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-A_{120}, VEGF-A_{164}, and VEGF-A_{188}, 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 $NaIO_4$/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-μm 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 phophotungstic 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 $VEGF_{120}$, rhPlGF (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 μg of VEGFR-1/Fc chimeric protein (R & D Systems) under the conditions described above. Finally, three consecutive injections of 5 μg 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 10^5 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 $VEGF_{120}$, VEGF-E, and rhPlGF. 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 ± 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. 2*A*). 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. 2*B*). 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. 2*C*).

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. 3*A*), 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. 3*A*). 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. 3*B*). 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. 3*B*). 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

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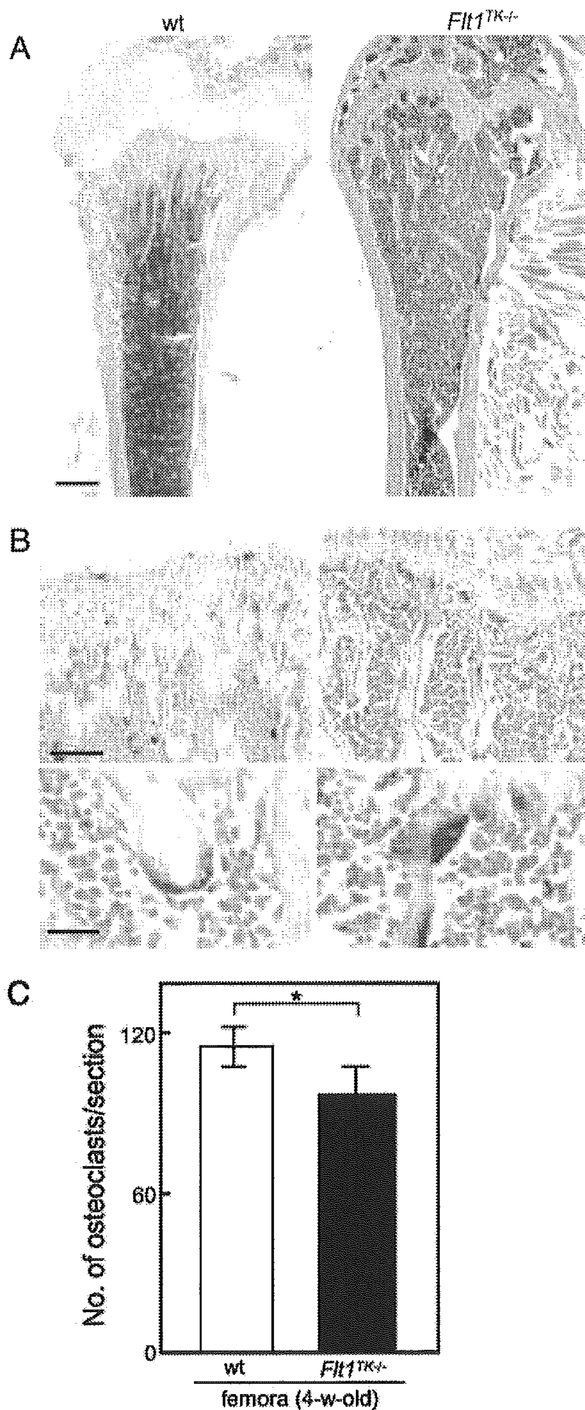

Fig. 1. Bone histology and number of osteoclasts in femora of 4-wk-old WT and $Flt1^{TK-/-}$ mice. (*A*) Longitudinal sections of femora were stained with hematoxylin/eosin. (Scale bar: 0.5 mm.) (*B*) Red-stained cells on the bone trabeculae are TRAP-positive osteoclasts. (*Lower*) High-magnification image of multinucleated osteoclasts. (Scale bars: *Upper*, 100 μm; *Lower*, 25 μm.) (*C*) The numbers of osteoclasts in the sections of the median portion of femora were counted. The TK domain deficiency showed a mild reduction in number of osteoclasts (*, $P < 0.05$).

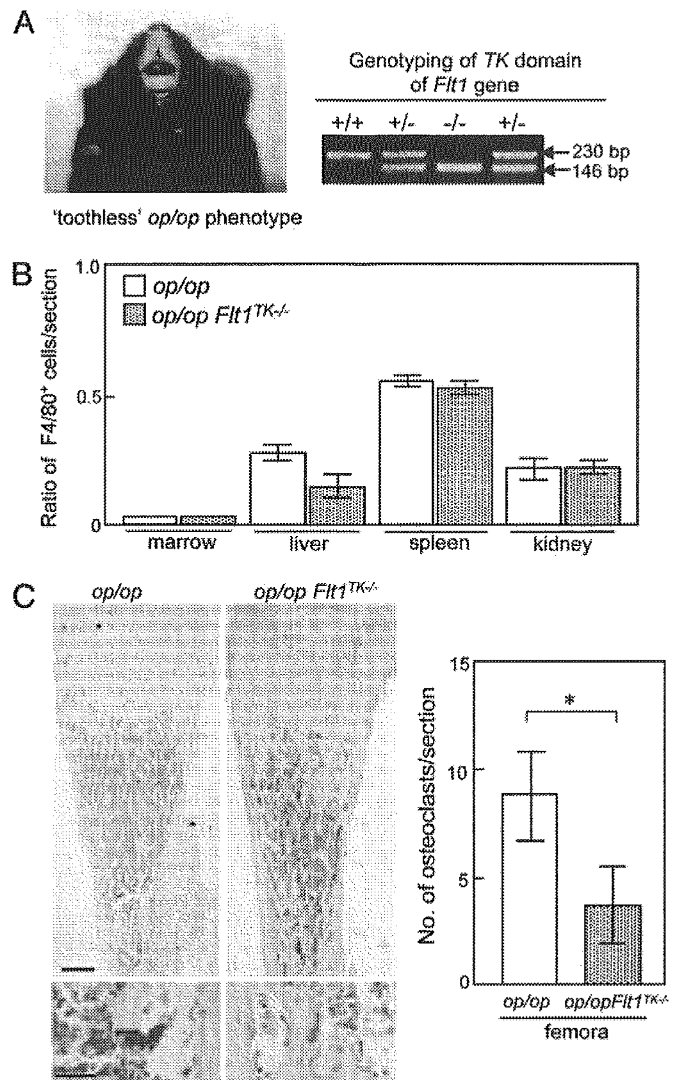

Fig. 2. Phenotype of $op/opFlt1^{TK-/-}$ mice. (*A*) Double mutant $op/opFlt1^{TK-/-}$ mice exhibit an *op/op* toothless phenotype with the genotype of $Flt1^{TK-/-}$ (−/−) from a litter resulting from the interbreeding of $op/+Flt1^{TK+/-}$ and $op/+Flt1^{TK+/-}$ mice. Tail DNA was isolated and analyzed by PCR according to our previous study (21). (*B*) The ratio of numbers of macrophages with F4/80 immunoreaction in various tissues of $op/opFlt1^{TK-/-}$ mice to WT mice. (*C*) The $op/opFlt1^{TK-/-}$ femur exhibits a more severe osteopetrosis. The small red spots are osteoclasts. TRAP-positive cells containing nuclei, as shown in the high-magnification image, were counted as osteoclasts. The number of osteoclasts in the sections of 2-wk-old $op/opFlt1^{TK-/-}$ mice femora significantly (*, $P < 0.01$) decreased compared with that in age-matched *op/op* mice. (Scale bars: *Upper*, 0.5 mm; *Lower*, 25 μm.)

for the mild and transient recruitment of osteoclasts in $op/opFlt1^{TK-/-}$ mice. To test this hypothesis, we used ligands specific for VEGFR-1 and VEGFR-2 in *op/op* and $op/opFlt1^{TK-/-}$ mice. rhCSF-1 was used as a control. The administration of rhPlGF, a VEGFR-1-specific ligand (26, 27), efficiently restored osteoclast formation in *op/op* mice but not in $op/opFlt1^{TK-/-}$ mice (Fig. 4*A*), although the osteoclasts were mostly small, and the degree was half that with rhCSF-1 (Fig. 4*A*). These results support our basic idea that VEGFR-1 is an important mediator for osteoclast formation. We next examined whether VEGF-E, a VEGFR-2-specific ligand, leads to osteoclastogenesis in $op/opFlt1^{TK-/-}$ mice. The VEGF-E administrations induced small osteoclasts in both *op/op* and $op/opFlt1^{TK-/-}$ mice. Similar results were obtained from the administration of $VEGF_{120}$, which binds both receptors (Fig. 4*A*).

These activities of VEGFs were also observed in an *in vitro* osteoclast formation assay in which spleen cells were cocultured with OP9 osteoclastogenesis-supportive stromal cells (Fig. 4*B*).

