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

Hisashi Takasu,1,2 Atsuko Sugita,2 Yasushi Uchiyama,2 Nobuyoshi Katagiri,1 Makoto Okazaki,2 Etsuro Ogata,3 and Kyoji Ikeda1

1Department of Bone and Joint Disease, Research Institute, National Center for Geriatrics and Gerontology, Obu, Japan. 2Pharmaceutical Research Laboratory, Chugai Pharmaceutical Co. Ltd., Gotemba, Japan. 3Cancer Institute Hospital, Japanese Foundation for Cancer Research, Tokyo, Japan.

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 D3 [1α,25(OH)2D3] 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)2D3 potently and dose-dependently inhibited their differentiation into multinucleate osteoclasts induced by RANKL. Among signaling molecules downstream of RANK, 1α,25(OH)2D3 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)2D3 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)2D3, 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 alfalcaldiol, a prodrug metabolized to the natural vitamin D hormone 1α,25-dihydroxyvitamin D3 [1α,25(OH)2D3], 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)2D3 in ovariectomy models. In order to define the molecular pathway(s) that VDR acts upon, we examined the effects of 1α,25(OH)2D3 in a genetic model of osteoporosis due to constitutive activation of RANK signaling.

Results

1α,25(OH)2D3 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)2D3 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)2D3, 1α,25-dihydroxyvitamin D3; 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.

Citation for this article: J Clin Invest. 116:528–535 (2006). doi:10.1172/JCI24742.
area (Figure 1B) in bone sections, down to levels in heterozygous mice used as a control. The suppressive effect of 1α,25(OH)2D3 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 1α,25(OH)2D3 caused a dose-dependent amelioration of bone loss at the tibia (Figure 1D). The small pharmacological doses of 1α,25(OH)2D3 used in the current study (0.05–0.2 µg/kg) did not induce hypercalcemia (data not shown). These results suggest that 1α,25(OH)2D3 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 1α,25(OH)2D3 administration in vivo (9), we hypothesized that 1α,25(OH)2D3 suppresses bone resorption by interfering with signaling through RANK receptors on osteoclast precursor cells.

1α,25(OH)2D3 inhibits osteoclast development by acting directly on osteoclast precursor cells in bone marrow. In order to examine whether 1α,25(OH)2D3 counters osteoclastogenic signaling emanating from RANK receptors, we isolated osteoclast progenitor cells from mouse bone marrow and examined the effects of 1α,25(OH)2D3 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 1α,25(OH)2D3 resulted in a dose-dependent reduction in the number of osteoclasts formed (Figure 2, A and B). 1α,25(OH)2D3 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-
cells derived from VDR KO mice (Figure 5A). Treatment with 1α,25(OH)2D3 alone had no effect. These results suggest that 1α,25(OH)2D3 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 1α,25(OH)2D3 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 1α,25(OH)2D3 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 1α,25(OH)2D3 (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 1α,25(OH)2D3 inhibited the induction of these target molecules of the c-Fos transcription factor (Figure 7A); this finding can be taken as evidence that 1α,25(OH)2D3, 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 1α,25(OH)2D3 with osteoclastogenesis.

In order to prove that 1α,25(OH)2D3-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 1α,25(OH)2D3 to suppress osteoclast formation. Forced expression of c-Fos protein abrogated the suppressive effect of 1α,25(OH)2D3 on osteoclastogenesis completely at 10–8 M and partially at 10–7 M (Figure 7B).

