

VEGF receptor 1 signaling is essential for osteoclast development and bone marrow formation in colony-stimulating factor 1-deficient mice

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VEGF receptor 1 (VEGFR-1/Flt-1) is a high-affinity tyrosine kinase (TK) receptor for VEGF and regulates angiogenesis as well as monocyte/macrophage functions. We previously showed that the osteoclast deficiency in osteopetrotic *Csf1^{op}/Csf1^{op} (op/op)* mice is gradually restored in an endogenous, VEGF-dependent manner. However, the molecular basis of the recovery is still not clear. To examine which VEGFR is important and to clarify how colony-stimulating factor 1 (CSF-1) and VEGF signals interact in osteoclastogenesis, we introduced a VEGFR-1 signaling deficiency (*Flt1^{TK-/-}*) into *op/op* mice. The original *Flt1^{TK-/-}* mice showed mild osteoclast reduction without bone marrow suppression. The double mutant (*op/opFlt1^{TK-/-}*) mice, however, exhibited very severe osteoclast deficiency and did not have numbers of osteoclasts sufficient to form the bone marrow cavity. The narrow bone marrow cavity in the *op/opFlt1^{TK-/-}* mice was gradually replaced with fibrous tissue, resulting in severe marrow hypoplasia and extramedullary hematopoiesis. In addition to osteoclasts, osteoblasts also decreased in number in the *op/opFlt1^{TK-/-}* mice. These results strongly suggest that the interaction of signals by means of VEGFR-1 and the CSF-1 receptor plays a predominant role not only in osteoclastogenesis but also in the maintenance of bone marrow functions.

osteoblast | hematopoiesis | hematopoietic niche | osteopetrosis | *op/op* mouse

Osteoclasts are terminally differentiated cells derived from the monocyte/macrophage lineage and serve critical functions in bone resorption. The differentiation, activation, and survival of osteoclasts are primarily regulated by colony-stimulating factor 1 (CSF-1)/macrophage colony-stimulating factor, whose biological effects are mediated through a cell surface receptor, CSF-1R/c-Fms (1, 2). The role of CSF-1 in osteoclast biology was first revealed in the osteopetrotic (*Csf1^{op}/Csf1^{op}*, hereafter abbreviated as *op/op*) mouse, which has a recessive mutation in the *Csf1* gene (3). The *op/op* mouse exhibits a severe deficiency of osteoclasts, monocytes, and tissue macrophages owing to a lack of CSF-1 function (4). Interestingly, however, the defect is evident only in juvenile mice. Osteoclasts gradually appear in *op/op* bone and correct the osteopetrosis spontaneously. In addition, a single administration of CSF-1 protein resulted in long-term, active bone resorption in *op/op* mice (5, 6). These findings suggest that some alternative factor (or factors) support and maintain osteoclastogenesis in the absence of CSF-1. We demonstrated that the administration of VEGF-A ameliorated osteoclastogenesis and bone resorption and that treatment with an antagonist for VEGF-A suppressed the spontaneous recruitment of osteoclasts in *op/op* mice (7). These results indicate that VEGF is a candidate cytokine to

substitute for CSF-1 in the osteoclast development in *op/op* mice.

VEGF-A is a key regulator of physiological angiogenesis and hematopoiesis (8, 9) and has been implicated in the establishment of epiphyseal vascularization and endochondral bone formation (10, 11). VEGF-A belongs to a gene family of growth factors (the VEGF family) that includes VEGF-A, placenta growth factor (PlGF), VEGF-B, VEGF-C, and VEGF-D (12). Also, an orf virus-derived VEGF, VEGF-E, has been identified (13). VEGF-A has multiple spliced isoforms, including VEGF-A₁₂₀, VEGF-A₁₆₄, and VEGF-A₁₈₈, in mice (12). VEGF-A binds to tyrosine kinase (TK) receptors, VEGF receptor 1 (VEGFR-1/Flt-1) and VEGFR-2 (Flk-1/KDR), subsequently serving as key mediators for angiogenesis (14, 15). PlGF and VEGF-B bind only to VEGFR-1. VEGF-C and VEGF-D bind to VEGFR-3 and regulate lymphatic angiogenesis. VEGF-E is a specific ligand to VEGFR-2 (13–15).

