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STUDIES OF THE INTERNALIZATION OF VITAMIN D_3 METABOLITES BY CULTURED OSTEOGENIC SARCOMA CELLS AND THEIR APPLICATION TO A NON-CHROMATOGRAPHIC CYTORECEPTOR ASSAY FOR 1,25-DIHYDROXYVITAMIN D_3

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SUMMARY: Cultured osteogenic sarcoma (OS) cells have been used here to study the internalization of $1,25(OH)_2D_3$ and other major metabolites of D_3 by cells. Intact OS cells incubated for 1h at 37°C in medium containing $[^3H]1,25(OH)_2D_3$ at low concentrations (0.16 to 1.6nM) take up and retain this hormone with high affinity ($K_d=3.3x10^{-10}M$) similar to that found for the hormone-receptor interaction in cytosol preparations. Vitamin D_3 and its major metabolites such as $25(OH)D_3$ or $24,25(OH)_2D_3$, even at supraphysiological concentrations, are not internalized by the cells when small amounts of plasma D binding protein (DBP) or human alpha-globulin are added to the incubation medium. This phenomenon can be exploited to develop a non-chromatographic cytoreceptor assay for $1,25(OH)_2D_3$.

INTRODUCTION

The biologically active metabolite of vitamin D_3, 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$], exerts its effects by binding to a receptor system present in the target cells of the vitamin (1, 2). Receptors for $1,25(OH)_2D_3$ are proteins with a sedimentation coefficient of 3.3 to 3.7S that bind to chromatin (3, 4) and DNA (5, 6) and are presumed to modulate the expression of genetic information.

The affinity of the receptor protein for $1,25(OH)_2D_3$, as it has been reported in cytosol preparations from all the tissues studied ($K_d=0.5\text{-}1x10^{10}M^{-1}$), corresponds with the circulating levels of the hormone ($10^{-10}M$). In studies performed in cytosol preparations, other analogs of D_3, such as 25-hydroxy D_3 [$25(OH)D_3$] and 24,25-dihydroxy D_3 [$24,25(OH)_2D_3$], have also been shown to interact with the receptor but with an affinity of 2 to 3 orders of magnitude less than the affinity of $1,25(OH)_2D_3$ (1, 2). Nevertheless, considering that the circulating levels of $25(OH)D_3$ are $10^{-7}M$ and of $24,25(OH)_2D_3$, $10^{-8}M$, these two metabolites could substitute in vivo for $1,25(OH)_2D_3$ at the receptor level. However, such a possibility is not consistent with the tightly controlled plasma levels of $1,25(OH)_2D_3$ and the existence of receptor systems in D_3 target cells

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of high affinity and specificity for this hormone. There is no definitive evidence which resolves the question of whether metabolites other than $1,25(OH)_2D_3$ have significant interaction with this receptor system in vivo. It is also unclear whether D_3 metabolites which are carried in plasma bound to D binding protein (DBP) are capable of interacting with target cells (7). We have here tested the hypothesis that major metabolites of vitamin D_3 other than $1,25(OH)_2D_3$ may not have access to the receptor because of their higher affinity to plasma carrierproteins versus their relatively lower affinity for the intracellular receptor.

In order to study the translocation of vitamin D_3 metabolites across cell membranes and their subsequent binding to the receptor system in intact cells we have employed cultured rat osteogenic sarcoma (OS) cells (8). This model has been previously shown to possess a receptor-like macromolecule with high affinity and specificity for $1,25(OH)_2D_3$ whose biochemical characteristics were indistinguishable from those of the receptor of normal target tissues for the hormone (6). Experience gained from these studies has been applied to the development of a cytoreceptor assay for $1,25(OH)_2D_3$.

MATERIALS AND METHODS

Materials: 1α,25-dihydroxy [26,27-^{3}H]vitamin D3 (160 Ci/mmol) was purchased from New England Nuclear. Crystalline $1,25(OH)_2D_3$, $25(OH)D_3$, and $24,25(OH)_2D_3$ were generously given by Dr. M. Uskoković of the Hoffmann-LaRoche Co. Vitamin D_3 was purchased from Sigma. Semipurified human D-binding globulin (70% pure) was generously provided by Dr. J.G. Haddad (9). Human alpha-globulin fraction IV was purchased from Miles Lab., Inc.

Cells: Osteogenic sarcoma (OS) cells are cultured in our laboratory as described elsewhere (6). Confluent cells freed by trypsinization were washed and subsequently suspended in minimal essential medium (MEM) with Hank's salts and 25mM Hepes.

