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Frequency of 1,25-Dihydroxyvitamin D_3 Receptor in Human Breast Cancer[1]

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ABSTRACT

Receptors for 1,25-dihydroxyvitamin D_3 have been shown to exist in cultured breast cancer cells and in primary breast cancers. It is reported here that 1,25-dihydroxyvitamin D_3 receptor (1,25-DR) was present in 80% of 54 unselected breast cancers. The concentration of 1,25-DR in the 43 receptor-positive tumors was 1.9 ± 0.4 fmol/mg protein (S.E.). There was no correlation between 1,25-DR presence or concentration and the age of the patient or the concentration of estrogen, progesterone, androgen, or glucocorticoid receptors. 1,25-DR was also found in two of three renal cortical carcinomas but only in three of 14 gastrointestinal tract carcinomas. The relatively low concentration of 1,25-DR in these breast cancers, compared with that found in cultured breast cancer cells, is partially explained by incomplete "exchange" with occupied receptors. Since the serum vitamin D-binding protein is not precipitated from serum itself or from tissue homogenates using the polyethylene glycol method, artifactual 1,25-DR levels due to the inevitable contamination of tissue specimens with this protein can be excluded.

These findings indicate that 1,25-DR is not a nonspecific marker of cancer. The high frequency of 1,25-DR in the breast cancers may be related to the calcium-transporting ability of breast cancer cells which allows them to grow as osteolytic metastases.

INTRODUCTION

Breast cancer is a common disease, affecting one in 14 women in the United States at some time of their life (4). While earlier detection and improved treatment have prolonged survival, it has been estimated that fully one-third of women still die within 5 years of diagnosis, most of them with skeletal metastases (22). We have reported recently the presence of receptors for 1,25-$(OH)_2D_3$[3] in several cultured breast cancer cell lines (10, 12), in normal rabbit mammary tissue, and in a small series of human breast cancers (11, 13).

The presence of 1,25-DR in normal breast tissue suggests that breast cancer 1,25-DR may be a characteristic retained from normal breast cells. The presence of specific, high-affinity 1,25-DR in various tissues has been taken to indicate that they are target organs for 1,25-$(OH)_2D_3$ action (2, 3, 18). The possibility that breast tissue may be 1,25-$(OH)_2D_3$ responsive is supported by the recent report that lactating rat milk calcium secretion may be vitamin D dependent (15).

It has been proposed recently that the ability of cancer cells to grow in particular sites depends upon their possessing specialized functions which are essential for growth in such situations (21). Several human breast cancer cell lines have been shown recently to resorb devitalized bone in culture (6). This specialized function may be related to the facility with which breast cancer cells grow in this specialized site, *i.e.*, as bony metastases. We have shown that each of these breast cancer cell lines also possesses specific receptor for 1,25-$(OH)_2D_3$. Although there is as yet no evidence for a causal relationship between 1,25-DR and bone-resorptive activity, we have postulated that 1,25-DR-positive breast cancer cells may be 1,25-$(OH)_2D_3$ responsive. The effect of 1,25-$(OH)_2D_3$ on breast cancer cell growth and function *in vitro* is being examined in our laboratory at present. However, the relationship of 1,25-DR to clinical course can only be determined in a long-term prospective study. The tumors studied in this report are part of the initial phase of such a study. The age of the patient and the presence of other steroid receptors have been correlated with the level of 1,25-DR for each tumor. The possibility that the presence of 1,25-DR is more a marker of cancer than of hormonal sensitivity has been approached by testing a variety of other primary non-target organ cancers for the presence of 1,25-DR. The clinical course of these patients, in particular the development of hypercalcemia and bony metastases, will be followed prospectively and correlated with the 1,25-DR level in each case.

