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Stimulation of Osteoclast Formation by 1,25-Dihydroxyvitamin D Requires Its Binding to Vitamin D Receptor (VDR) in Osteoblastic Cells: Studies Using VDR Knockout Mice

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ABSTRACT

Previous studies have shown that 1,25-dihydroxyvitamin D [1,25$(OH)_2$D] plays important roles in the formation of osteoclasts through its actions on osteoblastic cells. We have generated mice lacking vitamin D receptor (VDR) by gene targeting (VDR-/-). These mice had tartrate-resistant acid phosphatase (TRAP)-positive osteoclasts, and exhibited similar levels of parameters for bone resorption to those in wild type mice. The present studies were undertaken to clarify whether effects of 1,25$(OH)_2$D on osteoclast formation require VDR in osteoblasts, and to examine mechanisms of the formation of osteoclasts without VDR-mediated actions using VDR-/- mice. When wild-type calvarial osteoblasts and spleen cells were co-cultured with 1,25$(OH)_2$D, TRAP-positive osteoclasts were formed regardless of the genotypes of spleen cells. In contrast, when osteoblasts from VDR-/- mice were co-cultured, no osteoclasts could be formed even with wild-type spleen cells. Parathyroid hormone and interleukin-1α stimulated osteoclast formation by co-cultures from VDR-/- mice, and the generated osteoclasts showed resorbing activity. These results demonstrate that VDR-mediated actions of 1,25$(OH)_2$D in osteoblasts are essential for osteoclast formation by 1,25$(OH)_2$D, and that functionally intact osteoclasts can be formed without 1,25$(OH)_2$D actions under stimulations by other agents. It is suggested that osteoclastic bone resorption can be maintained without 1,25$(OH)_2$D actions by other stimulatory agents.

Vitamin D plays a pivotal role in the regulation of intestinal and renal calcium transport (1, 2). Vitamin D also has effects on bone to stimulate the formation of osteoclasts and the synthesis of matrix proteins (3-5). Thus, deficient actions of vitamin D result in hypocalcemia, hypophosphatemia and rickets/osteomalacia. Vitamin D exerts most of these actions via the binding of its active metabolite, 1,25-dihydroxyvitamin D [1,25$(OH)_2$D] to vitamin D receptor (VDR). The VDR-1,25$(OH)_2$D complex binds to its responsive elements on its target genes, and modifies the transcription of those genes to develop its actions. However, there is a possibility that some of the actions of 1,25$(OH)_2$D may be mediated by a non-genomic mechanism (6), and may not require the binding to VDR.

Although 1,25$(OH)_2$D is shown to play an important role in the formation of osteoclasts (7), it has been unclear whether the effect is mediated by its binding to VDR. Formation of osteoclasts requires cell-to-cell contact between hematopoietic osteoclast progenitors and stromal cells, and 1,25$(OH)_2$D acts on stromal cells to stimulate osteoclast formation (7-9). Because osteoclast precursors as well as stromal cells posses VDR, and because osteoclast-like cells can be formed without the presence of stromal cells under certain conditions (10), the question also remains whether VDR-mediated actions of 1,25$(OH)_2$D on stromal cells are required for osteoclast formation.

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We have generated mice lacking VDR (VDR-/-) by gene targeting (11). These mice exhibited low serum calcium and phosphate, and typical features of rickets similar to those of vitamin D-dependent rickets type II. Because osteoclasts are present on the bone surface of these mice, 1,25$(OH)_2$D may stimulate osteoclast formation without VDR. Alternatively, because factors such as parathyroid hormone (PTH) and interleukin (IL)-1 also stimulate osteoclast formation (12), and because PTH secretion is elevated in vitamin D-dependent rickets, osteoclasts may be formed under the stimulation by these factors in VDR-/- mice.

