1,25-Dihydroxyvitamin D_3 Stimulates Rat Osteoblastic Cells to Release a Soluble Factor That Increases Osteoclastic Bone Resorption

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Abstract

Although 1,25-dihydroxyvitamin D_3 stimulates osteoclastic bone resorption in vivo and in organ culture, the mechanism by which it effects this stimulation is unknown. We have recently found that the agent does not stimulate resorption by osteoclasts mechanically disaggregated from bone and incubated on slices of cortical bone. This suggests that the osteoclasts were removed by disaggregation from the influence of some cell type, present in intact bone, that mediates hormone responsiveness. We therefore tested the ability of osteoblastic cells derived from neonatal rat calvariae and of cloned, hormone-responsive osteosarcoma cells (UMR106) to restore hormone responsiveness to unresponsive populations of osteoclasts. We found that osteoblastic cells from both sources induced a two- to fourfold stimulation of osteoclastic bone resorption in the presence of 1,25-dihydroxyvitamin D_3. Stimulation was observed at concentrations of 10^{-10} M and above. Actinomycin D and cycloheximide did not affect bone resorption by osteoclasts incubated alone, but abolished the capacity of osteoblastic cells to stimulate osteoclastic resorption in the presence of 1,25-dihydroxyvitamin D_3. When calvarial cells or osteoblastlike UMR cells were incubated with the hormone, they produced a factor in cell-free supernatants that stimulated bone resorption by disaggregated osteoclasts. These experiments suggest that 1,25-dihydroxyvitamin D_3 stimulates bone resorption through a primary action on osteoblastic cells, that are induced by the hormone to produce a factor that stimulates osteoclastic bone resorption.

Introduction

1,25-Dihydroxyvitamin D_3 ($1,25(OH)_2D_3$)[1] is essential for mineralization of bone and cartilage, and defective mineralization is the most notable pathological feature of deficiency of the hormone. The main mechanism through which $1,25(OH)_2D_3$ induces bone and cartilage calcification is believed to be indirect, through an increase in the concentration of calcium and phosphate in the serum (1–3).

$1,25(OH)_2D_3$ also stimulates bone resorption in vivo (4–6). Unlike its effect on mineralization, its effect on bone appears to be direct, because increased osteoclastic resorption is induced in organ cultures of bone in vitro (7, 8).

The mechanism by which $1,25(OH)_2D_3$ stimulates bone resorption is unknown. Receptors have been identified in osteoprogenitor cells and osteoblasts but not in osteoclasts (9, 10). Either receptors are present in osteoclasts in numbers sufficient for stimulation of function but insufficient for identification, or the hormone stimulates osteoclastic resorption indirectly. Indirect stimulation may occur through interaction with other bone cells that possess receptors. Alternatively it has been suggested that precursors of osteoclasts, unlike mature cells, may possess receptors that stimulate osteoclastic precursors to differentiate into osteoclasts (10).

We have recently developed methods whereby osteoclasts can be disaggregated from bone and sedimented and widely dispersed onto slices of devitalized cortical bone (11). The osteoclasts excavate the bone surface, and the degree of excavation can be quantified in the scanning electron microscope after culture. Using this system, we found that $1,25(OH)_2D_3$ failed to stimulate osteoclastic bone resorption (12). This suggests that our cultures were deficient in some cell type, present in bone, that mediates the effect of $1,25(OH)_2D_3$ on osteoclastic bone resorption. In an attempt to identify the mechanism by which $1,25(OH)_2D_3$ acts on bone, we elected to repeat the experiment in the presence of osteoblastic cells to determine whether these were capable of mediating the stimulation of osteoclastic bone resorption induced by the hormone in intact bone.

Methods

$1,25(OH)_2D_3$ was a gift from Dr. J. O'Riordan. Cloned, hormone-responsive rat osteosarcoma cells (UMR106, passage 42, see reference 13) were obtained from Dr. T. J. Martin. The tissue culture medium used was Hepes-buffered medium 199 (Flow Laboratories, Irvine, UK) for cell isolation and sedimentation, and Eagle's minimum essential medium containing Hanks' salts (MEM) (Flow Laboratories) supplemented with 200 μg/ml streptomycin (Gibco, Grand Island, NY), benzylpenicillin (100 IU/ml; Gibco), and 10% heat-inactivated fetal calf serum (FCS; Gibco) for subsequent cell culture. All incubations were made at 37°C in 5% CO_2 in humidified air.