---

**Figure 3**
1α,25(OH)2D3 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 1α,25(OH)2D3 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 1α,25(OH)2D3 at 10–8 M is indicated by “+”. Note that the presence of 1α,25(OH)2D3 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.
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, 1α,25(OH)2D3. 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 1α,25(OH)2D3 (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-methyl-1-cyclohexanediol (Figure 9A), has a binding affinity for the VDR that is approximately 84% of that of 1α,25(OH)2D3; it also has a very short half-life in the circulation [less than 1 hour versus 8–10 hours for 1α,25(OH)2D3 when administered orally], presumably because of its very low affinity for vitamin D–binding protein [0.3% of that of 1α,25(OH)2D3]. We determined the doses of DD281 that had an effect on calcium absorption similar to the effect of 1α,25(OH)2D3 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 1α,25(OH)2D3 (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 1α,25(OH)2D3] 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 1α,25(OH)2D3 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 1α,25(OH)2D3 at the lumbar spine (Table 1). Neither drug caused hypercalcemia, although the higher dose of 1α,25(OH)2D3 raised serum calcium concentrations slightly but significantly, only when compared with those in vehicle-treated OVX mice (Table 2). Thus, DD281 was superior to 1α,25(OH)2D3 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 5
1α,25(OH)2D3 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 1α,25(OH)2D3. 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 1α,25(OH)2D3, and vehicle, respectively. β-Actin protein served as a loading control (A).

Figure 6
1α,25(OH)2D3 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 35S-methionine followed by chasing with cold methionine for the indicated times in the absence or presence of 1α,25(OH)2D3 treatment. Note that the degradation of c-Fos protein was not accelerated by 1α,25(OH)2D3 (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 35S-methionine in the absence or presence of 1α,25(OH)2D3. Labeled c-Fos protein was immunoprecipitated. Note that the biosynthesis of c-Fos protein stimulated by RANKL was inhibited by 1α,25(OH)2D3 treatment. β-Actin protein served as a loading control.
of thyroparathyroidectomized rats (31). Alternatively, under pathological conditions with excessive osteoclast generation, vitamin D hormone–induced (PTH-induced) RANKL expression in the bones may be downregulated in stromal/osteoblastic cells, relative to that in hematopoietic cells, which would mask the pro-osteoclastogenic action of vitamin D. Further studies are required to determine the relative contribution of stromal versus hematopoietic cells to the in vivo regulation of bone resorption through the VDR. The major action of vitamin D is stimulation of intestinal calcium absorption; and the therapeutic effects of vitamin D on bone, whether active or plain vitamin D₃, are believed to be indirect, through stimulation of intestinal calcium absorption, correction of a negative calcium balance, and normalization of the sustained PTH secretion frequently seen in elderly patients (33). In order to gain some insight into the role of PTH suppression in vitamin D action on bone, we previously examined the effects of 1α,25(OH)₂D₃ and its analog [22-oxa-1α,25(OH)₂D₃] on bone resorption in parathyroidectomized rats rendered hypercalcemic with constant PTH-related protein infusion (34). Under these “PTH clamp” conditions, we observed that 1α,25(OH)₂D₃ and 22-oxa-1α,25-dihydroxyvitamin D₃ were capable of inhibiting bone resorption (34). In agreement with these previous findings, we have demonstrated in this study that vitamin D hormone acts directly on hematopoietic cells in bone marrow, through VDR expressed in osteoclast progenitors of the monocyte/macrophage lineage, thereby inhibiting their terminal differentiation into mature osteoclasts. Thus, hematopoietic cells that receive the RANKL signal through the RANK receptor are important target cells of vitamin D action in vivo. We further investigated the mechanism by which 1α,25(OH)₂D₃ modulates the developmental program of hematopoietic precursor cells and inhibits their differentiation into mature osteoclasts. This process is tightly regulated by extracellular signals, including RANKL as an essential cytokine, as well as by negative regulators, such as OPG and other inhibitory molecules (35, 36). Our find-

Figure 7
c-Fos protein as a target of anti-osteoclastogenic action of 1α,25(OH)₂D₃. 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 the indicated doses of 1α,25(OH)₂D₃. (A) Northern blot analysis of c-Fos target genes, i.e., NFATc1 and IFN-β, in RANKL-treated osteoclast precursor cells without or with 1α,25(OH)₂D₃ for 24 hours (10⁻⁷ M). (B) Forced expression of c-Fos (indicated by “+”) by a retroviral vector abrogated the suppressive effect of 1α,25(OH)₂D₃ on osteoclast development. Osteoclast precursor cells isolated from bone marrow were infected with a retroviral vector encoding c-Fos, and cultured with M-CSF and RANKL for 3 days in the absence or presence of 1α,25(OH)₂D₃. Data are expressed as a percentage of the value for vehicle-treated cultures without retroviral infection (–). *P < 0.05 versus vector-infected group, n = 6.

