* mice. Osteoclasts predominantly expressed VEGF receptor 1 (VEGFR-1), and activity of recombinant human placenta growth factor 1 on osteoclast recruitment was comparable to that of rhVEGF, showing that the VEGF signal is mediated through VEGFR-1. The rhM-CSF–induced osteoclasts died after injections of VEGFR-1/Fc chimeric protein, and its effect was abrogated by concomitant injections of rhM-CSF. Osteoclasts supported by rhM-CSF or endogenous VEGF showed no significant difference in the bone-resorbing activity. *op/op* mice undergo an age-related resolution of osteopetrosis accompanied by an increase in osteoclast number. Most of the osteoclasts disappeared after injections of anti-VEGF antibody, demonstrating that endogenously produced VEGF is responsible for the appearance of osteoclasts in the mutant mice. In addition, rhVEGF replaced rhM-CSF in the support of in vitro osteoclast differentiation. These results demonstrate that M-CSF and VEGF have overlapping functions in the support of osteoclastic bone resorption.

Key words: osteoclasts • vascular endothelial growth factor • vascular endothelial growth factor receptor • macrophage colony-stimulating factor • osteopetrosis

Mice homozygous for the recessive mutation, osteopetrosis (*op*), on chromosome 3 have a severe deficiency of osteoclasts, monocytes, and macrophages in various organs (1–3). The deficiency is caused by the absence of functional macrophage colony-stimulating factor (M-CSF or CSF-1) as a result of a single basepair insertion within the coding region of the M-CSF gene (4) and can be cured by injections of recombinant human (rh)M-CSF (5–8). Direct action of M-CSF on osteoclast lineage cells is demonstrated by the expression of the receptor for M-CSF, c-Fms, in osteoclasts both in vitro (9) and in vivo (10). These findings indicate that M-CSF plays an essential role in the differentiation of osteoclasts, as well as macrophages in some organs, under physiological conditions.

However, severe osteopetrosis in *op/op* mice is evident only during their youth and is progressively corrected in association with an increase of osteoclasts (1, 2, 11). We found that when injected at high doses (>5 μg/mouse), only a single injection of rhM-CSF is sufficient to induce a synchronous wave of osteoclast recruitment, survival, and active bone resorption for a prolonged period in *op/op* mice (12, 13). These observations have suggested the presence of other regulatory factor(s) that are responsible for osteoclastic bone resorption in the absence of M-CSF. In this context, controversial data have been reported on the effects of GM-CSF on the cure of osteopetrosis in *op/op* mice, whereas several in vitro studies have suggested a role of this cytokine in osteoclast differentiation (14–16). Wiktor-Jedrzejczak et al. (17) and Nilsson et al. (18) reported that GM-CSF can correct macrophage deficiencies but fails to resolve osteopetrosis. Very recently, Myint et al. (19) reported that GM-CSF and/or IL-3 at low doses can induce osteoclast development in *op/op* mice.

c-Fms is one of the eight members of the platelet-derived growth factor receptor (PDGFR) family (20). As specific receptors for vascular endothelial growth factor (VEGF), two receptor tyrosine kinases of the PDGFR family, VEGFR-1/Flt-1 and VEGFR-2/KDR/Flk-1, as well as

neuropilin-1, have been identified (21). In contrast to endothelial cells which express all of the VEGFRs, monocyte/macrophage lineage cells predominantly express VEGFR-1 (21–23). VEGFR-1 mediates chemotactic response of the cells to VEGF or placenta growth factor 1 (PlGF-1), which shows high homology to VEGF and is expressed in umbilical vein endothelia and placenta (21–26).

Taking note of the close lineage relationship between macrophages and osteoclasts, we show in this study that VEGF can fully compensate for the deficiency of M-CSF in *op/op* mice in osteoclastic bone resorption, manifesting a unique type of redundancy in cytokine signaling by using different ligand–receptor combinations. Furthermore, we present evidence that endogenously produced VEGF is responsible for the age-related resolution of osteopetrosis in *op/op* mice.