Cell Incubation: [^{3}H]$1,25(OH)_2D_3$ and unlabelled sterols in ethanol were pipetted into polycarbonate tubes. The sterols were subsequently dried under N_2. 0.15 ml of cell suspension (0.5 to $1x10^6$ cells) was then added into each tube. Incubations were carried out for 1h at 37°C with continuous vortexing. The tubes were then placed on iced H_2O for 10 minutes and the cells pelleted by centrifugation for 5 minutes at 2000 rpm. The incubation medium was then aspirated and the cells were washed two times with 3.5 ml isotonic buffer (0.25M sucrose, 0.025M KCl, 0.005M $MgCl_2$, 0.001M EDTA, 0.012M thioglycerol, 0.05M Tris-HCl, and 1 mg/ml bovine serum albumin, pH 7.4). Then 1 ml of hypertonic buffer (0.3M KCl, 0.0005M dithiothreitol, 0.0015M EDTA, and 0.01M Tris-HCl, pH 7.4) was added and cells were sonicated. Finally, the tubes were centrifuged for 1/2 hour at 40,000 RCF and the supernatant was counted in 10 ml of scintillation liquid.

Saturation analysis of internalization: 0.15 ml aliquots of cell suspension ($0.5x10^6$ cells) were incubated as above with increasing concentrations of $[^3H]1,25(OH)_2D_3$ (0.16 to 1.6nM) alone and in the presence of 200-fold molar excess of unlabelled $1,25(OH)_2D_3$.

Effect of DBP on the $25(OH)D_3$ internalization: 0.15 ml aliquots of cell suspension ($0.5x10^6$ cells) were incubated in medium containing $[^3H]1,25(OH)_2D_3$ (0.32 nM) alone and with increasing amounts (10-50 ng) of unlabelled $25(OH)D_3$. Parallel incubations were performed in the same medium enriched with a 70% pure preparation of human DBP; each of these series of incubations contained, respectively, the equivalent of 4.3, 8.6, and 12.9 µg of pure DBP per tube.

Displacement curve of $[^3H]1,25(OH)_2D_3$ internalized in OS cells by unlabelled D_3 and its major metabolites: 0.15 ml aliquots of cell suspension ($1x10^6$ cells/tube) in medium enriched with 4 mg/ml of human alpha-globulin were incubated with constant amounts of $[^3H]1,25(OH)_2D_3$ (20 pg = 0.32nM) and unlabelled $25(OH)D_3$ (20 ng). The displacement of $[^3H]1,25(OH)_2D_3$ internalized under these conditions was examined in the presence of added unlabelled $1,25(OH)_2D_3$ (4 pg - 4 ng), D_3 (20, 40 ng), $25(OH)D_3$ (10, 20, 40 ng), and $24,25(OH)_2D_3$ (10, 20, 40 ng).

RESULTS AND DISCUSSION

The $[^3H]1,25(OH)_2D_3$ internalized by 0.5 million OS cells incubated for 1h at 37°C with the hormone is shown in Figure 1. Non-specific uptake, i.e., that