The present studies were undertaken to clarify the role of VDR-mediated actions of 1,25$(OH)_2$D on osteoclast formation, and to clarify whether the effects of 1,25$(OH)_2$D on osteoclast formation are mediated via osteoblastic cells or osteoclast precursor cells using VDR-/- mice. The results show that the presence of VDR in osteoblastic cells is required for the stimulation of osteoclast formation by 1,25$(OH)_2$D, and that osteoclasts can be formed under the stimulation by PTH or IL-1α in the absence of VDR-mediated actions of 1,25$(OH)_2$D.

Materials and Methods

Materials and Animals: 1,25$(OH)_2D_3$ and human PTH(1-34) were provided by Chugai Pharmaceutical Co. (Tokyo, Japan) and Asahi Chemical Co. (Tokyo, Japan), respectively. VDR-/- mice were produced and maintained as described (11). They were fed a standard laboratory chow (Japan Clea, Chiba, Japan) containing 1.2% calcium and 1.0% phosphorus.

Histomorphometric and histochemical analyses: Bone tissues were analyzed before (3-week old) and after weaning

(7-week old). Calcein (8 mg/kg body weight) was injected 14 and 7 days before sacrifice. Undecalcified tibiae were fixed in 99.5% ethanol and embedded in methyl methacrylate. Frontal sections (5-μm thick) were stained by modified Villanueva-Goldner's trichrome method, and bone histomorphometry of the metaphyseal secondary cancellous bone area was performed. Bone volume per tissue volume (BV/TV), trabecular thickness (Tb.Th), osteoid volume per total volume (OV/TV), osteoid surface per bone surface (OS/BS), osteoid thickness (O.Th), osteoblast surface per bone surface (Ob.S/BS), osteoblast surface per osteoid surface (Ob.S/OS), eroded surface per bone surface (ES/BS), eroded depth (E.De) and osteoclast number per bone perimeter (N.Oc/B.Pm) were determined. These abbreviations are derived from the recommendation of American Society for Bone and Mineral Research Histomorphometry Nomenclature Committee (13). Undecalcified tibiae were fixed with 4% paraformaldehyde in the mixtures of JB-4 (Polysciences Inc., Warrington, PA) and phosphate-buffered saline, pH 7.4, at 4°C, and embedded in methyl methacrylate. Alkaline phosphatase (ALP) was stained by incubating sections with naphthol AS-BI phosphate (Sigma Chemical Co.) in N,N-dimethyl formamide at pH 8.5. Tartrate-resistant acid phosphatase (TRAP) was stained with naphthol AS-MX phosphate (Sigma Chemical Co.) in N,N-dimethyl formamide in sodium tartrate at pH 5.0.

Co-culture analyses: Osteoclast formation was evaluated by co-cultures of calvarial osteoblasts with spleen cells from newborn mice (8). Genotypes of these mice were determined by Southern blot analysis using tail tissues (11). Isolated osteoblasts were cultured for 1 week. Then, spleen cells were obtained by mincing spleens and pipetting, and were co-cultured with osteoblasts. Osteoblasts (10^4 cells/well) and spleen cells ($5x10^5$ cells/well) from either VDR+/+ or -/- mice were cultured in α-MEM containing 10% fetal bovine serum. After 8 days, cells were fixed with 10% formaldehyde and stained for TRAP by reacting with fast red violet LB salt (Sigma Chemical Co.) and naphthol AS-MX phosphate in N,N-dimethyl formamide in sodium acetate buffer, pH 5.0, with 10 mM sodium tartrate. The number of TRAP-positive multinucleated cells (MNCs) with more than three nuclei was counted. Co-cultures were also performed on dentine slices to evaluate the resorbing activity of the generated osteoclasts (14). After 8 days, the slices were ultrasonicated and stained with hematoxylin.