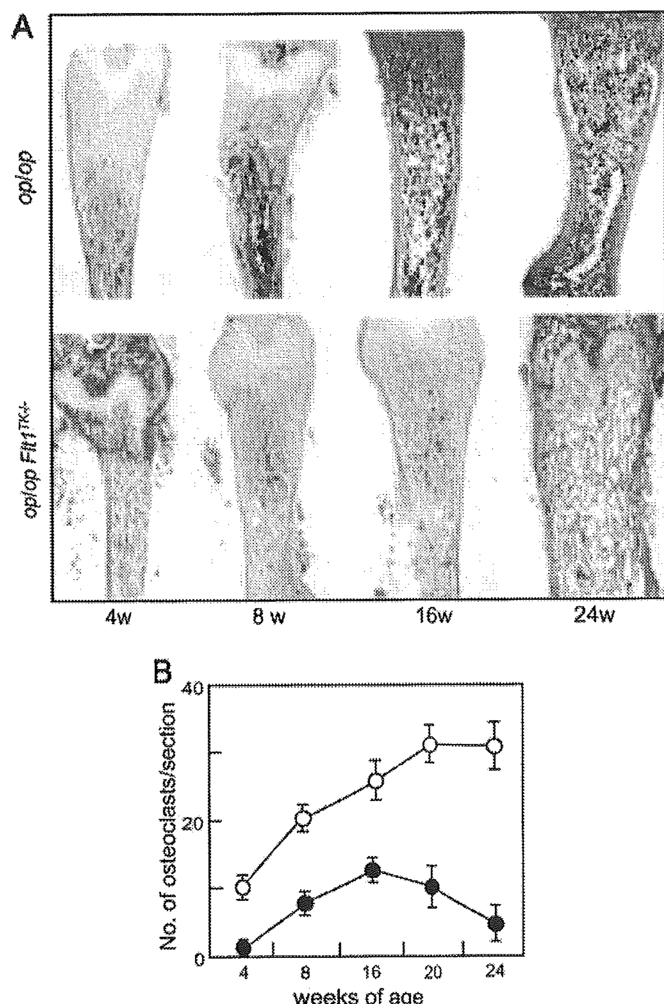

Fig. 3. *op/opFlt1*$^{TK-/-}$ mice cannot recruit enough osteoclasts to form bone marrow. (*A*) Growth changes of femora in *op/op* and *op/opFlt1*$^{TK-/-}$ mice. Longitudinal sections of femora were stained with hematoxylin/eosin. Bone trabeculae in *op/op* mice decreased with aging, whereas those of *op/opFlt1*$^{TK-/-}$ mice remained until 24 wk of age. (*B*) Changes in numbers of TRAP^{+} osteoclasts in *op/op* (open circles) and *op/opFlt1*$^{TK-/-}$ (filled circles) mice.

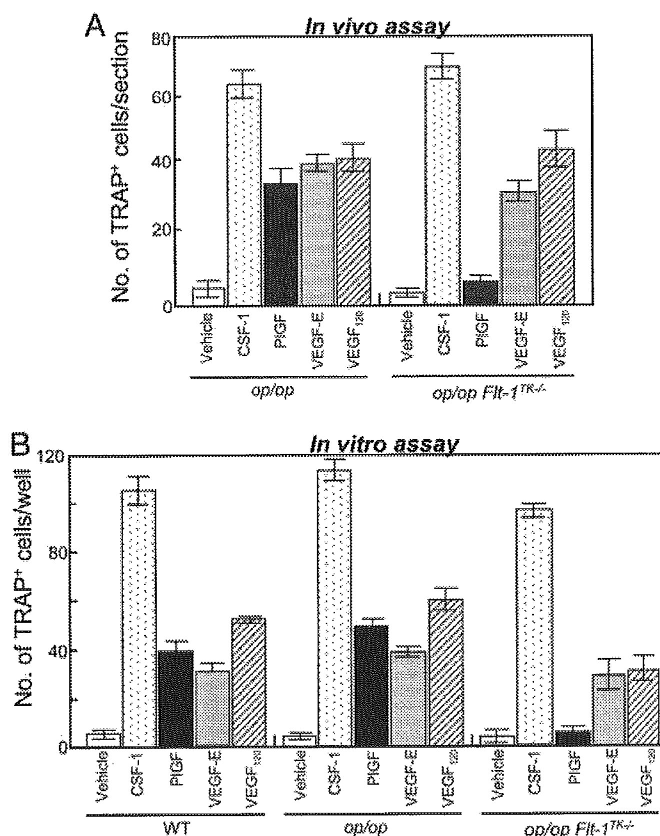

Fig. 4. Osteoclast-induction activity of rhCSF-1, recombinant mouse VEGF$_{120}$, rhPlGF, and VEGF-E in both mutant mice. (*A*) rhCSF-1 and VEGFs were injected in a single dosage of 5 μg into <3-wk-old *op/op* and *op/opFlt1*$^{TK-/-}$ mice, and mice were killed 4 d after the injection. Osteoclasts in longitudinal sections of the median portion of femora were counted. (*B*) *In vitro* assay of osteoclastogenic activity of rhCSF-1 and various VEGFs. Spleen cells of *op/op* and *op/opFlt1*$^{TK-/-}$ mice were cultured for 6 d with OP9 stromal cells in the presence of each cytokine in 96-well plates. The cultures were stained for TRAP activity, and TRAP-positive cells were counted.

Although PlGF-induced osteoclast-like cells were smaller than those induced by CSF-1 treatment, they have multiple nuclei. The cells induced with VEGF-E were the smallest, mostly with only a few nuclei. These features were essentially the same without OP9 feeder cells (see the supporting information, which is published on the PNAS web site); however, the survival rate of the cells was lower compared with that in the OP9 feeder system. These findings suggest that VEGFR-2 might play some role in the development of osteoclasts in *op/op* background mice. In our previous study, however, VEGFR-2 was under detectable levels in monocyte/macrophage lineage cells (18). A low level of VEGFR-2 might induce a differentiation signal, or VEGFR-2 could be up-regulated during the culture period, because a recent study showed that VEGFR-2-expression in monocytes/macrophages, initially undetectable, was induced by VEGF stimulation (28).

Our findings indicate that exogenous PlGF has an osteoclastogenic activity, raising the question of whether endogenous PlGF (or VEGF-B) plays a role in the activation of VEGFR-1 and up-regulates osteoclastogenesis *in vivo*. However, recent studies provided evidence that PlGF-deficient mice are normally developed and healthy without clear abnormality (29). VEGF-B-deficient mice are also basically healthy, with normal morphology except in the heart. Some VEGF-B$^{-/-}$ mice showed an enlarged heart (30, 31). These findings suggest that endogenous PlGF and VEGF-B have only a minor effect, if any, on the osteoclastogenesis by means of activation of VEGFR-1 and that the major signal is generated by means of endogenous VEGF-A.

Another question is why the endogenous VEGF-A cannot rescue osteoclastogenesis by means of VEGFR-2 in *op/opFlt1*$^{TK-/-}$ mice. We suggest two reasons. The first is a quantitative point. Concentration of endogenous VEGF-A is known to be significantly low compared with that of exogenous VEGF-A. Furthermore, the affinity of VEGFR-2 to VEGF-A is one order weaker than that of VEGFR-1 (14, 27). Thus, the endogenous VEGF-A may not be sufficient to stimulate VEGFR-2 at high levels. The second is a qualitative point. Our *in vitro* studies (Fig. 4*B*) indicate that the VEGFR-2-specific ligand induced osteoclast-like cells with only a few nuclei. Therefore, VEGFR-2 signaling might be qualitatively insufficient to generate a full differentiation signal in osteoclast precursor cells.

Myelofibrosis in *op/opFlt1*$^{TK-/-}$ Mice. Another striking feature of *op/opFlt1*$^{TK-/-}$ mice is the significant increase in fibrous tissues instead of hematopoietic cells in the marrow cavity. In 8-wk-old

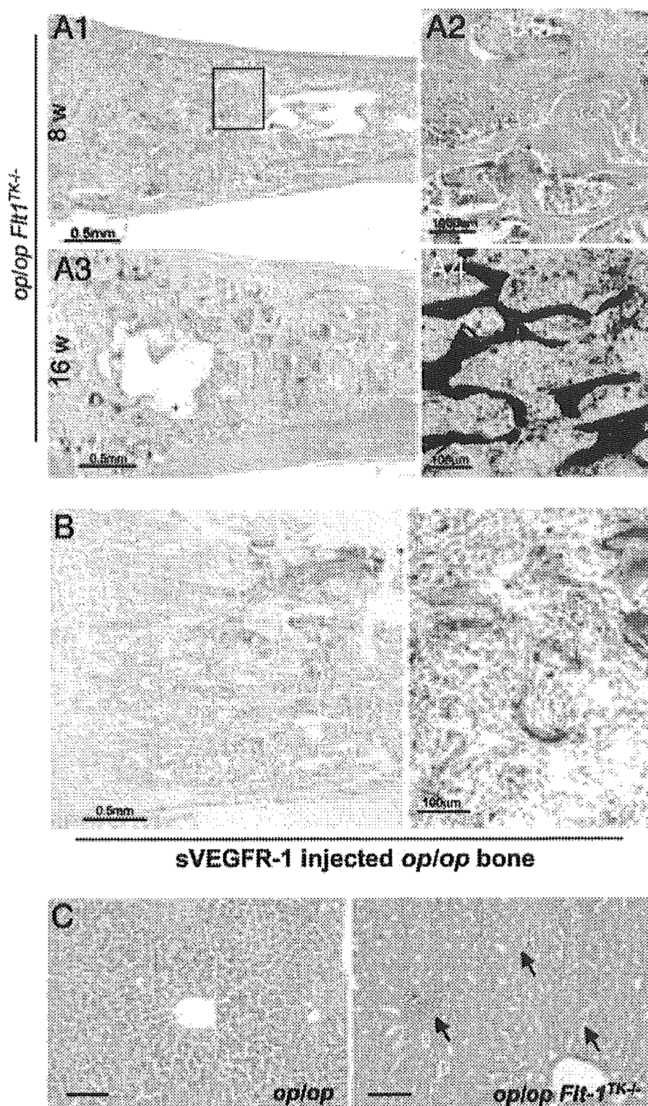

Fig. 5. Severe myelofibrosis in *op/opFlt1*$^{TK-/-}$ mice. (*A1* and *A2*) Myelofibrosis was found in the diaphysial region of the marrow cavity in 8-wk-old mice. A high-magnification image of the boxed area shows the junction of normal marrow and myelofibrosis. (*A3*) Myelofibrosis filled the bone 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 μm.)