Slices of cortical bone were used as substrates for osteoclastic resorption. Bovine femoral cortical bone was cleaned of adherent soft tissues and cut into slices 0.3 × 0.4 × 0.01 cm with a low-speed saw (Isomet, Buehler, IL). The slices were cleaned by ultrasonication for 20 min in sterile distilled water, washed in alcohol, and stored dry at room temperature.

Preparation of osteoblastic cells. Calvarial osteoblasts were obtained from neonatal female Wistar rats of the St. George's Hospital Medical School colony. Calvariae were dissected free of periosteum and associated soft tissues and incubated in medium 199 containing collagenase (1 mg/ml, type II, Sigma Chemical Co., St. Louis, MO) at 37°C for 100 min. The tissue fragments were then agitated, and suspended cells were removed and centrifuged at 250 *g* for 3 min. The cells were resuspended in MEM/FCS (1 to 3 × 10^5 cells/ml) and incubated in 50-mm Falcon tissue culture dishes (Sterilin, Teddington, UK) at 37°C. When confluent

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1. *Abbreviations used in this paper:* MEM, minimum essential medium; $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D_3.

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(always within 5 d), culture medium was removed and replaced with trypsin-EDTA (Gibco) for 5 min at 37°C. After incubation the cells were agitated, removed, washed, and resuspended in MEM/FCS (10^6 cells/ml) for addition to bone slices as below. Percentage of alkaline phosphatase–positive cells was determined histochemically.

Osteoblastlike UMR 106 cells were incubated to confluence in MEM/FCS and similarly suspended in trypsin-EDTA, washed, and resuspended in MEM/FCS at 10^6 cells/ml for addition to bone slices.

Comparison of the effect of 1,25(OH)$_2$D$_3$ on disaggregated osteoclasts incubated with and without osteoblastic cells. Osteoclasts were disaggregated from rats as previously described (11). Neonatal female Wistar rats were killed by decapitation. Femurs and tibias were removed, freed of adherent soft tissues, and cut across their epiphyses. The bones of each rat were curetted with a scalpel blade into 1 ml of medium 199 and vigorously agitated with a pipette. Larger fragments were allowed to sediment for 10 s before the suspension was added to bone slices in the well of a Sterilin 100 × 18 mm multiwell dish. The cells were incubated at 37°C for 15 min, after which the bone slices were removed and washed vigorously in medium 199. Each bone slice was then placed in a 16-mm diameter tissue culture well (Gibco) containing 750 μl of MEM/FCS. The calvarial cell suspension (0.75 ml per well) was added to alternate wells (0.75 ml MEM/FCS was added to the remainder). 1,25(OH)$_2$D$_3$ or vehicle was then added to a final concentration of 10^{-8} M. Because of variability in osteoclast numbers between different suspensions, the experiments were designed in such a way that all variables (±1,25[OH]$_2$D$_3$, ± calvarial cells) received osteoclasts from the same suspension. As additional controls, the calvarial cell suspension (0.75 ml) was sedimented onto bone slices without osteoclasts and incubated in the presence and absence of 1,25(OH)$_2$D$_3$.

Bone resorption was assessed after 24 h incubation, when the majority of osteoclasts have formed excavations in the surface of the bone slices (12, 14), the extent of which was assessed by morphometric means (15). Cells were removed from bone slices by immersion in sodium hypochlorite for 10 min. The bone slices were then dehydrated in alcohol, sputter-coated with gold, and the entire surface of each slice was examined in an S90 scanning electron microscope (Cambridge Instruments, Cambridge, UK). Bone resorption was measured by transferring the outline of each osteoclastic excavation to a microcomputer through a digitizing tablet. This enabled computation of the number of pits on each bone slice and their mean plan area of resorption, as previously described (15).

Alkaline phosphatase histochemistry was performed on the osteoblastic populations by substitution of 6-mm glass coverslips for bone slices. After incubation for 24 h the cells were fixed in 10% formal calcium (30 s), and the presence of alkaline phosphatase was demonstrated by the naphthol AS-B1 method (16). Fibroblasts from neonatal rat skin, obtained and incubated in an identical manner to calvarial cells, were used as controls.

Identical experiments were performed substituting UMR106 cells for calvarial cells.

Sensitivity of cocultures to 1,25(OH)$_2$D$_3$. Calvarial cells were added to osteoclasts on bone slices as previously described. For each experiment osteoblastic cells and osteoclasts from the same suspension were incubated together in serial dilutions of 1,25(OH)$_2$D$_3$ for 24 h. Bone resorption was quantified as above, and resorption of 1,25(OH)$_2$D$_3$-treated slices was expressed as a ratio of resorption of control (1,25(OH)$_2$D$_3$-free) slices. Ratios derived from consecutive experiments were pooled.