VEGFR-1 is expressed in monocytes and regulates their activation and chemotaxis (16, 17). We also revealed that monocyte/macrophage lineage cells including osteoclasts express VEGFR-1 (7, 18), indicating that, at the very least, VEGFR-1 is involved in osteoclastogenesis. In addition, recent studies suggested that VEGFR-2 is also expressed to some extent in mature osteoclasts (19, 20). To determine the function of the VEGF-VEGFR system in osteoclast development and activity, we introduced a VEGFR-1 TK domain-deficient mutation (*Flt1^{TK-/-}*) (21) into *op/op* mice. The double mutant *op/opFlt1^{TK-/-}* mice showed an extensive osteoclast deficiency compared with *op/op* mice and could not recruit numbers of osteoclasts sufficient to expand the marrow cavity, resulting in bone marrow fibrosis and extramedullary hematopoiesis.

Materials and Methods

Mice. The *Flt1^{TK-/-}* mice used in this study are described in ref. 21. Female *Flt1^{TK-/-}* mice with a C57BL/6 background were mated with male *op/op* homozygous mice (The Jackson Laboratory) having the B6C3Fe-a/a-*Csf1^{op}/Csf1^{op}* background. Double heterozygotes (*op/+Flt1^{TK+/-}*) of the subsequent generation were used for further breeding. The resulting mice, which were

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Abbreviations: CSF-1, colony-stimulating factor 1; VEGFR, VEGF receptor; PlGF, placenta growth factor; TK, tyrosine kinase; TRAP, tartrate-resistant acid phosphatase; ALP, alkaline phosphatase; rh, recombinant human.

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deficient in one gene and heterozygote for another, were used for breeding in parallel. Mice with the *op/op* phenotype were identified by the absence of incisor eruption and/or PCR analysis of tail DNA samples. Mice with the *Flt1^{TK-/-}* genotype were identified by PCR analysis and/or Southern blot analysis of the same DNA samples as described in ref. 21. The offspring were *op/op* and double mutant *op/opFlt1^{TK-/-}* and served as the subjects in this study. All animal experiments were approved by the National Center for Geriatrics and Gerontology's institutional animal experimentation committee.

Histological Analysis. Mice (4–24 wk old) were anesthetized and perfused with a periodate-lysine-paraformaldehyde (PLP) solution (4% paraformaldehyde/0.01 M NaIO₄/0.075 M lysine in 0.05 M phosphate buffer, pH 7.4). Bone, spleen, liver, and kidney organ blocks were postfixed for 10 h in the PLP solution. After being rinsed with the buffer, soft tissues were embedded in paraffin. Bones were decalcified in a 10% EDTA solution in 1 mM PBS (pH 7.4) for 2 wk at 4°C and embedded in paraffin. These samples were sectioned (3- to 7- μ m thick) and stained with hematoxylin/eosin or toluidine blue for histological and pathological observations. Longitudinal serial sections of the median portion of whole femora were stained for tartrate-resistant acid phosphatase (TRAP) activity and counterstained with hematoxylin as described in ref. 7. TRAP-positive cells on bone surfaces that contained more than two nuclei were counted as osteoclasts. To identify the type of fibers in the myelofibrosis, sections of *op/opFlt1^{TK-/-}* femora were stained with Azan stain, silver stain, periodic acid-methenamin-silver stain, Masson's trichrom stain, van Gieson stain, and phosphotungstic acid-hematoxylin stain to diagnose the changes in *op/opFlt1^{TK-/-}* bone marrow.

