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1,25-Dihydroxyvitamin D_3 Receptors in Human Epithelial Cancer Cell Lines[1]

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ABSTRACT

Specific, high-affinity cytosolic receptors for 1,25-dihydroxyvitamin D_3 have been demonstrated in five human cancer cell lines. The cell lines were derived from tumors of breast, lung, cervix, and melanotic and amelanotic melanomas. Binding affinity (K_d) of the receptors for 1,25-dihydroxyvitamin D_3 were all approximately 0.2 nM, and receptor content ranged from 21 to 174 fmol/mg cytosol protein. The receptors from all five cell lines sedimented at 3.2S on sucrose density gradients and exhibited preferential affinity for 1,25-dihydroxyvitamin D_3 compared to other vitamin D metabolites.

INTRODUCTION

The detection of steroid hormone receptors in a variety of cancers is currently being investigated as a means of ascertaining whether tumors have the potential to be hormonally dependent (20, 28). In addition, knowledge of the receptor status of tumors may provide information related to the state of tumor differentiation or the expected treatment response (21). Breast cancers have been extensively investigated for estrogen receptors (20-22, 28) and, more recently, similar studies with androgen receptors in prostate cancer (12, 15) and glucocorticoid receptors in leukemia (18) have been reported. $1,25(OH)_2D_3$,[3] the active metabolite of vitamin D_3, has a mode of action analogous to that of steroid hormones (14), and recent studies have characterized receptors in various mammalian (2, 4, 9, 16, 27) and human (8, 26) target cells. Receptors for $1,25(OH)_2D_3$ have been described in osteosarcoma cells (19), breast cancer tissue (7, 10), and malignant melanoma (3). As a major regulator of calcium transport, $1,25(OH)_2D_3$ action may alter multiple cell properties and may influence the behavior of a wide variety of tumors. The possible role of $1,25(OH)_2D_3$ as a modulator of cancer cell growth has recently been raised (3, 10). We therefore thought that it was important to examine a number of additional malignant human cell lines for the presence of $1,25(OH)_2D_3$ receptors and to compare the properties of these tumor receptors both to one another and to receptors in normal tissues. Our results indicate that such receptors are indeed present in a variety of human epithelial malignant cell lines and that the properties of these receptors are similar to those of receptors present in normal mammalian target organs.

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[3] The abbreviations used are: $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; 25-OH-D_3, 25-hydroxyvitamin D_3; 1α-OH-D_3, 1α-hydroxyvitamin D_3; $24,25(OH)_2D_3$, 24,25-dihydroxyvitamin D_3; KTEDM, 300 mM KCl, 10 mM Tris-HCl, 1 mM EDTA, 5 mM dithiothreitol, and 10 mM sodium molybdate, pH 7.4.

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MATERIALS AND METHODS

Steroids. 1α,25-Dihydroxy[23,24-^{3}H]cholecalciferol (82 and 120 Ci/mmol) was obtained from Amersham Corp. (Arlington Heights, Ill.). Crystalline $1,25(OH)_2D_3$, 25-OH-D_3, 1α-OH-D_3, and $24,25(OH)_2D_3$ were gifts from Dr. M. Uskokovic (Hoffmann-LaRoche Inc., Nutley, N. J.).