Results

Histochemical and histomorphometric analyses

As reported previously, there were no apparent histological abnormalities in bones of VDR-/- mice before weaning at 3 weeks (11). At 7 weeks, there was a marked increase in osteoid tissues in the primary spongiosa of VDR-/- mice (Figure 1). Histological features of VDR+/- mice were not different from those in VDR+/+ mice. Prehypertrophic and hypertrophic chondrocytes were stained for ALP, and the layers of ALP-positive chondrocytes were thicker in VDR-/- mice than those in VDR+/+ or +/- mice (Figure 2). ALP staining of osteoblasts on the surface of cancellous bones was much weaker and sparse

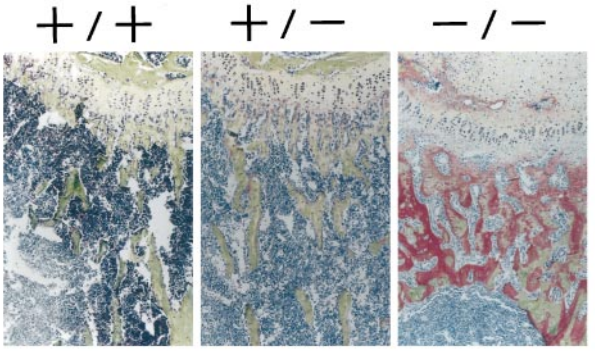

Figure 1. Histological analyses of tibiae.
Tibiae of 7-week old mice were stained by modified Villanueva-Goldner's trichrome method. The red staining is osteoid and the green is calcified bone. +/+: wild type, +/-: heterozygous and -/-: homozygous mice for vitamin D receptor knockout.

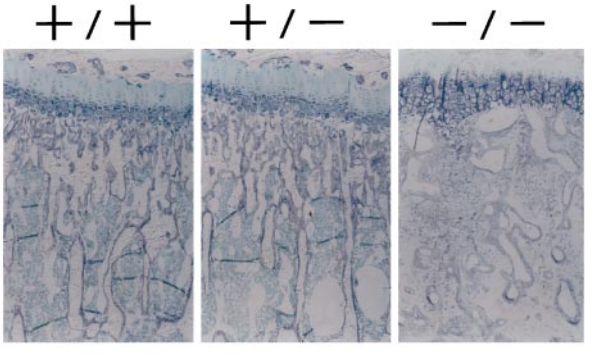

Figure 2. Alkaline phosphatase staining of tibiae.
Tibiae from 7-week old mice were stained for alkaline phosphatase (ALP). The dark blue staining is ALP-positive osteoblasts. +/+: wild type, +/-: heterozygous and -/-: homozygous mice for vitamin D receptor knockout.

in VDR-/- mice (Figure 2). Osteoclasts were seen in VDR-/- as well as in VDR+/+ and +/- mice. In VDR-/- mice, osteoclasts were present mainly in the secondary spongiosa, with less osteoclasts in the primary spongiosa (Figure 3).

At 7 weeks, BV/TV, Tb.Th, OV/BV, OS/BS and O.Th were markedly increased in VDR-/- mice (Table 1). Ob.S/OS was decreased in VDR-/- mice due to a marked increase in OS/BS. Because calcein staining was vague and irregular due to a marked impairment of bone mineralization, mineral apposition rate and bone formation rate could not be calculated in VDR-/- mice. In contrast, ES/BS, E.De and N.Oc/B.Pm were not significantly different among the three groups, showing that osteoclasts in VDR-/- mice can effectively resorb bone. At 3 weeks, no difference was seen in any of these parameters between VDR-/- and VDR+/+ or +/- mice (data not shown).

Osteoclast formation and function in vitro

In order to examine whether $1,25(OH)_2D$ can stimulate osteoclast formation without VDR, and whether the effect is

Table 1. Histomorphometric analyses of tibial secondary spongiosa of 7-week old mice.