Immunohistochemistry. Sections of several tissues of 4- and 8-wk-old mice were immunohistochemically stained with rat anti-mouse F4/80 Ab for detection of mature macrophages (22) and anti-mouse alkaline phosphatase (ALP) Ab (23). The primary Abs were detected with the streptavidin-biotin complex by using a Vectastain kit (Vector Laboratories) for macrophages and osteoblasts according to the manufacturer's instructions. Normal rabbit IgG was used as a control for the antibodies.

Cytokine Injections. Five micrograms of recombinant human (rh) CSF-1 (provided by Morinaga Milk Industry, Tokyo), recombinant mouse VEGF₁₂₀, rhPIGF (both from R & D Systems), or orf virus-derived VEGF-E (13) was i.p. injected into 21-d-old *op/op* and *op/opFlt1^{TK-/-}* mice. The mice were killed 4 d after the injections. As a control, vehicle (0.1 M PBS) was injected similarly as above.

A group of 7-wk-old *op/op* mice received three consecutive injections of 5 μ g of VEGFR-1/Fc chimeric protein (R & D Systems) under the conditions described above. Finally, three consecutive injections of 5 μ g of rhCSF-1 were given to 7-wk-old *op/opFlt1^{TK-/-}* mice at 24-h intervals. All of these mice were killed 5 d after the last injection.

Osteoclast Formation in Vitro. Spleen cells of 4-wk-old mice were passed through a Sephadex G-10 column (Amersham Pharmacia Biotech) as described in ref. 7. Cells were plated in 96-well plates at a density of 10⁵ cells per well onto confluent OP9 stromal cells, which were established from *op/op* mouse calvaria (24), and cultured for 6 d in α -MEM supplemented with 10% FBS in the presence of 10 ng/ml rhCSF-1, 50 ng/ml recombinant mouse VEGF₁₂₀, VEGF-E, and rhPIGF. The cultures were fixed with 4% paraformaldehyde and stained for TRAP. TRAP-positive multinucleated (three or more nuclei) cells were scored as osteoclasts under the microscope.

Statistical Analysis. Values are expressed as the mean \pm standard deviation. Significant differences between groups were determined with Student's *t* test in STAT VIEW 5.0 (SAS Institute, Cary, NC).

Results and Discussion

A Mild Reduction of Osteoclasts in *Flt1^{TK-/-}* Mice. We previously showed that endogenous VEGF-A substituted for CSF-1 in osteoclast development during the adult stage in CSF-1-deficient *op/op* mice and that osteoclasts expressed VEGFR-1 (7). Furthermore, ovariectomized *op/op* mice exhibited an increased number of osteoclasts accompanied by up-regulation of VEGF-A and VEGFR-1 mRNA expression (25). Thus, before crossing the *op/op* mice with *Flt1^{TK-/-}* mice, which undergo a basically normal development including angiogenesis (21), we examined the effects of VEGFR-1 signaling deficiency on the osteoclast formation *in vivo* by using *Flt1^{TK-/-}* mice. These mice displayed a mild reduction in numbers of TRAP-positive multinucleated osteoclasts and bone trabeculae just below the growth plate in long bones compared with that in WT mice (Fig. 1). Although the number of osteoclasts was sufficient for bone morphogenesis, these results suggest that VEGFR-1 signaling is partly implicated in physiological osteoclastogenesis.

***op/op* Mice Lacking a VEGFR-1 TK Domain Show Severe Bone Marrow Cavity Occlusion.** To clarify the roles of VEGFR-1 in osteoclast formation in more detail, the *Flt1^{TK-/-}* mice were bred with the *op/op* mice (Fig. 2A). The *op/op* mice lacking the VEGFR-1 TK domain (*op/opFlt1^{TK-/-}*) showed no significant difference in body weight or skeletal size compared with *op/op* mice (data not shown). F4/80-positive macrophage numbers were similarly reduced in marrow, liver, spleen, and kidney in both *op/op* and *op/opFlt1^{TK-/-}* mice (Fig. 2B). However, the limb bones in 2-wk-old *op/opFlt1^{TK-/-}* mice exhibited a more severe osteopetrosis with a decrease in the number and size of osteoclasts compared with those in *op/op* mice (Fig. 2C).