Cell Culture. Five human cancer cell lines were examined. Hs0578T is a breast cancer cell line of epithelial origin which produces casein and lacks estrogen receptors (13). A549 is a carcinoma of the lung which arose from type II alveolar epithelial cells and which synthesizes surfactant (17). Huk-HeLa cells were originally derived from human kidney but recently were found to be overgrown by HeLa cervical carcinoma cells as has been reported for a number of cell lines originating in many different laboratories (23). Two melanoma cell lines were also examined: G-361, a human melanoma cell line (American Tissue Culture Collection, CRL 1424) which produces melanin *in vitro*; and Hs695T, a metastatic amelanotic melanoma cell line, which has retained morphological properties of melanocytes (6). All the cells were grown as monolayers in glass roller bottles using Eagle's minimal essential medium or Dulbecco's modification of that medium (Grand Island Biological Co.) containing 10% fetal bovine serum. All 5 cell lines have been shown to produce tumors when injected into immunodeficient animals (5, 11) and, where such tumors have been examined histologically, their appearance was consistent with the tumor type from which the cell line was derived (5). An additional tumor cell studied was the Cloudman S91 mouse melanoma cell line (American Tissue Culture Collection; CCL 53.1, clone M-3) which was grown in Ham's medium supplemented with 20% calf serum. Several nonmalignant fibroblast cell lines were also examined. Human skin fibroblasts, derived from infant foreskin, were maintained as described previously (8). WI-38 cells, from a fibroblast cell line derived from normal human lung, were obtained from V. J. Cristofalo (Wistar Institute) and were maintained in F-17 medium (Grand Island Biological Co.) supplemented with 10% newborn calf serum.

[^{3}H]-$1,25(OH)_2D_3$ Binding Assay. All cells were grown to confluence, and fresh medium was added 24 hr before the experiment. Cell monolayers were rinsed with phosphate-buffered saline (NaCl 8 g/liter, KCl 0.2 g/liter, Na_2HPO_4 0.94 g/liter, KH_2PO_4, 0.2 g/liter) and scraped from the glass with a rubber policeman; in some experiments, cells were detached by trypsinization. Cells were washed 3 times and then were sonicated in a hypertonic buffer (KTEDM) containing 300 mM KCl, 10 mM Tris-HCl, 1 mM EDTA, 5 mM dithiothreitol, and 10 mM sodium molybdate, pH 7.4. High-speed supernatants of the cell extracts were obtained by centrifugation at 144,000 × *g* for 30 min and designated KTEDM extracts. It has been shown previously that 0.3 M KCl extracts contain both cytosolic receptors and any receptor in the nuclear compartment (25). Aliquots of the KTEDM extracts (200 µl) were incubated with the appropriate steroids for 3 hr at 0°. Bound and free steroids were separated by dextran-coated charcoal or by hydroxylapatite (4, 9, 27). Parallel incubations contained 1000-fold molar excess of radioinert $1,25(OH)_2D_3$ for determination of nonspecific binding, which was always less than 15%.

Sedimentation Analysis. KTEDM extracts were incubated with a saturating concentration (1.3 nM) of [^{3}H]-$1,25(OH)_2D_3$ with or without radioinert steroids for 3 hr at 0°. Free steroid was removed by charcoal adsorption. Aliquots (200 µl) of the KTEDM extracts were layered onto linear 5 to 20% sucrose gradients prepared in KTEDM buffer and centrifuged at 2° for 18 hr at 257,000 × *g*. The ^{14}C-proteins, carbonic anhydrase (3.0S), ovalbumin (3.7S), and bovine serum albumin (4.4S) (New England Nuclear, Boston, Mass.), were used as internal markers.

Protein Determination. Protein concentration was assayed by the Coomassie blue dye method of Bradford (1).

RESULTS

Sedimentation Analysis. We used the technique of sedimentation analysis on sucrose density gradients to examine the 1,25(OH)$_2$D$_3$-binding components present in KTEDM extracts from 5 human malignant cell lines. The findings were qualitatively the same in each case. Extracts from breast, lung, and HeLa cells all reproducibly demonstrated a [^{3}H]-1,25(OH)$_2$D$_3$-binding peak in the 3.2S region. The binding could be eliminated by an excess of radioinert 1,25-(OH)$_2$D$_3$ confirming the specific nature of this binding component (Chart 1). Similarly, KTEDM extracts from both the melanin-producing and amelanotic human melanoma cell lines contained a specific 3.2S binder for [^{3}H]-1,25(OH)$_2$D$_3$. No specific binding could be demonstrated with extracts from the Cloudman mouse melanoma cell line (Chart 2).