	+/+	+/-	-/-
BV/TV	13.1 ± 0.4	10.9 ± 0.4	50.3 ± 4.4*
Tb.Th	28.9 ± 2.8	25.6 ± 1.4	62.4 ± 3.8*
OV/BV	4.34 ± 2.28	3.94 ± 0.03	62.6 ± 1.5*
OS/BS	21.6 ± 11.0	19.8 ± 0.5	70.5 ± 1.9*
O.Th	2.87 ± 0.23	2.56 ± 0.23	26.9 ± 1.8*
Ob.S/BS	16.5 ± 7.7	15.9 ± 0.8	19.4 ± 2.0
Ob.S/OS	78.8 ± 4.9	80.3 ± 6.3	27.4 ± 2.2*
ES/BS	26.4 ± 6.2	26.7 ± 0.3	25.6 ± 3.0
E.De	6.78 ± 0.35	7.30 ± 1.14	7.88 ± 0.22
N.Oc/B.Pm	321 ± 72	318 ± 11	423 ± 27

+/+: wild type, +/-: heterozygous and -/-: homozygous mice for vitamin D receptor knockout. Data are means ± SD for four or five mice. *$p < 0.05$ vs wild type mice.

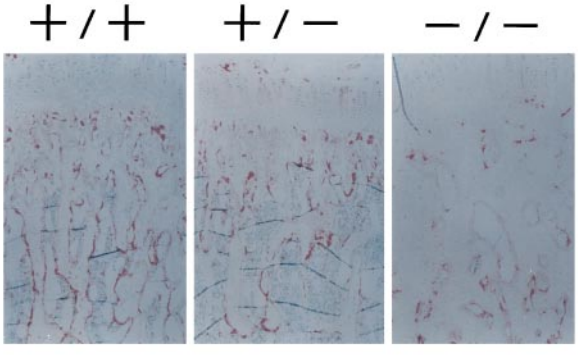

Figure 3. Tartrate-resistant acid phosphatase staining of tibiae. Tibiae from 7-week old mice were stained for tartrate-resistant acid phosphatase (TRAP). The red staining is cytoplasmic TRAP in osteoclasts. +/+: wild type, +/-: heterozygous and -/-: homozygous mice for vitamin D receptor knockout.

mediated via osteoblasts or osteoclast precursors, we co-cultured osteoblastic cells with spleen cells from VDR-/- and wild-type mice in the presence of 10^{-8}M $1,25(OH)_2D_3$. When both cells were prepared from VDR+/+ mice, $1,25(OH)_2D_3$ stimulated TRAP-positive MNC formation, whereas when cells were prepared from VDR-/- mice, osteoclasts were not formed even in the presence of $1,25(OH)_2D_3$ (Table 2). When we prepared osteoblasts from wild-type mice and spleen cells from VDR-/- mice, TRAP-positive MNCs were formed. In contrast, when osteoblasts from VDR-/- mice were co-cultured with spleen cells from wild-type mice, no TRAP-positive osteoclasts could be formed in the presence of $1,25(OH)_2D_3$. Thus, the stimulation of osteoclast formation by $1,25(OH)_2D$ requires the presence of VDR in osteoblasts.

Because osteoclast formation can be stimulated by other factors, we performed co-cultures in the presence of hPTH(1-34) or IL-1α. When osteoblasts and spleen cells were co-cultured

Table 2. Effects of $1,25(OH)_2D_3$, hPTH(1-34) and IL-1α on osteoclast formation in vitro.

Osteoblast	Spleen	$1,25(OH)_2D_3$	hPTH(1-34)	IL-1α
+/+	+/+	83.0 ± 10.4	50.0 ± 5.0	70.0 ± 3.8
+/+	-/-	89.0 ± 7.2	59.8 ± 5.3	64.8 ± 5.0
-/-	+/+	5.0 ± 1.2*	62.5 ± 2.8	70.8 ± 4.8
-/-	-/-	3.0 ± 1.0*	64.5 ± 7.5	79.8 ± 15.3

Calvarial osteoblasts and spleen cells obtained from wild type (+/+) or vitamin D receptor knockout (-/-) mice were co-cultured with 10^{-8} M $1,25(OH)_2D_3$, 2.5×10^{-8} M hPTH(1-34) or 1.5×10^{-10} M IL-1α for 8 days. TRAP-positive MNCs per well were counted. Data are means ± SD for four wells.
*$P < 0.05$ vs $1,25(OH)_2D_3$-treated group using wild type cells.

