Measurement of 1,25-Dihydroxyvitamin D₃ Receptors in Breast Cancer and Their Relationship to Biochemical and Clinical Indices[1]

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ABSTRACT

Both normal and malignant breast tissue contain the specific receptor for 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$). A recent study has shown its presence in 80% of surgically removed breast tumors, although only at low levels. We have measured the 1,25-$(OH)_2D_3$ receptor in breast tumors from 68 patients and have found it at similar frequency (75%) but at much higher concentrations (range, <1 to 30 fmol/mg protein). This receptor has the same characteristics as that measured in classic 1,25-$(OH)_2D_3$ target tissues and was distinguished by sucrose gradient centrifugation from plasma contaminants. Complete case histories and follow-up were available on 56 of these patients, and 1,25-$(OH)_2D_3$ receptor status (<8 or ≥8 fmol/mg protein) was not related to the level of estrogen receptors, menopausal status, T-stage or histology of tumors, or presence of ^{99m}Tc phosphate hot spots on bone scans. The lack of relationship betwen the level of 1,25-$(OH)_2D_3$ receptors and other prognostic indicators suggests its potential as a new independent variable for assessing breast cancer patients. However, at this stage, 1,25-$(OH)_2D_3$ receptor status did not result in any significant difference in probability of survival or metastasis-free survival. Assessment of the importance of this variable for treatment or outcome must await an increased number of patients and a longer time since surgery.

INTRODUCTION

Breast cancer is characterized by its high incidence, poor prognosis and, in at least 80% of advanced cases, secondary bone involvement (14, 16). The measurement of steroid receptors has become a routine part of the management of the disease, and the presence of estrogen receptors has been correlated with favorable response to endocrine therapy and improved survival prospects (18, 20, 30).

Specific binding of 1,25-$(OH)_2D_3$[4] was first demonstrated in breast using a continuous human breast cancer cell line (MCF7) (7). It has since been found in other human breast cell lines (9) and recently, at an incidence of 80% in a series of surgically removed breast tumors (8).

The 1,25-$(OH)_2D_3$ receptor is also found in lactating (but not virgin) breast tissue (16) where, as in its classic target tissues of intestine, bone and kidney (25), it appears to mediate the trans-

epithelial transport of calcium (13). The effects of 1,25-$(OH)_2D_3$ on neoplastic breast tissue in vivo are unknown, although in vitro we have found that physiological doses of 1,25-$(OH)_2D_3$ stimulate the growth of a human breast cancer cell line (T47D) (10), whereas high concentrations of the hormone inhibit cell division (5, 11).

The previous study on the frequency of the 1,25-$(OH)_2D_3$ receptor in human breast cancer detected the receptor in 80% of the tumors examined but found no relationships between its presence and any of the other parameters examined, including other steroid hormone receptors (8). However, the 1,25-$(OH)_2D_3$ receptor was detected only at extremely low levels (mean concentration, 1.9 ± 0.4 (S.D.) fmol/mg protein) using a single saturating dose assay. We have improved the techniques for cytosol preparation, measured the 1,25-$(OH)_2D_3$ receptor by saturation analysis, and have found much higher concentrations. We have therefore reexamined the relationship between the level of the receptor and other biochemical and clinical indices.

MATERIALS AND METHODS

[^3H]-1,25-$(OH)_2D_3$ (specific activity, 148 to 163 Ci/mmol), [^3H]-25-OHD₃ (specific activity, 110 Ci/mmol), and [2,4,6,7-^3H]estradiol (specific activity, 85 to 110 Ci/mmol) were purchased from Amersham International, Buckinghamshire, England. Nonradioactive 25-OHD₃ and 1,25-$(OH)_2D_3$ were the gift of Dr. R. S. B. Ehsanullah, Roche Products, Welwyn Garden City, Hertfordshire, England. All reagents were of analytical grade and were obtained through commercial suppliers.

Patients. Tumors were obtained for 1,25-$(OH)_2D_3$ receptor analysis from 68 patients with primary breast cancer. Estrogen receptor analysis was carried out on most of these samples, and full history, staging, histology, and follow-up were available in 56 patients.

Patients presenting with primary breast cancer were staged using a standard set of investigations (3) including ^{99m}Tc phosphate bone scanning and treated by mastectomy or local excision and radiotherapy. They were followed up at 3-month intervals until metastatic disease was present.

Tumors. Breast tumors were snap frozen in liquid nitrogen upon removal, and stored in liquid nitrogen for up to 1 year before assay. The only requirements for inclusion in the study were that samples should have histological confirmation of the presence of breast carcinoma tissue and be at least 1 g in weight. This enabled receptor measurements for both 1,25-$(OH)_2D_3$ and estrogen to be made.

