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 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 retrovirial 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 osteoporosis (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.

Figure 1

$1\alpha_{25}(\text{OH})_2\text{D}_3$ inhibits bone resorption in OPG KO mice. OPG homozgyous KO (−/−) mice were treated orally with the indicated doses of $1\alpha_{25}(\text{OH})_2\text{D}_3$ 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. $^*P < 0.05$ versus OPG KO group with vehicle treatment, $^{*P} < 0.01$ versus heterozygous control group, $n = 6$ each group.

Figure 2

$1\alpha_{25}(\text{OH})_2\text{D}_3$ 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 $1\alpha_{25}(\text{OH})_2\text{D}_3$ for 3 days (A). Note that the development of TRAP-positive multinucleate osteoclasts induced by RANKL was markedly inhibited by cotreatment with $1\alpha_{25}(\text{OH})_2\text{D}_3$. (B) The inhibitory effect of $1\alpha_{25}(\text{OH})_2\text{D}_3$ 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 VDRs 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.


area (Figure 1B) in bone sections, down to levels in heterozygous mice used as a control. The suppressive effect of $1\alpha_{25}(\text{OH})_2\text{D}_3$ 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 $1\alpha_{25}(\text{OH})_2\text{D}_3$ caused a dose-dependent amelioration of bone loss at the tibia (Figure 1D). The small pharmacological doses of $1\alpha_{25}(\text{OH})_2\text{D}_3$ used in the current study (0.05–0.2 µg/kg) did not induce hypercalcemia (data not shown). These results suggest that $1\alpha_{25}(\text{OH})_2\text{D}_3$ 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 $1\alpha_{25}(\text{OH})_2\text{D}_3$ administration in vivo (9), we hypothesized that $1\alpha_{25}(\text{OH})_2\text{D}_3$ suppresses bone resorption by interfering with signaling through RANK receptors on osteoclast precursor cells.

$1\alpha_{25}(\text{OH})_2\text{D}_3$ inhibits osteoclast development by acting directly on osteoclast precursor cells in bone marrow. In order to examine whether $1\alpha_{25}(\text{OH})_2\text{D}_3$ counters osteoclastogenic signaling emanating from RANK receptors, we isolated osteoclast progenitor cells from mouse bone marrow and examined the effects of $1\alpha_{25}(\text{OH})_2\text{D}_3$ 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 $1\alpha_{25}(\text{OH})_2\text{D}_3$ resulted in a dose-dependent reduction in the number of osteoclasts formed (Figure 2, A and B). $1\alpha_{25}(\text{OH})_2\text{D}_3$ 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-
tive responses to M-CSF. As shown in Figure 3A, treatment with 1α,25(OH)₂D₃ between 10⁻⁹ M and 10⁻⁷ M did not affect M-CSF-dependent cell proliferation, suggesting that 1α,25(OH)₂D₃ mainly acts at the latter differentiation stage. Also consistent with this notion are the results that treatment of bone marrow cultures with 1α,25(OH)₂D₃ 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 1α,25(OH)₂D₃ throughout the 6-day culture period did not result in further inhibition of osteoclastogenesis. Thus, although 1α,25(OH)₂D₃ 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 1α,25(OH)₂D₃.

When osteoclast precursor cells were isolated from the bone marrow of VDR-deficient mice, the suppressive effect of 1α,25(OH)₂D₃ 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 1α,25(OH)₂D₃ 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 1α,25(OH)₂D₃.