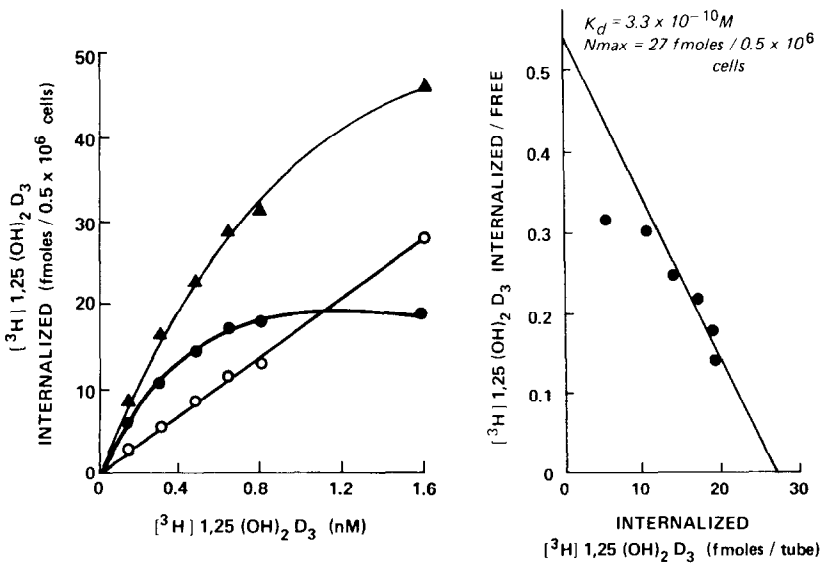

Figure 1. Saturation analysis of $[^3H]1,25(OH)_2D_3$ internalization by intact OS cells. Left - aliquots of OS cell suspension ($0.5x10^6$ cells/0.15 ml of medium) were incubated with increasing concentrations of $[^3H]1,25(OH)_2D_3$ (▲ —— ▲) alone or in the presence of 200-fold excess of unlabelled $1,25(OH)_2D_3$ (o—— o) for 1h at 37°C. At the end of incubation cells were washed, sonicated, spun, and the supernatant was counted for tritium. Non-specific internalization (that unaffected by the 200-fold excess of unlabelled hormone) was subtracted from total internalization to yield specific internalization (•—— •). Right - Scatchard analysis of specific internalization. Each point represents the mean of duplicate determinations.

remaining in the presence of 200-fold molar excess of unlabelled $1,25(OH)_2D_3$ is also depicted. The difference between total and non-specific has been used to determine the amount of specifically internalized ligand. In order to obtain a quantitative measure of the translocation process of the hormone from the medium to the interior of the cells we have performed Scatchard analysis of the specifically internalized hormone. Internalized over non-internalized [^{3}H]-$1,25(OH)_2D_3$, i.e., that remaining outside the cells, has been plotted against the internalized hormone. The plot yielded a straight line, indicating a single class of binding sites inside the cells. We have calculated an apparent dissociation constant (K_d) of the hormone-intracellular binder interaction of $3.3x10^{-10}$M and a total concentration of 27 fmoles per $0.5x10^6$ cells or 32,500 binding sites per cell. This corresponds closely to the K_d of the hormone-receptor interaction determined in cytosol preparations of OS cells ($2x10^{-10}$M) (6). This evidence therefore suggests that $1,25(OH)_2D_3$ like other steroid hormones (10), diffuses freely across cell membranes and is specifically retained inside its target cells because of its high affinity for the receptor protein. Under the conditions of our experiments most of the ligand was probably retained in the nucleus of the intact cell but was eventually recovered in the supernatant of cell sonicate because of the high salt content (0.3M KCl) of the sonication solution. The pellets of the cell sonicate contained only small amounts of [^{3}H]$1,25(OH)_2D_3$ which were not displaceable by unlabelled $1,25(OH)_2D_3$.

Figure 2 illustrates the effect of supraphysiological concentrations of unlabelled $25(OH)D_3$ on the [^{3}H]$1,25(OH)_2D_3$ uptake by OS cells in the absence and presence of semipurified human DBP. Over a range of 500-2500-fold molar excess $25(OH)D_3$ could displace up to 60% of total [^{3}H]$1,25(OH)_2D_3$ taken up by the cells. However, when increasing amounts of semipurified DBP were added into the incubation medium the displacement of [^{3}H]$1,25(OH)_2D_3$ by $25(OH)D_3$ became lesser in a dose-dependent fashion until it was completely prevented by the equivalent of 12.9 µg of pure DBP. In the presence of 12.9 µg of DBP the initial [^{3}H]$1,25(OH)_2D_3$ internalized by the cells was reduced to only 70% of that internalized in the

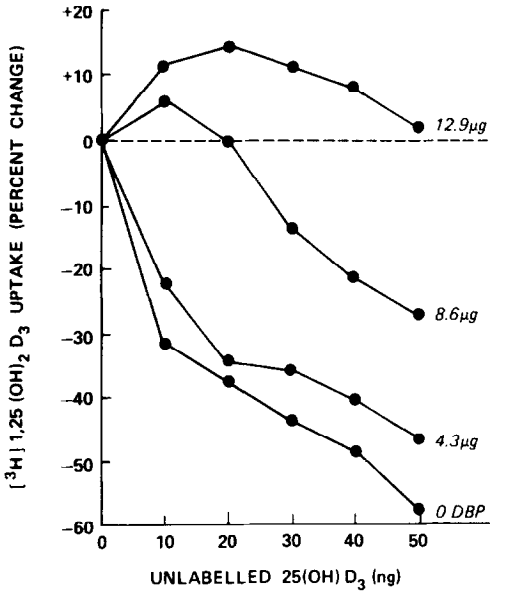

Figure 2. Displacement of [^{3}H]1,25(OH)$_2$D$_3$ internalized in OS cells by unlabelled 25(OH)D$_3$, in the absence and presence of human DBP. Aliquots of OS cells suspension (0.5×10^6 cells/0.15 ml of medium) were incubated with [^{3}H]1,25(OH)$_2$D$_3$ (0.32×10^{-9}M) and increasing amounts of unlabelled 25(OH)D$_3$. Incubations were performed in the absence of DBP and in the presence of increasing amounts of DBP. Percentage changes of initial [^{3}H]1,25(OH)$_2$D$_3$ [that internalized by cells in the absence of unlabelled 25(OH)D$_3$] are illustrated. Points represent means of duplicate determinations.