Sensitivity of 1,25(OH)$_2$D$_3$-responsiveness to inhibition by actinomycin D and cycloheximide. Osteoclasts were cultured alone or with UMR cells, as previously described, for 24 h in the presence or absence of 1,25(OH)$_2$D$_3$ or vehicle, and actinomycin (5 μM) or cycloheximide (50 μM) (or the appropriate concentration of vehicle).

Effect of medium conditioned by osteoblastic cells on osteoclastic bone resorption. UMR106 cells or calvarial cells were incubated to confluence as described above. Medium was removed and replaced with fresh MEM/FCS for osteoblastic cells, or serum-free MEM for UMR cells. The cells were incubated for 24 h in the presence of 1,25(OH)$_2$D$_3$ (10^{-8} M) or vehicle. After incubation, supernatant was removed, centrifuged, and 1,25(OH)$_2$D$_3$ added to the 1,25(OH)$_2$D$_3$-free supernatant. The supernatants were substituted for the cell suspensions used in the previous experiments.

Statistics. Gabriel's one-way analysis of variance was used throughout (17), except for Table I (one-sample *t* test after log-transformation of the data).

Results

Only the population of cells derived from mechanical disaggregation of neonatal rat bone, which contained recognizable (multinucleate) osteoclasts, was associated with excavations in the bone surface after incubation. This population does not consist of pure osteoclasts but results in a sparse population of 5–20 multinucleate cells accompanied by 100–200 mononuclear cells per bone slice, widely scattered across the bone surface (14). The figure shows that this population of cells was without response to 1,25(OH)$_2$D$_3$.

Hormone responsiveness was restored to unresponsive osteoclastic populations by coculture with calvarial cells or UMR cells (Fig. 1). This stimulation by 1,25(OH)$_2$D$_3$ was highly significant for both sources of osteoblastic cells but UMR cells, which contained a higher proportion of alkaline phosphatase-positive cells (85 vs. 65% for calvarial cells; fibroblasts, 0%) (Table II), caused significantly greater stimulation of bone resorption.

Table I. Resorption by Osteoclasts Incubated with Supernatant from UMR106 or Calvarial Osteoblastic Cells Cultured with Either 1,25(OH)$_2$D$_3$ or Vehicle

Incubation conditions	Excavations per bone slice*	Plan area resorbed per bone slice*	Bone resorption
		$\mu m^2 \times 10^{-3}$	*treated/control*
OC+S(UMR)	4.4±1.0	10.1±3.4	1.83±0.04‡
OC+S(UMR, 1,25(OH)$_2$D$_3$)	11.1±3.4	20.2±6.5	
OC+S(OB)	14.1±2.3	17.4±4	3.17±0.77§
OC+S(OB, 1,25(OH)$_2$D$_3$)	28.6±2.8‖	43.0±4.8‖	

1,25(OH)$_2$D$_3$ (10^{-8} M) was added to control supernatants before addition to osteoclasts. For both groups (UMR and OB) results are from four consecutive experiments with four bone slices for each variable per experiment. OC, osteoclasts; OB, osteoblasts; S, supernatant. * Mean±SE. ‡ $P < 0.001$. § $P < 0.05$ for treated to control ratio mean±SE using a one-sample *t* test after log transformation of the data. pH of incubation medium was measured at the end of the experiment and was 6.9±0.07 and 6.89±0.05 for test and control supernatant, respectively (mean±SE). ‖ $P < 0.01$ using Gabriel's one-way analysis of variance.

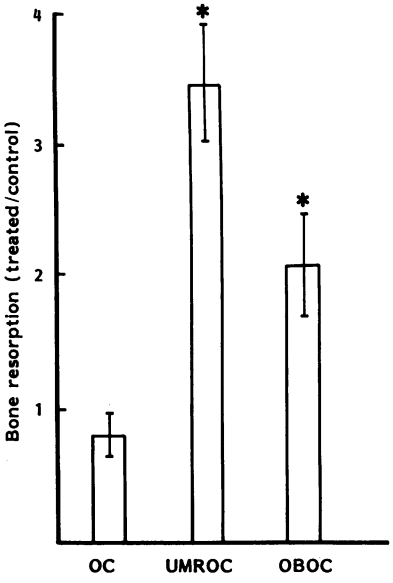

Figure 1. Bone resorption by disaggregated osteoclasts (*OC*) incubated alone or in the presence of osteoblasts or UMR106 cells. Osteoblastic cells incubated without osteoclasts showed no bone resorption with or without $1,25(OH)_2D_3$. Results expressed as ratio (mean±SE, six experiments) of bone resorbed in the presence of $1,25(OH)_2D_3$ (10^{-8} M) to bone resorbed in its absence. *$P < 0.05$ (Gabriel's one-way analysis of variance).