Materials and Methods

Mice. *op/op* mice and their normal littermates (+/?) were raised in our animal facility as described previously (6, 12). Mice of *op/op* genotype were identified at 11 d of age by the absence of incisor eruption. Male ddY mice were obtained from Saitama Experimental Animals Supply (Sugito, Saitama, Japan).

Injection of Cytokines, Antibody, and/or VEGFR-1/Fc Chimeric Protein into op/op Mice. 5 μg of either rhM-CSF (Austral Biologicals), rhVEGF165 (Genzyme Corp.), rhVEGF121 (Genzyme Corp.), or rhPlGF-1 (R&D Systems) was intraperitoneally injected into 12-d-old *op/op* mice, and the mice were killed 3 or 7 d after the injection. AFS98 rat anti–mouse c-Fms mAb (27) was intraperitoneally injected at a dosage of 750 μg/mouse into mutant mice both 2 h before and 24 h after the cytokine injection, and the mice were killed 3 d after cytokine injection.

In another series of experiments, *op/op* mice were pretreated with a single injection of rhM-CSF at 12 d of age. Starting at 4 d after the pretreatment, 5 μg of either VEGFR-1/Fc chimeric protein (R&D Systems) and/or rhM-CSF was intraperitoneally injected six times at 12-h intervals. The mice were killed 7 d after the pretreatment. As a control for the chimeric protein, human IgG1 (ICN Pharmaceuticals) was injected similarly as above. rhM-CSF alone or together with VEGFR-1/Fc was also consecutively injected six times at 12-h intervals into the mutant mice starting at 12 d of age without pretreatment. Mice were killed 3 d after the onset of the treatment.

Five consecutive injections of 100 μg of goat anti–mouse VEGF polyclonal antibody (R&D Systems) at 12-h intervals were given to 2-mo-old *op/op* mice. As a control, goat IgG (Santa Cruz Biotechnology) was injected similarly as above. The last group of mice received a single dose of 5 μg rhVEGF165. All of these mice were killed 3 d after the onset of the treatments.

Histological Observations. *op/op* mice were anesthetized with ether and perfused with 4% periodate-lysine-paraformaldehyde fixative solution (pH 7.4) through descending aorta. Femurs were decalcified in 10% EDTA (pH 7.0) for 4 d and embedded in paraffin. Longitudinal sections (7 μm thick) of the median portion of whole femurs were stained for tartrate-resistant acid phosphatase (TRAP) activity as described previously (12, 13) and counterstained with hematoxylin. TRAP-positive cells with two or more nuclei were counted as osteoclasts. Some sections were stained by Mallory's azan staining.

Immunohistochemical Staining for VEGFRs. Femurs of 2–3-wk-old +/? or *op/op* mice were fixed and embedded in paraffin as described above. Sections (5 μm thick) of the femurs were immunohistochemically stained with rabbit anti–mouse VEGFR-1

polyclonal antibody (Santa Cruz Biotechnology) or AVAS12 rat anti–mouse VEGFR-2 mAb (28), using Vectastain Elite ABC kits (Vector Laboratories), and counterstained with hematoxylin. Normal rabbit IgG (Santa Cruz Biotechnology) and rat IgG2a (Santa Cruz Biotechnology) were used as controls for the polyclonal and monoclonal antibodies, respectively.