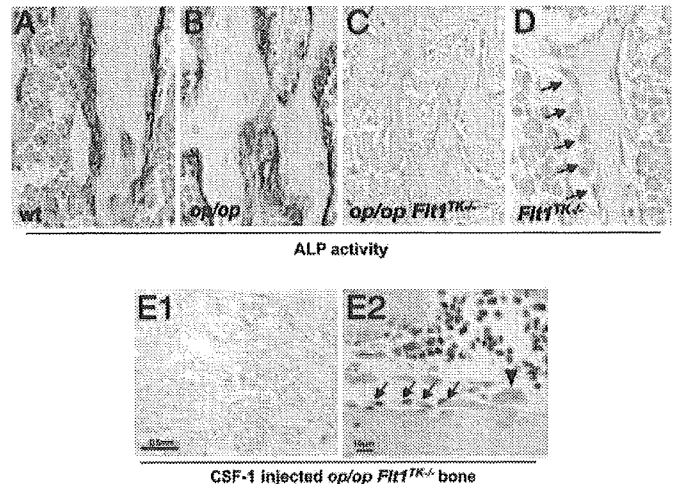

Fig. 6. Immunohistochemistry of ALP activity in four mouse genotypes: WT, *op/op*, *op/opFlt1*$^{TK-/-}$, and *Flt1*$^{TK-/-}$. (*A–D*) Cells stained brown are ALP-positive osteoblasts. The ALP activity of the *op/opFlt1*$^{TK-/-}$ and *Flt1*$^{TK-/-}$ mice is extremely weak compared with that of the 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 trabeculare. (*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.

mice, fibrous tissue was initially observed in the diaphysial regions of the femora, although hematopoietic cells still occupied the intratrabecular spaces in the epiphysial region (Fig. 5*A1* and *A2*). With aging, the fibrous tissue gradually expanded to the whole marrow cavity, resulting in marked decreases in marrow cellularity, including osteoclasts (Fig. 5*A3*). Histological analysis indicated that the fibrous tissue consisted of reticular fiber-like fibrils (Fig. 5*A4*). 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. 5*B*).

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 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. 1*A*). Furthermore, a number of small hematopoietic foci were found in the liver of *op/opFlt1*$^{TK-/-}$ mice (Fig. 5*C*). 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 extramedually 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. 6 *A–C*). Osteoblasts were significantly decreased on the surface of bone trabeculae, adjacent to myelofibrosis (Fig. 6*C*). 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. 6*D*). 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. 6*E*). Moreover, rhCSF-1 treatment also prevented marrow alterations (Fig. 6*E2*). 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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REVIEW ・ JAOB・Lion Dental Research Award ・

Osteoclast・forming Activity of Vascular Endothelial Growth Factor

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Key words ・ osteoclast ・ VEGF ・ VEGF receptor ・ Flt・1 ・ Flk・1

Abstract ・ Colony・stimulating factor・1・ CSF・1 ・is an essential regulator of the differentiation, proliferation and survival of macrophage lineage cells including bone・resorbing osteoclasts. We have demonstrated that vascular endothelial growth factor ・ VEGF ・, a known angiogenic factor, can act as a substitute for CSF・1 function in osteoclastogenesis through the VEGF receptor・1. Osteopetrotic Csf1^{op}・Csf1^{op} ・ op・op ・ mice exhibit severe osteoclast deficiency owing to the lack of CSF・1 function. However, the deficiency is gradually reversed with aging, suggesting the existence of an alternative factor supporting osteoclastogenesis. We have found that the administration of VEGF to op・op mice induces a sufficient number of osteoclasts to ameliorate the osteopetrosis. Estrogen deficiency induces the acceleration of osteoclastic bone resorption mediated by the upregulation of bone・resorbing factors including CSF・1. Ovariectomized op・op mice exhibited upregulation of VEGF expression and an increase in number of osteoclasts. VEGF antagonists inhibited both spontaneous osteoclast recruitment in the aging op・op mice and estrogen deficiency・dependent increases in osteoclasts in OVX・op・op mice. These results clearly demonstrate an ability of osteoclastogenic activity of VEGF.

Introduction

・Osteoclast biology has been extensively studied over the last decade. It has been established that hematopoietic growth factor colony・stimulating factor・1・ CSF・1, also known as M・CSF ・is essential for the proliferation, differentiation, and survival of osteoclasts derived from monocyte・macrophage lineage cells[1,2]・. The biological effects of CSF・1 are mediated through a cell・surface tyrosine kinase receptor c・Fms, which is one of the eight members of the platelet・derived growth factor receptor ・ PDGFR ・family[3]・. The critical role of CSF・1 in osteoclastogenesis has been proven in studies using osteopetrotic Csf1^{op}・Csf1^{op} ・ op・op ・ mice. Mice homozygous for a recessive op mutation on chromosome 3 exhibit a severe deficiency of osteoclasts, monocytes and tissue macrophages owing to a lack of functional CSF・1[4・6]・. Yoshida, et al.[7]・ revealed that the loss of CSF・1 function in op・op mice is caused by a point mutation within the cording region of the Csf1 gene. The administration of the recombinant human CSF・1 ・ rhCSF・1 ・ reversed the defects in op・op mice[8・10]・. The expression of c・Fms in osteoclasts demonstrated the direct action of CSF・1 on osteoclast lineage cells[11,12]・. However, severe osteopetrosis in op・op mice is evident only in juvenile mice. With aging, cells stained by tartrate・resistant acid phosphatase ・TRAP・, an osteoclast marker, appear spontaneously in op・op mice bones and correct the osteopetrosis. In addition, only a single dose of rhCSF・1 ・ ・5 ・g・body ・ is sufficient to induce not only osteoclastogenesis but also continued

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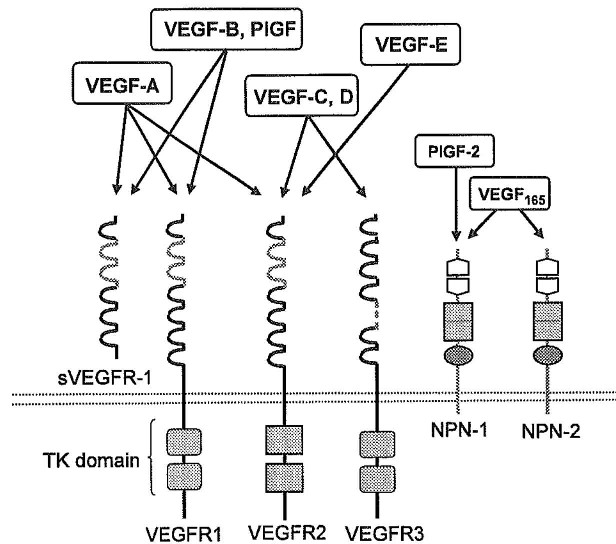

Fig. 1 • VEGF families and their interactions with VEGF receptors. Osteoclasts and preosteoclasts express both VEGFR1 and VEGFR2.

active bone resorption in op·op mice[13,14]. These results suggest the existence of an alternative factor supporting osteoclastogenesis and survival in op·op mice.

• Although evidence from op·op mice reveals an essential role for CSF·1 in osteoclast biology, it simultaneously raises the question • what induces osteoclastogenesis in op·op mice?. Granulocyte·macrophage colony·stimulating factor• GM·CSF •has a function similar that of CSF·1 in the development of macrophage linage cells. However, GM·CSF is not responsible for the correction of osteoclast deficiency in the op·op mice[15,16]. We have previously demonstrated that congenital osteoclast deficiency in op·op mice can also be ameliorated by administration of a recombinant human vascular endothelial growth factor • rhVEGF •[17,18].

VEGF and Its Receptors

• VEGF is a key regulator of the growth and differentiation of vascular and lymphatic endothelial cells[19], and is also known as vascular permeability factor • VPF •[20]. VEGF belongs to the PDGF supergene family and includes several members including VEGF·A, placenta growth factor • PlGF •, VEGF·B, VEGF·C, and VEGF·D. In addition, Orf·virus·derived VEGF·like polypeptide, VEGF·E, has been identified[21]. Human VEGF·A has multiple spliced isoforms including $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$[22·24]. In mice and rats, VEGF·A isoforms are shorter by one amino acid[19,25]. $VEGF_{121}$ fails to bind to heparin, while $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$ are heparin·binding proteins[26]. Recently, two other splice variants, $VEGF_{145}$ and $VEGF_{183}$, were identified in humans[27,28].