MATERIALS AND METHODS

Aprotinin (Trasylol) was obtained from Bayer Pharmaceuticals, Botany, New South Wales, Australia. Polyethylene Glycol 4000 was obtained from BDH Chemicals Australia, Port Fairy, Victoria, Australia. Hydroxylapatite (DNA grade) was obtained from Bio-Rad Laboratories, Richmond, Calif. Trizma, diethylstilbestrol, and dexamethasone were obtained from Sigma Chemical Co., St. Louis, Mo. Triamcinolone acetonide was obtained from Squibb & Son, Melbourne, Victoria, Australia. Nonradioactive R5020 and R1881 were obtained from New England Nuclear, Boston, Mass. [2,4,6,7-^{3}H]Dexamethasone (47 Ci/mmol), [17α-*methyl*-^{3}H]R5020 (87 Ci/mmol), [17α-*methyl*-^{3}H]R1881 (87 Ci/mmol), and 1,25-$(OH)_2$-[26,27-*methyl*-^{3}H]D_3 (160 Ci/mmol) were all obtained from New England Nuclear. 1,25-$(OH)_2$-[23,24-^{3}H]D_3 (80 to 110 Ci/mmol) and 25-OH-[26(27)-*methyl*-^{3}H]D_3 (9.6 Ci/mmol) were obtained from the Radiochemical Centre, Amersham, England. 1,25-$(OH)_2D_3$ and 25-OH D_3 were generous gifts from Dr. M. Uskoković, Hoffmann-La Roche Inc., Nutley, N. J. Instagel scintillation fluid was obtained from the Packard Instrument Co., Downers Grove, Ill.

Breast and other tumors were obtained at surgery from unselected patients. Tumors were cooled in ice, frozen in liquid nitrogen, and stored at −70° until assay. Cytosol was prepared from T47 D breast cancer cells as described previously (10–12).

1,25-$(OH)_2D_3$ Receptor Assay. Tumors were rinsed in ice-cold phosphate-buffered saline containing 0.137 M NaCl, 0.0081 M Na_2HPO_4, and 0.0015 M KH_2PO_4 and then homogenized in 0.05 M potassium phosphate (pH 7.2) buffer containing 0.3 M KCl, 0.002 M EDTA, 0.002

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[3] The abbreviations used are: 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; 1,25-DR, receptor for 1,25-dihydroxyvitamin D_3; R5020, 17,21-dimethyl-19-nor-4,9-pregnadiene-3,20-dione; R1881, 17β-hydroxy-17α-methylestra-4,9,11-triene-3-one; 25-OH D_3, 25-hydroxyvitamin D_3; DBP, serum vitamin D-binding protein.

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M dithiothreitol, and 1000 KIU of aprotinin per ml. The homogenate was then sonicated and centrifuged at 30,000 × g for 1 hr at 4° to yield a high-speed supernatant for assay with isotope and reagents as described previously (11–13). Briefly, the supernatant (1 ml of 1 to 10 mg of protein per ml) was incubated with 0.05 nM (9,000 to 18,000 dpm) tritiated 1,25-$(OH)_2D_3$ (80 to 160 Ci/mmol) with and without 25 nM unlabeled 1,25-$(OH)_2D_3$ or 25-OH D_3 for 2 hr at 25°. The concentration of tritiated 1,25-$(OH)_2D_3$ of 0.05 nmol was selected after due consideration of the K_d of 6 to 20 pmol found for the cultured breast cancer cells (10–13). With some tumor cytosols, binding was studied using 0.05 and 0.5 nM tritiated 1,25-$(OH)_2D_3$. There was no significant increase in specific binding using the higher radioligand concentration. Similarly, the temperature and time of incubation were selected as those required for the 1,25-DR from breast cancer cell cytosol to reach binding equilibrium. Longer times or higher temperatures apparently led to receptor degradation. Polyethylene glycol precipitation of bound hormone was used to effect bound and free separation (7–9). 1,25-$(OH)_2D_3$ receptor detection limit is 0.3 fmol/mg protein based on a statistically significant difference (≥250 dpm) between maximum and nonspecific binding in triplicate determinations and the lack of a significant effect of unlabeled 25-OH D_3 on tritiated 1,25-$(OH)_2D_3$ binding at that level.