In Vitro Generation of Osteoclasts. rhVEGF165 and rhM-CSF were dissolved in fetal bovine serum (FBS) at concentrations of 2 μg/ml and 400 ng/ml, respectively. Wells of 96-well plates were coated with 5 μl of either of the cytokine solutions or FBS and air dried for 30 min. Bone marrow cells obtained from tibias and femurs of 5–8-wk-old male ddY mice were passed through a Sephadex G-10 (Amersham Pharmacia Biotech) column as described by Ly and Mishell (29). Nonadherent cells were plated at a density of 10^5 cells/well into the cytokine-coated wells and cultured with α-MEM supplemented with 15% FBS in the absence or presence of 100 ng/ml of recombinant human receptor activator of nuclear factor κB ligand (rhRANKL; PeproTech) for 7 d. The final concentrations of rhVEGF165 and rhM-CSF were 100 and 20 ng/ml, respectively. The cultures were fixed with 4% paraformaldehyde and stained for TRAP as described above. The nonadherent bone marrow cells were also inoculated onto dentine slices with a diameter of 5 mm, placed in the wells of 24-well plates, similarly as described above, and cultured for 7 d. The slices were examined by backscattered electron microscopy as described previously (30).

Results and Discussion

To examine whether VEGF can compensate for the absence of functional M-CSF in *op/op* mice in the support of osteoclast recruitment, we first injected either rhM-CSF, rhVEGF165, rhVEGF121, or rhPlGF-1 into 12-d-old *op/op* mice. As shown in Table I, a single 5-μg injection of any of these factors was sufficient for the osteoclast recruitment in the mutant mice, although the number of osteoclasts recruited by rhVEGFs or rhPlGF-1 was 60–70% of that by rhM-CSF. The antagonistic anti–c-Fms mAb, AFS98 (27), decreased osteoclast recruitment by rhM-CSF to ~25%, but not that by rhVEGFs or rhPlGF-1, confirming that c-Fms mediates the response of osteoclast precursor cells to M-CSF, but not the response to VEGFs or PlGF-1.

As shown in Fig. 1 A, osteoclasts were strongly stained with rabbit anti–mouse VEGFR-1 polyclonal antibody, whereas endothelial cells were weakly positive for VEGFR-1. In contrast, osteoclasts were not stained with AVAS12 anti–mouse VEGFR-2 mAb (28), while endothelial cells were positively stained for VEGFR-2 (Fig. 1 B). Neither normal rabbit IgG (Fig. 1 C) nor rat IgG2a (data not shown) stained any cell types. rhM-CSF–induced osteoclasts in *op/op* mice showed the same staining pattern as described above (data not shown). These results demonstrate that osteoclasts predominantly express VEGFR-1, in a manner similar to monocyte/macrophage lineage cells (22, 23). VEGF121 does not bind neuropilin-1 (21). PlGF-1 binds VEGFR-1, but not VEGFR-2 or neuropilin-1 (21, 22–25). The results that both rhVEGF121 and rhPlGF-1 showed activities comparable to rhVEGF165 in the support of osteoclast recruitment (Table I) confirm that the response of osteoclast precursor cells to VEGF is mediated by VEGFR-1.

Next, we examined the capacity of VEGF and M-CSF to

Table I. *Capacity of rhM-CSF, rhVEGFs, and rhPlGF-1 to Recruit Osteoclasts in op/op Mice*

Cytokine	AFS98	No. of osteoclasts/section
		(mean ± SD)
None	−	3 ± 2
rhM-CSF	−	60 ± 6
rhM-CSF	+	14 ± 9
rhVEGF165	−	42 ± 1
rhVEGF165	+	43 ± 7
rhVEGF121	−	37 ± 4
rhPlGF-1	−	37 ± 2
rhPlGF-1	+	35 ± 2

Cytokines were injected at a single dosage of 5 μg into 12-d-old *op/op* mice, and mice were killed 3 d after the injection. AFS98 anti–c-Fms mAb was injected at a dosage of 750 μg/mouse both 2 h before and 24 h after the cytokine injection. Osteoclasts in the longitudinal sections of the median portion of whole femurs were counted. Results represent the mean ± SD of six sections from three mice.

support the survival of mature osteoclasts by neutralizing VEGF endogenously produced in *op/op* mice with injections of VEGFR-1/Fc chimeric protein. Consistent with our previous observations (12, 13), osteoclast number reached a plateau at 3 d after a single rhM-CSF injection and was maintained up to day 7 (Tables I and II). Consecutive injections of the chimeric protein at 12-h intervals during days 4–6 decreased osteoclasts to ∼25%, whereas injections of human IgG1 did not affect osteoclast number (Table II). In contrast, when rhM-CSF was injected together with VEGFR-1/Fc, osteoclast number increased to the levels observed in mice consecutively injected with rhM-CSF alone. These results indicate that survival of osteoclasts recruited after a single rhM-CSF injection was supported by endogenously produced VEGF in *op/op* mice and that M-CSF can support the survival of mature osteoclasts without the help of VEGF.