1,25-$(OH)_2D_3$ Receptor Assay. Samples were chopped with a blade over ice while still frozen and freeze-crushed after cooling in liquid nitrogen using a Braun Mikrodismembrator (F. T. Scientific Instruments, Tewkesbury, Gloucestshire, England). The resultant powder was weighed and taken up in 3 ml ice-cold Tris buffer [10 mM Tris-HCl, pH 7.4 at 4°, 0.3 M KCl, 1 mM (ethylenebis[oxyethenenitrile])tetraacetic acid, 1.5 mM dithiothreitol, 10 mM sodium molybdate, and Trasylol (200 KIU/ml)]. Cytosols were prepared by centrifugation at 88,000 × g for 1 hr at 4° in the Ti70 rotor of a Beckman L8-70 ultracentrifuge. Protein was estimated by the method of Lowry (21).

1,25-(OH)₂D₃ receptor concentration was determined by saturation analysis as described previously (22). Briefly, 200-μl aliquots of cytosol were incubated for 2 hr at 25° in duplicate with increasing amounts of [³H]-1,25-(OH)₂D₃ (final concentration, 50 to 750 pM) in the presence or absence of 100-fold excess of nonradioactive hormone. No significant degradation of receptor occurred at this temperature. A similar excess of 25-OHD₃ was used in parallel incubations to ensure specificity of displacement for 1,25-(OH)₂D₃. Free hormone was pelleted with dextran-coated charcoal, and bound radioactivity was determined by liquid scintillation counting. Eel liver, a 1,25-(OH)₂D₃-receptor-positive tissue (22) was included in each assay as a high pool.

Discontinuous 5 to 20% sucrose gradients (w/v, in the same Tris buffer) were prepared, allowed to equilibrate at room temperature, and cooled on ice before use. Tumor cytosols were labeled with [³H]1,25-(OH)₂D₃ or [³H]-25-OHD₃ with or without 100-fold excess nonradioactive competitors and applied to the gradients as described previously (22). They were centrifuged at 288,000 × g for 14 hr at 4° in the SW55 Ti rotor of a Beckman L8-70 ultracentrifuge.

Estrogen Receptor Measurement. Estrogen receptors were quantitated by a modification of the method of McGuire et al. (23). Aliquots of breast tissue cytosol were incubated with known concentrations of [³H]estradiol in the presence or absence of excess diethylstilbestrol and unbound steroid absorbed by dextran-coated charcoal. The results were computed using regression analysis and Woolf linearization (19). Using this assay it has been found that a patient with estrogen receptor <10 fmol/mg cytosol protein is unlikely to respond to endocrine therapy (2), and this is defined as an estrogen receptor negative. Patients with tumors containing receptor levels ≥30 fmol/mg protein frequently respond to such treatment.

Measurement of Both Receptors on the Same Cytosol. Usually the tumors were separated into 2 pieces for measurement of 1,25-(OH)₂D₃ and estrogen receptors. However, to guard against the inevitable heterogeneity of each sample, 20 tumors were first homogenized by freeze-crushing as described above, and the resultant powders were divided into 2 and kept over liquid nitrogen until assay. They were reconstituted in the appropriate buffer and, specific binding of each hormone was measured as before.

Statistical Methods. The relationship of 1,25-(OH)₂D₃ receptor with estrogen receptor was assessed using a product-moment correlation coefficient (after transforming the data logarithmically); the relationship of 1,25-(OH)₂D₃ receptor status to other prognostic factors was examined using simple contingency tables. Overall survival and metastasis-free survival in the 1,25-(OH)₂D₃ receptor-positive and -negative groups were examined using life tables (actuarial survival curves), and the 2 groups were compared using the logrank test (26).

RESULTS

1,25-(OH)₂D₃ Receptor Assay. 75% of the 68 tumor cytosols assayed contained greater than 1 fmol/mg protein of the 1,25-(OH)₂D₃ binding protein, as determined by Scatchard analysis of saturation plots (Table 1). The equilibrium dissociation constant for this binding protein was $1.72 \pm 1.53 \times 10^{-10}$ M. Eel liver was included in each assay as a high pool, and analysis of values obtained for 1,25-(OH)₂D₃ receptor concentrations in these cytosols showed an interassay coefficient of variation of 25% (R_C = 37.0 ± 9.2 fmol/mg protein; n = 13).

