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THE INFLUENCE OF ALBUMIN ON VITAMIN D METABOLISM IN FETAL CHICK OSTEOBLAST-LIKE CELLS

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SUMMARY Incubation of osteoblast-like cells with [3H]25-(OH)D3 and varying bovine serum albumin (BSA) concentrations resulted in a dramatic change in the accumulation of 1,25-(OH)2D3 and 24,25-(OH)2D3 in the medium. At 0.1% BSA 1,25-(OH)2D3 formation was transient and 24,25-(OH)2D3 was the main product after 3 h. At 2% BSA accumulation of 1,25-(OH)2D3 was sustained whereas 24,25-(OH)2D3 formation was suppressed. At low BSA levels added [3H]1,25-(OH)2D3 was rapidly metabolized to 1,24,25-(OH)3D3 and more polar metabolites. The effect of increasing BSA concentrations on 25-(OH)D3 metabolism was mimicked by addition of cycloheximide. This indicates that high BSA levels prevent the induction of 24-hydroxylase activity in this system, probably by lowering of the free 25-(OH)D3 concentration. The accumulation of 1,25-(OH)2D3 from 25-(OH)D3 not only depends on the 1α-hydroxylase activity, but also on the further metabolism of 1,25-(OH)2D3 by 24-hydroxylation.

The synthesis of 1,25-$(OH)_2D_3$ from 25-$(OH)D_3$ has long been considered to be confined to the kidney (1). More recent studies, however, showed evidence that cells from a number of other tissues also have the ability to convert 25-$(OH)D_3$ to 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$. This has been observed in fetal chicken calvarial cells (2,3), chicken and rabbit cartilage (4), human bone cells and human osteogenic sarcoma (5), chick chorioallantoic membrane (6) and rat placenta (7). That extrarenal 1,25-$(OH)_2D_3$ production occurs in humans is supported by the observation of low but significant levels of 1,25-$(OH)_2D_3$ in anephric patients (8).

Since knowledge about the production of vitamin D_3 metabolites by bone cells and its regulation may be crucial for understanding the effect of vitamin D on the skeleton, we studied the conversion of vitamin D metabolites in fetal chicken osteoblast-like cells (OB-cells). In this paper we report that primary cultures of OB-cells are capable to convert 25-$(OH)D_3$ to more polar metabolites,

Abbreviations employed are: 25-hydroxy-vitamin D3 (25-(OH)D3); 1,25-dihydroxy-vitamin D3 (1,25-(OH)2D3; 24,25-dihydroxy-vitamin D3 (24,25-(OH)2D3); 1,24,25-trihydroxy-vitamin D3 (1,24,25-(OH)3D3).

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including $1,25\text{-(OH)}_2D_3$ and $24,25\text{-(OH)}_2D_3$, and that addition of increasing concentrations of bovine serum albumin (BSA) or of cycloheximide profoundly change $25\text{-(OH)}D_3$ metabolism.

MATERIALS AND METHODS [26,27-3H] 25-(OH)D3 (19.2 Ci/mmol), [23,24-3H] 1,25-(OH)2D3 (85 Ci/mmol) and [23,24-3H]24,25-(OH)2D3 (64 Ci/mmol) were obtained from Amersham. Standards of 25-(OH)D3, 1,25-(OH)2D3 and 24,25-(OH)2D3 were donated by Hoffmann-La Roche (Mijdrecht, The Netherlands) and Duphar (Weesp, The Netherlands). 1,24,25-(OH)3D3 was a generous gift of Dr. R. Bouillon (Catholic University, Louvain, Belgium), The following materials were from Sigma: BSA (fraction V), collagenase type I, α-tosyl-lysyl-chloromethane and HEPES. Eagle's MEM, embryonic extract (EE), penicilline and streptomycine sulphate were obtained from Flow Laboratories. Fetal calf serum (FCS) and the culture dishes (35 mm) were purchased from Greiner. The other reagents were of analytical grade.