We also examined the bone resorption in the femurs of *op/op* mice that had received either a single rhM-CSF injection only or consecutive injections of VEGFR-1/Fc and rhM-CSF in addition to the single rhM-CSF injection. Osteoclasts in the
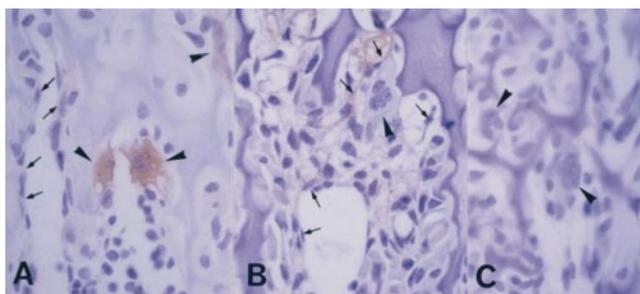

Figure 1. Immunohistochemical staining of femur sections for VEGFRs. Longitudinal sections of femurs of 3-wk-old +/? mice were stained with either anti–VEGFR-1 polyclonal antibody (A), AVAS12 anti–VEGFR-2 mAb (B), or rabbit IgG (C). Arrowheads indicate osteoclasts, and arrows indicate endothelial cells. Original magnifications: ×238.

Table II. *Effect of Injections of VEGFR-1/Fc Chimeric Protein on the Survival of rhM-CSF–recruited Osteoclasts in op/op Mice*

Treatment	No. of osteoclasts/section
	(mean ± SD)
None	59 ± 9
VEGFR-1Fc	15 ± 5
Human IgG1	65 ± 9
VEGFR-1/Fc and rhM-CSF	87 ± 9
rhM-CSF	81 ± 8

op/op mice were pretreated with a single injection of 5 μg rhM/CSF at 12 d of age. 5 μg each of VEGFR-1/Fc and/or rhM-CSF or 5 μg human IgG1 was consecutively injected six times at 12-h intervals into the mice during 16–18 d of age. Osteoclasts in the longitudinal sections of the median portion of whole femurs were counted. Results represent the mean ± SD of six sections from three mice.

former group of mice are thought to perform their functions with the support of endogenous VEGF, whereas those in the latter rely on exogenous rhM-CSF. As reported previously (12), resorption of a massive amount of bone trabeculae and replacement with bone marrow in femurs were apparent by 7 d after a single rhM-CSF injection (Fig. 2, A and B). Bone resorption was also similarly observed in the latter group of mice (Fig. 2 C). These observations show that both M-CSF and VEGF can support the bone-resorbing function of osteoclasts.

The above finding that VEGF is endogenously produced at levels sufficient for the survival of mature osteoclasts and expression of their functions prompted us to confirm that rhM-CSF can induce osteoclast recruitment without the help of endogenous VEGF. As shown in Table III, twice the number of osteoclasts were recruited by multiple injections of rhM-CSF compared with a single injection. Con-
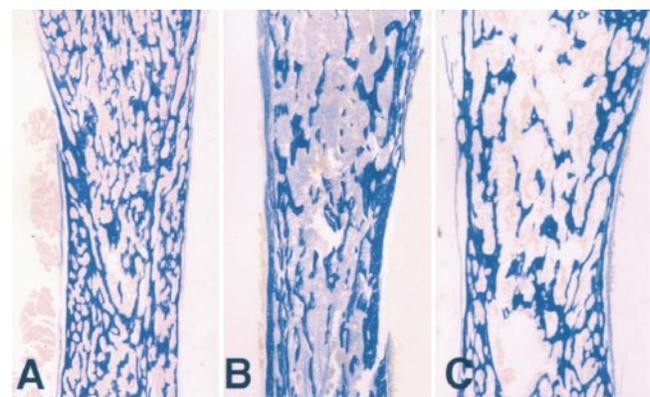

