No 1,25-Dihydroxyvitamin D_3 Receptors on Osteoclasts of Calcium-deficient Chicken Despite Demonstrable Receptors on Circulating Monocytes

J. Merke, G. Klaus, U. Hügel, R. Waldherr, and E. Ritz
Department of Internal Medicine and Pathology, University of Heidelberg, D-6900 Heidelberg, Federal Republic of Germany

Abstract

1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) is known to stimulate osteoclastic bone resorption in vivo and whole organ bone culture systems in vitro. It has not been established whether $1,25(OH)_2D_3$ acts directly on osteoclasts or whether its action on osteoclasts is mediated via other bone cells (e.g., osteoblasts) or recruitment of osteoclast precursor cells. Circulating monocytes have been characterized as osteoclast precursors. In the present study, vitamin D_3-replete chicken on a calcium-deficient diet were studied. Circulating monocytes, whole bone cell preparations, and isolated osteoclasts (differential sedimentation) were examined for presence of $1,25(OH)_2D_3$ receptors. Reversible, specific, and saturable binding of $[^3H]$-$1,25(OH)_2D_3$ to a 3.5 S macromolecule was demonstrated in nuclear fractions of monocytes (maximal binding capacity, 48 fmol/mg protein; dissociation constant, 1.3×10^{-10} M) and of whole bone cell preparations. $1,25(OH)_2D_3$ receptors were not demonstrable in osteoclast preparations (70% pure; detection threshold, 2 fmol/mg protein). Data are consistent with indirect action of $1,25(OH)_2D_3$ on osteoclastic bone resorption.

Introduction

1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$)[1] is known to stimulate osteoclastic bone resorption in vivo (1) and bone cultures in vitro (2). Bone cells are known to have receptors for $1,25(OH)_2D_3$ (3) but thaw-mounted autoradiography failed to show $1,25(OH)_2D_3$-binding to osteoclasts (4). It has therefore been proposed that $1,25(OH)_2D_3$ acts on osteoclastic bone resorption by interaction with other bone cells, e.g., osteoblasts, (5) or by recruitment of osteoclast precursor cells (6). Cogent experiments have shown that peripheral monocytes may function as osteoclast precursors and can be induced to resorb bone in vitro (7, 8). Recently, a method has been described which permits osteoclast preparations of high yield and purity to be obtained (9, 10). Using this procedure we examined circulating monocytes, whole bone cell fractions, and isolated osteoclasts of calcium-deficient chicken in order to provide direct evidence for $1,25(OH)_2D_3$ receptor status of osteoclasts and their presumed precursor cells.

Methods

Chemicals. 1,25-dihydroxy-[26,27-*methyl*-^{3}H]cholecalciferol (158 Ci/mM) and 25-hydroxy-[23,24-^{3}H]cholecalciferol (85 Ci/mM) were obtained from Amersham Corp. (Arlington Heights, IL). Radiochemical purity was determined by high performance liquid chromatography, 93–98%, with no other D metabolites demonstrable. Radioinert, chromatographically pure 1α-25-dihydroxyvitamin D_3 (1α-$25(OH)_2D_3$), 25-hydroxyvitamin D_3 ($25(OH)D_3$), 1α-hydroxyvitamin D_3 (1α-$(OH)D_3$), and 24R,25-dihydroxyvitamin D_3 ($24R,25(OH)_2D_3$) were obtained from Duphar Co. (Amsterdam, the Netherlands). Bovine serum albumin (BSA) was [^{14}C]methylated (20 μCi/mg protein), ovalbumin was [^{14}C]methylated (20 μCi/mg protein), and γ-globulin was [^{14}C]methylated (20 Ci/mg protein) (all from New England Nuclear, Dreieich, Federal Republic of Germany). Hydroxyapatite, dithiothreitol (DTT), and Triton-X-100 were obtained from Sigma Chemical Co. (Munich, Federal Republic of Germany). Ficoll-Hypaque, colloidal polyvinylpyrolidone-coated silica, and Percoll were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Hank's balanced salt solution, free of $NaHCO_3$; minimal essential medium; 1 M Hepes buffer; 20 mM L-glutamine; penicillin/streptomycin, 10,000 U/10,000 μg per ml; RPMI 1640 with 2 g/liter $NaHCO_3$, glutamin free; fetal calf serum (FCS); phosphate-buffered saline (PBS); and bovine albumin were purchased from Seromed/Biochrom KG (Berlin, Federal Republic of Germany). Aprotinin (Trasylol) was obtained from Bayer Co. (Leverkusen, Federal Republic of Germany) and phenylmethylsulfonyl fluoride (PMSF) from Sigma Chemical Co.