absence of DBP and unlabelled 1,25(OH)$_2$D$_3$ at 5- to 10-fold excess was capable of displacing over 50% of [^{3}H]1,25(OH)$_2$D$_3$. This data suggests that 25(OH)D$_3$ also could diffuse across cell membranes and compete at its high levels with 1,25(OH)$_2$D$_3$ for the receptor sites; however, it is prevented from doing so by being bound with high affinity to DBP. This view is supported by the dramatic reduction of the uptake of [^{3}H]1,25(OH)$_2$D$_3$, in the presence of serum, observed in OS cells (15) and in perfused dog tibia (16). In contrast, 1,25(OH)$_2$D$_3$, because of higher affinity for the receptor and only low affinity for plasma binding proteins, has ready access to the receptor sites. Hence, because of these differential affinities, the hormonal message of 1,25(OH)$_2$D$_3$ can be delivered to its target cells without any interference by the other major metabolites of D$_3$.

These studies have been adapted to the development of a novel assay for 1,25(OH)$_2$D$_3$ based on the internalization of the hormone by cultured cells. Currently

used methods for this measurement (11-14) require the chromatographic separation of $1,25(OH)_2D_3$ from other metabolites of vitamin D_3 because the binding proteins used are not selective enough to distinguish the hormone at its relatively low plasma concentrations from other metabolites which are present at much higher levels in plasma. The experiments described above however have pointed out that such specificity for $1,25(OH)_2D_3$ over other D_3 metabolites can be obtained by the combination of the intracellular receptor along with the intact membrane and the plasma binding proteins. These principles have been employed in the experiment illustrated in Figure 3. Here the semipurified human DBP employed earlier has been replaced by human alpha-globulin since this preparation contains DBP (9). The amount of $[^3H]$-$1,25(OH)_2D_3$ internalized by OS cells was displaceable by unlabelled $1,25(OH)_2D_3$