Figure 8
Screening for vitamin D (VD) analogs that reduce c-Fos protein and inhibit osteoclast differentiation more potently than 1α,25(OH)₂D₃. The effects of 1α,25(OH)₂D₃ (open circles) and its analogs (DD281, DD280, and DD208) on the c-Fos protein level in osteoclast precursor cells (A and B) and the formation of TRAP-positive multinucleate osteoclasts (C) at the indicated concentrations are shown. The lower bands in A show β-actin as an internal control for protein loading. Filled circles, rectangles, and triangles (B and C) represent DD281, DD280, and DD208, respectively. Data are expressed as a percentage of the value for vehicle-treated cultures.
A novel vitamin D analog, DD281, inhibits osteoclast differentiation and increases BMD more potently than 1α,25(OH)2D3 in vivo. (A) Structures of 1α,25(OH)2D3 and its analog, DD281. (B) Ovariectomized (OVX) C57BL/6J mice were treated orally with the indicated doses of 1α,25(OH)2D3 or its analog DD281 for 4 weeks, and urinary calcium excretion was determined for the final 24 hours. *P < 0.05 versus OVX group with vehicle treatment, **P < 0.05 versus sham group, n = 8 each group. (C) BMD at the lumbar vertebrae was determined. *P < 0.05 versus OVX group with vehicle treatment, **P < 0.005 versus OVX group with vehicle treatment, ***P < 0.0005 versus OVX group with vehicle treatment, **P < 0.05 versus sham group, n = 8 each group.

Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>1α,25(OH)2D3</th>
<th>DD281</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oc.S/BS (%)</td>
<td>0.0125</td>
<td>0.05</td>
</tr>
<tr>
<td>13.3 ± 0.7</td>
<td>10.5 ± 1.4</td>
<td>11.1 ± 0.4</td>
</tr>
<tr>
<td>0.25 ± 0.2</td>
<td>2.2 ± 0.3</td>
<td>2.4 ± 0.2</td>
</tr>
</tbody>
</table>

Eleven-week-old OVX mice (n = 8 each group) were treated with 1α,25(OH)2D3 or DD281 at the indicated doses for 4 weeks. Bone histomorphometry was performed at the lumbar spine. Oc.S/BS, bone surface covered by osteoclasts; N.Oc/BS, number of osteoclasts, corrected for bone surface. *P < 0.05 versus OVX group, **P < 0.005 versus OVX group, *P < 0.05 versus 1α,25(OH)2D3-treated group.
50–60% humidity. The mice were allowed free access to tap water and commercial standard rodent chow (CE-2) containing 1.20% calcium, 1.08% phosphate, and 240 IU/100 g vitamin D3 (CLEA Japan Inc.). After acclimation, various doses of 1α,25(OH)2D3 or vehicle (medium chain triglyceride) were administered orally 5 times a week for 6 weeks.

Nine-week-old female C57BL/6J mice were purchased from Japan SLC Inc. and ovariopectomized (OVX) after a 1-week acclimation. Sham-operated mice served as the control. OVX mice were treated orally with 1α,25(OH)2D3 (0.0125–0.05 µg/kg body weight once daily), its analog DD281 (5–10 µg/kg body weight twice daily), or vehicle (medium chain triglyceride) 5 times a week for 4 weeks. Urine was collected during the final 24 hours, and blood was drawn to obtain serum. *P < 0.05 versus OVX group.

All experiments were performed in accordance with Chugai Pharmaceutical Co.'s ethical guidelines for animal care, and the experimental protocols were approved by the animal care committee of the company and by the Animal Experimentation Ethics Committee of the National Center for Geriatrics and Gerontology.