Experimental protocol. 1-yr-old White Leghorn hens (2 kg) were kept on a commercial diet (3% Ca) and egg deposition was controlled for at least 15 d. Animals were kept in single cages and exposed to a regular day/night cycle (night, 9 p.m.–3 a.m.). Subsequently, hens were given a low calcium diet (0.17%) from Altromin Co. (Lage, Federal Republic of Germany). Egg deposition ceased on day 3 of the diet. On day 8, serum calcium was 2.4±0.12 (SD) mM/liter (n = 8) vs. 5.4±0.23 in controls. Animals were killed on day 8 of the low calcium diet.

Osteoclast preparation. Osteoclasts were isolated from medullary bone of the femura and tibiae immediately after exsanguination under sterile conditions using laminar flow. Bone fragments were first gently washed with 2–3 changes of ice-cold PBS and then scraped with sharpened needles. Cell suspensions were collected and allowed to sediment in PBS test tubes (5°C) for several minutes in order to eliminate bone debris. The suspension was then centrifuged at low speed (15 min, 3000 *g*). Cell pellets were resuspended in PBS. Osmotic shock (0.2% NaCl for 30 s followed by 1.6% NaCl for 30 s) was used to remove erythrocytes. Osteoclasts were then isolated by unit gravity sedimentation (5°C, sterile conditions, laminar flow). Test tubes were half filled with 50% FCS in PBS and overlayered with a cell suspension in PBS. After 45 min, the

Address reprint requests to Dr. Ritz, Department of Internal Medicine, University of Heidelberg, Bergheimer Strasse 58, D-6900 Heidelberg, Federal Republic of Germany.

Received for publication 11 June 1985 and in revised form 13 September 1985.

1. *Abbreviations used in this paper:* 1α-$25(OH)_2D_3$, 1α-25-dihydroxyvitamin D_3; 1α-$(OH)D_3$, 1α-hydroxyvitamin D_3, $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; $24R,25$-$(OH)_2D_3$, 24R,25-dihydroxyvitamin D_3; $25(OH)D_3$, 25-hydroxyvitamin D_3; DTT, dithiothreitol; FCS, fetal calf serum; N_{max}, maximal binding capacity; PMSF, phenylmethylsulfonyl fluoride; TED, hypertonic buffer.

J. Clin. Invest.

0021-9738/86/01/0312/03 $1.00
Volume 77, January 1986, 312–314

upper fraction was discarded, the underlying solution centrifuged, and the pellet resuspended in cold PBS with 1% BSA. This procedure was repeated four times. In the final preparation osteoclasts constituted ≥70% of all cells as evaluated by phase-contrast microscopy and acid phosphatase stain. Vitality control (trypan blue exclusion) showed >95% vital cells. Transmission electron micrographs showed reasonable preservation of subcellular structures.

Whole bone fractions were prepared by removing medullary bone and bone marrow mechanically and by flushing with cold PBS, respectively. Bone was subsequently homogenized with a Polytron homogenizer (Jahnke and Kunkel, Stauffen, Federal Republic of Germany) in cold PBS and the homogenate was used for binding studies.

