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PARTIAL CHARACTERIZATION OF A SPECIFIC HIGH AFFINITY BINDING MACROMOLECULE FOR 24R,25 DIHYDROXYVITAMIN D_3 IN DIFFERENTIATING SKELETAL MESENCHYME

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Cytosol preparations and cells from 6-day old cultured differentiating chick limb-bud mesenchyme, which consist of a high proportion of chondrocytes, were shown to specifically bind 24R,25 dihydroxycholecalciferol. Nuclei from identical cultures also showed specific binding for 24R,25 dihydroxycholecalciferol. On the contrary, similar preparations of limb-bud mesenchyme cells (6-day old cultures) pretreated on day one by 5-bromodesoxyuridine which induced a fibroblast phenotypic expression, failed to show any specific binding for either 24R,25 or 1α,25 dihydroxycholecalciferol. Pronase treatment of the cytosol indicated that the receptor was protein-like in nature. The chromatographic properties of the protein-receptor on diethylaminoethyl cellulose and Sephadex G-100 columns were similar to those of the protein receptor found for 1α,25 dihydroxycholecalciferol. This report is the first demonstration that a cytosol protein receptor for 24R,25 dihydroxycholecalciferol exists in developing skeletal tissue. 24,25 dihydroxyvitamin D_3 but not any of the other metabolites was shown to induce DNA synthesis after 24 h by almost two-fold and protein synthesis after 5 h by 240%. These results suggest an important physiological role for 24R,25 dihydroxyvitamin D_3 in the development of skeletal tissue.

INTRODUCTION

1α,25-dihydroxyvitamin D_3($1,25(OH)_2D_3$) is active in the control of calcium homeostasis and metabolism in the intestine (1), kidney (2) and bone (3). Specific cytoplasmic receptors for this metabolite exist in those organs (2, 5-8) and in the pituitary (9).

Recently it was shown that 24R,25 dihydroxyvitamin D_3 ($24,25(OH)_2D_3$, another vitamin D metabolite which has little effect on calcium metabolism,

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in conjunction with 1,25 $(OH)_2D_3$ plays a significant role in bone formation in embryonic cartilage (10), in regenerating endochondral bone (12-14) and in chondrocyte cultures (15). We therefore believed that embryonic osteogenic tissue could be a target for 24,25$(OH)_2D_3$ although no cytoplasmic receptor has been demonstrated for this metabolite. However, specific nuclear uptake of 24,25 $(OH)_2D_3$ was observed in cartilage cells (15). In vitro micro-mass cultures of embryonic limb-bud mesenchyme cells can undergo chondrogenesis within four days, and their differentiation and calcification may be regulated by exogenous manipulation of the growth medium (16,17). Using the above model system for chondrogenesis, this communication demonstrates the presence of specific proteinaceous cytosol receptors for 24,25$(OH)_2D_3$ in these cells.

MATERIALS AND METHODS

Micro-mass cultures of embryonic chick limb-bud mesenchymal cells were prepared as described previously (17). On the 6th day of culture 1.2 nM{^{3}H}24R,25$(OH)_2D_3$ was added to the culture with or without 100-fold excess of either 25$(OH)D_3$ or 1,25$(OH)_2D_3$ or 24,25$(OH)_2D_3$, and incubated for 10 minutes at 37°C. The medium was removed and cells were harvested by scraping in cold homogenizing buffer (0.25M Sucrose, 0.01M Tris-HCl, 0.005M dithiothreitol, 0.005M EGTA, 0.003M $MgCl_2$, pH 7.4) and sonicated for 10 sec. Nuclei were isolated by centrifugation at 800 x g and washed twice. Nuclear integrity was examined by microscopy and protein to DNA ratio which was 1.7. Cytosol was prepared by centrifugation of the supernatant for 10 min at 39,000 x g. Cytosol was also prepared from cells not subjected to prior incubation, and incubated with 1.2nM{^{3}H}24,25$(OH)_2D_3$ with or without 100-fold excess of the metabolites for 90' at 0°C. Cytosol was also prepared from cultures treated on day one with BrdU (5-bromo-2'-desoxyuridine) which convert the cells to fibroblasts (17,26).

Specific binding of vitamin D metabolites in the cytosol was determined by a modified charcoal dextran method (18). Protein was determined by the Lowry method (19). Nuclear pellet was dissolved in 0.3N NaOH and radioactivity and DNA content (20) were determined.