Saturation Analysis. KTEDM cell extracts from various cell lines were incubated with increasing concentrations (0.065 to 1.3 nM) of [^{3}H]-1,25(OH)$_2$D$_3$ for 3 hr at 0°. In all cell lines examined, Scatchard analysis of [^{3}H]-1,25(OH)$_2$D$_3$ binding yielded linear plots, indicating a single class of binding sites. Two such plots, obtained with KTEDM extracts of HeLa and breast cells, are shown in Chart 3, and data from all cell lines are summarized in Table 1. The apparent dissociation constants were very similar in all the cell lines examined (~0.2 nM), and Scatchard analysis of [^{3}H]-1,25(OH)$_2$D$_3$ binding indicated a range of receptor content from 21 to 174 fmol/mg protein. No specific binding could be detected with KTEDM extracts from WI-38 fibroblasts (lung) whereas normal human skin fibroblasts did possess high-affinity receptors for 1,25(OH)$_2$D$_3$ (Table 1).

Specificity of 1,25(OH)$_2$D$_3$ Binding in Cancer Cells. In order to determine the hormonal specificity of this receptor, the ability of 2 physiologically less active vitamin D$_3$ metabolites, 25-OH-D$_3$ and 24,25(OH)$_2$D$_3$, and the synthetic analog 1α-OH-D$_3$ to compete for [^{3}H]-1,25(OH)$_2$D$_3$-binding sites was analyzed by sucrose density gradient techniques. KTEDM extracts of HeLa cells were incubated with 1.3 nM [^{3}H]-1,25(OH)$_2$D$_3$ with or without a 5-fold molar excess of various vitamin D$_3$ metabolites. Sedimentation of the extracts bound with [^{3}H]-1,25(OH)$_2$D$_3$ yielded a single binding peak sedimenting at 3.2S. A 5-fold molar excess of radioinert 1,25(OH)$_2$D$_3$ caused a substantial reduction of [^{3}H]-1,25(OH)$_2$D$_3$ binding in the 3.2S peak (Chart 4). The same concentration of 25-OH-D$_3$, 24,25(OH)$_2$D$_3$, and 1α-OH-D$_3$ produced only a negligible decrease in the height of the 3.2S peak (Chart 4*B*). Similar results were previously found with KTEDM extracts of Hs695T melanoma cells (3). As shown

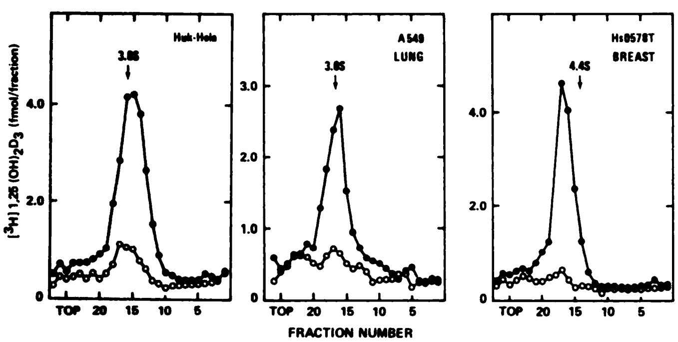

Chart 1. Sucrose density gradient analyses of [^{3}H]-1,25(OH)$_2$D$_3$ binding in KTEDM extracts of 3 human cancer cell lines. ●, binding of 1.3 nM [^{3}H]-1,25(OH)$_2$D$_3$; ○, plus 1.3 μM radioinert 1,25(OH)$_2$D$_3$ to assess nonspecific binding.