• VEGF receptor 1 • VEGFR1·Flt·1 • and VEGF receptor 2 • VEGFR2·Flk·1·KDR • are high·affinity receptos for VEGF·A and function as key mediators for angiogenesis • Fig. 1 •. These receptors have seven immunoglobulin • Ig •·like domains in their extracellular regions and an ·70 amino·acid·long tyrosine kinase • TK • domain in the cytoplasmic regions[29·32]. The fundamental structure of VEGFRs is very similar to that of PDGFR family members such as PDGFR·c·Fms·c·kit·Flt·3, although the PDGFRs have five instead seven Ig·like domains in their extracellular domains. VEGFR1 has a high affinity for $rhVEGF_{165}$[33] and as a decoy receptor for VEGF negatively regulates, at least in some circumstances, angiogenesis[34]. In addition, the VEGFR1 gene encodes an alternatively spliced soluble form of VEGFR1 • sVEGFR1 • lacking the seventh Ig·like domain and the cytoplasmic TK domain[29,31,35]. sVEGFR1 also has a high affin-

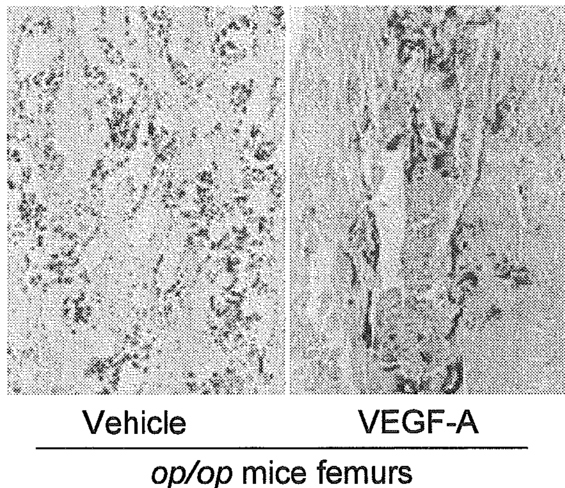

Fig. 2 • Exogenous VEGF•A administration in op•op mice recovering osteoclastogenesis. Cells colored black in the photograph on the right are osteoclasts induced by VEGF•A.

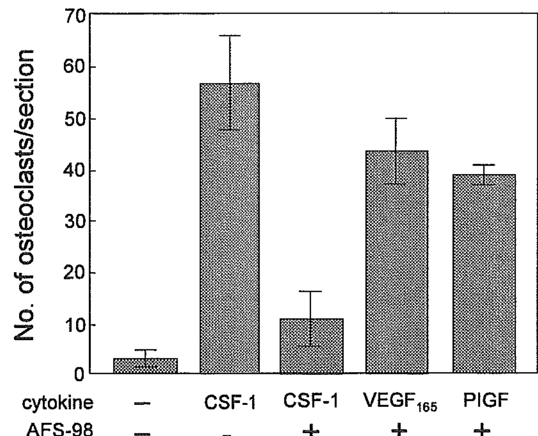

Fig. 3 • AFS98 anti•c•Fms mAb was administered at a dose of 750 •g•body at both 2h before and after 24h after cytokine administration. The numbers of TRAP• cells in the longitudinal sections of whole femurs were counted.

ity for VEGF•A. Although VEGFR2 has a lower affinity for VEGF than does VEGFR1[36]•, it serves as a major signaling receptor for endothelial cell proliferation and differentiation. The homozygous deletion of any of these receptor genes in mice results in embryonic lethality attributable to deficiencies or abnormalities in vasculogenesis and angiogenesis[37•39]•. In addition to these receptors, $VEGF_{165}$•specific receptor, neuropilin•1 • *NPN*•1 •, has been identified[40]•. *NPN*•1 is a known receptor for the semaphorin•collapsin family involved in neuronal cell guidance • it enhances the binding activity of $VEGF_{165}$ to VEGFR2 and $VEGF_{165}$•mediated chemotaxis. Interestingly, it has been reported that VEGFR1 is expressed in monocyte•macrophage lineage cells and is involved in signal transduction for cell migration[41,42]•.

VEGF Rescues Osteoclast•deficiency in op•op Mice

• To date, VEGF is the only cytokine, excluding CSF•1, which is able to induce osteoclasts in op•op mice by exogenous administration • Fig. 2 •. This discovery has revealed the existence of a unique type of redundancy in cytokine signaling through different ligand•receptor systems in osteoclastogenesis. Dose•response studies demonstrated that a single injection of 5 •g of $rhVEGF_{165}$ is sufficient to correct osteopetrosis in op•op mice[18]•. Either $rhVEGF_{121}$ or rhPlGF•1, a specific ligand of VEGFR1, was effective at inducing osteoclastogenesis[17]•. The number of osteoclasts induced by any of these factors was •70 • of that induced by rhCSF•1. *N*eutralization with anti•c•Fms antibody significantly decreased the rhCSF•1•dependent osteoclast induction in op•op mice, but not the rhVEGF•A and rhPlGF•dependent osteoclastogenesis • Fig. 3 •, suggesting the presence of VEGF•A•specific receptors on osteoclast precursors. We have detected the expression of VEGFR1, but not VEGFR2, on the osteoclasts in both normal and op•op mice[17,18]•. $VEGF_{121}$ does not bind *NPN*•1[43]•. PlGF•1 binds VEGFR1, but not VEGFR2 or *NPN*•1[42•44]•. Our results, showing that the osteoclastogenic activities of $rhVEGF_{121}$ and rhPlGF•1 are comparable to that of $rhVEGF_{165}$, support the hypothesis that the response of osteoclast precursors to VEGF is directly mediated, at least in part, by VEGFR1. *N*akagawa, et al.[45]• demonstrated that VEGFR2 is directly involved in osteoclastic bone resorption. Recently, we confirmed immunohistochemically the expression of VEGFR2 on op•op osteoclasts. These results do not exclude the possibility that VEGFR2 is also involved in osteoclastogenesis. Further studies will be

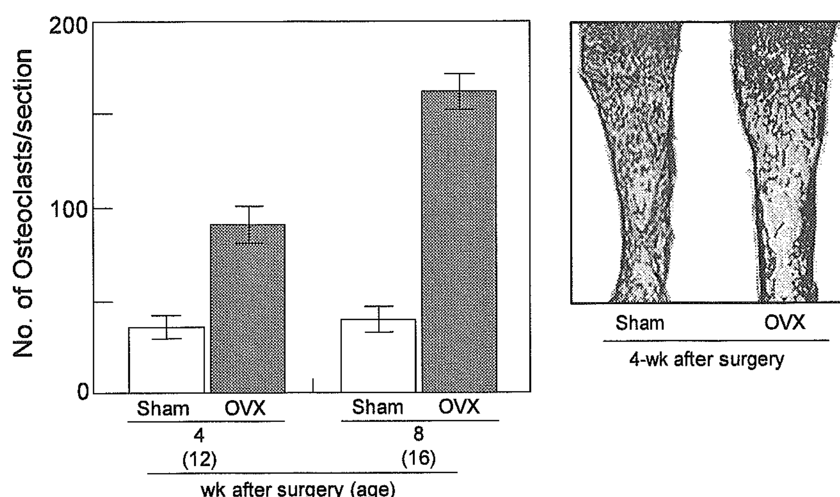

Fig. 4 ▪ OVX•induced osteoclastogenesis and bone resorption in op•op mice. Mice were ovariectomized at 8•week•old, and euthanized at 4 or 8 weeks after surgery.

required to elucidate the VEGFR signaling mechanisms involved in osteoclastogenesis.

▪ The receptor activator of nuclear factor• ▪B ligand ▪ RANKL ▪ plays a crucial role in the terminal differentiation of osteoclasts in cooperation with CSF•1[46,47]▪. The osteoclastogenic activity of VEGF was further confirmed by in vitro studies. We cultured mouse bone marrow hematopoietic cells in the presence of rhVEGF$_{165}$ and•or rhRANKL. No TRAP▪ cells appeared in the presence of rhVEGF$_{165}$ alone. The combination of rhVEGF$_{165}$ and rhRANKL resulted in the formation of supported TRAP▪ cells, although the cell size was smaller than those generated in the presence of rhCSF•1 and rhRANKL[17]▪. Bone•resorbing activity of these cells has been confirmed by scanning electron microscopy[17]▪.

▪ Osteopetrosis in op•op mice is progressively corrected with aging owing to spontaneous osteoclast recruitment. We examined whether osteoclast recruitment in op•op mice depends on the endogenous production of VEGF. A significantly larger number of osteoclasts was observed in the femurs of ▪2•month•old op•op mice as compared with 2•week•old mice. Five consecutive injections of 100 ▪g goat anti•VEGF polyclonal antibody at 12•hr intervals significantly decreased the osteoclast number in 2•month•old mice, indicating that VEGF is responsible for spontaneous osteoclast recruitment in the absence of functional CSF•1 in op•op mice.