In order to clarify the mechanism by which 1α,25(OH)₂D₃ inhibits osteoclastogenic signaling in precursor cells, we investigated the effects of 1α,25(OH)₂D₃ on molecules that are known to transmit signals from the RANK receptor. Western blot analysis revealed that 1α,25(OH)₂D₃ 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, 1α,25(OH)₂D₃ even at 10⁻⁷ M did not inhibit their phosphorylation (Figure 4B). In contrast, 1α,25(OH)₂D₃ 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 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 10⁻³ M and partially at 10⁻² M (Figure 7B).
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 protein 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,3S,5Z)5-[(2-ethyl-2-hydroxybutyl)thio]ethyl}thio]ethyl]-3,3a,5,6,7,7a-hexahydro-7a-methyl-4H-inden-4-ylidene]ethylidene}-4-methylen-1,3-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-
min D has been controversial (25, 26). It is generally believed that 1α,25(OH)2D3 stimulates bone resorption, based on the fact that it was found to be a bone-resorbing hormone in a classic experiment using bone organ cultures (27), and the fact that 1α,25(OH)2D3 induces the expression of RANKL, an essential cytokine for osteoclast development (28). In the present study we have provided biochemical as well as histological evidence that vitamin D hormone can exert a pharmacological action to inhibit bone resorption under pathological conditions with excessive osteoclast generation. The exact reason for these contrasting effects of 1α,25(OH)2D3 on bone resorption is not clear at the present time. 1α,25(OH)2D3 at high concentrations is known to induce the expression of RANKL in stromal/osteoblastic cells in vitro, which is assumed to favor osteoclastogenesis. In sharp contrast, as demonstrated in the current study, 1α,25(OH)2D3 can act on hematopoietic lineage cells and potently inhibit their differentiation into mature osteoclasts. 1α,25(OH)2D3 has also been shown to inhibit osteoclast differentiation from human PBMCs (29). We (7, 8, 30) and others (31) have previously reported that when vitamin D hormone is administered in vivo at pharmacological doses in animal models with excessive bone resorption, it actually reduces osteoclast number and suppresses bone resorption. This has been proven in a recent clinical trial, in which ED-71, a vitamin D analog, reduced a bone resorption marker and increased BMD in osteoporotic patients with hypercalcemia were administered (9). Others have reported that 1α,25(OH)2D3 within a certain dose range inhibits parathyroid hormone–induced (PTH-induced) RANKL expression in the bones of thyroparathyroidectomized rats (31). Alternatively, under pathological conditions with elevated bone resorption, VDR signaling 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 D, 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 findings show that vitamin D regulates hematopoietic cell function through 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.
ings that \( \alpha,25(\text{OH})_2\text{D} \) inhibited bone resorption in mice lacking OPG rule out the possibility that the antiresorptive action is mediated through OPG. It is also unlikely that \( \alpha,25(\text{OH})_2\text{D} \) interferes with the binding of RANKL to its receptor RANK and inhibits its overall signaling, since some intracellular signals activated downstream of RANK, such as I\text{k}B kinase and JNK, were not affected by \( \alpha,25(\text{OH})_2\text{D} \). Instead, our data suggest that, among known signaling molecules involved in osteoclast development downstream of the RANK receptor, c-Fos protein is a key target molecule of VDR. In addition, our experiments revealed that the reduction in the level of c-Fos protein took place with only a modest decrease in its mRNA level. Taken together with the results of the pulse-labeling experiments, this finding suggests that VDR, classically viewed as a transcription factor, inhibits the translation of c-Fos protein, although the underlying molecular mechanism remains elusive. The effect of \( \alpha,25(\text{OH})_2\text{D} \) on c-Fos protein in osteoclast precursors contrasts with the reported effect of \( \alpha,25(\text{OH})_2\text{D} \) on c-Fos gene transcription in osteoblasts and other cell types (37, 38). Thus, there may exist cell type–specific regulatory mechanisms that modulate the expression of c-Fos gene expression in response to \( \alpha,25(\text{OH})_2\text{D} \). Since a number of other proteins involved in RANK signaling, such as RANK itself, JNK, p38 MAPK, I\text{k}B kinase, p65 and p52 subunits of NF-\kappa B, and c-Jun, were not affected by \( \alpha,25(\text{OH})_2\text{D} \), it is conceivable that the inhibitory effect of the VDR is specific to c-Fos protein in osteoclast precursor cells.

Importantly, the suppression of c-Fos protein through VDRs is critical for the pharmacological action of vitamin D, since we showed that forced expression of c-Fos blocked the suppressive effect of vitamin D on osteoclast formation. The identification of novel synthetic vitamin D analogs that were capable of inhibiting osteoclast development more efficiently than the natural hormone, \( \alpha,25(\text{OH})_2\text{D} \), in parallel with greater suppression of c-Fos protein, lends further support to our concept that c-Fos-suppressing activity is important for the antiresorptive function of vitamin D. This mechanism may also underlie the activity of other recently developed bone-selective vitamin D analogs (39, 40). It should be noted, however, that DD281 was not completely bone-selective and increased urinary calcium excretion above that in the vehicle-treated mice. Therefore, careful monitoring of urinary and serum calcium levels would be required if its clinical utility were to be tested.