Cytosol was also prepared from confluent cultures of bone cells that were prepared as described previously (21), and assayed for binding activity. Ion exchange chromatography on DEAE cellulose (22) was performed on 0.5ml cytosol preparation, using a 5ml syringe as a column, previously equiliberated with sucrose-free homogenizing buffer. A gradient of 0 - 0.6M KCl in the same buffer was used for elution. 1.4 ml fractions were collected and radioactivity, protein content (23) and salt content (conductivity) were determined. Gel filtration of 0.8 ml cytosol preparation was performed on standardized Sephadex G-100 column equilibrated with sucrose-free homogenizing buffer containing 0.005M KCl (22). Molecular weight was determined by the method of Gelotte (24).

TABLE 1

SPECIFICITY OF {^{3}H}24R,25(OH)$_2$D$_3$ NUCLEAR AND CYTOSOL UPTAKE

Metabolites[a]	Quantities of {^{3}H}24R,25 Dihydroxyvitamin D$_3$[c]			
	1[b] nuclei	2 cytosol	3 cytosol	4 cytosol
I	0.25 ± 0.026(3)	1012.3 ± 105.8(3)	686.6 ± 68.0(8)	168.2
I + II	0.24 ± 0.035(3)	952.3 ± 137.8(3)	642.4 ± 9.3(3)	168.5
I + III	0.26 ± 0.015(3)	1179.3 ± 68.5(3)	686.9 ± 13.3(3)	161.5
I + IV	0.10 ± 0.023(3)	148.3 ± 34.4(3)	111.6 ± 25.9(8)	199.7

a. I = 1.2nM {^{3}H}24R,25-dihydroxycholecalciferol;
II = 120nM 25-hydroxycholecalciferol;
III = 120nM 1α,25-dihydroxycholecalciferol;
IV = 120nM 24R,25-dihydroxycholecalciferol

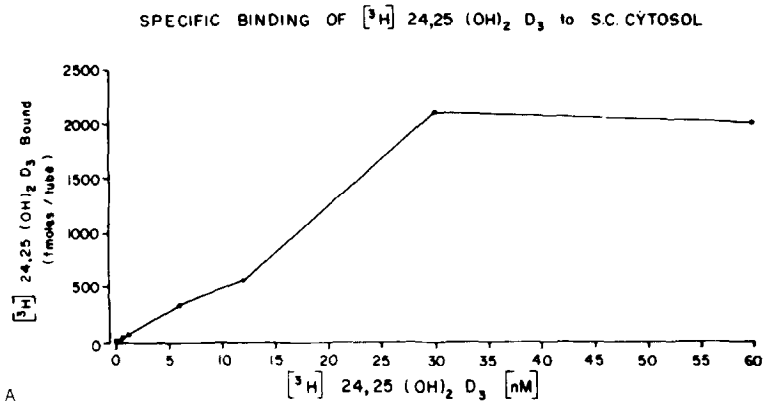
b. Column 1 = nuclei from incubation of chondrocytes and metabolite, {^{3}H}fmols/μgDNA Column 2 = cytosol from incubation of chondrocytes and metabolite, {^{3}H}fmols/mg protein; Column 3 = cytosol prepared from chondrocytes and then incubated with metabolite, {^{3}H}fmols/mg protein; Column 4 = cytosol prepared from bone cells (21) and then incubated with metabolite, {^{3}H}fmols/mg protein.

c. Average of duplicate analyses, where no statistical analysis is indicated. Number of experiments in parentheses.

RESULTS

Table 1 demonstrates that cytosol (column 2) and nuclear (column 1) preparations, from intact chondrocytes after incubation with {^{3}H}24,25(OH)$_2$D$_3$, binds specifically this ligand, and the binding can be competed only by 100-fold excess concentration of non radioactive 24,25(OH)$_2$D$_3$ and not by 25(OH)D$_3$ or 1,25(OH)$_2$D$_3$. In addition, cytosol preparations from intact cells with no prior incubation with any metabolite shows similar results (Table 1, column 3), namely only 24,25(OH)$_2$D$_3$is an effective competitor. In contrast, cytosol prepared from cultured rat bone cells (Table 1, column 4), does not demonstrate any specific binding of 24,25(OH)$_2$D$_3$. Identical experiments using {^{3}H}1, 25(OH)$_2$D$_3$indicated no specific cytoplasmic or nuclear binding of this metabolite in chondrogenic cells, but specific binding to intestinal mucosa (26).