Figure 2. Resorption of bone trabeculae in the femurs of *op/op* mice by osteoclasts with the support of endogenous VEGF or exogenous rhM-CSF. The treatment was started at 12 d of age, and the mice were killed at 19 d of age. Longitudinal sections of femurs were stained by Mallory's azan. Each micrograph represents a group of femurs from three animals. (A) No injection; (B) a single injection of 5 μg rhM-CSF at 12 d of age; (C) a single injection of 5 μg rhM-CSF at 12 d of age, and six consecutive injections of 5 μg each of VEGFR-1/Fc chimeric protein and rhM-CSF at 12-h intervals during 16 and 18 d of age. Original magnifications: ×20.

Table III. *Effect of VEGFR-1/Fc Injection on Osteoclast Recruitment by rhM-CSF in op/op Mice*

Treatment	No. of injections	No. of osteoclasts/section
		(mean ± SD)
None	0	3 ± 2
rhM-CSF	1	56 ± 9
rhM-CSF	6	108 ± 11
rhM-CSF and VEGFR-1/Fc	6	101 ± 7

The treatments of *op/op* mice began at 12 d of age. They received either a single injection of 5 μg rhM-CSF or six consecutive injections of 5 μg rhM-CSF alone or together with 5 μg VEGFR-1/Fc at 12-h intervals, and were killed at 3 d after the onset of the treatments. Osteoclasts in the longitudinal sections of the median portion of whole femurs were counted. Results represent the mean ± SD of six sections from three mice.

comitant injections of VEGFR-1/Fc with rhM-CSF did not affect osteoclast recruitment. These results are the first unequivocal demonstration of the capacity of M-CSF to support in vivo osteoclast differentiation.

It became clear that M-CSF supports osteoclast differentiation in cooperation with osteoclast differentiation factor (ODF)/osteoprotegerin ligand (OPGL)/TNF-related activation-induced cytokine (TRANCE)/RANKL (31, 32). We examined whether rhVEGF165 can replace rhM-CSF in osteoclast generation in in vitro culture of nonadherent bone marrow cells. Consistent with previous observations (31, 32), no TRAP-positive cells appeared in the presence of rhM-CSF or rhRANKL alone (data not shown). rhVEGF165 alone also failed to support the osteoclast differentiation (Fig. 3 A). A combination of rhVEGF165 and rhRANKL supported the generation of TRAP-positive cells (Fig. 3 B), al-
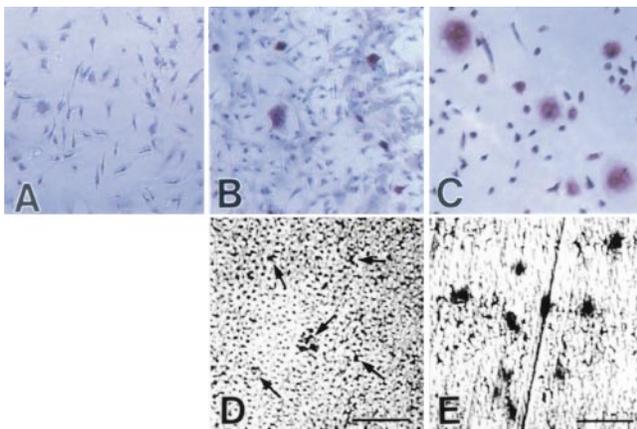
though the cells were significantly smaller in size than those generated in the presence of rhM-CSF and rhRANKL (Fig. 3 C). Consequently, the osteoclasts supported by rhVEGF165 and rhRANKL formed smaller resorption lacunae than those supported by rhM-CSF and rhRANKL (Fig. 3, D and E). These results demonstrate that VEGF can indeed support osteoclast differentiation in cooperation with ODF/OPGL/TRANCE/RANKL.