Cell culture and incubation procedures. Osteoblast-like cells (OB-cells) were isolated from 18 day-old chick embryo calvaria with collagenase (2 mg/ml isolation medium (9)) as previously described (10). The isolated cells were washed with 15% (v/v) FCS in Hanks-HEPES solution, centrifuged, and then suspended in 10 ml cultivation fluid consisting of Eagle's MEM (buffered with Earle's BSS, pH 7.4), 10% FCS, 5% EE, 0.1 mg/ml glutamine, 0.05 mg/ml ascorbic acid, 100 units/ml penicilline and 100 μg/ml streptomycine. The cells were then seeded in petri dishes and cultured in a moist atmosphere of 5% CO2 in air (37 C) until the cultures reached confluency (5-6 days). Media were changed every two days with 1 ml of the above-mentioned medium, without EE. At least 16 h prior to incubation with substrate the growth medium was replaced by Eagle's MEM (Ca++ 1,25 mM) containing no FCS, but BSA in concentrations ranging from 0.1 to 2%. The reaction was started by the addition of the radioactive vitamin D metabolites dissolved in ethanol (final concentration <0.1%) as indicated in the legends of the figures. The incubations (0.5-3 h) were carried out with the culture dishes placed on a slightly angled, slowly rotating plate, which enhanced the rate of metabolism considerably. At the end of the incubations the medium was removed and extracted with diethyl ether. The lipid extract was subjected to high pressure liquid chromatography (HPLC) on a 0.46 x 25 cm CPtmSpher Silica column (Chrompack) using hexane-isopropanol (91 : 9) as the solvent at a flow rate of 1.8 ml/min or on a 0.46 x 25 cm Zorbax CN column (Du Pont) using hexane-isopropanol-methanol (94 : 5: 1) as the solvent at a flow rate of 1.3 ml/min. The radioactive vitamin D metabolites were identified by their retention time after calibration with standard 25-(OH)D3, 24,25-(OH)2D3, 1,25-(OH)2D3 and 1,24,25-(OH)3D3. Aqueous periodate treatment of putative 1,25-(OH)2D3 and 24,25-(OH)2D3 was performed according to Tanaka et al. (11). At 0.1-2% BSA the radioactivity recovered from the incubation medium varied from 60-90% for [3H]25-(OH)D3 and from 75-90% for [3H]1,25-(OH)2D3, respectively. The DNA content of the cultures was determined according to the method of Johnson-Wint et al. (12). Because of the narrow range of the DNA content per dish among different cultures (15 $\pm$ 2.3 μg) the results were expressed as pmol per sample or, where indicated, as the percentage radioactivity recovered.

Binding of vitamin D3 metabolites at different BSA concentrations. Tritiated 25-(OH)D3, 24,25-(OH)2D3 or 1,25-(OH)2D3 (0.5-10 nM) were incubated for 1 h at 37C with different BSA concentrations (0.1-4%) in serum-free medium. At the end of the incubations protein-bound and free vitamin D were separated by charcoal-dextran adsorption (13), and the bound fraction was counted for radioactivity.

RESULTS

Identification of $1,25\text{-(OH)}_2D_3$ and $24,25\text{-(OH)}_2D_3$ formed by the OB-cells. We first examined the ability of primary cultures of OB-cells to metabolize

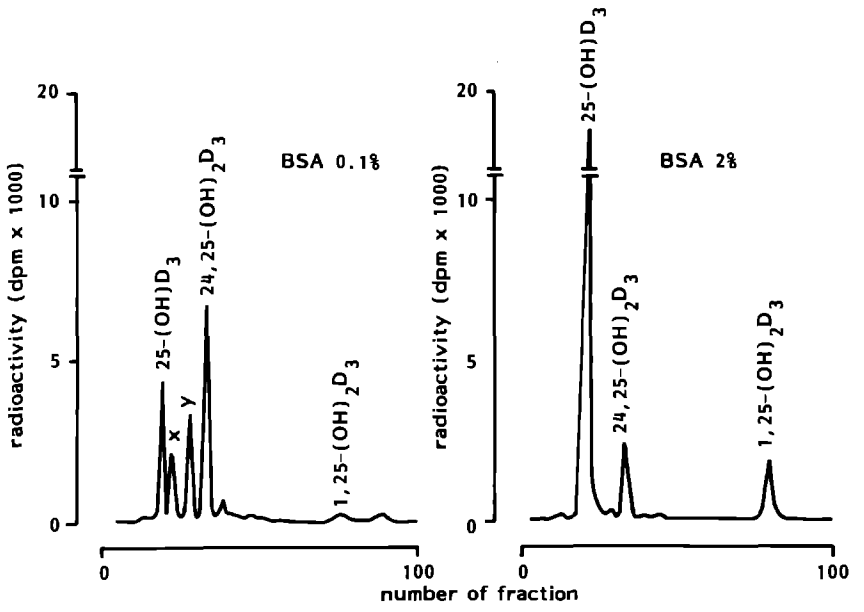

Fig.1 Representative metabolite profiles at 0.1% and 2% BSA after 3 h incubation of OB-cells with 10 nM [3H]25-(OH)D_3. (CPtmSpher Silica column; hexane-isopropanol = 91 : 9; fraction volume 0.45 ml).