Bone analysis. For bone analysis, right femurs and lumbar vertebrae were dissected and stored in 70% ethanol. BMD was measured by dual-energy x-ray absorptiometry (DEXA-600EX, ALOKA Inc.). Left femurs and lumbar vertebrae were fixed in 4% paraformaldehyde for bone histomorphometry as described previously (7, 8). Each sample was sectioned, and then stained for tartrate-resistant acid phosphatase (TRAP). Histomorphometric parameters were measured at Niigata Bone Science Institute (Niigata, Japan).

Biochemical analysis. Serum and urinary calcium concentrations were determined using an autoanalyzer (Hitachi 7170). Urinary deoxypyridinoline was measured with a PYRILINKS-D assay kit (Metra Biosystems Inc.).

Osteocalcogenic assay in vitro. Bone marrow cells were isolated from the tibiae and femurs of 6- to 9-week-old male C57BL/6J mice (SLC) and VDR KO mice (kindly provided by Shigeki Kato, University of Tokyo, Tokyo, Japan). All cells were plated in culture dishes containing α-MEM/10% heat-inactivated FBS/1% antibiotics and incubated for 12 hours. Non-adherent cells were separated and cultured for 3 days with M-CSF (30 ng/ml), and then those that became adherent were used as osteoclast precursor cells. Cells were treated with RANKL (40 ng/ml) in the absence or presence of 1α,25(OH)2D3 for 3 days, fixed in 4% paraformaldehyde, and stained for TRAP. Multinucleate (>3 nuclei), TRAP-positive cells were counted as osteoclasts. Osteoclast precursor cells were cultured in the presence of 30 ng/ml M-CSF without or with increasing doses of 1α,25(OH)2D3 for 3 days, and cell proliferation was assessed using a Cell Counting Kit-8 (Dojindo Laboratories).

Western and Northern analyses. Whole-cell extracts were isolated from osteoclast precursors, and protein concentrations were determined by use of a Bio-Rad Protein Assay kit (Bio-Rad Laboratories). Equivalent amounts of protein were loaded for 4–20% SDS-PAGE, and proteins transferred onto the membrane were detected with an ECL Plus Western blotting detection system (Amersham Biosciences). Phosphorylation of JNK, p38 MAPK, and IkB kinase was evaluated using kits from Cell Signaling Technology. Total RNA was isolated with TRIzol reagent (Invitrogen Corp.), according to the manufacturer’s instructions. For Northern analysis, equal amounts of total RNA (10 µg/lane) were fractionated on a 1.5% agarose gel. The specific mRNAs were detected by hybridization of Hybrid N+ nylon membranes (Amersham Biosciences) with 32P-labeled cDNA probes for mouse c-Fos, IFN-β, and NFATc1. For quantitative RT-PCR, total RNA (1 µg) was reverse transcribed using SuperScript III (Invitrogen Corp.), and samples were analyzed using a LightCycler (Roche Diagnostics Corp.). The primers included 5′-ACCT-GTTCGTAACACACCA-3′ and 5′-ACAAACACACTTACCGGTTT-3′ for c-Fos, 5′-GGACATTGGCATGATGAAGG-3′ and 5′-CTCAGACT-GTCTCTCAAAGGC-3′ for VDR, and 5′-TGCTGCCATTTGATGAT-3′ and 5′-CTCACAGCTTTGTAGAC-3′ for EF-1α. The amount of c-Fos and VDR mRNA was corrected by that of EF-1α mRNA.

Pulse-labeling and pulse-chase labeling experiments. For pulse labeling, osteoclast precursor cells were stimulated with RANKL for 24 hours in the absence or presence of 1α,25(OH)2D3 at 10−7 M and then radiolabeled for 1 hour with culture medium containing 150 µCi/ml of a 35S-methionine and cysteine mixture (Amersham Biosciences). In the case of pulse-chase labeling, RANKL-treated osteoclast precursor cells were similarly radiolabeled and then incubated in the presence of nonradioactive medium containing 10 mM cold methionine and 10 mM cold cysteine for various periods of time. Cell extracts were immunoprecipitated with anti-c-Fos antibody, and the precipitates were then subjected to SDS-PAGE. After the gels had been dried, autoradiography was performed.