Monocytes were isolated from heparinized chick whole blood by slow speed centrifugation (300 *g*, 10 min, 4°C) and subsequent centrifugation (800 g/20 min, 22°C) on Ficoll-Hypaque density gradient (*d* = 1.077) as described previously (11). Cells harvested from interphase were washed twice in Hank's balanced salt solution at 4°C. Cell viability (≥95%) was ascertained by trypan blue exclusion. 1×10^6 mononuclear cells/ml were incubated in plastic petri dishes with RPMI 1640 (37°C, 5% CO_2/95% air). Nonadherent lymphocytes were removed after 1 h by gently rinsing with fresh warm medium. After addition of ice-cold (4°C) RPMI 1640, adherent cells, i.e., a monocyte-enriched population (85% peroxydase positive), were harvested with a rubber policeman. Cell suspensions were washed thrice with RPMI without FCS or BSA. The final yield was 9×10^5 monocytes/animal.

Cytosolic preparation. The method described previously was used (11, 12); in brief, the material was homogenized in 4 vol (wt/vol) of KCl (0.3 M)-containing hypertonic buffer (TED): 10 mM Tris/HCl, 1.5 mM EDTA, 1 mM DTT, and 10 mM Na-molybdate, pH 7.4, at 4°C using a Polytron homogenizer. The homogenate was centrifuged at 5,000 *g* for 10 min at 4°C and subsequently at 105,000 *g* for 60 min at 4°C (Ultracentrifuge; Beckman Instruments, Inc., Fullerton, CA).

Nuclear preparation. The method described previously was used (12); in brief, the material was homogenized in 4 vol (wt/vol) of KCl-free buffer (TED) (10 mM Tris HCl, 1.5 mM EDTA, 1 mM DTT, 10 mM sodium molybdate, pH 7.4; 4°C) using a Polytron homogenizer in the absence or presence of aprotinin (100 KU/ml) and PMSF (10^{-5} M). Homogenate was centrifuged (5000 *g;* 10 min; 4°C) to yield the nuclear pellet. A crude chromatin fraction was prepared by resuspending the pellet three times in the above buffer with 0.5% Triton-X-100, pH 7.4, followed by centrifugation (10,000 *g;* 10 min). The resulting crude chromatin pellet was then extracted with 0.4 M KCl-TED for 45 min with frequent mixing. Chromatin extract was centrifuged (5,000 *g*/10 min) and resulting supernatant subsequently centrifuged at 100,000 *g* for 1 h. In one control experiment, to check receptor recovery in the osteoclast preparation, one measured aliquot of intestinal mucosa cells (1.2 mg protein/ml), prepared as described previously (12), was homogenized directly and another aliquot was first added to osteoclasts (0.6 mg protein/ml) and subsequently homogenized.

Sucrose density gradient analysis and hydroxylapatite assay were performed as described in detail previously (11). In brief, linear, 5–20% sucrose density gradient in TED buffer (4 ml), containing 0.4 M KCl, was made using a self-designed gradient former. Nuclear samples (0.2 ml) were incubated with 1 nM [^{3}H]1,25$(OH)_2D_3$ alone or in combination with 100-fold molar excess of 1,25$(OH)_2D_3$; 25$(OH)D_3$; 1α-$(OH)D_3$; and 24R,25$(OH)_2D_3$. The samples (1–3 mg protein) were then carefully layered on top of preequilibrated (2 h, 4°C) gradients and centrifuged (255,000 *g*, 21 h, 4°C, SW-60 rotor, Beckman Instruments, Inc.). 7-drop fractions were collected. Sedimentation rate for proteins was calculated using ^{14}C-labeled ovalbumin (3.7 S) or γ-globulin (7.3 S).