In no case was 25-OHD₃ in 100-fold excess able to compete with [³H]1,25-(OH)₂D₃ for binding in these cytosols. Sucrose gradient analysis of [³H]-1,25-(OH)₂D₃-labeled cytosols showed that binding was to a single 3.7S peak, which could be abolished by coincubation with 100-fold excess 1,25-(OH)₂D₃ but not 25-OHD₃ (Chart 1). When cytosols were similarly incubated with [³H]-25-OHD₃, a large peak of radioactivity was found in the 6S

Table 1
Concentration of 1,25-(OH)₂D₃ receptors in breast tumor cytosols

Cytosols (200 μl; 0.5 to 2 mg protein) were incubated in duplicate for 2 hr at 25° with increasing amounts (0.01 to 0.13 pmol) of [³H]-1,25-(OH)₂D₃ in the absence or presence of 100-fold excess nonradioactive hormone. Scatchard analysis (28) of specific binding was used to generate the receptor concentrations (R_C).

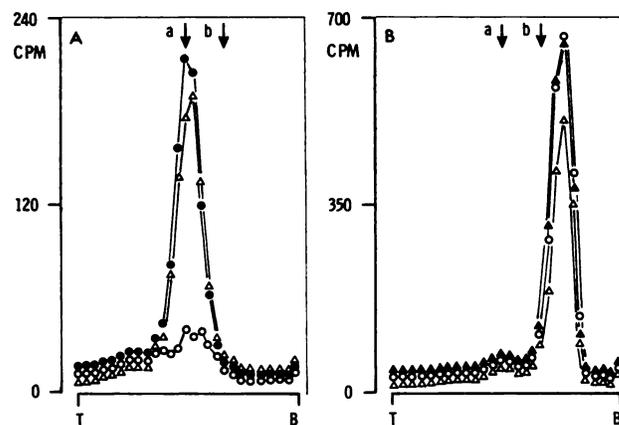| R_C fmol/mg protein	No. of tumors	%
1	17	25
2–4	7	10
4–8	16	24
8–15	15	22
15–30	13	19
Total	68	100

Chart 1. Sucrose gradient analysis of [³H]-1,25-(OH)₂D₃ and [³H]-25-OHD₃ binding in human breast cancer cytosols. Cytosols were incubated with (A) [³H]-1,25-(OH)₂D₃, or (B) [³H]-25-OHD₃ alone (●, ▲), or with 100-fold excess nonradioactive 1,25-(OH)₂D₃ (O) or 25-OHD₃ (△). Unbound steroid was precipitated with charcoal, and the labeled cytosols were applied to 5 to 20% sucrose gradients and spun at 288,000 × g for 14 hr at 4°. The gradients were divided into 150-μl fractions from the bottom and counted by liquid scintillation. T, top; B, bottom fraction. Sedimentation markers, run in parallel gradients, were [¹⁴C]ovalbumin (3.7S;a) and [¹⁴C]bovine serum albumin (4.4S;b).

region (Chart 1). This peak was slightly reduced by coincubation with excess 25-OHD₃, but 1,25-(OH)₂D₃ produced no ablative effect.

Relationship to Estrogen Receptor Content. 63% of the tumors examined were classed as estrogen receptor positive (R_C > 10 fmol/mg protein). No correlation was observed between the levels of the 1,25-(OH)₂D₃ and estrogen receptors in these tumors (Chart 2). Similarly, no relationship was observed between either receptor in the 20 tumors which were homogenized prior to division for assay. Consequently, these have been included in the larger group.

Relationship to Other Indices. Complete histories and follow-up were available in 56 of the original 68 cases, and so possible relationships between 1,25-(OH)₂D₃ receptor status and other factors were investigated using this number of patients. The 56 cases were divided into 1,25-(OH)₂D₃ receptor-positive and -negative groups at a cutoff point of 8 fmol/mg protein, chosen on grounds of assay precision. This division gave roughly equal numbers in each group. No relationships were found between 1,25-(OH)₂D₃ receptor status and menopausal status, tumor histology (infiltrating ductal or other), T-stage of tumor, number of positive nodes, or presence of hot spots on bone scans (Table 2). Similar results were obtained if higher or lower cutoff values of receptor concentration were used (data not shown).
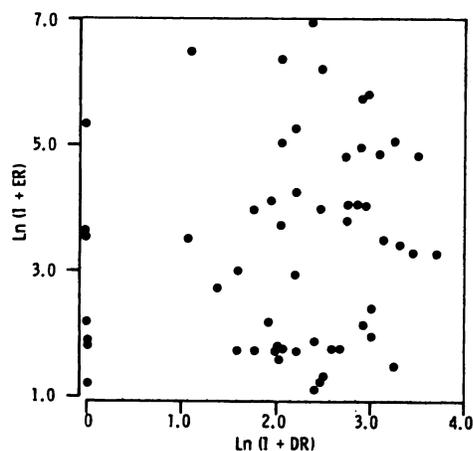
Chart 2. Relationship between receptor levels for estrogen and 1,25-(OH)₂D₃ in human breast cancer cytosols. Cytosolic concentrations of the receptors were determined as described in "Materials and Methods" and then transformed logarithmically. *ER*, estrogen receptor concentration; *DR*, 1,25-(OH)₂D₃ receptor concentration, both in fmol/mg protein. There is no significant correlation between the levels of the 2 receptors, $r = 0.16$.