Retrosial expression of c-Fos protein. A retroviral vector encoding mouse c-Fos (pBabe-c-Fos, kindly provided by K. Matsuo, Keio University, Tokyo, Japan) was used to transfect Plat-E retrovirus packaging cells (a gift from T. Kitamura, University of Tokyo, Tokyo, Japan). The culture media were collected 48 hours after the transfection and kept as retrovirus stocks. Osteoclast precursor cells were exposed to the retrovirus in the presence of polybrene (8 µg/ml) for 1 day and were subsequently treated with RANKL and 1α,25(OH)2D3 for 4 days in the presence of puromycin (1.6 µg/ml). Osteoclast differentiation was evaluated as described above.

Statistics. Data were expressed as the means ± SEM. Statistical analysis was carried out by ANOVA, using Statistical Analysis System software (SAS Institute Inc.). The significance of differences was determined using 2-tailed Student’s t test and Dunnett’s multiple test. A value of P < 0.05 was considered to indicate a significant difference.

### Table 2

<table>
<thead>
<tr>
<th>Operation</th>
<th>Treatment</th>
<th>Sham</th>
<th>Vehicle</th>
<th>Vehicle</th>
<th>1α,25(OH)2D3</th>
<th>0.0125</th>
<th>0.05</th>
<th>5</th>
<th>DD281</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>Calcium (mg/dl)</td>
<td>9.63 ± 0.13</td>
<td>9.34 ± 0.11</td>
<td>9.09 ± 0.09</td>
<td>9.69 ± 0.09</td>
<td>9.19 ± 0.05</td>
<td>9.31 ± 0.11</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Phosphorus (mg/dl)</td>
<td>8.35 ± 0.39</td>
<td>7.50 ± 0.41</td>
<td>6.36 ± 0.24*</td>
<td>8.19 ± 0.47</td>
<td>6.39 ± 0.21</td>
<td>7.23 ± 0.19</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urine</td>
<td>Phosphorus/Creatinine</td>
<td>6.71 ± 0.45</td>
<td>6.74 ± 0.44</td>
<td>7.75 ± 0.32</td>
<td>7.10 ± 0.52</td>
<td>6.28 ± 0.56</td>
<td>6.32 ± 0.32</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Eleven-week-old OVX mice (n = 8 per group) were treated with 1α,25(OH)2D3 or DD281 at the indicated doses for 4 weeks. Urine was collected during the final 24 hours, and blood was drawn to obtain serum. *P < 0.05 versus OVX group.
Acknowledgments

We thank Shigeaki Kato and Toshio Kitamura (University of Tokyo) for providing VDR KO mice and retrovector vectors, respectively; Koichi Matsuo, Sunao Takeshita, and Ken Watanabe (National Center for Geriatrics and Gerontology) for valuable suggestions; Mie Suzuki (National Center for Geriatrics and Gerontology) for expert technical assistance; and Akemi Ito (Niigata Bone Science Institute) for discussion on the data of bone histomorphometry. This study was supported in part by grants from the program Comparative Research on Aging and Health of the Ministry of Health, Labor, and Welfare of Japan (to K. Ikeda) and by a grant from the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation of Japan (MF-14 to K. Ikeda).

Received for publication February 14, 2005, and accepted in revised form November 8, 2005.

Address correspondence to: Kyoji Ikeda, Department of Bone and Joint Disease, Research Institute, National Center for Geriatrics and Gerontology, 36-3 Gengo, Morioka, Obu, Aichi 474-8522, Japan. Phone and Fax: 81-562-46-8094; E-mail: kikeda@nils.go.jp.