Next, we examined histological changes of femora in mice aged 4–24 wk. In *op/op* mice, the original osteopetrosis gradually ameliorated and marrow cellularity increased between the ages of 8 and 24 wk (Fig. 3A), as shown previously (7). In contrast, in *op/opFlt1^{TK-/-}* mice, the osteoclastic bone resorption did not recover throughout the observation period (6 mo). The osteopetrotic phenotype remained in *op/opFlt1^{TK-/-}* mice even at the 24-wk-old stage, although the thickened growth plate that is one of the features of osteopetrotic mice had been replaced with bone trabeculae (Fig. 3A). These results suggest that the VEGF-dependent osteoclastic bone resorption system does not function sufficiently in *op/opFlt1^{TK-/-}* mice.

Then, we compared the number of osteoclasts in femora of *op/op* and *op/opFlt1^{TK-/-}* mice. Small numbers of TRAP-positive osteoclasts were observed in 4-wk-old *op/op* femora, and numbers gradually increased during the observation period (Fig. 3B). Although osteoclasts were hardly detectable in 4-wk-old *op/opFlt1^{TK-/-}* femora, a small number appeared in 8-wk-old bone. The number of osteoclasts transiently increased to a lesser extent at 16 wk, returning to a barely detectable level again at 24 wk (Fig. 3B). In age-matched WT mice, 100 or 200 osteoclasts were observed during the observation period. These results clearly indicate that the TK domain in VEGFR-1 plays a pivotal role in the recruitment of osteoclasts in mice with a CSF-1-deficient background. The transient appearance of osteoclasts in the *op/opFlt1^{TK-/-}* bones, however, might suggest other signaling pathway(s).

Exogenous VEGFs Rescue Osteopetrosis in *op/opFlt1^{TK-/-}* Mice. Given our previous observation that the administration of neutralized Ab against VEGF-A completely inhibited osteoclast development in *op/op* mice (7), VEGFR-2 may be responsible

