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Calcitonin and 1,25-Dihydroxyvitamin D_3 Receptors in Human Breast Cancer Cell Lines[1]

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ABSTRACT

Five human breast cancer cell lines (MCF 7, T 47D, BT 20, MDA 157, and MDA 231) and a human breast epithelial cell line (HBL 100) have been found to contain specific high-affinity receptors for 1,25-dihydroxyvitamin D_3. K_d values ranged from 0.6 to 2.0 × 10^{-11} M and receptor concentration from 31 to 150 fmol/mg cytosol protein. Two of the breast cancer lines (MCF 7 and T 47D) contain specific high-affinity receptors for calcitonin and a calcitonin-responsive adenylate cyclase, which have been characterized with the aid of salmon, eel, and human calcitonins and in several substituted analogues of human calcitonin. The 1,25-dihydroxyvitamin D_3 receptor may reflect a normal property of the breast cell. Breast cancer cell lines provide a useful source of 1,25-dihydroxyvitamin D_3 receptors. Their coexistence with a calcitonin receptor and biological response in some breast cancers offers the opportunity to investigate new aspects of breast cancer endocrinology.

INTRODUCTION

A human breast cancer cell line (MCF 7) has been found to have a specific calcitonin receptor and calcitonin-responsive adenylate cyclase, which we have characterized (14). The same cell line was also found to possess a specific macromolecular cytosol receptor for the calcium-transporting hormone, 1,25-$(OH)_2D_3$[3] (4, 5). Examination of a small series of breast cancers obtained at surgery suggested that 1,25-$(OH)_2D_3$ receptors might be common in this cancer, and the receptors were found also in pregnant and lactating rabbit breast (3). These observations led to the present study in which a series of 5 well-characterized human breast cancer cell lines (including MCF 7) and a human breast epithelial cell line (HBL 100) were investigated. In all cases, specific high-affinity receptors for 1,25-$(OH)_2D_3$ have been found and, in 2 cancer lines, receptors for calcitonin associated with a calcitonin-responsive adenylate cyclase were present.

MATERIALS AND METHODS

Cells and Cell Culture. The breast cancer cell lines BT 20, T 47D, MDA 157, and MDA 231 and the human breast epithelial cell line HBL 100 were obtained from the Breast Cancer Task Force, Washington, D. C., and the MCF 7 cells were obtained from Dr. C. McGrath, Michigan Cancer Foundation. The human lung cancer line (BEN cells) was grown as described previously (6, 9). Breast cell lines were grown in plastic culture bottles in Roswell Park Memorial Institute Tissue Culture Medium 1640 (Commonwealth Serum Laboratories, Parkville, Australia) containing 10% fetal calf serum, 10^{-7} M insulin (Novo porcine), and 10^{-7} M hydrocortisone hemisuccinate. Subculture was carried out by washing the cells, incubating for 1 to 2 min in 0.0125% trypsin in 0.02% EDTA/0.9% NaCl solution, shaking the cells free and washing in complete medium.

Adenylate Cyclase. Cell cultures at confluence were used in all assays. Cells were washed twice with Dulbecco's phosphate-buffered saline and once with 25 mM Tris-HCl, pH 8.2, containing 0.25 M sucrose, 1 mM dithiothreitol, and 1 mM EDTA. After the cells were scraped from the surface with a plastic policeman, they were homogenized with 6 strokes of a hand-held all-glass homogenizer in 1 ml of the same buffer, and the homogenate was used directly in the assay. All other additions to the adenylate cyclase assay were prepared in a buffer consisting of 25 mM Tris-HCl (pH 8.2), 4.5 mM Mg^{2+}, 30mM K^+, and 0.1% bovine serum albumin. Incubations of 100 μl were carried out with 0.1 mM [α-^{32}P]ATP (Radiochemical Centre, Amersham, United Kingdom) as substrate for min at 37°, using an ATP-regenerative system of phosphoenolpyruvate and pyruvate kinase. Cyclic AMP (0.1 mM) was included to inhibit breakdown of labeled cyclic AMP. The reaction was terminated by simultaneous boiling and addition of excess unlabeled ATP. The labeled product was purified by chromatography on Dowex and alumina as described previously (6, 10, 13). Protein concentrations were measured by the method of Hartree (8).