Finally, we examined whether progressive correction of osteopetrosis with age accompanied by an increase of osteoclasts in *op/op* mice (1, 2, 11) is due to endogenously produced VEGF. As shown in Fig. 4 A, a significantly larger number of small osteoclasts with 2–3 nuclei was observed in the femurs of 2-mo-old *op/op* mice (28 ± 1 osteoclasts/section) compared with those of 2-wk-old mutants (Tables I and III), even though the size of the femur sections of the older animals was ∼1.6 times larger than that of younger ones. In addition, TRAP-positive mononuclear cells were frequently observed in the marrow space. Five consecutive injections of 100 μg goat anti-VEGF polyclonal antibody at 12-h intervals
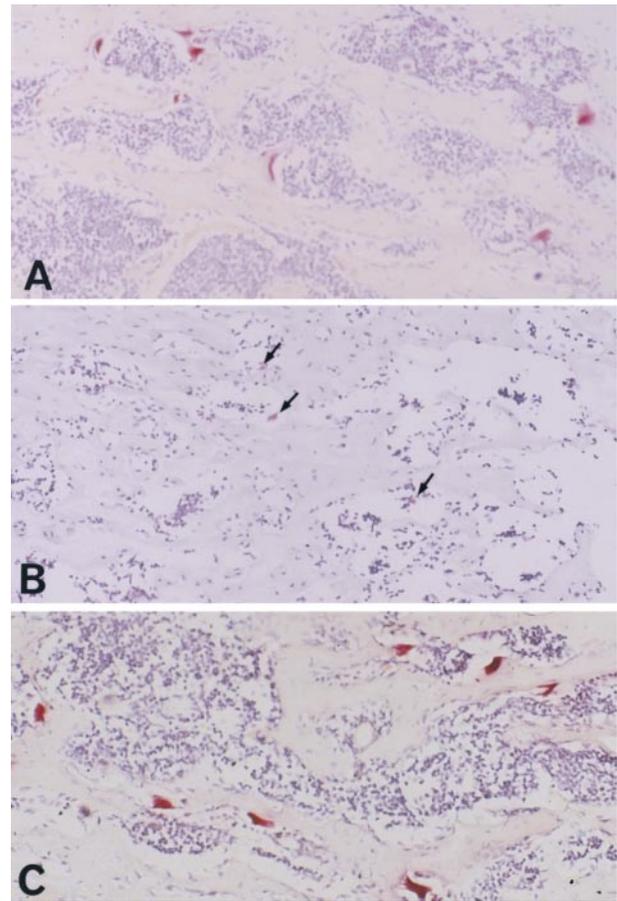
Figure 3. Ability of VEGF to support in vitro generation of osteoclasts. Nonadherent bone marrow cells were cultured in the presence of rhVEGF165 alone (A), rhVEGF165 and rhRANKL (B and D), or rhM-CSF and rhRANKL (C and E) in the wells of 96-well plates (A–C) or on dentine slices (D and E) for 7 d. The cultures were either stained for TRAP activity (A–C) or examined by backscattered electron microscopy (D and E). Arrows in D indicate small resorption lacunae. Original magnifications (A–C): ×25. Bars (D and E): 50 μm.