None of the control cultures, in which osteoblastic cells were incubated on bone slices without osteoclasts, showed evidence of bone resorption even in the presence of $1,25(OH)_2D_3$. This indicates that osteoblastic cells did not directly contribute to bone excavation but rather were induced by $1,25(OH)_2D_3$ to stimulate osteoclastic bone resorption. Osteoblastic cells restore hormone responsiveness to unresponsive populations of osteoclasts.

The sensitivity of cocultures of osteoblastic cells and disaggregated osteoclasts was assessed using calvarial cells after incubation to confluence. Significant stimulation was observed at concentrations of 10^{-10} M $1,25(OH)_2D_3$ and above (Table II). This compares with stimulation of resorption in organ culture observed at concentrations of $1,25(OH)_2D_3$ of 10^{-9} to 10^{-11} M (7, 8, 18).

Stimulation of bone resorption in the presence of UMR cells and $1,25(OH)_2D_3$ was abolished by cycloheximide and actinomycin D (Tables III and IV). Neither agent significantly influenced bone resorption by osteoclasts incubated alone, and both agents were also present in cocultures of UMR cells and osteoclasts incubated with and without the hormone (Tables III and IV). These results suggest that the inhibitors were acting to prevent $1,25(OH)_2D_3$ stimulation of osteoclastic bone resorption through an effect on UMR cells rather than to directly interfere with osteoclastic function, and that UMR cells mediate $1,25(OH)_2D_3$ stimulation of resorption through an RNA and protein synthesis–dependent mechanism.

We found that supernatants from both calvarial cells and UMR cells incubated for 24 h with $1,25(OH)_2D_3$-stimulated bone resorption by osteoclasts compared with supernatants from osteoblastic cells incubated without the hormone (but to which hormone was added before incubation with osteoclasts) (Table I).

Table II. Sensitivity of Cocultures of Calvarial Cells and Osteoclasts to $1,25(OH)_2D_3$

Incubation conditions	$1,25(OH)_2D_3$ concentration	Plan surface area per bone slice
	M	*treated/control*
OC	0	0.79±0.1 (6)
OBOC	−7	2.19±0.28 (5)*
OBOC	−8	3.05±0.54 (5)*
OBOC	−9	1.92±0.18 (5)*
OBOC	−10	2.03±0.31 (4)*
OBOC	−11	0.96±0.15 (4)
OBOC	−12	1.09±0.19 (3)

Results expressed as mean±SE of consecutive experiments (number in parentheses), each experiment consisting of four bone slices per variable. OC, osteoclast; OB, calvarial cells.
* Treated to control ratio significantly greater than calvarial cell-free control ($P < 0.02$). (Gabriel's one-way analysis of variance).

Table III. Bone Resorption by Osteoclasts Incubated Alone, in Presence of UMR Cells, and in Presence and Absence of 10^{-8}M $1,25(OH)_2D_3$ and 5 μm Actinomycin D

Incubation conditions	Excavations per bone slice	Plan surface area resorbed per bone slice
		$\mu m^2 \times 10^{-3}$
OC	3.5±1.1	7.3±2.3
OC + $1,25(OH)_2D_3$	4.1±2.1	7.7±4.5
UMROC	8.0±3.3	10.8±3.4
UMROC + $1,25(OH)_2D_3$	19.6±6.4*	33.1±12.2*
OC + AD	5.3±0.8	10.4±2.0
OC + $1,25(OH)_2D_3$ + AD	5.8±1.4	10.4±2.6
UMROC + AD	9.3±2.3	18.0±4.8
UMROC + $1,25(OH)_2D_3$ + AD	6.9±1.7	12.5±2.6

Results expressed as mean±SE of three consecutive experiments (four bone slices per group in each experiment). OC, osteoclasts; UMROC, osteoclasts in presence of UMR cells; AD, actinomycin D.
* $P < 0.05$ compared with all other groups (Gabriel's one-way analysis of variance).

