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DEMONSTRATION AND CHARACTERIZATION OF 1,25-DIHYDROXY-VITAMIN D_3 RECEPTORS IN HUMAN MONONUCLEAR BLOOD CELLS

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Summary: Circulating human mononuclear blood cells were studied for the presence of specific 1,25-dihydroxyvitamin D_3 (calcitriol) binding macromolecules. Cells were isolated by density gradient centrifugation and characterized by surface markers. Specific reversible high affinity binding by a 3.5 S macromolecule was demonstrated in malignant B-cells and circulating monocytes. In monocytes specific calcitriol binding was found both in the presence and absence of vitamin D_3 to saturate the vitamin D_3 binding serum protein. No specific calcitriol binding was found in resting B or T lymphocytes. The data suggest a role of calcitriol in the control of mononuclear blood cell proliferation/differentiation.

Introduction: In recent years, 1,25-dihydroxyvitamin D_3 (calcitriol) receptors have been demonstrated not only in classical target organs of vitamin D, but also in a variety of endocrine organs (1,2,3) and tumor cell lines (4,5,6).

It was the purpose of the present study to examine several human blood cell types for the presence of calcitriol receptors. The data clearly demonstrates specific high affinity calcitriol binding macromolecules in malignant proliferating B-cells and in circulating monocytes.

Material and Methods:

Chemicals: 1,25-dihydroxy [26,27-methyl-^{3}H] cholecalciferol (158 Ci/mmol), 25-hydroxy [23,24-^{3}H] cholecalciferol (85 Ci/mmol) were obtained from Amersham/Buchler Co. (Braunschweig). Radiochemical purity by HPLC 93-98% with no other D metabolites demonstrable. Radioinert chromatographically pure calcitriol (1,25-dihydroxy-vitamin D_3), calcidiol (25-OH-vitamin D_3) and calciol (vitamin D_3) from Duphar Co. (Amsterdam). Bovine serum albumin [^{14}C] methylated (20 µCi/mg protein); ovalbumin [^{14}C] methylated (20 µCi/mg protein), y-globulin [^{14}C] methylated (20 Ci/mg protein). Hydroxyapatite, dithiothreitol, Triton X-100 from Sigma Co., Ficoll-Paque^R, colloidal PVP (polyvinylprolidone) coated silica Percoll^R by Pharmacia Fine Chemicals AB (Uppsala), Hank's solution free of $NaHCO_3$; MEM, 1 M Hepes buffer, 200 mM L-glutamine, penicillin/streptomycin 10,000 U/10,000 µg per ml, RPMI-Medium 1640 with 2 g/L $NaHCO_3$ glutamin free, FCS by Seromed/Biochrom KG, Berlin.

Assays: Lymphocyte preparation:

Human leucocytes were obtained from platelet donors (IBM 2997 Blood cell separator). After platelet removal (thrombocytapheresis), the buffy coat was suspended in Hank's solution (HBSS). Further purification by slow speed centrifugation (300 g, 10 min, 4°C) and subsequent centrifugation (800 g, 20 min, 22°C) on Ficoll - Paque^R density gradient (density 1.077)

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(7). Cells harvested from interface were washed twice in HBSS at 4°C. Cell viability (95%) ascertained by trypan blue exclusion (0.01% final concentration). Mononuclear cell population consisted of 55-75% T cells (E rosetting), 10-15% B cells (surface immunoglobulin) and 10-20% monocytes (esterase-positive) (8).

Separation of human blood monocytes from leucocytes: $3x10^6$ mononuclear cells/ml were incubated in plastic Petri dishes with RPMI-Medium 1640 (37°C, 5% CO_2/95% air). Non-adherent lymphocytes were removed after 12h by gently rinsing with fresh warm medium. After addition of ice-cold (4°C) RPMI-Medium 1640, adherent cells, i.e. a monocyte enrichted population (75-85% peroxidase-positive; ref. 9) were harvested with a rubber policeman (10).

Preparation of T and B lymphocytes: Lymphocytes were prepared from citrated blood using Ficoll-Hypaque^R density gradient (11). One volume of lymphocytes 5-10 x 10^6/ml) was mixed with two volumes of 1% SRBC (sheep red blood cells) previously treated with neuraminidase and one volume of heat inactivated absorbed FCS (12). The mixture was incubated for 1h at 4°C for rosette formation and subsequently layered on Ficoll-Hypaque^R and centrifuged at 800 x g for 20 min. T-lymphocytes were enriched in the pellet. RBCL-buffer (red blood cell lysing buffer) was added to the pellet to lyse SRBC. A cell fraction containing 90-97% T cells was recovered after twice washing with 0.9% NaCl (viability 100% with trypan blue exclusion).