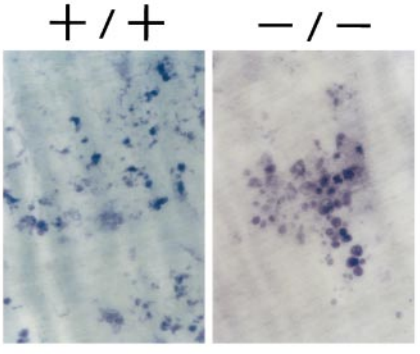

Figure 4. Pit formation by osteoclasts generated by co-cultures. Calvarial osteoblasts and spleen cells from wild type (+/+) and vitamin D receptor knockout (-/-) mice were co-cultured in the presence of 2.5×10^{-8}M hPTH(1-34) on dentine slices. After 8 days, they were ultrasonicated and stained with hematoxylin.

with 2.5×10^{-8}M hPTH(1-34) or 1.5×10^{-10}M IL-1α, osteoclasts were formed regardless of the genotypes of these cells (Table 2). In addition, osteoclasts generated from osteoblasts and spleen cells of VDR-/- mice could form resorption pits in the presence of hPTH(1-34) (Figure 4). These results are in agreement with the histological observations, and demonstrate that osteoclasts produced from VDR-/- cells can resorb bone.

Discussion

Stimulation of osteoclast formation by $1,25(OH)_2D$ requires cell-to-cell interaction between osteoblastic cells and osteoclast precursors, and $1,25(OH)_2D$ exerts these effects via its actions on osteoblastic cells (7-9). The present results using co-cultures of osteoblastic cells and spleen cells from VDR+/+ and -/- mice are in agreement with those observations, and demonstrate that the effect of $1,25(OH)_2D$ on osteoblastic cells to stimulate osteoclast formation requires the presence of VDR in osteoblastic cells but not in osteoclast precursor cells.

Despite the absence of $1,25(OH)_2D$ actions in VDR-/-

mice, there are abundant osteoclasts on bone surfaces, suggesting that osteoclast formation is stimulated by other factors in these mice. The present studies demonstrated that osteoclasts can be formed in the presence of hPTH(1-34) or IL-1α by co-cultures of osteoblastic cells and osteoclast precursors regardless of VDR genotypes. Because PTH levels are expected to be elevated in VDR-/- mice, the in vivo finding of the presence of osteoclasts in these mice can be explained by the stimulation of osteoclast formation by factors such as PTH. In addition, histomorphometric analyses as well as resorption pit assay in vitro revealed that osteoclasts formed in VDR-/- mice are functionally intact and effectively resorb bone. These results are consistent with the assumption that VDR-mediated actions of $1,25(OH)_2D$ are not essential for the formation and resorptive functions of osteoclasts in bone.

A secretory protein of the tumor necrosis factor receptor family, osteoprotegerin (OPG)/osteoclastogenesis-inhibitory factor (OCIF), has been cloned and is shown to inhibit osteoclast formation by interfering with the cell-to-cell interaction between osteoblastic cells and osteoclast progenitors (15, 16). Recently, a ligand for OPG/OCIF was cloned from mouse stromal cell cDNA library (17). The protein is a member of the membrane-associated tumor necrosis factor ligand family, and a recombinant soluble form containing the extracellular domain can induce osteoclast formation from spleen cells in the absence of osteoblast/stromal cells. Furthermore, expression of the OPG/OCIF ligand gene in mouse stromal cells is up-regulated by $1,25(OH)_2D$. Up-regulation of the gene is also observed in mouse primary osteoblasts by PTH, interleukin-11 and prostaglandin E_2 (17). In the light of these observations along with our present results, it is plausible to assume that $1,25(OH)_2D$ stimulates osteoclast formation by VDR-dependent up-regulation of OPG/OCIF ligand in osteoblasts.

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