Exchangeability of Occupied 1,25-DR. T47 D cytosol, prepared in the same buffer as for the tumors above, was incubated with 1.2×10^{-10} M tritiated 1,25$(OH)_2D_3$ with or without 7.7×10^{-8} M unlabeled 1,25$(OH)_2D_3$ for 2 hr at 25°. Following this preincubation, "maximum binding" and "nonspecific binding" cytosols were diluted 1:7 in buffer either alone or with 0.5×10^{-10} M or 1.2×10^{-8} M unlabeled 1,25-$(OH)_2D_3$. Finally, the diluted cytosols were incubated at 25° for 2 hr or at 4° for 16 hr. Bound hormone was precipitated with polyethylene glycol and counted as described above.

Contribution of DBP. DBP inevitably contaminates any human tissue obtained surgically. This binding protein is specific for 25-OH D_3 but has a significant although low affinity for 1,25-$(OH)_2D_3$. Hence, it is theoretically possible that it could contribute to the apparent level of 1,25-$(OH)_2D_3$ binding. In order to evaluate this possibility, 2 approaches were taken. In the first, serum was diluted 1:100 in tumor assay buffer, cytosol was prepared from a specimen of human striated muscle, and human serum was diluted 1:100 in the muscle cytosol. Each of these 3 preparations was incubated for 3 hr at 4° with 5×10^{-9} M tritiated 25-OH D_3 with or without 5×10^{-7} M unlabeled 25-OH D_3. Following this incubation, a polyethylene glycol precipitation was performed and counted as above on one set of tubes. With a second set of tubes, 0.5 ml of 0.15% dextran T-70:1.5% charcoal was added to each tube, which was mixed and centrifuged at 3100 rpm (2270 × g) for 25 min at 4°. The supernatant was decanted and counted with 10 ml of Instagel scintillant.

The second approach was to use excess unlabeled 25-OH D_3 as a competitor in the tritiated 1,25-$(OH)_2D_3$ binding studies on each tumor. 25-OH D_3 has a higher affinity for the DBP than does 1,25-$(OH)_2D_3$; however, in no cytosol with specific 1,25-$(OH)_2D_3$ binding did 25-OH D_3 compete at the concentration used, a 100-fold molar excess.

Other Steroid Receptor Assays. Tumors were trimmed and homogenized in 0.005 M sodium phosphate- 0.01 M Trizma buffer (pH 7.4) containing 0.0015 M EDTA, 0.0005 M dithiothreitol, and 10% glycerol. Homogenates were centrifuged at 50,000 × g for 30 min at 4° to yield a high-speed supernatant, aliquots of which were incubated for 19 hr at 4° with single near-saturating doses of tritiated steroid; duplicate determinations were made of total binding and of nonspecific binding in the presence of excess nonradioactive competitor. Estrogen receptor concentration was determined using 2 nM [^{3}H]estradiol ± 0.2 μM diethylstilbestrol; progesterone receptors were determined with 8 nM [^{3}H]R5020 ± 0.8 μM nonradioactive R5020; androgen receptors were determined with 8 nM [^{3}H]R1881 + 4 μM triamcinolone acetonide ± 0.8 μM nonradioactive R1881; and glucocorticoid receptors were determined with 10 nM [^{3}H]dexamethasone ± 1 μM nonradioactive dexamethasone. Steroid-receptor complexes were removed from solution at the end of incubation with hydroxylapatite, which was then washed twice with steroid-free medium, and the receptor-bound radioactivity was eluted with ethanol for counting.