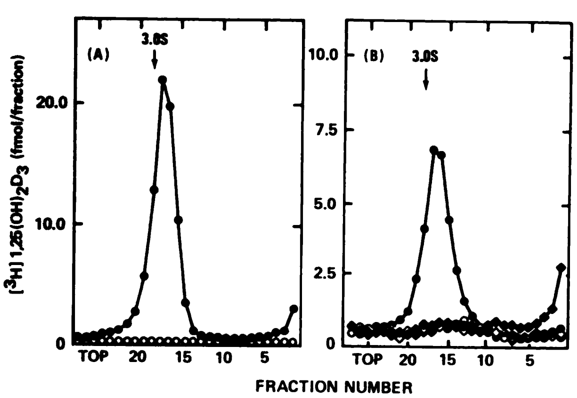

Chart 2. Sucrose density gradient analysis of [^{3}H]-1,25(OH)$_2$D$_3$ binding in KTEDM extracts of melanoma cells. *A*, amelanotic melanoma cell line Hs695T. ●, binding of 1.3 nM [^{3}H]-1,25(OH)$_2$D$_3$; ○, plus 1.3 μM radioinert 1,25(OH)$_2$D$_3$. *B*, melanotic cell line G-361. ●, binding of 1.3 nM [^{3}H]-1,25(OH)$_2$D$_3$; ○, plus 1.3 μM radioinert 1,25(OH)$_2$D$_3$. Cloudman mouse melanoma cell line. ◆, binding of 1.3 nM [^{3}H]-1,25(OH)$_2$D$_3$; ◇, plus 1.3 μM radioinert 1,25(OH)$_2$D$_3$.

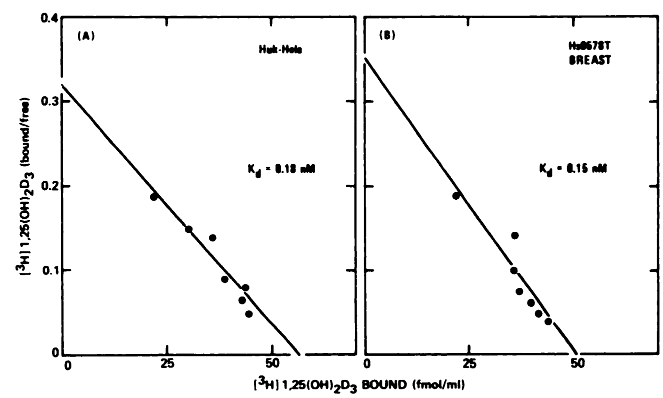

Chart 3. Scatchard analysis of specific [^{3}H]-1,25(OH)$_2$D$_3$ binding in KTEDM extracts. *A*, Huk-HeLa cells (protein concentration, 2.9 mg/ml; *B*, breast cancer cells (protein concentration, 1.4 mg/ml).

Table 1

1,25(OH)$_2$D$_3$ receptors in cultured human cell lines

Values for equilibrium dissociation constant (K_d) and total mean receptor content were determined by Scatchard analysis (n = 2 or 3).

Cell line	Tissue of origin	K_d (nM)	Receptor content (fmol/mg cytosol protein)
Cancer cells			
Hs695T	Melanoma (amelanotic)	0.18	174 (172–176)[a]
G361	Melanoma (melanotic)	0.12	21 (15–26)
Huk-HeLa	Cervical carcinoma	0.22	31 (19–43)
Hs0578T	Breast carcinoma	0.12	40 (35–45)
A549	Lung carcinoma	[b]	50 (46–54)
Normal cells			
Human skin fibroblasts	Infant foreskin	0.27	42 (30–50)
WI-38	Normal lung fibroblast	[b]	0

[a] Numbers in parentheses, range.