Calcitonin Binding. Experiments were carried out in intact cells, as described for the study of calcitonin receptors in a human lung cancer cell line (6). After standard subculture procedure and 2 extra washes in Dulbecco's phosphate-buffered saline, cells were resuspended in a buffer containing 15 mM Tris, 120 mM NaCl, 4 mM KCl, 1.6 mM $MgSO_4$, 2 mM NaH_2PO_4, 10 mM glucose, and 0.1% human serum albumin, (pH 7.4). Cells were counted in a hemocytometer. Binding was carried out in 10-ml plastic tubes in a total volume of 0.5 ml (300 μl of cell suspension, 20,000 to 40,000 cpm of ^{125}I-labeled salmon calcitonin, and varying concentrations of unlabeled peptides). Incubations were carried out in duplicate at 20° for 1 h with continuous agitation. Duplicate 200-μl samples were taken from each tube and layered onto 100 μl of chilled buffer in microfuge tubes which were centrifuged at 10,000 × *g* for 2 min in a Beckman Model B microfuge. The tips containing the pellets were excised, and bound and free radioactivity was measured in a Packard Automatic scintillation well counter with an efficiency of 70%.

Synthetic salmon calcitonin (gift of Armour Pharmaceutical

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[3] The abbreviations used are: 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3 (1, 25-dihydroxycholecalciferol); cyclic AMP, cyclic adenosine 3′:5′-monophosphate.

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Co., Kankakee, Ill.) was labeled with ^{125}I as described previously (6, 9), using 2 μg chloramine-T for 10 sec. Purified labeled hormone was stored in 25% acetone in 1% acetic acid in small samples and could be used in binding experiments for at least 4 weeks when stored at −20°. Synthetic human calcitonin and all its analogs were provided by Dr. W. Rittel, Ciba-Geigy, Basel, and [aminosuberic acid^{1,7}]-eel calcitonin was provided by Dr. J. Murase, Toyo Joyo Company, Japan.

1,25-$(OH)_2D_3$ Binding. Cells were grown for at least 24 hr before study at confluence in medium containing no added insulin or cortisol and fetal calf serum which had been treated with charcoal to remove steroid hormones (11). Cell monolayers were washed with Dulbecco's phosphate-buffered saline and scraped into 0.05 M phosphate buffer, pH 7.25, containing 0.4 M KCl, 0.002 M dithiothreitol, 0.1% gelatin, and 1000 kIU of Trasylol per ml. After sonication for 60 sec at 20 kHz and 50 watts, the high-speed supernatant was obtained by centrifugation at 300,000 × g_{max} in the SM 50.1 head of a Beckman L5-75 ultracentrifuge. One-ml cytosol preparations (from about 10^6 cells), adjusted to 0.2 M KCl, were incubated with 0.04 pmol of 1,25-dihydroxy[23,24-^3H]cholecalciferol (110 Ci/mmol; Radiochemical Centre) for 2 hr at 25°. Bound hormone was separated from free hormone by precipitation with polyethylene glycol (2, 4) and counted after solubilization in Soluene 350.

Scatchard analysis was carried out using varying amounts of ^3H-labeled 1,25-$(OH)_2D_3$ from 0.005 to 0.05 pmol. Nonspecific binding tubes were identical except for the addition of 0.1 nmol of unlabeled 1,25-$(OH)_2D_3$. For each breast cell line, the effect of other steroids (testosterone, estradiol, progesterone, cortisol) was tested by addition of up to 10^{-4} M.

RESULTS

In all cases, specific high-affinity receptors for 1,25-$(OH)_2D_3$ were found (Table 1). The affinity constants were uniform among the various cell lines and low enough to be consistent with the possibility of an *in vivo* action of circulating 1,25-$(OH)_2D_3$. Scatchard analysis of binding of 1,25-$(OH)_2D_3$ to cytosol from the breast cancer lines indicated a range of receptor concentrations which was of the same order as that found in chicken intestinal cytosol. The highest concentration of receptors was found in cytosol from the T 47D cell line (Chart 1). The other major metabolites of vitamin D_3, 25-hydroxyvitamin D_3 and 24,25-dihydroxyvitamin D_3, competed for binding to cytosol receptor from each cell line with the same relative efficacy as found with MCF 7 cells (4) and chicken intestinal cytosol receptor (2). In all cases, the unrelated steroids, testosterone, estradiol, progesterone, and cortisol showed no competition for binding at concentrations of up to 10^{-4} M. Ultracentrifugal analysis of cytosol preparations after incubation with ^3H-labeled 1,25-$(OH)_2D_3$ indicated a 3.7S macromolecular receptor identical with that found for MCF 7 cells (4).