'Osteoporosis' in the op•op Mice

▪ Estrogen deficiency leads to bone loss through the action of an increased number of osteoclasts. Many studies in ovariectomized ▪ OVX ▪ animals have implicated increases in various cytokines such as CSF•1, IL•1, IL•6, TNF• ▪, and PGE$_2$[48,49]▪. These cytokines accelerate bone resorption mainly through the proliferation of presoteoclasts. Estrogen replacement in OVX animals suppresses the expression of these cytokines in osteoclastogenesis•supportive bone marrow stromal cells, monocytes, and lymphocytes[48]▪. In addition, recent studies indicated that high concentrations of TNF• ▪ promote osteoclastogenesis independently of RANKL[50,51]▪.

▪ Over ten years ago, we discovered that OVX led to a large amount of osteoclastogenesis in op•op mice ▪ Fig. 4 ▪. Although we had not been able to identify the type of factor▪ s ▪ that substitute for CSF•1 in OVX•op•op mice, the results suggested that the factor▪ s ▪ were modulated by estrogen deficiency. It has been shown that estrogen and other steroid hormones regulate VEGF production in several tissues. Again, we have attempted to identify the regulator of

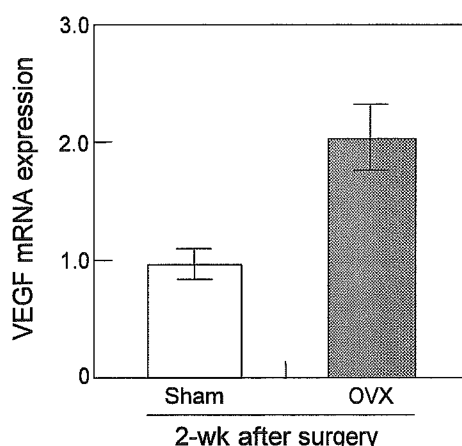

Fig. 5 • Expression of VEGF mRNA upregulated in OVX•op•op bone. Interestingly, the expression of the mRNAs of VEGFR1 and RANKL was also elevated in OVX•op•op mice.

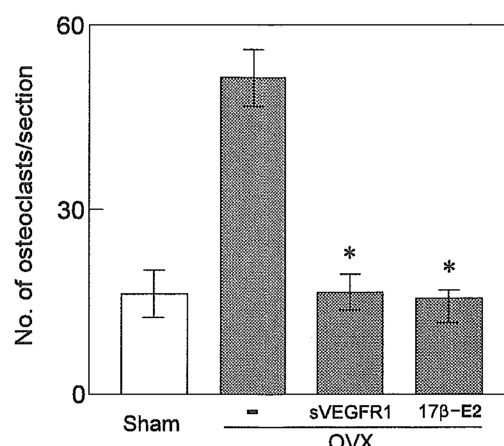

Fig. 6 • Estrogen deficiency•dependent increase in osteoclast number suppressed by the administration of soluble VEGFR1 chimeric protein or 17 • •estradiol • 17 • • E2 •.

osteoclastogenesis in OVX•op•op mice[52]. VEGF expression was elevated in bone in OVX•op•op mice compared with sham•operated mice • Fig. 5 •. The enhanced expression of the mRNA for VEGFR1, RANKL and IL•6, but not for TNF• • and IL•1•, was also observed in bones from OVX mice[52]. It has been shown that IL•6 acts as a stimulator for the production of RANKL and VEGF[53]. The upregulation of VEGFR1 expression suggests an increase in the number of osteoclast precursors. TNF• • and IL•1• are mainly produced by monocytes in bone marrow. op•op mice exhibit a severe deficiency of these cells •therefore, the upregulation of TNF• • and IL•1• is not observed in op•op mice. Neutralization experiments revealed that the increase in osteoclasts in OVX•op•op mice is prevented by soluble chimeric VEGFR1 protein or anti•VEGF antibody and by replacement with 17 • •estradiol • Fig. 6 •. These results strongly suggest that the upregulation of VEGF•VEGFR1 in OVX•op•op mouse bone is an important determinant in the increase in the number of osteoclasts by estrogen deficiency. IL•6 may contribute by augmenting the production of VEGF and RANKL. Estrogen deficiency•dependent hypoxia may be involved in upregulation of VEGF expression in bone[54]. The downregulation of estrogen production leads to hypoxia, a known key regulator of VEGF production[55], in several organs through decreased nitric oxide synthesis[56]. The details of mediators in VEGF•dependent osteoclastogenesis are not well established.

Role of VEGF in Endochondral Bone Formation

• VEGF plays an important role in endochondral bone formation. Hypertrophic chondrocytes in the epiphysial growth plate express VEGF[57,58]. The administration of soluble chimeric VEGFR1 protein completely suppresses blood vessel invasion into the growth plate in mice. Furthermore, the recruitment of osteoclasts and the resorption of terminal chondrocytes are substantially decreased, concomitant with impaired trabecular bone formation[57]. Mice with the conditional deletion, by means of Cre•loxP•system, of a single VEGF•A allele in collagen type • • expressing cells exhibited impaired bone vascularization and mineralization when embryos survive until day E17.5[59]. A similar phenotype was observed in mice expressing only a single $VEGF_{120}$ isoform • $VEGF^{120/120}$ •[60]. Although $VEGF^{120/120}$ mice proceed through all stages of embryogenesis, they show skeletal abnormalities including impaired vascularization and deformity of the epiphysial cartilage. Moreover,

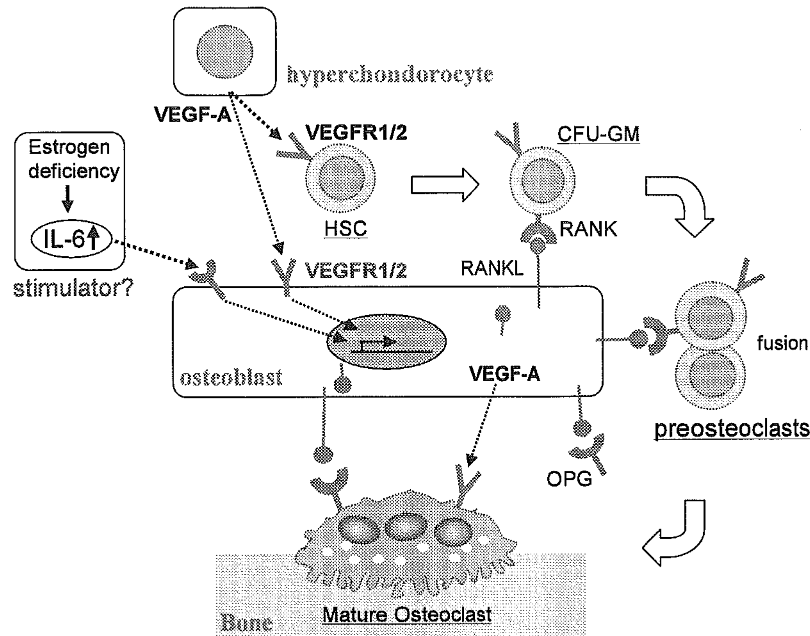

Fig. 7 • Proposed model of VEGF action in osteoclastogenesis. VEGF is produced by hyperchondrocytes and osteoblasts. OPG • osteoprotegerin, HSC • hematopoietic stem cells, CFU•GM • granulocyte•macrophage colony•forming unit.

$VEGF^{120/120}$ mice have a significantly reduced number of osteoclasts. These results indicate that the heparin•binding type of VEGF•A is an important regulator of endochondral ossification. Subsequently, it was shown that mice exclusively expressing $VEGF_{188}$ • $VEGF^{188/188}$ • also exhibit several bone anomalies. Taken together, these results suggest a crucial role for $VEGF_{164}$ during bone development[61]•. It has been reported that no bone anomalies are observed in $VEGF^{164/164}$ mice[61]•.

• VEGF may contribute to bone formation via a direct effect on osteoblasts. In vitro studies demonstrate that $rhVEGF_{165}$ binds to osteoblasts and stimulates migration, accumulation of PTH•dependent cAMP and upregulation of alkaline phosphatase activity[62]•. Several studies demonstrated the expression of VEGFR1 and VEGFR2 on osteoblasts[63]•. $VEGF^{120/120}$ mice exhibit reduced osteoblast activity in endochondral bone and intramembranous bone[60]•. VEGF•A appears to have multiple functions during bone development.

Conclusions and Future Perspectives

• We have demonstrated that VEGF and PlGF can induce osteoclastogenesis and substitute for CSF•1 function • Fig. 7 •. Several other studies support our findings[45,57,60]•. In addition, we have demonstrated the possibility that VEGF is implicated in estrogen deficiency•dependent bone loss. Although osteoclasts and their precursor cells express VEGFR1 and VEGFR2, our recent experiments suggest that VEGFR1 plays a predominant role in osteoclastogenesis • unpublished data •. However, several questions remain. VEGFR1 signaling is extremely weak. How such a weakly signaling receptor can play a critical role in osteoclastogensis is unclear. This is likely to be addressed in future studies.

• Gene targeting strategies have revealed roles for VEGF in bone development. VEGF is required for angiogenesis and cartilage matrix resorption during endochondral ossification. Taken together with our

findings, these results suggest a critical role for VEGF • mediated blood vessel invasion in coupling cartilage resorption with bone formation, and provide new insights into the regulation of bone development and remodeling.