Figure 4. Dependence of osteoclasts in the femurs of 2-mo-old *op/op* mice on endogenously produced VEGF. The mice were killed 3 d after the onset of the treatment. Longitudinal sections of femurs were stained for TRAP activity and counterstained with hematoxylin. Each micrograph represents a group of femurs from three animals. Arrows in B indicate mononuclear TRAP-positive cells. (A) No injection; (B) five consecutive injections of 100 μg anti-VEGF polyclonal antibody at 12-h intervals; (C) a single injection of 5 μg rhVEGF165. Original magnifications: ×103.

significantly decreased osteoclast number (4 ± 2 osteoclasts/ section; Fig. 4 B). Injections of goat IgG did not affect osteoclast number (data not shown). VEGFR-1/Fc injections according to the protocol applied to 2-wk-old mutant mice (Table II) failed to show any noticeable effect on osteoclast number (data not shown). A single injection of 5 μg rhVEGF165 induced further recruitment of osteoclasts (64 ± 5 osteoclasts/section; Fig. 4 C), indicating that VEGF levels in the femurs of 2-mo-old *op/op* mice are still insufficient to recruit osteoclasts at maximum level. These results demonstrate that VEGF is responsible for the spontaneous osteoclast recruitment in the absence of functional M-CSF in *op/op* mice. Changes in osteoclast number with the age and difference in the amount of VEGFR-1/Fc required to neutralize endogenous VEGF activity in 2-wk- and 2-mo-old animals suggest higher levels of VEGF production in older mutant mice, although the possibility that sensitivity of osteoclast precursors to VEGF changes with age cannot be ruled out.

This study demonstrates that M-CSF and VEGF can play almost entirely overlapping roles in osteoclastic bone resorption. The presence of either of the cytokines was sufficient to support all the processes of osteoclastic bone resorption, i.e., the differentiation of osteoclasts and their survival and active bone resorption, representing a unique type of redundancy of cytokine signaling. However, osteoclasts generated in vitro with the support of rhVEGF165 and rhRANKL were significantly smaller in size and formed smaller resorption lacunae compared with those supported by rhM-CSF and rhRANKL. Osteoclasts observed in 2-mo-old *op/op* mice had only two to three nuclei. Nevertheless, our data indicated that progressive correction of osteopetrosis in *op/op* mice is due to endogenously produced VEGF.

It has been well established that VEGF is a key regulator of vasculogenesis (21). Osteoclastic bone resorption and concomitant bone marrow formation are closely associated with active neovascularization (8, 33), and osteoblasts have been reported to produce VEGF (34). Our results indicate that VEGF is produced in *op/op* mice at levels sufficient for the survival and functioning of mature osteoclasts, but not for their recruitment at maximal levels. The finding that mice lacking a single VEGF allele die in utero with aberrant blood vessel formation in the yolk sac and embryo indicates that VEGF is produced at threshold levels for endothelial cell proliferation (35, 36). Furthermore, mice expressing the VEGFR-1 lacking the tyrosine kinase domain (26) had no appreciable abnormality in osteoclastic bone resorption (M. Shibuya, The University of Tokyo, personal communication). Therefore, M-CSF seems to play a dominant role in osteoclastic bone resorption under physiological conditions.

Macrophages from mice with kinase-deficient VEGFR-1 exhibit a defect in their migratory response to VEGF (26). The common feature of predominant expression of VEGFR-1 in monocytes and macrophages (21–23) and in osteoclasts may provide further support for the view of shared origin of these cells. We found previously that multiple injections of rhM-CSF are required for macrophage recruitment in the femurs of *op/op* mice (12, 13). In the present study, we also failed to find any sign of macrophage recruitment in the femurs after a single injection of rhVEGFs or rhPlGF-1 (data not shown). These observations may suggest that macrophage lineage cells are less sensitive to M-CSF, VEGFs, and PlGF-1 compared with osteoclast precursors or more probably that macrophage precursors are more strictly dependent on the continuous presence of M-CSF.

The function of VEGFR-1 as a mediator of mitogenic response of endothelial cells to VEGF has yet to be clearly identified, although unequivocal evidence for such a role of VEGFR-2 has accumulated (21). The phenotypes of the mice with VEGFR-1 deficiency (37) and those expressing kinase-deficient VEGFR-1 (29) strongly suggest the role of VEGFR-1 in the negative regulation of endothelial growth in embryonic angiogenesis. Therefore, it is of interest to compare the VEGFR-1 signaling in osteoclasts and their precursor cells with that in endothelial cells.

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