Table IV. Bone Resorption by Osteoclasts Incubated Alone, in Presence of UMR Cells, and in Presence and Absence of 10^{-8} M $1,25(OH)_2D_3$ and 50 μm Cycloheximide

Incubation conditions	Excavations per bone slice	Plan surface area resorbed per bone slice
		$\mu m^2 \times 10^{-3}$
OC	17.9±2.7	22.9±4.3
OC + $1,25(OH)_2D_3$	15.3±1.2	18.5±3.3
UMROC	32.3±5.7	53.5±9.5
UMROC + $1,25(OH)_2D_3$	56.6±11.9*	114.4±24.0*
OC + CX	9.3±2.2	15.3±4.4
OC + $1,25(OH)_2D_3$ + CX	11.1±1.4	16.8±2.5
UMROC + CX	19.6±2.0	30.8±4.2
UMROC + $1,25(OH)_2D_3$ + CX	17.7±3.5	27.9±6.4

Results expressed as mean±SE of two consecutive experiments (four bone slices per group in each experiment). CX, cycloheximide.
* $P < 0.05$ compared with all other groups (Gabriel's one-way analysis of variance).

Discussion

We have found that while osteoclasts mechanically disaggregated from neonatal rat long bone do not respond to $1,25(OH)_2D_3$ if incubated alone, they do so if incubated in the presence of osteoblastic cells derived from rat calvaria, or in the presence of cloned, hormone-responsive osteosarcoma cells. Direct induction by the hormone of the differentiation of increased numbers of osteoclastic cells from precursors was unlikely to account for this stimulation (10). Precursors may have been present in the osteoblastic or osteoclastic cell suspensions, but there was no stimulation of bone resorption in osteoclastic populations incubated without osteoblastic cells, and there was no evidence of bone resorption when calvarial osteoblastic cells or osteosarcoma cells were incubated alone on bone slices. These experiments thus suggest that $1,25(OH)_2D_3$ does not act directly on either osteoclastic precursors or mature osteoclasts.

This conclusion was supported by the coculture and supernatant experiments. Incubation of osteoblastic cells with disaggregated osteoclasts restored hormonal responsiveness to otherwise nonresponsive cells. Moreover, we found that osteoblastic cells incubated in the presence of $1,25(OH)_2D_3$ produced a supernatant factor that stimulated osteoclastic bone resorption. This demonstrates that $1,25(OH)_2D_3$ acts to stimulate bone resorption through a primary action on osteoblasts, which release a soluble factor that stimulates osteoclastic resorption.

Neither cycloheximide nor actinomycin D had any effect on bone resorption by disaggregated osteoclasts. Both, however, abolished production of stimulatory factor by osteosarcoma cells, suggesting that UMR-cell responsiveness to $1,25(OH)_2D_3$ depends, like many other cellular responses to the hormone, on de novo RNA and protein synthesis (19).

Bone resorption in osteoblast-osteoclast cocultures was stimulated by concentrations of $1,25(OH)_2D_3$ of 10^{-10} M and above. This suggests that physiological concentrations of $1,25(OH)_2D_3$ may be capable of influencing osteoclastic bone resorption. We presume that the absence of dose responsiveness is related to the variability of the technique and the steep range of $1,25(OH)_2D_3$ concentrations used. Although the extent to which the sensitivity of our cocultures reflects that of intact bone is unknown, the degree of sensitivity of the cocultures to $1,25(OH)_2D_3$ suggests that the interactions we have observed have physiological significance.

Our results are consistent with receptor studies showing that $1,25(OH)_2D_3$ receptors are present in osteoprogenitor and osteoblastic cells but not in osteoclasts (9, 10). The major effect of increased levels of $1,25(OH)_2D_3$ in vivo is hypercalcemia; the osteoclast contributes to this with increased levels of bone resorption. Therefore it is initially curious that whereas $1,25(OH)_2D_3$ receptors have been identified in almost every tissue examined (20), they are absent from the cell type that contributes to the major effect of increased hormonal levels. A likely explanation is that osteoclasts are derived from wandering, hematogenous cells (21), and direct stimulation of the function of these may lead to random increase in bone resorption. Instead, increased osteoclastic resorption is mediated by cells of the osteoblastic lineage, which may have access to physiochemical and morphogenetic information derived from the adjacent and subjacent cells with which they make intimate contact through profuse cytoplasmic processes. This may enable only those osteoclasts in the most appropriate sites to be stimulated to increased resorption. We have found that parathyroid hormone, like $1,25(OH)_2D_3$, also increases osteoclastic bone resorption through a primary hormonal interaction with osteoblastic cells (14), which it similarly induces to produce a supernatant factor that stimulates osteoclasts (22). It is possible that the same factor mediates the stimulation caused by both hormones, and it may be that regulation of osteoblastic production of the osteoclast-stimulating factor is a final common pathway through which diverse local and systemic hormonal stimuli influence osteoclastic resorption.

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