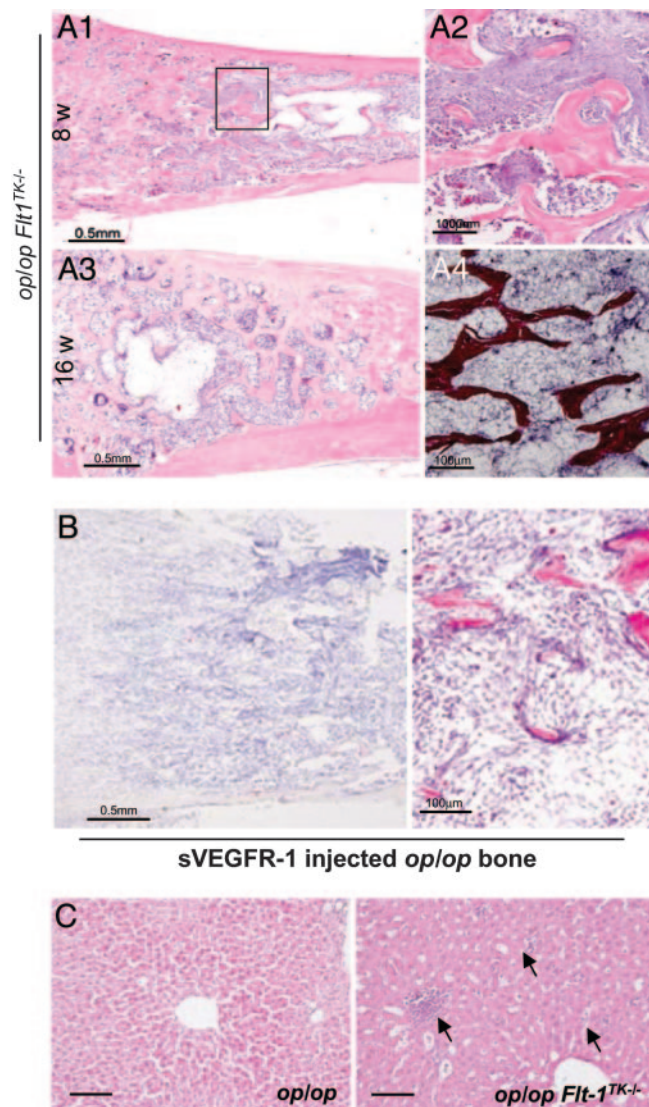


Fig. 5. Severe myelofibrosis in *op/opFlt1^{TK-/-}* mice. (A1 and A2) Myelofibrosis was found in the diaphysal region of the marrow cavity in 8-wk-old mice. A high-magnification image of the boxed area shows the junction of normal marrow and myelofibrosis. (A3) Myelofibrosis filled the bone marrow space in 16-wk-old mice. (A4) A high-magnification image shows silver staining for myelofibrosis. (B) TRAP and histology of the femur of 8-wk-old *op/op* mice treated with soluble VEGFR-1/Fc chimeric protein, indicating very few osteoclasts. (C) Hematoxylin/eosin-stained livers of *op/op* and *op/opFlt1^{TK-/-}* mice. Small hematopoietic foci were observed in *op/opFlt1^{TK-/-}* mice (Right, arrows) but not in *op/op* mice (Left). (Scale bars: 100 μ m.)

mice, fibrous tissue was initially observed in the diaphysal regions of the femora, although hematopoietic cells still occupied the intratrabecular spaces in the epiphysal region (Fig. 5A1 and A2). With aging, the fibrous tissue gradually expanded to the whole marrow cavity, resulting in marked decreases in marrow cellularity, including osteoclasts (Fig. 5A3). Histological analysis indicated that the fibrous tissue consisted of reticular fiber-like fibrils (Fig. 5A4). Furthermore, we found that the phenotype obtained on administration of soluble VEGFR-1 chimeric protein, an efficient VEGF inhibitor, into *op/op* mice mimicked the marrow phenotype of *op/opFlt1^{TK-/-}* mice (Fig. 5B).

Recent studies suggest that a portion of hematopoietic stem cells (HSCs) express VEGFR-1 (32) and that HSCs require VEGFR-1 signaling for their recruitment and mobilization in marrow (33). According to these reports, the decreased marrow

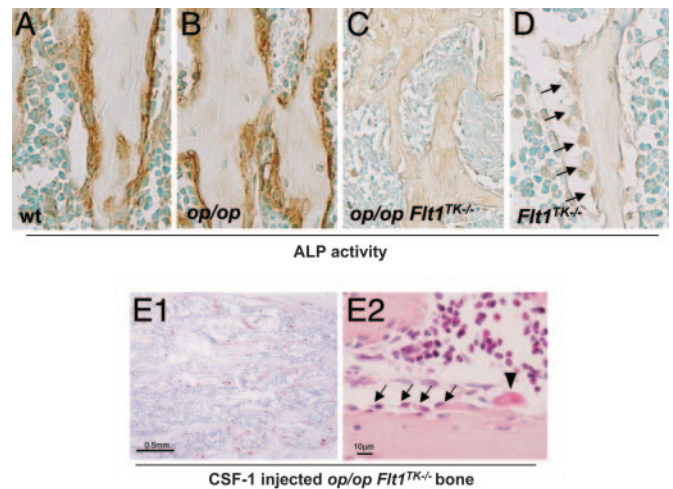


Fig. 6. Immunohistochemistry of ALP activity in four mouse genotypes: WT, *op/op*, *op/opFlt1^{TK-/-}*, and *Flt1^{TK-/-}*. (A–D) Cells stained brown are ALP-positive osteoblasts. The ALP activity of the *op/opFlt1^{TK-/-}* and *Flt1^{TK-/-}* mice is extremely weak compared with that of the WT and *op/op* mice. Although the ALP activity is weak, the osteoblasts in the *Flt1^{TK-/-}* mice retain the morphology of active-phase cells (D). However, in the *op/opFlt1^{TK-/-}* mice, most osteoblasts disappeared from the bone trabeculae. (E) TRAP activity (E1) and histology (E2) of the bone marrow in the femur of 8-wk-old *op/op* mice treated with rhCSF-1. Administration of rhCSF-1 prevented changes in the marrow and osteoblast reduction in the *op/opFlt1^{TK-/-}* mice. Arrows, osteoblasts; arrowhead, osteoclast.