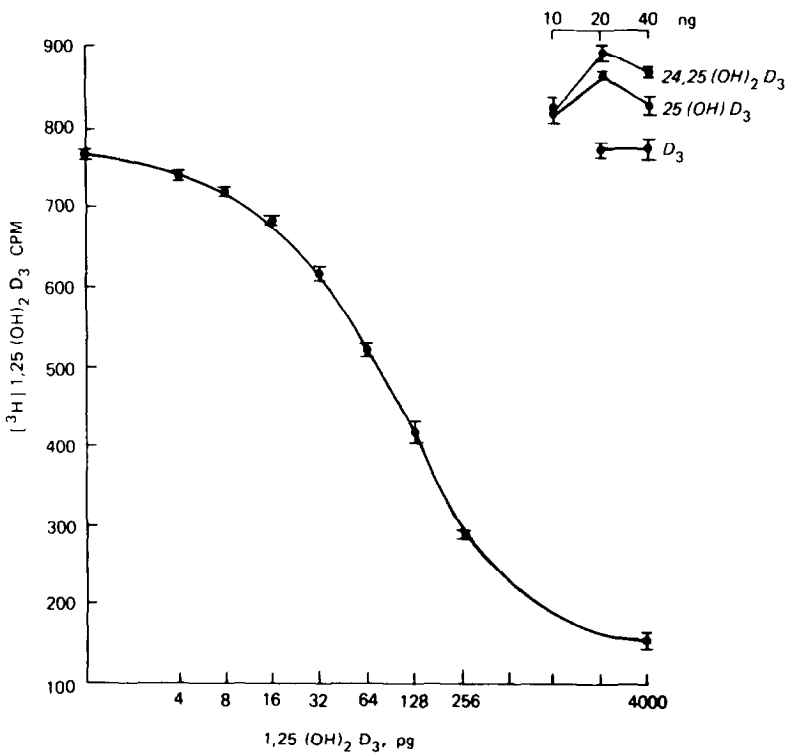

Figure 3. Standard curve of the cytoreceptor assay for $1,25(OH)_2D_3$. Cell suspension ($1x10^6$ cells/0.15 ml of medium) were incubated with constant amount of $[^3H]1,25(OH)_2D_3$ (20 pg) and unlabelled $25(OH)D_3$ (20 ng). The displacement of $[^3H]1,25(OH)_2D_3$ by increasing amounts of added unlabelled $1,25(OH)_2D_3$ (4-4000 pg) vitamin D_3 (20, 40 ng), $25(OH)D_3$ (10, 20, 40 ng), and $24,25(OH)_2D_3$ (10, 20, 40 ng) is shown. Note different scale for the later 3 substances. Each point represents the mean of triplicate determinations. Vertical bars represent ± SEM.

over a range of 4-4000 pg, in spite of the presence of 20 ng of $25(OH)D_3$. Vitamin D_3, $24,25(OH)_2D_3$, and additional amounts of $25(OH)D_3$ did not interfere with the displacement of the ligand by $1,25(OH)_2D_3$. This competition provides a standard curve for the sensitive measurement of $1,25(OH)_2D_3$ even in the presence of high concentrations of other vitamin D_3 metabolites. The detection limit of the cytoreceptor assay curve is comparable to that of the most sensitive currently available assays for the hormone (11-14).

The principles described could be employed for the assay of other hormones, such as steroids or even peptides, or any substances which are specifically internalized by certain cells. We thus believe that this method is a novel approach to assay procedures in general. Complete evaluation of the non-chromatographic cytoreceptor assay for $1,25(OH)_2D_3$ in small volumes of extracted plasma is currently in progress in our laboratory.

REFERENCES

1. Haussler, M.R., and McCain, T.A. (1977). New Engl. J. Med. 297, 974-983; 1041-1050.
2. Norman, A.W., and Wecksler, W.R. (1978). In Receptors in Hormone Action, (O'Malley, B.W., and Birnbaumer, L., eds.), Volume 2, pp 533-571, Academic Press, New York.
3. Brumbaugh, P.F., and Haussler, M.R. (1975). J. Biol. Chem. 250, 1588-1594.
4. Proscol, D.A., Okamura, W.H., and Norman, A.W. (1975). J. Biol. Chem. 250, 8382-8388.
5. Chandler, J.S., Pike, J.W., and Haussler, M.R. (1979). Biochem. Biophys. Res. Commun. 90, 1057-1063.
6. Manolagas, S.C., Haussler, M.R., and Deftos, L.J. (1980). J. Biol. Chem. 255, 4414-4417.
7. Haddad, J.G., Jr. (1979). Clin. Orth. Rel. Res. 142, 249-261.
8. Martin, T.J., Ingleton, P.M., Coulton, L.A., and Melick, R.A. (1979). Clin. Orth. Rel. Res. 140, 247-254.
9. Haddad, J.G., Jr., and Walgate, J. (1976). J. Biol. Chem. 251, 4803-4809.
10. Peck, E.J., Jr., Burgner, J., and Clark, J.G. (1973). Biochemistry 12, 4596-4603.
11. Brumbaugh, P.F., Haussler, D.H., Bursac, K.M., and Haussler, M.R. (1974). Biochemistry 13, 4091-4097.
12. Eisman, J.A., Hamster, A.J., Kream, B.E., and DeLuca, H.F. (1976). Arch. Biochem. Biophys. 176, 235-243.
13. Clemens, T.L., Hendy, G.N., Graham, R.F., Baggioloni, E.G., Uskoković, M.R., and O'Riordan, J.L.H. (1978). Clin. Sci. Mol. Med. 54, 329-332.
14. Schaefer, P.C., and Goldsmith, R.S. (1979). In Vitamin D Basic Research and Its Clinical Applications, (Norman, A.W., et al., eds.), Walter deGruyter, pp 205-212.
15. Manolagas, S.C., Larsen, M., Meler, D., LaFrance, M., Haussler, M.R., and Deftos, L.J. (1980). In Proceedings of the 62nd Annual Meeting of the Endocrine Society, p. 286, Abstract 845.
16. Olgaard, K., Finco, D., Martin, K., Schwartz, J., Haddad, J.G., Jr., Avioli, L.V., Klahr, S., and Slatopolsky, E. (1980). In Proceedings of the 2nd Annual Scientific Meeting of the American Society for Bone and Mineral Research, p. 39A.