[b] For undetermined reasons, possibly relating to the high mucin content, we have been unable to obtain satisfactory Scatchard plots with A549 lung cells. In this case, receptor content was estimated by single-point analyses using a saturating concentration of [^{3}H]-1,25(OH)$_2$D$_3$. No specific receptor binding could be detected in WI-38 cells.

in Chart 5, the receptor in both the breast cancer cell line Hs0578T and the lung cancer cell line A549 also preferentially bound the dihydroxymetabolite. When KTEDM extracts of these 2 cell lines were incubated with 1.3 nM [^{3}H]-1,25(OH)$_2$D$_3$, the inclusion of a 5-fold molar excess of radioinert 1,25(OH)$_2$D$_3$ markedly reduced the height of the 3.2S peak, whereas 25-OH-D$_3$ was a less effective competitor at the same concentration. The profile found with the lung sample is somewhat unusual in the relatively weaker response to 5-fold 1,25(OH)$_2$D$_3$ and the somewhat stronger competition exhibited by 25-OH-D$_3$. We have attributed these findings, as well as our inability to obtain adequate Scatchard plots, to the large concentration of mucinous material synthesized by these cells (17) and present in the cytosol preparations.

DISCUSSION

These experiments demonstrate the presence of a specific, high-affinity binder for 1,25(OH)$_2$D$_3$ in 5 human epithelial cancer cell lines. The 3.2S sedimentation coefficient and the preferential binding of 1,25(OH)$_2$D$_3$ compared to 25-OH-D$_3$ or 24,25(OH)$_2$D$_3$ indicate that this binder is the 1,25(OH)$_2$D$_3$ receptor and not the 25-OH-D$_3$ plasma binder found in all tissues (4). The properties, including affinity, hormonal specificity, and sedimentation coefficient, are virtually identical to those we and others have obtained for the 1,25(OH)$_2$D$_3$ receptor in a variety of mammalian target organs (2, 4, 16, 27), indicating a similarity between the malignant and normal cell receptors.

The concentration of binding sites in these tumors varied from 21 to 174 fmol/mg protein. However, we do not wish to emphasize the quantitative aspects of these data since recent findings in our laboratory indicate that the receptor content of cultured cells varies extensively at different points in the culture cycle (2). In that study, primary cultures of normal bone cells exhibited a 4-fold alteration in 1,25(OH)$_2$D$_3$ receptor content which directly correlated with the rate of cell proliferation. The cells in the present study were not examined for receptor fluctuation related to cell growth.

The demonstration of receptors in these tumor cells raises the question of whether the receptor is present in the normal counterpart or whether the findings in malignant cells represent a cancer-related alteration. We and others demonstrated 1,25(OH)$_2$D$_3$ receptors in normal breast tissue (4, 7). Although we have been able to show 1,25(OH)$_2$D$_3$ receptors in human (8) and mouse (4) skin, it is not yet clear whether normal melanocytes possess receptors. However, both cultured human skin fibroblasts and keratinocytes are receptor positive (8). Samples of total mouse lung do not contain detectable receptors (4). However, the type II alveolar cell, the origin of the tumor from which cell line A549 was derived, represents a minority fraction of the total lung cell population (24), and detection of a receptor limited to this cell would be difficult. Again, whereas total mouse uterus appears negative for receptor (4), good data are not available regarding normal cervix. Since the origin of all 5 tumor cell lines is an epithelial layer, all may be involved in transport of calcium, which is the best understood function of 1,25(OH)$_2$D$_3$. It remains to be determined whether the nonmalignant counterparts of these cell types are target tissues for the hormone.

As with estrogen receptors in breast cancer, the demonstration of 1,25(OH)$_2$D$_3$ receptor in these tumor cells may be significant in either of 2 ways: (*a*) the demonstration of receptors may merely prove to be a marker of cellular differentiation providing an additional measurable protein gene product to characterize the tumor; (*b*) the presence of receptors indicates that the cell is potentially responsive to 1,25(OH)$_2$D$_3$, and thus tumor activity may be altered in the presence of the hormone. The cellular pattern of growth and metabolism may be regulated either by 1,25(OH)$_2$D$_3$ itself or by local shifts in calcium transport mediated by the hormone. In this regard, our studies on the effect of 1,25(OH)$_2$D$_3$ on the *in vitro* growth of malignant melanoma cell line Hs695T indicate that these melanoma cells are functionally responsive to the hormone. Inclusion of 1,25(OH)$_2$D$_3$ in the tissue culture medium leads to a marked inhibition of cell proliferation (3). The present findings in the Cloudman melanoma emphasize that not all melanoma cell lines are receptor positive.