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c-Fos protein as a target of anti-osteoclastogenic action of vitamin D, and synthesis of new analogs

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Although active vitamin D drugs have been used for the treatment of osteoporosis, how the vitamin D receptor (VDR) regulates bone cell function remains largely unknown. Using osteoprotegerin-deficient mice, which exhibit severe osteoporosis due to excessive receptor activator of NF-κB ligand/receptor activator of NF-κB (RANKL/RANK) stimulation, we show herein that oral treatment of these mice with 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$] inhibited bone resorption and prevented bone loss, suggesting that VDR counters RANKL/RANK signaling. In M-CSF–dependent osteoclast precursor cells isolated from mouse bone marrow, 1α,25$(OH)_2D_3$ potently and dose-dependently inhibited their differentiation into multinucleate osteoclasts induced by RANKL. Among signaling molecules downstream of RANK, 1α,25$(OH)_2D_3$ inhibited the induction of c-Fos protein after RANKL stimulation, and retroviral expression of c-Fos protein abrogated the suppressive effect of 1α,25$(OH)_2D_3$ on osteoclast development. By screening vitamin D analogs based on their c-Fos–suppressing activity, we identified a new analog, named DD281, that inhibited bone resorption and prevented bone loss in ovariectomized mice, more potently than 1α,25$(OH)_2D_3$, with similar levels of calcium absorption. Thus, c-Fos protein is an important target of the skeletal action of VDR-based drugs, and DD281 is a bone-selective analog that may be useful for the treatment of bone diseases with excessive osteoclastic activity.

Introduction

Excessive osteoclastic bone resorption plays a central role in the pathogenesis of age-related bone loss and microstructural deterioration, leading to fragility fractures (1). Mutinucleated osteoclasts are generated from hematopoietic precursor cells through the action of M-CSF and receptor activator of NF-κB ligand (RANKL) (2–4). These cytokines are produced by osteoclastogenesis-supporting marrow stromal cells and act on osteoclast precursor cells that express their receptors, c-fms and receptor activator of NF-κB (RANK), respectively. These cell-surface receptors transmit osteoclastogenic signals through intracellular kinase cascades that culminate in the activation of transcription factors c-Fos/AP-1 and NF-κB in the nucleus. Accordingly, mice deficient in c-Fos, NF-κB, RANK, RANKL, or M-CSF cannot generate osteoclasts and exhibit osteopetrosis (2–4).

Osteoclasts thus formed fuse with one another and mature into multinucleated, functional osteoclasts that undergo cytoskeletal reorganization and produce effector molecules involved in acidification, degradation of matrix proteins, and expression of hormone/cytokine receptors. Disruption of c-Src, chloride channels, proton pump, or cathepsin K results in the generation of osteoclasts with impaired bone-resorbing function (2). Bisphosphonates, currently most widely used for the treatment of osteoporosis, are known to interfere with the bone-resorbing activity of mature osteoclasts rather than with their differentiation from hematopoietic precursors (5, 6), although the precise target molecules remain to be identified.

Vitamin D hormone, acting through the nuclear vitamin D receptor (VDR), has been used to generate osteoclasts, based on its ability to induce RANKL expression in marrow stromal cells; and it is generally recognized as a bone-resorbing agent (3). Contrary to this belief, we previously demonstrated in estrogen-deficient rats and mice with accelerated bone resorption that alfacalcidol, a prodrug metabolized to the natural vitamin D hormone 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$], and its analog ED-71 reduced the number of osteoclasts, thereby potently suppressing bone resorption in vivo (7–9). Osteoclast activation in estrogen deficiency involves diverse mechanisms, including the production of bone-resorbing cytokines in the bone microenvironment (10, 11) in addition to estrogen's direct effect on osteoclasts and their precursors (12). It is, therefore, difficult to identify the target cell and molecule of 1α,25$(OH)_2D_3$ in ovariectomy models. In order to define the molecular pathway(s) that VDR acts upon, we examined the effects of 1α,25$(OH)_2D_3$ in a genetic model of osteoporosis due to constitutive activation of RANK signaling.

Results

1α,25$(OH)_2D_3$ inhibits bone resorption in osteoprotegerin KO mice. Osteoprotegerin (OPG) is a decoy receptor of RANKL that belongs to the TNF receptor family (13), and mice lacking OPG exhibit excessive bone resorption as a result of constitutive activation of RANKL/RANK signaling (14). Oral administration of 1α,25$(OH)_2D_3$ to *OPG* homozygous KO mice caused a dose-dependent reduction in the osteoclast number (Figure 1A) and in osteoclast surface

Nonstandard abbreviations used: BMD, bone mineral density; 1α,25$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; OPG, osteoprotegerin; OVX, ovariectomized; RANK, receptor activator of NF-κB; RANKL, RANK ligand; TRAP, tartrate-resistant acid phosphatase; VDR, vitamin D receptor.

Conflict of interest: E. Ogata is a member of the board of Chugai Pharmaceutical Co., which manufactures active vitamin D derivatives for the treatment of bone diseases.

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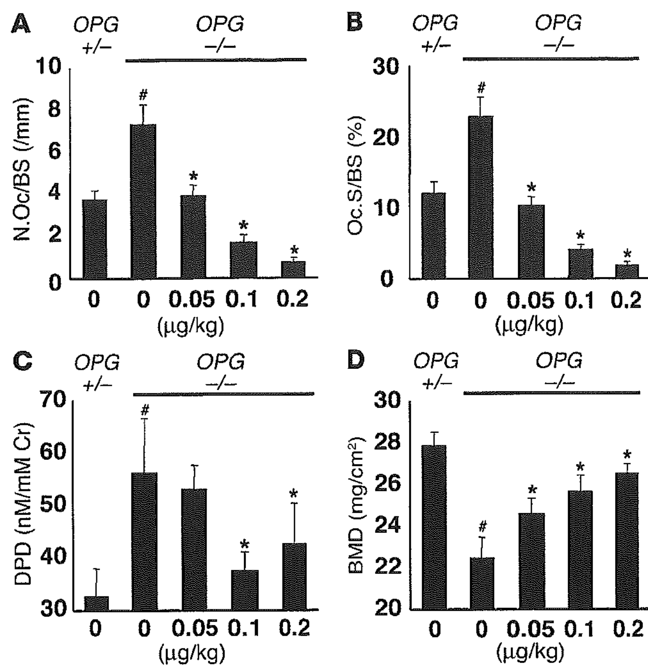

Figure 1
$1\alpha,25(OH)_2D_3$ inhibits bone resorption in *OPG* KO mice. *OPG* homozygous KO (–/–) mice were treated orally with the indicated doses of $1\alpha,25(OH)_2D_3$ for 6 weeks, and osteoclast number (corrected for bone surface; N.Oc/BS (**A**), bone surface covered by osteoclasts (Oc.S/BS) (**B**), urinary deoxypyridinoline excretion (DPD; corrected for creatinine [Cr]) (**C**), and BMD (**D**) at the left femur were determined as described in Methods. Heterozygous (+/–) littermates served as the control. $^{*}P < 0.01$ versus *OPG* KO group with vehicle treatment, $^{\#}P < 0.01$ versus heterozygous control group, $n = 6$ each group.

area (Figure 1B) in bone sections, down to levels in heterozygous mice used as a control. The suppressive effect of $1\alpha,25(OH)_2D_3$ on bone resorption was also demonstrated by a reduction in the urinary level of a biochemical marker of bone resorption, deoxypyridinoline (Figure 1C). As reported previously (14), OPG-deficient mice had a markedly reduced bone mineral density (BMD) as a result of excessive bone resorption, and oral administration of $1\alpha,25(OH)_2D_3$ caused a dose-dependent amelioration of bone loss at the tibia (Figure 1D). The small pharmacological doses of $1\alpha,25(OH)_2D_3$ used in the current study (0.05–0.2 μg/kg) did not induce hypercalcemia (data not shown). These results suggest that $1\alpha,25(OH)_2D_3$ acts as an inhibitor of bone resorption in vivo by countering the RANKL/RANK pathway. In light of our previous observations that the expression of RANKL in bone did not increase following $1\alpha,25(OH)_2D_3$ administration in vivo (9), we hypothesized that $1\alpha,25(OH)_2D_3$ suppresses bone resorption by interfering with signaling through RANK receptors on osteoclast precursor cells.

$1\alpha,25(OH)_2D_3$ inhibits osteoclast development by acting directly on osteoclast precursor cells in bone marrow. In order to examine whether $1\alpha,25(OH)_2D_3$ counters osteoclastogenic signaling emanating from RANK receptors, we isolated osteoclast progenitor cells from mouse bone marrow and examined the effects of $1\alpha,25(OH)_2D_3$ on RANKL-induced osteoclastogenesis. In the presence of M-CSF and RANKL, the murine cultures gave rise to numerous multinucleated giant cells (Figure 2A) that were capable of forming resorption pits on dentine slices (data not shown). Treatment of the same cultures with $1\alpha,25(OH)_2D_3$ resulted in a dose-dependent reduction in the number of osteoclasts formed (Figure 2, A and B). $1\alpha,25(OH)_2D_3$ caused a significant reduction in the osteoclast number at a concentration as low as 10^{-9} M and inhibited the formation of osteoclasts by 70% at 10^{-8} M (Figure 2B).