Cytosol preparation of mononuclear cells: Adapting the method described before (1) freshly prepared enriched whole lymphocytes, purified T and B cell lymphocyte or monocyte preparations were sonicated (15-s bursts, Sonofier cell disruptor). After suspension of cells in four volumes (w/v) of KCl containing hypertonic buffer were sonicated or centrifuged at 5000 x g for 10 min at 4°C to yield a crude supernatant fraction. The buffer (KTEDMo) contained: 0.4 M KCl/HCl, 1.5 mM EDTA, 1 mM dithiothreitol, 10 mM Na-molybdate, pH 7.4, 4°C). A purified cytosol fraction was prepared by centrifugation at 105,000 x g for 60 min at 4°C (Beckman Instruments Ultracentrifuge). The cytosol was meticulously kept at 4°C throughout. Cytosol protein after Lowry (13).

Sucrose density gradient analysis: Linear 5-20% sucrose density gradients in KTEDMo-buffer (4 ml) were made by using a self designed gradient former (1). Cytosol samples (0.2 ml) were carefully layered on top of pre-equilibrated (2 h, 4°C) gradients and centrifuged (255,000 x g, 21 h, 4°C, SW-60 rotor Beckman Instruments Co.). 7-drop fractions were collected. The sedimentation rate (in Svedberg units, S) for proteins was calculated using [^{14}C]labeled ovalbumin (3.7 S) or gamma-globulin (7.3 S).

Results

Calcitriol binding in cytosol of human whole lymphocyte and B or T lymphocyte-enrichted preparations

When cytosolic fractions (0.2 ml/10^8 cells/ 1.9 mg protein) of whole lymphocyte-enriched preparation (approx. 85% B + T lymphocytes) were incubated with [^{3}H] calcitriol and subsequently fractioned on sucrose density gradients, neither specific binding for [^{3}H] calcitriol nor a typical tritium peak at 3.5 S as evidence of the calcitriol receptor complex could be demonstrated. Similarly negative findings were obtained with the cytosolic fractions of the enriched T-cell population or B-cell population. Negative results were confirmed in 8 independent experiments.

In addition, mononuclear cells of leucemic patients (CML, n=2; prelymphocytic leukemia, n=1) and Burkitt lymphoma with terminal B-cell leukemia (n=1) were

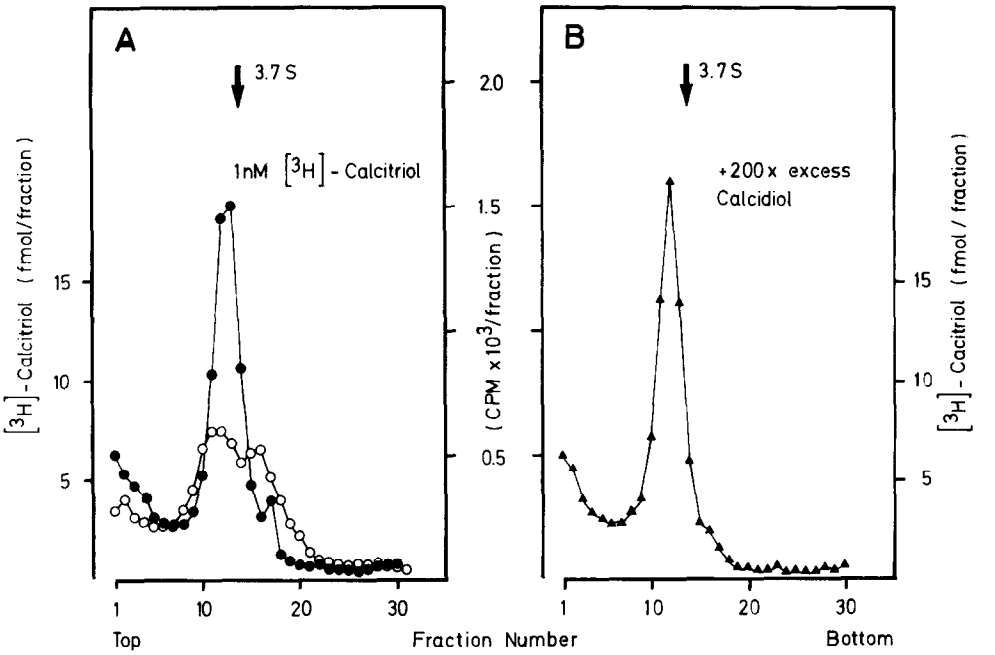

fig. 1 : Sucrose density gradient analysis of [^{3}H] calcitriol in the cytosolic fraction ($1x10^7$ cells/0.2 ml/0.3 mg protein) of an enriched lymphocyte preparation of a patient with leukemic Burkitt lymphoma.
Panel 1A. Incubation of 1 nM [^{3}H] calcitriol in the absence and presence of 200-fold molar excess of radioinert calcitriol; arrow= [^{14}C] ovalbumin at 3.7 S as marker.
Panel 1B. 1 nM [^{3}H] calcitriol in the presence of 200-fold molar excess of radioinert calcidiol.

examined. With the exception of Burkitt leukemia (see fig. 1) specific binding for calcitriol was not demonstrable.