RESULTS

1,25-DR and Other Steroid Receptor Concentrations. The patients from whom the breast tumors were obtained ranged in age from 20 to 84 years with a mean age of 56.4 ± 12.3 years (S.D.). Receptor for 1,25-$(OH)_2D_3$ was detected in 80% of these initial 54 tumors (Chart 1). Of the tumors in which 1,25-DR was not detectable, 3 of 12 were from premenopausal and 8 of 36 were from postmenopausal women. Six tumors were from perimenopausal women. For the entire group, the concentration of 1,25-DR was 1.9 ± 0.4 fmol/mg protein (S.E.); this was not different from 1.6 ± 0.3 and 1.6 ± 0.3 for the tumors from the pre- and postmenopausal women considered separately (Chart 2). There was no correlation between age and 1,25-DR concentration, nor was there any correlation between 1,25-DR concentration and that of the other steroid hormone receptors. Apart from the correlation of glucocorticoid and estrogen receptor concentrations ($r = 0.44$; $p < 0.01$), there were no significant correlations among the concentrations of the other steroid hormones or between any of those concentrations and the ages of the patients. When the data from the small premenopausal group (12 patients) were analyzed separately, 2 significant correlations emerged. The concentrations of estrogen receptors ($r = 0.62$; $p < 0.05$) and progesterone receptors ($r = +0.65$; $p < 0.05$) were found to correlate with the age of the premenopausal patients. As shown in Table 1, 1,25-DR was found in 2 renal cortical carcinomas

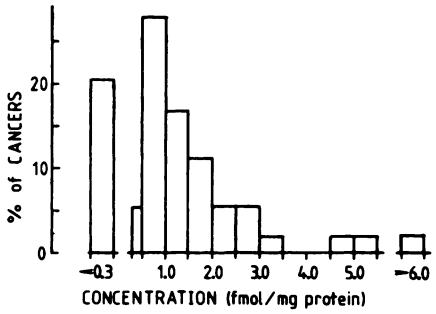

Chart 1. Concentration of 1,25-DR in breast cancer specimens. The number of tumors in each 1,25-DR concentration range is expressed as a percentage of the 54 tumors examined.

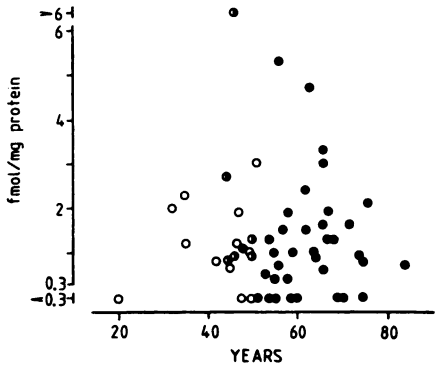

Chart 2. Age distribution of 1,25-DR concentration. Menopausal status of the patient is indicated: ○, premenopausal; ◑, perimenopausal; and ●, postmenopausal.

but was not found in a pleomorphic renal adenocarcinoma. Furthermore, 1,25-DR was found in only 3 of 19 (16%) unselected non-target organ tumors.

Partial Exchangeability of Occupied 1,25-DR. As seen in Chart 3, the 1,25-DR which had been prelabeled with tritiated 1,25-(OH)$_2$D$_3$ was relatively refractory to displacement with unlabeled 1,25-(OH)$_2$D$_3$. At a concentration (0.5×10^{-10} M) equivalent to that used for tritiated 1,25-(OH)$_2$D$_3$ in the normal assay, only 23% was displaced at 25° for 2 hr, and there was no displacement at 4° for 16 hr. Even at the relatively high concentration of 1.2×10^{-8} M unlabeled 1,25-(OH)$_2$D$_3$, only 50% and 20% displacement of tritiated 1,25-(OH)$_2$D$_3$ occurred.

Contribution of DBP. Specific binding of 25-OH-[^{3}H]D$_3$ in the serum, muscle cytosol, and cytosol-serum combination was demontrated using the dextran-charcoal separation method (Chart 4). However, when the polyethylene glycol method was used with the muscle cytosol preparation, there was no specifically bound 25-OH-[^{3}H]D$_3$ precipitated (Chart 4). Further, with the samples containing serum, the addition of unlabeled 25-OH D$_3$ actually increased the precipitated radioactivity. This could be explained by displacement of radioactivity from the DBP onto some other low-affinity, high-capacity binding site which is precipitated by polyethylene glycol.