25-(OH)D_3 in a 0.1 and 2% BSA medium. After 3 h incubation and subsequent processing of the medium we obtained the typical elution profiles shown in Fig.1. No metabolites other than 25-(OH)D_3 were found if the incubations with [^{3}H]25-(OH)D_3 were performed without cells or with cells killed with glutaraldehyde. Of the polar metabolites formed two were identified as 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ on the basis of their comigration with authentic standards on HPLC in two different systems (Silica and Zorbax CN column). After periodate treatment 90-95% of the radioactivity in the 24,25-$(OH)_2D_3$ and 2-5% of that in the 1,25-$(OH)_2D_3$ region was lost.

25-(OH)D_3 metabolism at different medium BSA concentrations. Incubation of the OB-cells for 3 h with 25-(OH)D_3 at 0.1% BSA resulted in the accumulation of a large amount of 24,25-$(OH)_2D_3$ and a small amount of 1,25-$(OH)_2D_3$, while at 2% BSA a more pronounced 1,25-$(OH)_2D_3$ accumulation was accompanied by low levels of 24,25-$(OH)_2D_3$ (Fig.2). The accumulation of two other metabolites, denoted X and Y in Fig.1, is also lowered by raising the medium BSA concentration. Time course experiments demonstrated the transient nature of the accumulation of 1,25-$(OH)_2D_3$ in the presence of 0.1% BSA, while at 2% BSA an initially slower, but more sustained rise of this derivative was found (Fig.3). The decline of 1,25-$(OH)_2D_3$ after 2 h in the presence of 0.1% BSA was accompanied by an acceleration of the 24,25-$(OH)_2D_3$ accumulation. At 2% BSA the concentration of 24,25-$(OH)_2D_3$ in the medium remained low.

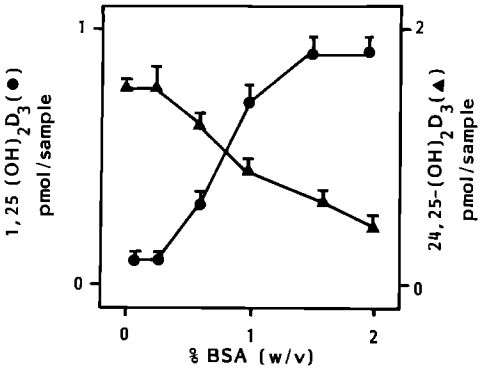

Fig.2 Accumulation of 1,25-(OH)2D3 (●) and 24,25-(OH)2D3 (▲) at 0.1-2% BSA after 3 h incubation with 10 nM [3H]25-(OH)D3. Values represent the means ± SEM of 3 different experiments.

1,25-$(OH)_2D_3$ metabolism at different medium BSA concentrations. Further 24-hydroxylation of the product may be the underlying mechanism for the transient accumulation of 1,25-$(OH)_2D_3$ at 0.1% BSA. To simulate the above-mentioned experiments, cells were incubated for 3 h with a low concentration of labeled 1,25-$(OH)_2D_3$ (0.5 nM) together with 10 nM unlabeled 25-$(OH)D_3$. Recently, we have shown that in OB-cells, 1,25-$(OH)_2D_3$ is rapidly converted to 1,24,25-$(OH)_3D_3$ and other more polar metabolites (14). In Fig.4 the influence of varying medium BSA concentrations on the accumulation of 1,24,25-$(OH)_3D_3$ is seen. At 0.1% BSA considerable conversion to 1,24,25-$(OH)_3D_3$ was found, which was progressively inhibited by increasing BSA concentrations. Non-ether-extractable radioactivity was affected in the same manner as the 1,24,25-$(OH)_3D_3$ accumulation.