The whole process of osteoclast development in murine cultures consists mainly of 2 phases: first, a stage of M-CSF–dependent growth of osteoclast progenitors, and then a latter phase of terminal differentiation induced by RANKL in the presence of M-CSF. The former process was assessed by isolation of osteoclast progenitor cells from bone marrow and measurement of their prolifera-

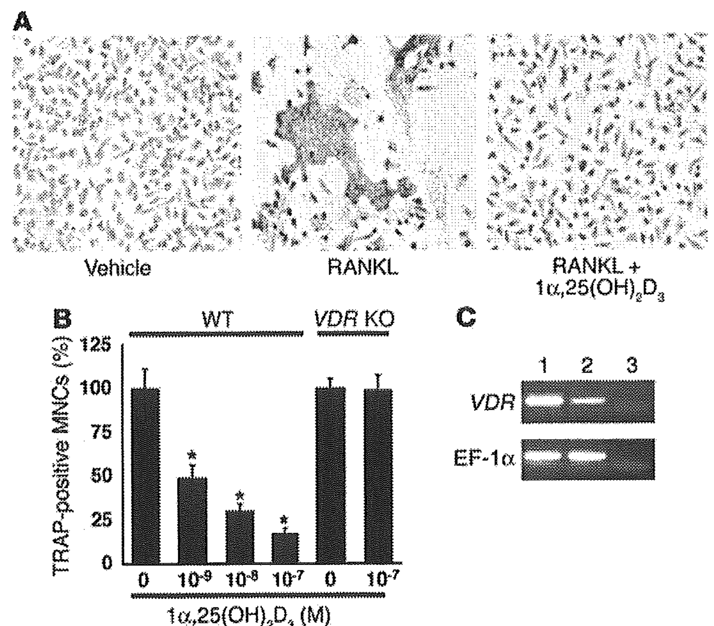

Figure 2
$1\alpha,25(OH)_2D_3$ inhibits osteoclast development through *VDR* by acting directly on osteoclast precursor cells in bone marrow. (A and B) Osteoclast precursor cells were isolated from the bone marrow of WT C57BL/6J and *VDR* KO mice (**B**) as M-CSF–dependent adherent cells, as described in Methods, and were further treated with RANKL (40 ng/ml) in the absence or presence of 10^{-7} M $1\alpha,25(OH)_2D_3$ for 3 days (**A**). Note that the development of TRAP-positive multinucleate osteoclasts induced by RANKL was markedly inhibited by cotreatment with $1\alpha,25(OH)_2D_3$. (**B**) The inhibitory effect of $1\alpha,25(OH)_2D_3$ on the formation of TRAP-positive multinucleate cells (MNCs) was dose-dependent and was not seen in marrow cultures derived from *VDR* KO mice, even at the highest dose of 10^{-7} M. Data are expressed as a percentage of vehicle-treated cultures. $^{*}P < 0.05$ versus vehicle group, $n = 6$. (**C**) Expression of *VDR*s in the intestine (lane 1) and osteoclast precursor cells (lane 2) as detected by RT-PCR. EF-1α mRNA served as control for PCR. Lane 3 contained water as a negative control.

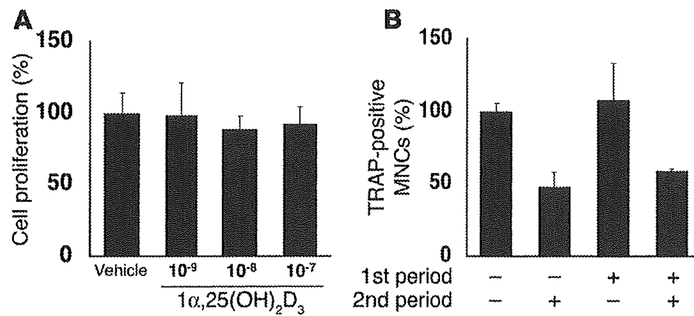

Figure 3

$1\alpha,25(OH)_2D_3$ inhibits RANKL-induced terminal differentiation into osteoclasts. (**A**) Osteoclast precursor cells were isolated from the bone marrow of WT C57BL/6J mice. These cells were cultured in the presence of 30 ng/ml M-CSF without or with increasing doses of $1\alpha,25(OH)_2D_3$ for 3 days, and cell proliferation was assessed as described in Methods. (**B**) Bone marrow cells were cultured with M-CSF for the first 3 days (1st period) and then with RANKL in addition to M-CSF for the latter 3 days (2nd period). The presence of $1\alpha,25(OH)_2D_3$ at 10^{-8} M is indicated by "+". Note that the presence of $1\alpha,25(OH)_2D_3$ only in the latter period was sufficient to inhibit the formation of TRAP-positive multinucleate cells, whereas its presence in the former M-CSF–dependent cell growth period failed to inhibit osteoclastogenesis.

tive responses to M-CSF. As shown in Figure 3A, treatment with $1\alpha,25(OH)_2D_3$ between 10^{-9} M and 10^{-7} M did not affect M-CSF-dependent cell proliferation, suggesting that $1\alpha,25(OH)_2D_3$ mainly acts at the latter differentiation stage. Also consistent with this notion are the results that treatment of bone marrow cultures with $1\alpha,25(OH)_2D_3$ only during the latter half (3 days) of the 6-day period was sufficient to inhibit osteoclast formation, whereas its presence in the former half period (3 days) during M-CSF-dependent growth failed to do so (Figure 3B). The presence of $1\alpha,25(OH)_2D_3$ throughout the 6-day culture period did not result in further inhibition of osteoclastogenesis. Thus, although $1\alpha,25(OH)_2D_3$ is well known for its antiproliferative activity in a variety of cell types (15), in this case the RANKL-dependent terminal differentiation step of osteoclast progenitor cells was specifically inhibited by $1\alpha,25(OH)_2D_3$.

When osteoclast precursor cells were isolated from the bone marrow of VDR-deficient mice, the suppressive effect of $1\alpha,25(OH)_2D_3$ on osteoclastogenesis was not observed at all, even at the highest dose of 10^{-7} M (Figure 2B). Taken together with the expression of VDR in the osteoclast precursor cells (Figure 2C), these data indicate that $1\alpha,25(OH)_2D_3$ acts directly on osteoclast precursors and inhibits their differentiation into mature osteoclasts and that this effect is mediated through the VDR.

c-Fos protein as a target of anti-osteoclastogenic action of $1\alpha,25(OH)_2D_3$. In order to clarify the mechanism by which $1\alpha,25(OH)_2D_3$ inhibits osteoclastogenic signaling in precursor cells, we investigated the effects of $1\alpha,25(OH)_2D_3$ on molecules that are known to transmit signals from the RANK receptor. Western blot analysis revealed that $1\alpha,25(OH)_2D_3$ did not affect the protein levels of the RANK receptor itself, TRAF6, p65 and p52 subunits of NF-κB, or c-Jun protein (Figure 4A and data not shown). Stimulation with RANKL caused activation of IκB kinase, p38, and JNK, through their phosphorylation; however, $1\alpha,25(OH)_2D_3$ even at 10^{-7} M did not inhibit their phosphorylation (Figure 4B). In contrast, $1\alpha,25(OH)_2D_3$ did inhibit the induction of c-Fos protein by RANKL in a dose-dependent manner, and this effect on c-Fos protein was not observed in cells derived from *VDR* KO mice (Figure 5A). Treatment with $1\alpha,25(OH)_2D_3$ alone had no effect. These results suggest that $1\alpha,25(OH)_2D_3$ blocked the induction by RANKL of c-Fos protein, a component of the AP-1 transcription factor, thereby antagonizing its transcription function in the nucleus.

Interestingly, the marked reduction in c-Fos protein took place with just a modest change in the level of c-Fos mRNA. Quantification of the c-Fos mRNA level by quantitative RT-PCR analysis revealed a substantial increase following RANKL stimulation, peaking at 6 hours, but $1\alpha,25(OH)_2D_3$ only modestly inhibited this increase in c-Fos mRNA at this time point (Figure 5B), suggesting that a posttranscriptional mechanism is involved in the VDR-mediated suppression of the c-Fos protein. Pulse-chase experiments revealed that c-Fos protein in osteoclast precursor cells turned over rapidly with an estimated half-life of less than 2 hours, as reported for other cell types (16), whereas treatment with $1\alpha,25(OH)_2D_3$ did not result in a further acceleration of c-Fos degradation (Figure 6, A and B). In pulse-labeling experiments, biosynthesis of c-Fos protein, which increased markedly after RANKL stimulation, was inhibited by cotreatment with $1\alpha,25(OH)_2D_3$ (Figure 6C).

Earlier targeted gene ablation experiments revealed a fundamental role of the Fos/AP-1 transcription factor in osteoclast development (17, 18), and recent studies identified its critical target molecules (19–21). As reported, stimulation with RANKL induced 2 notable c-Fos target genes, NFATc1 and IFN-β, which regulate osteoclast differentiation positively and negatively, respectively (Figure 7A). Simultaneous treatment with $1\alpha,25(OH)_2D_3$ inhibited the induction of these target molecules of the c-Fos transcription factor (Figure 7A); this finding can be taken as evidence that $1\alpha,25(OH)_2D_3$, by suppressing the level of c-Fos protein, functionally dampens its transcription activity. Thus, it is conceivable that suppression of c-Fos protein plays an important role in the functional interference of $1\alpha,25(OH)_2D_3$ with osteoclastogenesis.