MCF 7 cells had been shown to contain calcitonin receptors and a calcitonin-responsive adenylate cyclase (14). Each of the cell lines was screened by studying the effect of salmon calcitonin upon adenylate cyclase (Table 2). Preparations from T 47D cells responded with a pronounced increase in adenylate cyclase activity, which was concentration dependent, and the relative effects of salmon *versus* human calcitonin paralleled their effects in other biologically responsive systems (Chart 2). Of the other cell lines tested, none showed any consistent increase in adenylate cyclase activity in response to calcitonin (Table 2). The most comprehensive analysis of effects of various calcitonins and analogs on adenylate cyclase in breast cancer cells was carried out using MCF 7 cells (Table 3), in which a comparison was made with BEN cells, a human lung cancer cell line the calcitonin receptor and adenylate cyclase of which have been characterized in detail (6, 9). In this assessment and in the analysis of the comparative binding competition data also shown in Table 3, there is a satisfactory agreement between the 2 cell lines, and the relative potencies

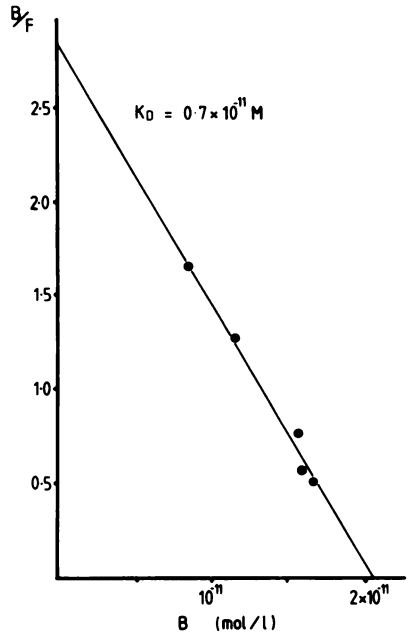

Chart 1. Scatchard analysis of binding of 1,25-$(OH)_2D_3$ to cytosol from T 47D cells (see "Materials and Methods"). Receptor concentration was 150 fmol/mg cytosol protein.

Table 1

1,25-$(OH)_2D_3$ receptors in breast cell lines

Cell line	K_d (× 10^{-11} M)	R_C (fmol/mg cytosol protein)
MCF 7	1.1	31
T 47D	0.7	150
BT 20	0.6	95
MDA 231	2.0	41
MDA 157	1.6	86
HBL 100	1.5	36
Chicken intestinal cytosol	5.8	180

Table 2

Adenylate cyclase response to calcitonin in breast cell lines

Cell line	Activity (pmol cyclic AMP/mg protein/min)	
	Basal	Salmon calcitonin (3 × 10^{-8} M)
MCF 7	31.6 ± 0.2[a]	97.4 ± 1.2[b]
T 47D	20.8 ± 0.2	132.4 ± 4.4[b]
BT 20	27.3 ± 0.6	25.1 ± 1.0
MDA 231	42.9 ± 0.3	43.0 ± 2.3
MDA 157	31.0 ± 1.0	36.2 ± 1.8
HBL 100	23.9 ± 0.8	21.7 ± 0.2

[a] Means ± S.E. of triplicate observations in individual experiments repeated at least 5 times.
[b] $p < 0.001$.

of the calcitonins resemble their relative potencies as calcium-lowering agents in the rat (12, 13, 16, 18).

Specific high-affinity binding of calcitonin to T 47D cells was found, with a receptor content (R_C) of 27,300 ± 2,100 (S.E.; 5 experiments)/cell and a dissociation constant (K_d) of 9.8 ± 1.2 × 10^{-10} M from the same experiments. An example is given in Chart 3. In experiments with MCF 7 cells, R_C = 5300 and K_d = 17.1 ± 2.2 × 10^{-10} M. The MCF 7 cells showed greater proteolytic activity towards labeled hormone than did T 47D cells under the conditions of binding experiments. As assessed by trichloroacetic acid precipitability, tracer degradation was 40% for T 47D and 80% for MCF 7 cells after 1 hr at 20° (results not shown). In the case of T 47D and MCF 7 cells, the percentage of labeled hormone bound by cells ranged from 5 to 25% in different experiments, with nonspecific binding always less than 1.5% of total radioactivity. Statistically significant specific binding was consistently found with the cell lines MDA 157 (0.29%), MDA 231 (0.58%) and HBL 100 (0.48%). BT 20 cells were not tested for calcitonin binding. These levels of specific binding were too low to allow studies of binding constants and receptor number, and indeed, their biological significance, if any, remains to be determined.