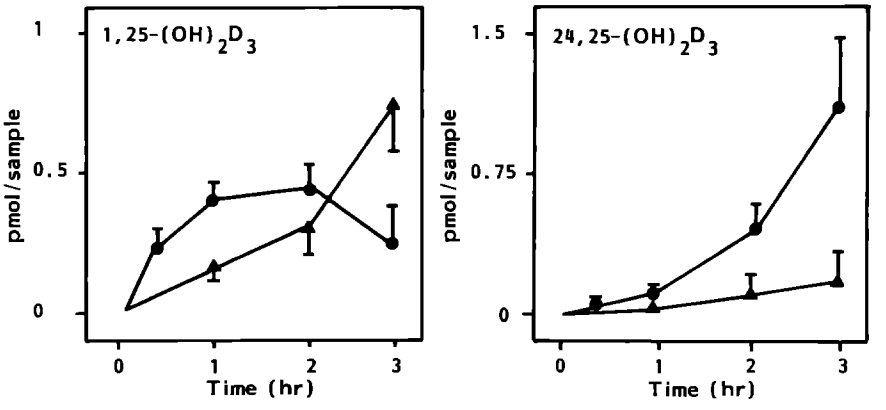

Fig.3 Effect of incubation time on the metabolism of [3H]25-(OH)D3 (10 nM) at 0.1% (●) and 2% (w/v) BSA (▲) after incubation for 0.5-3 h. Values represent the means ± SEM of 3 different experiments.

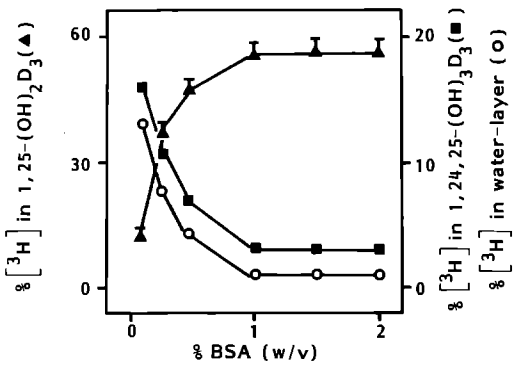

Fig.4 1,25-(OH)2D3 metabolism at 0.1-2% (w/v) BSA after 3 h incubation with 0.5 nM [3H]1,25-(OH)2D3 and 10 nM 25-(OH)D3. 1,25-(OH)2D3 (▲) and 1,24,25-(OH)3D3 (■) were separated by HPLC. The water-layer (○) represents the non-diethyl ether-extractable fraction. Values represent the means ± SEM of at least 2 experiments.

Effect of cycloheximide. To assess whether the 24-hydroxylase activity observed in our experiments requires de novo protein synthesis, we studied the metabolism of 25-(OH)D_3 in the presence and absence of cycloheximide. The experiments were performed at 0.1% BSA because of the pronounced 24,25-$(OH)_2D_3$ accumulation under these circumstances. Incubation of OB-cells with cycloheximide (1 µg/ml) for 3 h resulted in significantly lower levels of 24,25-$(OH)_2D_3$ and significantly higher levels of 1,25-$(OH)_2D_3$ (Table I). Under these circumstances a decline of the peaks X and Y (cf Fig.1) was found also (not shown).

Binding of vitamin D_3 metabolites at different BSA concentrations. The effect of BSA may be secondary to changes in the availability of 25-(OH)D_3 and its metabolites. Figure 5 shows the progressive increase in the binding of the

Table I

Effect of cycloheximide on 25-(OH)D_3 metabolism in OB-cells

	Accumulation of [^{3}H]25-(OH)D_3 metabolites	
Addition	1,25-$(OH)_2D_3$ pmol/sample	24,25-$(OH)_2D_3$ pmol/sample
none	0.13 ± 0.02	1.09 ± 0.04
cycloheximide	0.33 ± 0.09*	0.37 ± 0.02*

OB-cells were incubated for 3 h at 0.1% BSA with 10 nM [^{3}H]25-(OH)D_3 in the presence or absence of 1 µg/ml cycloheximide. The media of 5 parallel incubations per experiments were analysed as described under Materials and Methods. Values represent the means ± SEM of 2 experiments. (* $p < 0.001$ vs control)

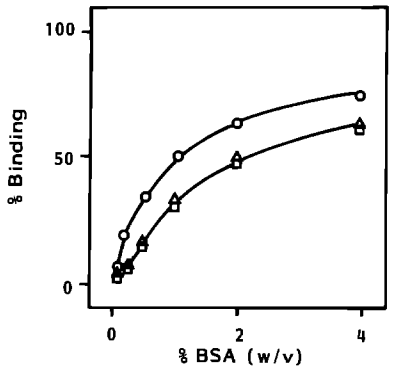

Fig.5 Binding of 25-(OH)D3, 24,25-(OH)2D3 and 1,25-(OH)2D3 to BSA. Serum-free medium with 0.1-4% (w/v) BSA was incubated for 1 h with 0.5 nM [3H]25-(OH)D3 (○), [3H]1,25-(OH)2D3 (□) or [3H]24,25-(OH)2D3 (△). Values represent the means ± SEM of at least 2 experiments.

different vitamin D derivatives to increasing BSA concentrations. Highest binding is observed with 25-(OH)D_3, while 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ have equal affinity for BSA. The binding of all three metabolites was not significantly influenced by increasing their concentration from 0.5 to 10 nM.