Table 2

Relationship of 1,25-(OH)₂D₃ receptor status to other parameters

Patients and tumors were assessed as described by Coombes et al. (2).

Variable	Total	1,25-(OH)₂D₃ receptor content (fmol/mg protein)	
		<8	≥8
Menopausal status			
Premenopausal	21	10	11
Postmenopausal	35	17	18
T-stage			
T0–T2	42	18	24
T3–T4	11	7	4
Unknown	3	2	1
Histology, primary			
Infiltrating ductal	40	18	22
Other	16	9	7
Nodes			
Negative	26	12	14
Positive	24	11	13
Unknown	6	4	2
Hot spots on bone scan			
None	46	20	26
>1	6	4	2
Unknown	4	3	1

Prognosis. We have examined the relapse rate of patients since the biopsy of the primary tumor. Over a 2-year period, 17 patients have relapsed, 11 of whom had 1,25-(OH)₂D₃ receptor <8 fmol/mg protein, and 6 had a content of >8 fmol/mg protein. Four patients have relapsed with skeletal metastases so far, 2 in each group. Only 8 patients have died, 6 of whom had 1,25-(OH)₂D₃ >8 fmol/mg protein. The logrank test showed no significant difference in overall survival ($p = 0.21$) between the 1,25-(OH)₂D₃ receptor-positive and -negative groups.

The survival curves are shown in Chart 3. In addition, no significant difference in survival or metastasis-free survival was seen by taking levels of 1,25-(OH)₂D₃ receptor higher or lower than 8 fmol/mg protein as the cutoff point between positive and negative groups (data not shown).
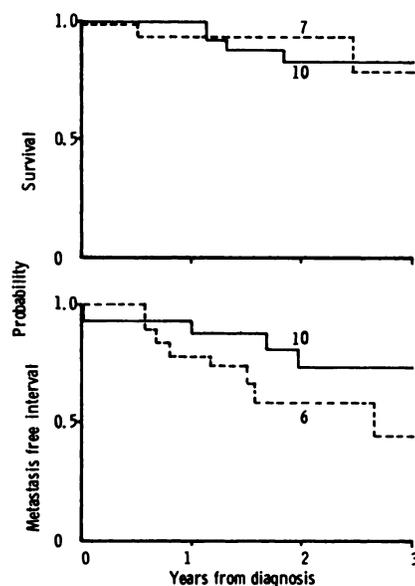

Chart 3. Relationship between 1,25-(OH)₂D₃ receptor status and actuarial survival in human breast cancer patients. Cytosolic concentration of 1,25-(OH)₂D₃ receptor was determined as described in "Materials and Methods," and then patients were divided into 1,25-(OH)₂D₃ receptor positive (——, $R_c > 8$ fmol/mg protein) and 1,25-(OH)₂D₃ receptor negative (‐ ‐ ‐ ‐, $R_c < 8$ fmol/mg protein). Probability of survival and metastasis-free survival was determined using actuarial survival curves, and the 2 groups were compared using the logrank test. There was no significant difference in either parameter between the 2 groups ($p = 0.21$). The numbers indicate the number of patients within each group after 2 years. The median follow-up on all patients was 1.6 years.

DISCUSSION

Our results confirm that the 1,25-(OH)₂D₃ receptor is present in human breast tumors and at concentrations higher than was thought previously. Use of Scatchard analysis has shown that this binding protein has an equilibrium dissociation constant very similar to those measured in numerous human and other tissues (1, 6, 10, 22, 25).