In conclusion, we demonstrated herein that the pharmacological action of \( \alpha,25(\text{OH})_2\text{D} \) is to mitigate excessive bone resorption, under pathological conditions such as osteoporosis; that it does so by acting on monocyte/macrophage–lineage cells in bone marrow; that the presence of VDRs is a prerequisite for this action; and that c-Fos protein is a key target molecule of VDR action. Based on these findings, we synthesized new vitamin D analogs, among which DD281, with potent antiresorptive activity relative to calcium absorption function, may warrant clinical trial for the treatment of bone diseases associated with excessive osteoclastic activity.

### Methods

**Reagents.** \( \alpha,25(\text{OH})_2\text{D} \) and its analogs, including DD208, DD280, and DD281, were synthesized at Chugai Pharmaceutical Co. Mouse RANKL and M-CSF were purchased from R&D Systems. Antibodies against RANK, TRAF6, p65, and p52 were purchased from Santa Cruz Biotechnology Inc. Anti–c-Jun and –c-Fos antibodies came from Sigma-Aldrich.

**Animal experiments.** Six-week-old female OPG homozygous and heterozygous KO mice were purchased from CLEA Japan Inc. and acclimated for 1 week under standard laboratory conditions at 24 ± 2°C and

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<th>Effects of ( \alpha,25(\text{OH})_2\text{D} ) and DD281 on osteoclast parameters in OVX mice</th>
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<td><strong>Treatment</strong></td>
<td><strong>( \alpha,25(\text{OH})_2\text{D} )</strong></td>
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<tr>
<td><strong>Dose (( \mu \text{g/kg body weight} ))</strong></td>
<td><strong>0</strong></td>
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<tr>
<td>Oc.S/BS (%)</td>
<td>13.3 ± 0.7</td>
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<tr>
<td>N.Oc/BS (no./mm)</td>
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| n | 8 each group |

Eleven-week-old OVX mice (\( n = 8 \) each group) were treated with \( \alpha,25(\text{OH})_2\text{D} \) 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. \(< \alpha,25(\text{OH})_2\text{D} \) versus vehicle, \(< \alpha,25(\text{OH})_2\text{D} \) versus DD281, \(< \alpha,25(\text{OH})_2\text{D} \) versus DD281.
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% phosphorus, and 2.40 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 ovariectomized (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 samples were centrifuged to obtain the serum.

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 40% 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 PIRILINKS-D assay kit (Metra Biosystems Inc.).

**Osteocalcogenes 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 (kindsly provided by Shigeaki 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. Nonadherent 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 µg/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 Hybond 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-GTTGCAAACACACCA-3′ and 5′-ACAACACACTCCATGGGTTT-3′ for c-Fos, 5′-GGACATTGGCATGATGAAGG-3′ and 5′-CTCAGACT-GTCTCTCAAGGC-3′ for VDR, and 5′-TGCTGCCATTGTTGATATGG-3′ and 5′-TCCACAGCCTTTGATGACC-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 presence or absence of 1α,25(OH)2D3 at 10−8 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.

**Retroviral 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.

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**Table 2**

<table>
<thead>
<tr>
<th>Operation</th>
<th>Sham</th>
<th>Vehicle</th>
<th>VDR</th>
<th>1α,25(OH)2D3</th>
<th>DD281</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calcium</td>
<td>9.63 ± 0.13</td>
<td>9.34 ± 0.11</td>
<td>9.09 ± 0.09</td>
<td>9.69 ± 0.04</td>
<td>9.19 ± 0.05</td>
</tr>
<tr>
<td>Phosphorus</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>
</tr>
<tr>
<td>Urine</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>
</tr>
</tbody>
</table>

Urine Phosphorus/Creatinine

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.
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