DISCUSSION

The observation that fetal chicken calvarial cells are able to metabolize 25-(OH)D_3 to 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ has been interpreted as a possible means of local regulation of the response to vitamin D (2). However, it was not clear to what extent the use of a protein-free medium might have influenced the ability of these cells to metabolize 25-(OH)D_3 via the different pathways. We performed our experiments in the presence of a range of BSA concentrations. We found that fetal chicken osteoblast-like cells metabolize 25-(OH)D_3 both at 0.1% and 2% BSA (Fig.1), although marked differences in the accumulation of 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ are seen (Fig.2). The striking differences in the time-course experiments at 0.1% and 2% BSA (Fig.3) suggest that factors such as the availability of the substrate to the enzymes and the rates of further conversion of the products alter the accumulation of 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$. The high free substrate concentration in the presence of 0.1% BSA (Fig.5) initially appears to result in a high rate of synthesis of the two dihydroxy-metabolites (Fig.3). However, once a significant production of 24,25-$(OH)_2D_3$ takes place the level of 1,25-$(OH)_2D_3$ falls probably due to further hydroxylation in the 24 position. This is supported by the observation that a considerable proportion of added 1,25-$(OH)_2D_3$ is converted to 1,24,25-$(OH)_3D_3$. The latter conversion is decreased at higher BSA concentrations (Fig.4), which is in agreement with the ultimately higher accumulation of 1,25-$(OH)_2D_3$ from 25-(OH)D_3 in the presence of 2% BSA.

It is clear from these results that 24-hydroxylation represents a major step in the metabolism of 25-(OH)D_3 and 1,25-$(OH)_2D_3$, which however is fully expressed only at low ambient albumin concentrations, i.e. high free substrate concentrations. It is also evident that once 24,25-$(OH)_2D_3$ is allowed to accumulate other metabolites (cf. the peaks X and Y in Fig.1) appear also. These compounds probably are the 24-oxo- and 23-hydroxy-derivatives of 25-$(OH)_2D_3$, recently identified in perfusion studies of rat kidney (15). For 1,25-$(OH)_2D_3$ we also propose further conversion by the C_{23}- and C_{24}-oxidation pathways, as the loss of tritium from 23,24-tritiated 1,25-$(OH)_2D_3$ into the medium appeared to be much higher than one would expect from 24-hydroxylation alone (Fig.4). This hypothesis is supported by the recent observation of such a pathway in intestinal 1,25-$(OH)_2D_3$ metabolism (16).

Other workers (2) using comparable substrate concentrations have concluded that the rates of production of 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ in fetal chicken calvarial cells are linear for up to 4 h. We observed that the induction of the 24-hydroxylase, which is dependent upon de novo protein synthesis (Table I), is one of the factors that cause the non-linearity in the accumulation of 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ in our experiments. The fact that incubation with cycloheximide also results in a higher level of 1,25-$(OH)_2D_3$ formed from 25-(OH)D_3 again indicates the formation of 1,24,25-$(OH)_3D_3$ at low BSA concentrations in the standard incubations. Because 1,25-$(OH)_2D_3$ is known as a potent inductor of 24-hydroxylase activity (2,17,18) it is attractive to speculate that the (preceeding) accumulation of this compound is responsible for the increase in 24-hydroxylase activity. Induction of 24-hydroxylase activity by 25-(OH)D_3 itself is, however, not excluded (19).

We do not know whether or not the 1α- and 24-hydroxylases are located in the same type of cell in our culture. It is important to note that although primary cultures of OB-cells retain several characteristics of osteoblasts (10), a significant admixture of other cells, especially fibroblasts, may occur. Although fibroblasts do not contain basal 1α- and 24-hydroxylase activities (2), a rapid induction of 24-hydroxylase activity by 1,25-$(OH)_2D_3$ in these cells has been reported (20,21). A similar induction could also play a role in our experiments. Therefore, the significance of 24-hydroxylation as a major mechanism of inactivation of 1,25-$(OH)_2D_3$ in bone tissue remains to be clarified. The main question raised by our study is to what extent the metabolism of 25-(OH)D_3 in OB-cell cultures reflect the in vivo situation. Our results strongly suggest that interpretation of such studies is not possible without knowledge of the effects of vitamin D-binding proteins.

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