Calcitriol binding in cytosol of normal human monocytes:

When the cytosolic fraction (0.2 ml/$2x10^7$ cells/1.2 mg protein) was incubated with [^{3}H] calcitriol and subsequently fractioned on sucrose density gradient centrifugation, [^{3}H] calcitriol was bound in the 3.5 S region as shown in fig. 2a, pointing to the presence of a typical calcitriol receptor binding macromolecule. Since previous authors (14) had problems with the determination of calcitriol binding resulting from the presence of plasma derived vitamin D binding protein, [^{3}H] calcitriol incubations were performed in the absence of up to 50,000 fold molar excess of calciol. Calciol caused a slight increase of tritium binding in the 3.5 S region, but no displacement of [^{3}H] calcitriol, i.e.no competition. Fig. 2b shows [^{3}H] calcitriol binding in the presence of a 20,000 fold molar excess of calciol. Specific [^{3}H] calcitriol binding in the absence or presence of calciol excess by normal human monocytes was confirmed in 3 independent experiments.
Scatchard plots yielded a K_D of 10^{-10}M. Binding capacity was 40 fmol/mg protein. Low binding capacity is further illustrated by fig. 2C, where as little

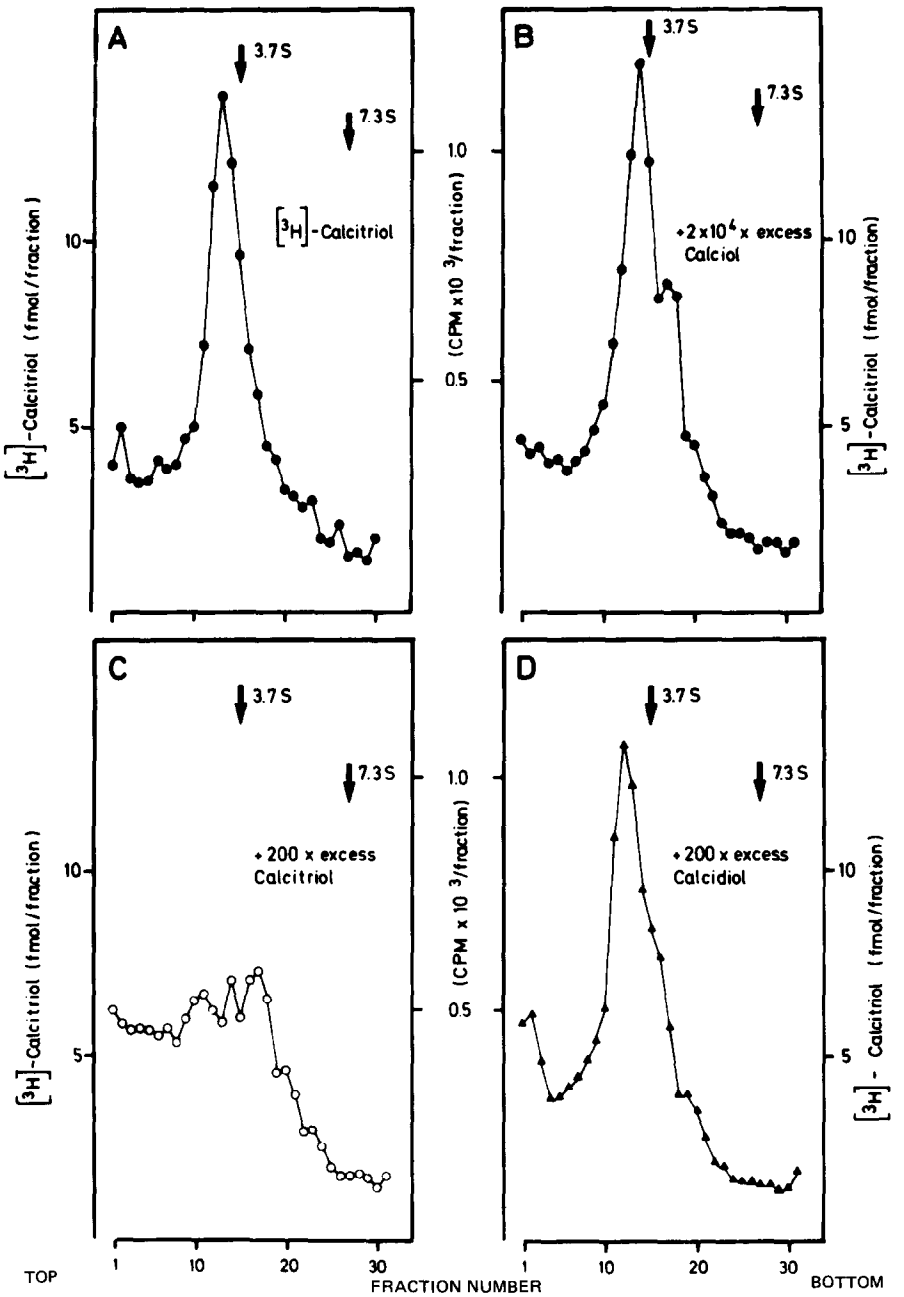

fig. 2 : Sucrose density gradient analysis of [^{3}H] calcitriol in the cytosolic fraction of normal human monocytes

[^{3}H] calcitriol binding to a 3.5 S binding macromolecule, reversible with addition of 200 x molar excess of calcitriol (1 C), but not affected by excess radioinert calciol (1 B) or calcidiol (1 D), respectively.

1 A. 1 nM [^{3}H] calcitriol without additions; arrow indicates [^{14}C] ovalbumin and [^{14}C]γ-globulin at 3.7 S and 7.3 S respectively.

1 B. 1 nM [^{3}H] calcitriol in the presence of 20,000 fold molar excess of radioinert calciol.

1 C. 1 nM [^{3}H] calcitriol in the presence of both 20,000 fold molar excess of radioinert calciol and 200-fold molar excess of radioinert calcitriol.

as 200-fold molar excess of radioinert calcitriol was able to totally displace the tritium peak at 3.5 S. High specificity of the calcitriol receptor is illustrated by the failure of 200-fold molar excess of calcidiol to affect [^{3}H] calcitriol binding.

Discussion: This study demonstrates the presence of specific calcitriol binding macromolecules in B-lymphocytes with malignant transformation (Burkitt lymphoma) and normal circulating human monocytes. This conclusion is in agreement with two communications which appeared while this study was in progress (14,15). No specific calcitriol binding could be demonstrated in resting whole lymphocyte preparations. T- and B-lymphocyte preparations were assessed separately and yielded equally negative results. Simultaneous independent controls were always done with chick intestinal mucosa (16) which uniformly showed calcitriol receptors thus validating the method used. Consequently, within the sensitivity of the method used (5 fmol/mg protein) resting lymphocytes do not phenotypically express calcitriol receptors.