Saturation analysis according to Scatchard was carried out as described (12). Nuclear extracts (0.1 ml/0.3 mg protein) were incubated with varying concentrations (0.1–10 nM) of [^{3}H]1,25$(OH)_2D_3$ in the absence (total binding) or presence (nonspecific binding) of 100-fold molar excess of unlabeled 1,25$(OH)_2D_3$ for 16 h at 4°C. Bound [^{3}H]1,25$(OH)_2D_3$ was determined using the above hydroxylapatite assay.

DNA-cellulose affinity chromatography of receptor bound [^{3}H] 1,25$(OH)_2D_3$. DNA-cellulose (0.3 mg DNA/ml) was prepared according to Alberts and Herrick (13) using highly polymerized calf thymus DNA (type I, Sigma Chemical Co.) and CF-11 cellulose (Whatman Chemical Seperation, Inc., Clifton, NJ). Each column was equilibrated in TED for 16 h at 4°C before use and run at a flow rate of 5 ml/h per cm^2. Nuclear extracts (0.6 ml/1.8 mg protein) were incubated for 2 h at 4°C with [^{3}H] 1,25$(OH)_2D_3$ before chromatography on DNA-cellulose. Samples were loaded onto the columns, washed with 3 vol of TED, and eluted in 10 vol with a linear gradient from 0.1 to 0.6 M KCl in TED.

Results

Specific saturable reversible binding of [^{3}H]1,25$(OH)_2D_3$ to circulating monocytes and whole bone fraction, but not to isolated osteoclasts, was found in eight independent experiments. Fig. 1 gives a representative example. Binding of 1,25$(OH)_2D_3$ to a 3.5 S macromolecule was observed in the nuclear fractions of monocytes and whole bone homogenates and this was specific, since it was reversed by 100-fold molar excess of nonlabeled 1,25$(OH)_2D_3$, but not by a 100-fold molar excess of 25$(OH)D_3$, or (not shown) 1α-$(OH)D_3$ and 24R,25$(OH)_2D_3$. Scatchard analysis revealed a dissociation constant of 1.3×10^{-10} M with a maximal binding capacity (N_{max}) of 48 fmol/mg protein for circulating monocytes. For whole bone fractions, the respective values were a dissociation constant of 1.8×10^{-10} M and an N_{max} of 56 fmol/mg protein. No binding was found in any of the osteoclast preparations, although the sensitivity of the method (determined with concomitant assay of chick intestinal mucosa; reference 14) was ≥2 fmol/mg protein. The results were similar whether or not homogenization and subsequent steps were carried out in the presence of aprotinin (100 KU/ml) and PMSF (10^{-5} M). Recovery of intestinal mucosa cell receptor, when added to the osteoclast preparation before the homogenization steps, was 96%.

The findings in the nuclear fractions were paralleled by similar results in the cytosol fractions (N_{max} in monocytes, 17 fmol/mg protein; N_{max} in whole bone fraction, 18 fmol/mg protein; no binding in osteoclasts).

[^{3}H]1,25$(OH)_2D_3$ liganded with its receptor from nuclear fractions of monocytes or whole bone homogenates, bound to

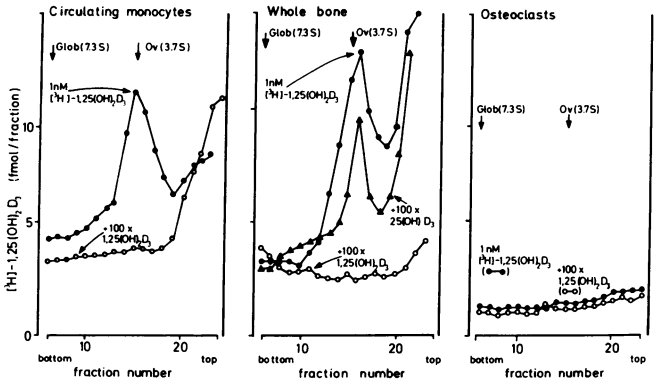

Figure 1. [^{3}H]1,25$(OH)_2D_3$ binding, analyzed by sucrose density gradient technique, in the nuclear fractions of circulating monocytes (*left*), whole bone fraction (*middle*), and isolated osteoclasts (*right*) of hypocalcemic chicken. Bovine gamma globulin and ovalbumin as markers at 7.3 or 3.7 S, respectively. The figure shows binding of [^{3}H]-1,25$(OH)_2D_3$ by a 3.5 S macromolecule in preparations of circulating monocytes in whole bone but not on osteoclasts. Binding is reversed by 100-fold molar excess of nonlabeled 1,25$(OH)_2D_3$, but not 100-fold molar excess of 25$(OH)D_3$.