The binding protein is selective for 1,25-(OH)₂D₃ and appears as a single 3.7S peak upon sucrose gradient centrifugation. This eliminates the possibility that the binding seen was due to the DBP, which forms a 6S complex with intracellular actin upon tissue homogenization (29). Incubation of cytosols with [³H]-25-OHD₃ showed that the 6S protein was present and in relatively high amounts in comparison with the 1,25-(OH)₂D₃ receptor but that under the conditions used the dihydroxylated metabolite was not bound to it. It is known that human DBP has a lower affinity for 1,25-(OH)₂D₃ than it does for 25-OHD₃ (17), and it appears that the tracer amounts of [³H]-1,25-(OH)₂D₃ used in these studies were not bound by the plasma vitamin D-binding protein-actin complex. This is not the case in rodents, where the 6S protein can mask low concentrations of cytosolic receptor (1). Thus, 1,25-(OH)₂D₃ receptor measurements can be performed in human normal and neoplastic tissue cytosols, without interference from plasma contamination.

The higher levels of receptor as measured here in comparison to the study of Eisman et al. (8) are probably attributable to improved recovery due to revised methods of cytosol preparation. Freeze-crushing can produce complete cellular disruption without any elevation of temperature. Other techniques may result in underestimation of the amount of 1,25-(OH)₂D₃ receptor

present due to incomplete homogenization of the tough tissue together with inactivation of the labile receptor by local heating. Like other steroid hormone receptors, the unoccupied 1,25-$(OH)_2D_3$ receptor is unstable, and it seems inevitable that the levels finally measured in a cytosol preparation reflect both the intrinsic receptor content of the tissue and the degree of degradation that occurs during homogenization and assay. However, given the heterogeneity of tumor samples, the levels of receptor measured here are appropriate, relative to those seen both in other 1,25-$(OH)_2D_3$ target tissues (1, 22, 25) and in breast cancer cell lines (7, 9, 11). Standardization of measurement techniques could speed the assessment of the importance of this parameter.

Estrogen receptors are routinely measured in surgically removed breast tumors. Patients with receptor-positive tumors will probably respond to endocrine therapy (23, 24) and will also survive longer (20, 30) than will those with estrogen-receptor-negative lesions. In common with Eisman *et al.* (8), we found no correlation between the levels of available receptors for estrogen and 1,25-$(OH)_2D_3$. In addition, we have shown that this lack of correlation cannot be ascribed to heterogeneity of individual tumors. There are other important characteristics of patients with primary breast cancer such as menopausal status, isotopic bone scan results, and tumors-nodes-metastasis staging and histological type of the tumor itself.

These parameters were all monitored on 56 patients in this study, and no relationship between them and 1,25-$(OH)_2D_3$ receptor status was found. Thus, although the number of patients is as yet relatively small, it appears that 1,25-$(OH)_2D_3$ receptor status represents a new and independent characteristic of breast tumors.

We have attempted to assess the importance of this characteristic by examining the probabilities of survival and metastasis-free survival in patients with 1,25-$(OH)_2D_3$ receptor-positive and -negative tumors. No difference in either parameter was seen, but given the relatively short time since primary surgery and the number of these patients this is inconclusive. Large differences in survival or metastasis-free survival would not be inconsistent with this data. Therefore, it is too early to say whether the presence of 1,25-$(OH)_2D_3$ receptors in their tumors influences survival or metastasis-free survival in breast cancer patients.

The pattern of development of metastases is a nonrandom event and may depend on properties of the original tumor cells which enable them to grow in selected tissue environments (27). Breast tumors metastasize with high frequency to bone, and it has been suggested that tumor invasion of bone involves both activation of osteoclasts and direct resorption by tumor cells (15). Certainly, breast cancer cell lines are capable of resorbing devitalized bone *in vitro* (4). In addition, such cell lines have been shown to be 1,25-$(OH)_2D_3$ receptor positive (9).

It is possible that the peculiar ability of these cells to grow in bone may be related to their possession of this receptor. It is not known whether the transformed cells also possess other parts of the poorly understood calcium-transporting mechanism that exists in classic vitamin D target tissue such as intestine, bone, kidney (25), and probably breast (13). Interestingly, the VX_2 carcinoma, a rabbit tumor which invades bone and is frequently used as a model for bone metastases (15) also possesses the 1,25-$(OH)_2D_3$ receptor protein (12).

We have shown that near-physiological concentrations of 1,25-$(OH)_2D_3$ stimulate the growth of a human breast cancer cell line (T47D) in culture (10). Higher doses of hormone inhibit growth

(11), and similar inhibition has been reported by Eisman *et al.* (5). Although *in vivo* effects remain unknown, it may be that the levels of 1,25-$(OH)_2D_3$ receptor present may enable us to gain some insight into new approaches for therapy of human breast tumors.

ACKNOWLEDGMENTS

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