Table 1

1,5-(OH)$_2$D$_3$ receptors in various tumors

Tumor origin and histology	1,25-(OH)$_2$D$_3$ receptor concentration (fmol/mg protein)
Gastrointestinal	
Squamous cell carcinoma	
Tongue (1)[a]	1.2
Esophagus (1)	0
Anal canal (1)	0
Adenocarcinoma	
Stomach (2)	0
Cecum (1)	0.4
Colon (5)	0
Rectum (2)	0
Gall bladder-liver metastasis (1)	0.5
Genitourinary tract	
Renal pelvis, transitional-cell carcinoma (1)	0
Prostate, carcinoma (2)	0
Prostate, benign hypertrophy (2)	0
Renal	
Cortical carcinoma	
Clear cell (1)	0.3
Clear cell (1)	0.3
Pleomorphic (1)	0

[a] Numbers in parentheses, number of tumors examined in each group.

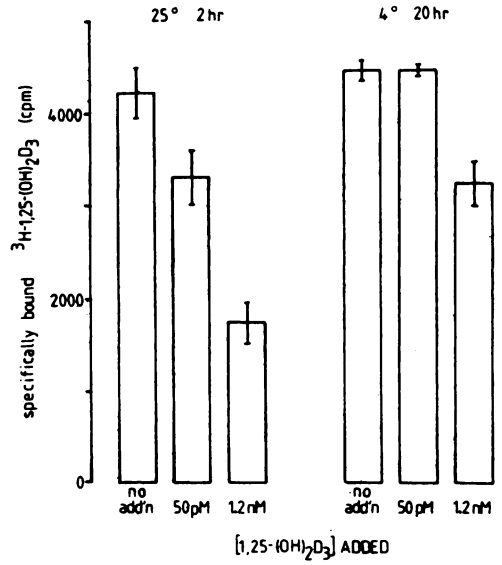

Chart 3. Partial exchangeability of occupied 1,25-DR. T47 D breast cancer cell cytosol was preincubated with tritiated 1,25-(OH)$_2$D$_3$ to occupy receptors. Subsequently, the cytosol was diluted and reincubated either alone or with concentrations of unlabeled 1,25-(OH)$_2$D$_3$ at 25° for 2 hr or at 4° for 16 hr as indicated. Persisting specific binding is expressed in cpm/incubation. Values are means of triplicate determinations; *bars*, S.E.

DISCUSSION

Before discussing the possible significance of the data presented, it is important to consider 2 possible methodological problems. The first problem is the potential contribution to 1,25-(OH)$_2$D$_3$ binding of DBP contamination. This can be dismissed on 2 grounds. (*a*) The DBP is not precipitated by polyethylene glycol as shown here. (*b*) Presumably because of the preceding behavior of DBP, unlabeled 25-OH D$_3$ at relatively low (100-fold) molar excess does not compete for tritiated 1,25-(OH)$_2$D$_3$ binding.

The second problem is that the levels of 1,25-DR are relatively low in the primary breast tumors when compared with either the levels of other steroid receptors in the same primary breast cancers or the 1,25-DR levels in the cultured human breast cancer cell lines. This discrepancy has several possible explanations. The first reason could be that the tumors were obtained from vitamin D-replete patients; hence, much of the 1,25-DR could already be occupied, and this 1,25-DR would be systematically underestimated because of incomplete exchange. We estimated the ability of low concentrations of unlabeled 1,25-(OH)$_2$D$_3$ to displace tritiated 1,25-(OH)$_2$D$_3$ which had already bound to the receptor. This situation is exactly analogous to the normal assay conditions where the same concentration of tritiated 1,25-(OH)$_2$D$_3$ is used to displace 1,25-(OH)$_2$D$_3$ from *in vivo* occupied receptors. On the basis of these studies, it can be estimated that only 20 to 25% of occupied receptors may be detected. By contrast, many of the patients from whom these tumors were obtained were post-