DNA as shown by DNA-cellulose affinity chromatography. [^{3}H] $1,25(OH)_2D_3$ bound in the holo-receptor complex eluted as a sharp peak at 0.27 M KCl. The results (data not shown) confirmed interaction with DNA of receptor-bound [^{3}H] $1,25(OH)_2D_3$ in monocytes and bone fractions, but not in osteoclasts.

Discussion

In the present study, osteoclasts which were highly purified, vital, and almost intact morphologically were obtained from medullary bone of calcium-deficient chicken. It could be demonstrated that mature osteoclasts do not phenotypically express a $1,25(OH)_2D_3$ receptor. The sensitivity of the method used was adequate to detect $\geq$2 fmol $1,25(OH)_2D_3$ bound/mg protein. Proteolytic receptor degradation by osteoclasts during the preparation is unlikely in view of similarly negative findings in the presence of protease inhibitors and in view of almost 100% recovery of $1,25(OH)_2D_3$ receptors when cells of known receptor content were added to the osteoclast preparation. Absence of specific binding of [^{3}H]$1,25(OH)_2D_3$ by osteoclasts is in agreement with previous autoradiographic findings (4). The negative finding in osteoclasts contrasts with the ready demonstrability of $1,25(OH)_2D_3$ receptors on circulating monocytes and in whole bone fractions, i.e., a complex mixture of cells containing, amongst others, osteoblasts. The demonstration of receptors on circulating monocytes and osteoblasts in chicken is in agreement with previous similar findings in other species.

Demonstration of $1,25(OH)_2D_3$ receptors on monocytes, i.e., putative osteoclast precursors, but not on fully differentiated osteoclasts, may have implications with respect to actions of $1,25(OH)_2D_3$ on osteoclastic bone resorption. $1,25(OH)_2D_3$ is apparently devoid of direct functional action on osteoclasts (15). $1,25(OH)_2D_3$ generally acts as a differentiation signal for various cell lines, e.g., leukemic human promyelocytic leukemia cells (HL-60) (6) which are transformed by $1,25(OH)_2D_3$ into bone resorbing cells. Similarly, $1,25(OH)_2D_3$ receptors are present on basal cells of the epidermis (16) but not on terminally differentiated keratinocytes (16). $1,25(OH)_2D_3$ suppresses basal cell proliferation and induces differentiation markers (17). Similarly, the above osteoclast data are compatible with the notion that under appropriate conditions $1,25(OH)_2D_3$ is required as a differentiation signal for osteoclast precursor cells while differentiated osteoclasts no longer interact with $1,25(OH)_2D_3$ and lose the receptor.

Acknowledgments

The authors acknowledge the most generous help of A. Zambonin-Zallone (Department of Anatomy, University of Bari, Bari, Italy) in establishing the osteoclast isolation procedure.

References

1. Holtrop, M. E., K. A. Cox, M. B. Clark, M. F. Holick, and C. S. Anast. 1981. 1,25-dihydroxycholecalciferol stimulates osteoclasts in rat bones in the absence of parathyroid hormone. *Endocrinology.* 108:2293–2301.

2. Raisz, L. G., C. L. Trummel, M. F. Holick, and H. F. DeLuca. 1972. 1,25-dihydroxycholecalciferol: a potent stimulator of bone resorption in tissue culture. *Science (Wash. DC).* 175:768–769.