Cytosol prepared from chondrogenic cells incubated with increasing concentrations of 24,25(OH)$_2$D$_3$ showed saturable binding reaching a plateau



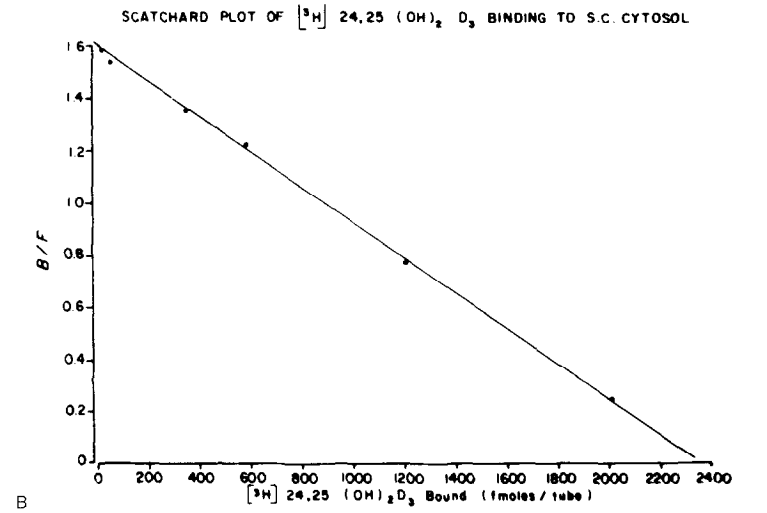


Figure 1:

A. Scatchard analysis (28) of {^{3}H}24,25$(OH)_2D_3$-binding in cytosol from limb-bud mesenchyme cells. Cells from sixth day micro-mass cultures were washed and disrupted by sonication for 10 sec. Cytosols 39,000g supernates were incubated with varying concentrations of {^{3}H}24,25$(OH)_2D_3$ at 0°C for 90 min. Bound and free steroid were separated by a charcoal-dextran adsorption method. To obtain specific bound counts, nonspecific binding performed in the presence of 100-fold excess of non-radioactive 24,25$(OH)_2D_3$ was subtracted from the total binding.

B. Scatchard plot analysis of the above data.

at 30nM (Fig. 1A). Scatchard plot analysis (Fig. 1B) yielded a straight line indicating a single class of noninteracting binding sites, with an apparent Kd of 0.21×10^{-9}M and Nmax of 2.35 fmol/μg cytosol protein.

Chondrocyte proliferation constitutes one of the most important phases in growth plate cartilage metabolism and differentiation. In this study

12nM of $24,25(OH)_2D_3$ but no other metabolites induced 2.05-fold increase in {^{3}H} thymidine incorporation into DNA after 24 h of treatment and 2.4-fold increase in {^{3}H} leucine incorporation into total protein after 5 h of treatment.

Treatment with BrdU which changes the chondrogenic phenotypic expression of limb-bud mesenchyme cells (17,27) into fibroblast-like cells caused loss of the cytoplasmic receptor sites for $24,25\text{-}(OH)_2D_3$.

Pronase treatment of the cytosol leads to abolishment of receptor activity indicating that the receptor is a protein.

DEAE-cellulose chromatography of cytosol prepared from cells after incubation with {^{3}H}$24,25(OH)_2D_3$ (Fig. 2A), or from untreated cells followed by incubation with the hormone (Fig. 2B), indicated a single radioactive peak eluting at ∿0.2M KCl. Sephadex G-100 gel filtration of the labelled cytosol gave a single radioactive peak which had a molecular weight of 110,000 daltons (Fig. 2C). Standard HPLC analysis of the chloroform-methanol extract of the peak from the DEAE cellulose yielded solely $24,25(OH)_2D_3$.