These data indicate that 1,25(OH)$_2$D$_3$ receptors are present in several human malignant cell lines. The possible effects of

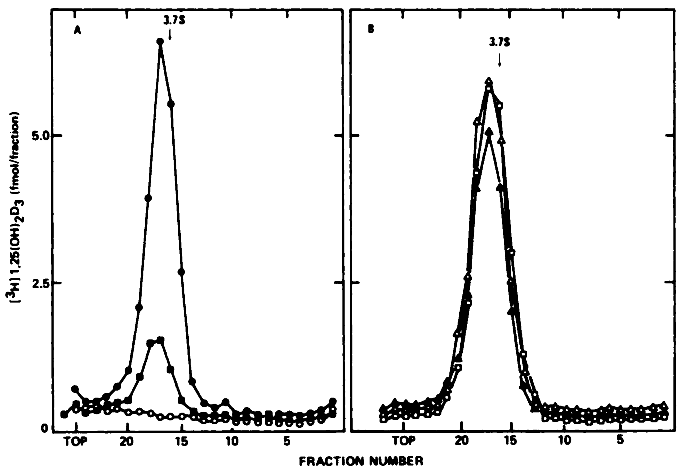

Chart 4. Sucrose gradient analysis of 1,25(OH)$_2$D$_3$ binding in KTEDM extracts of Huk-HeLa cells. Hormone specificity of the binding was assessed by inclusion of radioinert metabolites. [^{3}H]-1,25(OH)$_2$D$_3$ (1.3 nM) was present in all samples. *A*: ●, 1.3 nM [^{3}H]-1,25(OH)$_2$D$_3$ alone; ○, plus 1.3 µM 1,25(OH)$_2$D$_3$; ■, plus 6.5 nM 1,25(OH)$_2$D$_3$. *B*: ▲, plus 6.5 nM 25-OH-D$_3$; △, plus 6.5 nM 24,25(OH)$_2$D$_3$; □, plus 6.5 nM 1α-OH-D$_3$.

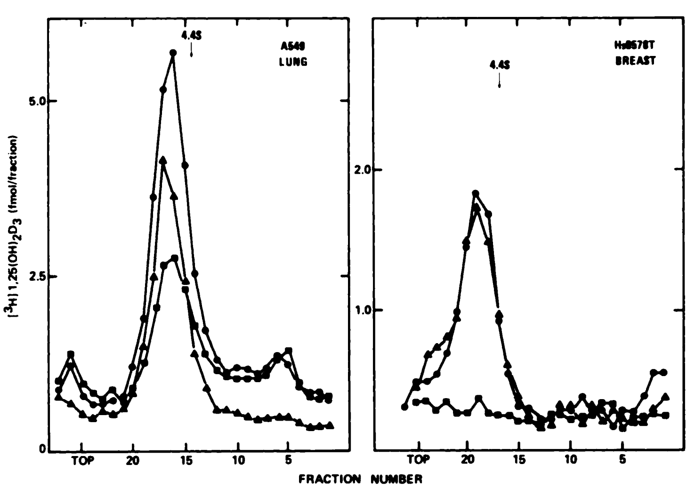

Chart 5. Sucrose gradient analysis of [^{3}H]-1,25(OH)$_2$D$_3$ binding in KTEDM extracts of lung and breast cancer cells. Hormone specificity of the binding was assessed by inclusion of radioinert metabolites. ●, 1.3 nM [^{3}H]-1,25(OH)$_2$D$_3$ alone; ■, plus 6.5 nM radioinert 1,25(OH)$_2$D$_3$; ▲, plus 6.5 nM radioinert 25-OH-D$_3$.

the hormone on tumor growth and the potential value of 1,25(OH)$_2$D$_3$ receptor determinations in patients with these tumors warrant further study.

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