3. Kream, B. E., M. J. S. Yamada, and H. F. DeLuca. 1977. A specific highaffinity binding macromolecule for 1,25-dihydroxyvitamin D_3 in fetal bone. *Science (Wash. DC).* 197:1086–1088.

4. Stumpf, W. E., S. Madhabananda, and H. F. DeLuca. 1981. Sites of action of $1,25(OH)_2$ vitamin D_3 identified by thaw-mount autoradiography. *In* Hormonal Control of Calcium Metabolism. D. V. Cohn, R. V. Talmage, and J. L. Matthews, editors. Excerpta Medica, Amsterdam. 222–226.

5. Key, L. L., D. L. Carnes, R. Weichselbaum, and C. S. Anast. 1983. Calcitriol stimulates osteosarcoma cells to produce a factor which increases monocyte-dependent bone resorption. *Calcif. Tissue Int.* 35:696*a*. (Abstr.)

6. Bar-Shavit, Z., S. L. Teitelbaum, P. Reitsma, A. Hall, L. E. Pegg, J. Trial, and A. J. Kahn. 1983. Induction of monocytic differentiation and bone resorption by 1,25-dihydroxyvitamin D_3. *Proc. Natl. Acad. Sci. USA.* 80:5907–5911.

7. Tinkler, S. M. B., D. M. Williams, J. E. Linder, and N. W. Johnson. 1983. Kinetics of osteoclast formation: the significance of blood monocytes as osteoclast precursors during 1α-hydroxycholecalciferol-stimulated bone resorption in the mouse. *J. Anat.* 137:335–340.

8. Kahn, A. J., C. C. Stewart, and S. L. Teitelbaum. 1978. Contact mediated bone resorption by human monocytes in vitro. *Science (Wash. DC).* 199:988–990.

9. Zambonin-Zallone, A., A. Teti, and M. V. Primavera. 1982. Isolated osteoclasts in primary culture: first observations on structure and survival in culture media. *Anat. Embryol.* 165:405–413.

10. Zambonin-Zallone, A., A. Teti, M. V. Primavera, L. Naldini, and P. C. Marchisio. 1983. Osteoclasts and monocytes have similar cytoskeletal structures and adhesion properties in vitro. *J. Anat.* 137:57–70.

11. Merke, J., S. Senst, and E. Ritz. 1984. Demonstration and characterization of 1,25-dihydroxyvitamin D_3 receptors in human mononuclear blood cells. *Biochem. Biophys. Res. Commun.* 120:199–205.

12. Merke, J., U. Hügel, and E. Ritz. 1985. Nuclear testicular 1,25-dihydroxyvitamin D_3 receptors in Sertoli cells and seminiferous tubules of adult rodents. *Biochem. Biophys. Res. Commun.* 127:303–310.

13. Alberts, B., and G. Herrick. 1971. DNA cellulose chromatography. *Methods Enzymol.* 21:198–217.

14. Merke, J., W. Kreusser, B. Bier, and E. Ritz. 1983. Demonstration and characterization of a testicular receptor for 1,25-dihydroxycholecalciferol in the rat. *Eur. J. Biochem.* 130:303–308.

15. Chambers, T. J., and C. J. Dunn. 1983. Pharmacological control of osteoclastic motility. *Calcif. Tissue Int.* 35:566–570.

16. Merke, J., D. Schwittay, G. Fürstenberger, M. Gross, F. Marks, and E. Ritz. 1983. Demonstration and characterization of 1,25-dihydroxyvitamin D_3 receptors in basal cells of epidermis of neonatal and adult mice. *Calcif. Tissue Int.* 37:257–267.

17. Smith, E. L., and M. F. Holick. 1985. 1,25-dihydroxyvitamin D_3 stimulates differentiation and 24-hydroxylase activity in cultured human keratinocytes. *Sixth Workshop on Vitamin D, Merano.* (Abstr.)