In order to prove that $1\alpha,25(OH)_2D_3$-mediated inhibition of c-Fos protein induction by RANKL was responsible for the suppressive effect of the hormone on osteoclast differentiation, we transfected osteoclast precursor cells with a retroviral vector encoding c-Fos protein and then examined them for the ability of $1\alpha,25(OH)_2D_3$ to suppress osteoclast formation. Forced expression of c-Fos protein abrogated the suppressive effect of $1\alpha,25(OH)_2D_3$ on osteoclastogenesis completely at 10^{-9} M and partially at 10^{-8} M (Figure 7B).

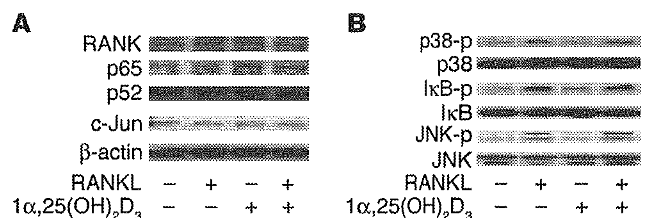

Figure 4

$1\alpha,25(OH)_2D_3$ fails to inhibit NF-κB and p38/JNK pathways in osteoclast precursor cells. Osteoclast precursor cells were isolated from the bone marrow of C57BL/6J mice as M-CSF–dependent adherent cells and were treated with RANKL (40 ng/ml) for 24 hours in the absence or presence of 10^{-8} M $1\alpha,25(OH)_2D_3$. Expression of RANK, p65, p52, and c-Jun proteins (**A**) and phosphorylation of IκB (IκB-p), p38 (p38-p), and JNK (JNK-p) (**B**) were analyzed by Western blotting after RANKL treatment for 15 minutes. β-Actin protein served as a loading control.

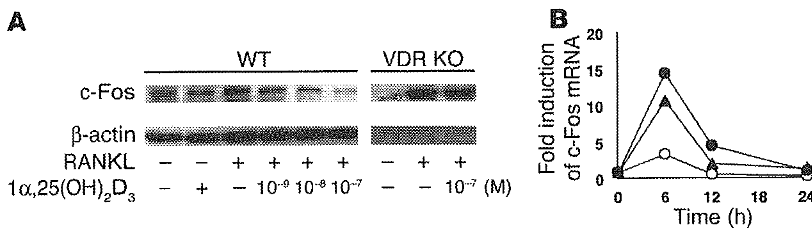

Figure 5
$1\alpha,25(OH)_2D_3$ inhibits expression of c-Fos protein induced by RANKL. Osteoclast precursor cells were isolated from the bone marrow of WT C57BL/6J and *VDR* KO mice as M-CSF–dependent adherent cells and were treated with RANKL (40 ng/ml) for 24 hours in the absence or presence of the indicated doses of $1\alpha,25(OH)_2D_3$. Western blotting for c-Fos protein (**A**) and quantitative RT-PCR analyses (**B**) were performed. RNA was isolated from osteoclast precursor cells at the indicated times after RANKL stimulation, and quantitative RT-PCR for c-Fos mRNA was performed using a LightCycler with EF-1α mRNA as a control. Filled circles, filled triangles, and open circles represent RANKL, RANKL plus $1\alpha,25(OH)_2D_3$, and vehicle, respectively. β-Actin protein served as a loading control (**A**).

Vitamin D analogs that reduce c-Fos protein and inhibit osteoclast differentiation more potently than the natural hormone. The finding that the suppression of c-Fos underlies the anti-osteoclastogenic function of the VDR suggests that the former activity can be used for screening vitamin D analogs for those with more potent antiresorptive function than the natural hormone, $1\alpha,25(OH)_2D_3$. By screening newly synthesized vitamin D compounds, we identified 2 analogs, DD280 and DD281, that reduced the level of c-Fos protein more potently than $1\alpha,25(OH)_2D_3$ (Figure 8, A and B). When the analogs were tested in murine bone marrow cultures, it was evident that, by reducing the level of c-Fos protein, these analogs caused more potent suppression of osteoclast development than the natural hormone (Figure 8C).

We tested one of the potent analogs, DD281, for its pharmacological activity in vivo. The major action of vitamin D hormone is to stimulate intestinal calcium absorption, and its therapeutically beneficial action in bone is often compromised by side effects, such as hypercalcemia and hypercalciuria, especially when the dosage is increased. DD281, which is chemically (1R,3S,5Z)-5-[(2E)-[(3aS,7aS)-1-[(1R)-1-[(2-ethyl-2-hydroxybutyl)thio]ethyl]-3,3a,5,6,7,7a-hexahydro-7a-methyl-4H-inden-4-ylidene]ethylidene]-4-methylene-1,3-cyclohexanediol (Figure 9A), has a binding affinity for the VDR that is approximately 84% of that of $1\alpha,25(OH)_2D_3$; it also has a very short half-life in the circulation [less than 1 hour versus 8–10 hours for $1\alpha,25(OH)_2D_3$ when administered orally], presumably because of its very low affinity for vitamin D–binding protein [0.3% of that of $1\alpha,25(OH)_2D_3$]. We determined the doses of DD281 that had an effect on calcium absorption similar to the effect of $1\alpha,25(OH)_2D_3$ by estimating urinary calcium excretion in ovariectomized (OVX), estrogen-deficient mice. As summarized in Figure 9B, the lower dose of DD281 (5 μg/kg body weight) or $1\alpha,25(OH)_2D_3$ (0.0125 μg/kg body weight) did not change the 24-hour urinary excretion of calcium, whereas the higher dose of each [10 μg/kg for DD281 and 0.05 μg/kg for $1\alpha,25(OH)_2D_3$] caused a similar increase in urinary calcium excretion. For the same degree of effect on calcium metabolism, DD281 prevented bone loss more significantly and more potently than $1\alpha,25(OH)_2D_3$ at the lumbar spine (Figure 9C). Also, DD281 reduced the osteoclast number and the bone surface covered by osteoclasts more significantly and more potently than $1\alpha,25(OH)_2D_3$ at the lumbar spine (Table 1). Neither drug caused hypercalcemia, although the higher dose of $1\alpha,25(OH)_2D_3$ raised serum calcium concentrations slightly but significantly, only when compared with those in vehicle-treated OVX mice (Table 2). Thus, DD281 was superior to $1\alpha,25(OH)_2D_3$ in antiresorptive and bone-protective effects while having the same effect on calcium metabolism as the natural hormone.

Discussion

Hypocalcemia and rickets/osteomalacia observed in VDR gene KO mice as well as in patients with vitamin D deficiency point to the physiological importance of VDR in maintaining calcium homeostasis and bone mineralization (22). Regarding the pharmacology, the importance of vitamin D as a nutrient for the prevention of osteoporosis is well recognized, especially in the elderly population, in which simple vitamin D deficiency is prevalent (23, 24). However, the utility of vitamin D hormone in osteoporotic patients, even in the setting of vitamin D sufficiency, and whether or not it has any peculiar properties in terms of bone action not shown by plain vita-

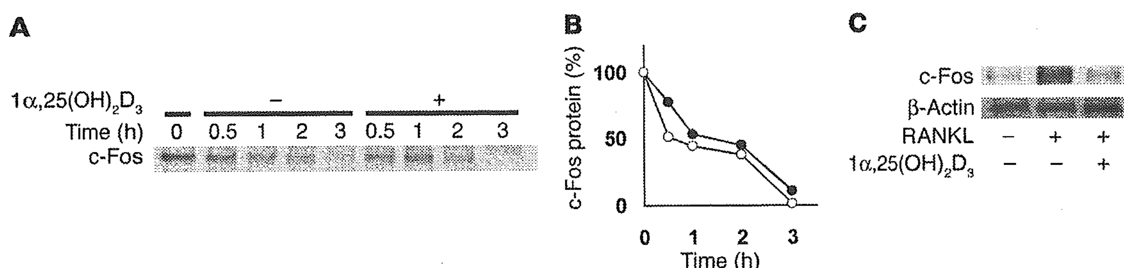

Figure 6
$1\alpha,25(OH)_2D_3$ inhibits translation of c-Fos protein in osteoclast precursor cells. Osteoclast precursor cells were isolated from the bone marrow of C57BL/6J mice as M-CSF–dependent adherent cells. (**A** and **B**) After RANKL stimulation for 24 hours, osteoclast progenitor cells were pulse-labeled for 30 minutes with ^{35}S-methionine followed by chasing with cold methionine for the indicated times in the absence or presence of $1\alpha,25(OH)_2D_3$ treatment. Note that the degradation of c-Fos protein was not accelerated by $1\alpha,25(OH)_2D_3$ (open circles), compared with that for vehicle-treated cells (filled circles). (**C**) After RANKL stimulation for 24 hours, osteoclast progenitor cells were pulse-labeled for 30 minutes with ^{35}S-methionine in the absence or presence of $1\alpha,25(OH)_2D_3$. Labeled c-Fos protein was immunoprecipitated. Note that the biosynthesis of c-Fos protein stimulated by RANKL was inhibited by $1\alpha,25(OH)_2D_3$ treatment. β-Actin protein served as a loading control.