DISCUSSION

Recently it has been shown that $24,25(OH)_2D_3$ itself is physiologically active in cartilage (11,16) and, together with $1,25(OH)_2D_3$ enhances embryonic bone formation (14) and fracture healing of endochondral bone (13). Nuclei from chondrocytes were reported to bind specifically $24,25(OH)_2D_3$ (15), which is confirmed by us. Parathyroid gland of rachitic chicks was also shown to contain both nuclear and cytoplasmic receptor for $24,25(OH)_2D_3$ (25). It was shown (1) that $1,25(OH)_2D_3$, like other steroid hormones, acts via binding to specific receptors. This report presents data clearly documenting proteinaceous cytoplasmic receptor specific for $24,25(OH)_2D_3$ in cartilage cells. This receptor

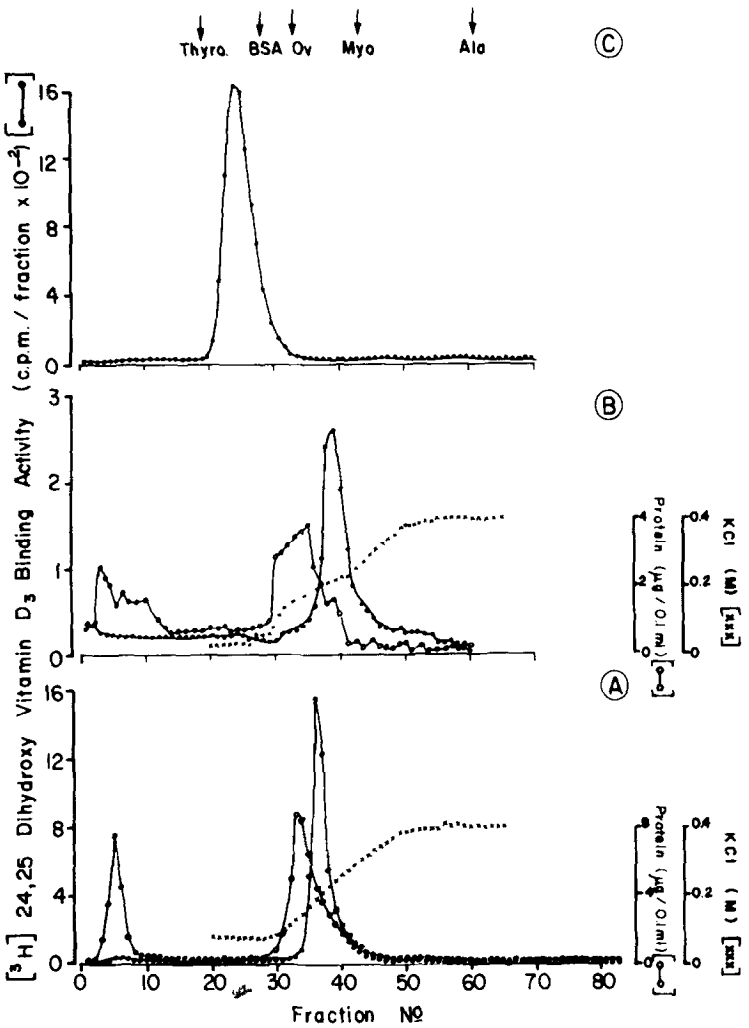


Figure 2:

A. DEAE cellulose profile of cytosol prepared from cells that had been incubated with {^{3}H}24,25$(OH)_2D_3$.
B. Chromatography of cytosol on DEAE cellulose that had been prepared from cells and then incubated with {^{3}H}24,25$(OH)_2D_3$.
C. Gel filtration of material from figure 1B on Sephadex G-100. Elution volumes of marker protein indicated by arrow. ●—● {^{3}H} CPM o—o protein xxxxx - gradient. See methods and materials for experimental details.

is ligand and cell specific. However, we have also demonstrated (26) that intestinal mucosa and bone cells contain specific receptors for 1,25$(OH)_2D_3$. It is interesting to note that the chromatographic properties (Fig. 2) of the cytoplasmic receptor for 24,25$(OH)_2D_3$ reported here are similar to those of the receptor for 1,25$(OH)_2D_3$ (9), and to those for 24,25$(OH)_2D_3$ in the chick parathyroid gland (25). We

present here evidence for the physiological role of $24,25(OH)_2D_3$ in bone metabolism in addition to $1,25(OH)_2D_3$. Chondrocyte proliferation is an important prequisite for skeletal growth and differentiation. Recently, it was shown that $24,25(OH)_2D_3$ induced both cytoplasmic and nuclear DNA polymerase in cultured rabbit growth plate chondrocytes (15). We also observed increased protein and DNA synthesis induced by this metabolite, suggesting its important role in bone growth.

Since we find that developing chondrocytes contain specific cytoplasmic receptors for $24,25(OH)_2D_3$, we suggest that this metabolite is important and necessary for the physiological transformation of embryonic mesenchymal cells into cartilage and bone.

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