In contrast, in malignantly transformed B-cells and normal human monocytes calcitriol binding was demonstrated which was specific, i.e. reversible with addition of radioinert calcitriol, selective, i.e. not affected by competition with other vitamin D metabolites, and of limited capacity, i.e. exhibiting finite capacity in the Scatchard plot and almost complete displacement with 200-fold molar excess of radioinert calcitriol.
The finding of specific calcitriol binding in circulating B-lymphocytes of a Burkitt lymphoma patient despite no demonstrable binding in resting lymphocytes argues for a role of calcitriol in the control of proliferation and differentiation of human B-cells, as recently postulated by others (17,18).

In past studies (14) serum vitamin D binding protein proved to be a problem when whole cells were used for calcitriol receptor studies. Our results of no major effect of 50,000 rsp. 20,000 molar excess of calciol in a cytosolic preparation demonstrates that this system is less susceptible to interference by contaminating serum vitamin D binding protein, suggesting that this preparation is more advantageous in this respect than whole cell preparations.

Several previous findings established an action of vitamin D_3 (metabolites) on macrophage/monocyte cells. Inflammatory and phagocytic functions of macrophages of vitamin D-deficient mice were defective but fully corrected by calcitriol repletion (19). Furthermore, calcitriol induced maturation of murine leukemia M 1 cell lines into macrophages (18). Similar maturation was found for the mononuclear cell line U 937 (20). In the HL 60 promyelocytic leukemia cell line, calcitriol reversibly modulated expression of c-myc oncogen

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1 D. 1 nM [^{3}H] calcitriol in the presence of 20,000-fold molar excess of radioinert calciol and 200-fold molar excess of radioinert calcidiol.

and induced expression of monocyte-like features (21,22). The present obsercation of calcitriol receptors in circulating monocytes demonstrates that monocytes, or a monocyte subpopulation, are target cells for calcitriol. This observation is of note because of the recently recognized role of macrophages as osteoclast precursors.
Demonstration of calcitriol receptors in human mononuclear blood cells would provide an easily accessible tool for in vivo analysis of calcitriol receptor status.

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