cellularity in *op/opFlt1^{TK-/-}* mice might be due to defective HSCs. However, the single-gene mutant *Flt1^{TK-/-}* mice showed no apparent defect in marrow cellularity (Fig. 1A). Furthermore, a number of small hematopoietic foci were found in the liver of *op/opFlt1^{TK-/-}* mice (Fig. 5C). Taken together, these findings indicate that HSCs do exist even in the *op/opFlt1^{TK-/-}* mice and are functional for extramedullary hematopoiesis.

A Possible Intercommunication Between Osteoclasts and Osteoblasts. Myelopoiesis is supported by marrow stromal cells, including osteoblasts, which produce various osteogenic and hematopoietic growth factors (34). Osteoblast-deficient mice, owing to a lack of the *Runx2/Cbfa1* gene, which encodes a transcription factor for osteoblastogenesis, exhibit an absence of marrow cells (35). Osteoblast deficiency induced by different genetic approaches also arrests marrow hematopoiesis and establishes extramedullary hematopoiesis (36). Increases in the number of osteoblasts correlate with the establishment of hematopoietic niches (37, 38). Therefore, next we examined the activity of osteoblasts in the *op/opFlt1^{TK-/-}* mice.

Immunostaining for ALP in the bone sections of *op/opFlt1^{TK-/-}* mice revealed a remarkable reduction in the immunoreaction compared with that of *op/op* and WT mice (Fig. 6A–C). Osteoblasts were significantly decreased on the surface of bone trabeculae, adjacent to myelofibrosis (Fig. 6C). The down-regulation of osteoblast activity may disrupt the hematopoiesis-supportive microenvironment in *op/opFlt1^{TK-/-}* mice, resulting in a reduction in marrow cellularity and an increase in fibrosis. Because marrow hematopoiesis was weakly initiated in young *op/opFlt1^{TK-/-}* mice, we tested for ALP activity of osteoblasts in *Flt1^{TK-/-}* mouse bone. Although many osteoblasts were observed in *Flt1^{TK-/-}* mice, ALP activity was extremely weak (Fig. 6D). Taken together, our data provide genetic evidence that VEGFR-1 signaling is important for osteoblast activity during bone formation.

Bone undergoes remodeling through the coordinated process of bone resorption and bone formation to maintain bone mass.

It is considered that this harmonious balance is modulated by coupling paracrine signaling between osteoclasts and osteoblasts (for review, see ref. 39). Thus, we hypothesized that the survival of osteoblasts in *Flt1^{TK-/-}* mice may be supported by the existence of osteoclasts. To test this, we lastly examined whether induction of osteoclasts would rescue the hypoplastic marrow in *op/opFlt1^{TK-/-}* mice. Administration of rhCSF-1 to 7-wk-old *op/opFlt1^{TK-/-}* mice restored not only osteoclasts but also osteoblasts (Fig. 6E). Moreover, rhCSF-1 treatment also prevented marrow alterations (Fig. 6E2). These findings suggest that osteoclasts are implicated in the survival of osteoblasts and that osteoblasts are crucial for construction of the marrow hematopoiesis-supportive microenvironment as described in refs. 36 and 37. Simultaneously, our findings demonstrate that CSF-1 plays an important role not only in bone remodeling but also in the organization of marrow structure.

In conclusion, we provided anatomical and genetic findings to

show the importance of the interaction of VEGFR-1 signaling and CSF-1 receptor signaling in mice. Lack of these signals induces a severe alteration in bone and marrow structure. These findings may contribute to further understanding of the interaction between bone cells and marrow cells.

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