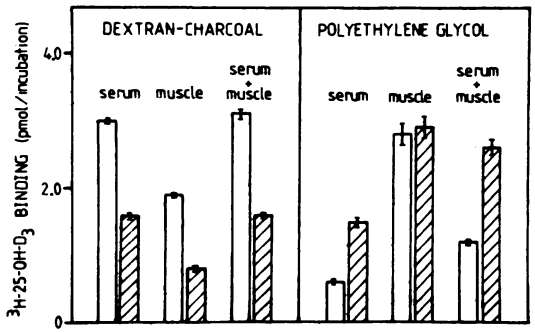

Chart 4. 25-OH-[^{3}H]D$_3$ binding as measured by charcoal-dextran and polyethylene glycol. Diluted human serum, human striated muscle cytosol, and a mixture of these 2 were incubated with 25-OH-[^{3}H]D$_3$ with or without excess unlabeled 25-OH D$_3$. 25-OH-[^{3}H]D$_3$ radioactivity, which did not bind to dextran-charcoal (*left*) or which was precipitated by polyethylene glycol (*right*), is expressed in pmol/incubation. Binding in the presence of 100-fold molar excess of unlabeled 25-OH D$_3$ is shown in the *hatched bar* in each case. Values are means of triplicate determinations; *bars*, S.E.

menopausal, so that conditions for detecting estrogen receptor are optimal. A second reason could be the heterogeneity of the cells in a primary cancer compared with the relative homogeneity of cultured breast cancer cells. This could not explain the discrepancy between actual amounts of the 1,25-$(OH)_2D_3$ and other steroid receptors in the same tumor specimens, but it could account for the difference between the 1,25-DR levels in the cultured cells and primary breast cancers. Finally, it may be relevant that the breast cancer cell lines are derived from metastatic cells. Since metastatic growth probably requires special properties (21), it may be that 1,25-DR-positive cells are more able to metastasize and hence have been selected.

For any tumor to grow in bone, it is clear that the surrounding bone in its path must be destroyed. The resorption may be mediated by activation of adjacent osteoclasts (14), for example, by tumor-derived prostaglandins (1, 5, 17, 20). It has also been suggested that tumors might invade bone without the mediation of osteoclasts (5, 16), and furthermore, breast cancer cells have been shown to resorb devitalized bone in culture (6). The discovery of receptors for 1,25-$(OH)_2D_3$ in breast cancers indicates that these tumors might be responsive to the hormone. It has yet to be established whether 1,25-$(OH)_2D_3$ has any effect on the bone-resorbing ability of these cells. The frequency of 1,25-DR in this initial series of breast cancer is high, and it seems unlikely that 1,25-DR is simply a marker of cancer in view of the relatively low incidence of 1,25-DR in the other cancers examined.

The lack of correlation of 1,25-DR concentration with the age or menopausal status of the patient or with the levels of the other steroid hormone receptors suggests that the receptor is not dependent on the presence or effect of those other hormones for its activity. This is in particular contrast with mouse calvarial cells, where glucocorticoids have been suggested to be necessary for the maintenance of 1,25-DR and effects of 1,25-$(OH)_2D_3$ in culture (19, 23). Although both 1,25-DR and bony metastases are common in breast cancer, it is not possible to correlate these 2 parameters in a retrospective study. It will be most important to determine the 1,25-DR status of bony metastases of breast and other metastatic cancers. However, there are no data on the presence or absence of 1,25-DR in the breast cancer cells of bony metastases, and such data will be extremely difficult to obtain. For these reasons, it was essential to start a prospective study on the relationship between 1,25-DR concentration and the clinical course of the patient, with particular reference to the occurrence of bony metastases and hypercalcemia in breast cancer. The patients described in this paper are the initial group of patients being followed in this way.

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