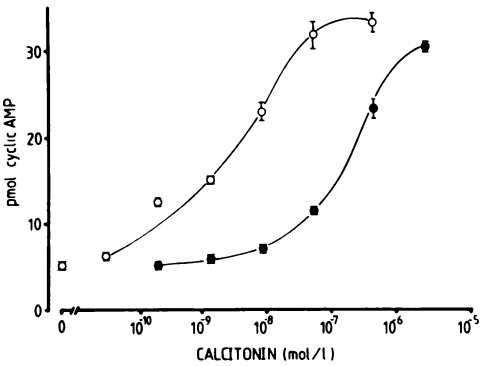

Chart 2. Effects of salmon (○) and human (●) calcitonins on adenylate cyclase activity in homogenates of T 47D cells.

Table 3

Relative efficacy of calcitonins and analogs in binding and in stimulation of adenylate cyclase

SCT[a] was assigned an arbitrary potency of 100 in each system; all other preparations were compared from standard curves (at least 5 points) in which the half-maximally effective concentration of calcitonin preparation is related to that of SCT in the same assay. For each analog, the substituted amino acid is indicated by its abbreviation, and its position in the molecule is indicated by the superscript number.

Hormone or analog	BEN cells		MCF 7 cells	
	Binding	Adenylate cyclase	Binding	Adenylate cyclase
SCT	100	100	100	100
HCT	7	5	5	3
[ASU^{1,7}]-ECT	156	250	100	168
Des-Tyr-SCT	100	100	NT	NT
Leu^{12}-HCT	12	14	12	12
Leu^{12,16,19}-HCT	23	26	10	30
Val^{8}-HCT	6	10	8	6
Gly^{8}-HCT	<0.2	<0.5	<0.5	<0
Arg^{24}-HCT	18	4	10	27.5
Asn^{26}-HCT	22	16	12	10
Thre^{27}-HCT	41	28	33	36
Asn^{26}-Thre^{27}-HCT	41	36	34	45
Lys^{11}-HCT	47	48	11	NT

[a] SCT, salmon calcitonin; HCT, human calcitonin; ASU, aminosuberic acid; ECT, eel calcitonin; NT, not tested.

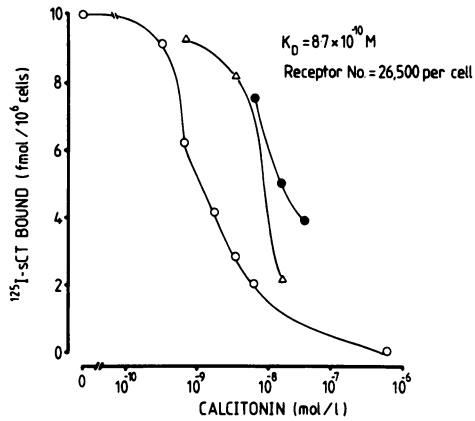

Chart 3. Binding of ^{125}I-labeled salmon calcitonin (^{125}*I-sCT*) to intact T 47D cells and competition by unlabeled salmon (○) and human (●) calcitonin, and Leu^{12}-human calcitonin (△).

DISCUSSION

These experiments, together with our published data (3–5), suggest that a receptor for 1,25-$(OH)_2D_3$ may reflect retention in the breast cancer cell of a property of the normal breast epithelial cell. This would imply that 1,25-$(OH)_2D_3$ normally acts upon the breast, most likely by influencing calcium and phosphorus transport during lactation. Retention of this property in the malignant cell may be related to the calcium- and phosphorus-transporting ability of these cells, which have been shown to be capable of resorbing bone directly *in vitro* (1), a property which is consistent with the view that established breast cancer metastases in bone can act directly, without the intervention of osteoclasts (7).

It is clear from our results that in some breast cancer cell lines a classical calcitonin receptor persists which mediates profound effects of the hormone on adenylate cyclase activity. It is less clear in the case of calcitonin whether this might reflect a normal calcitonin receptor in the breast. The finding of low specific binding and absent adenylate cyclase response in some lines, including the normal line HBL 100, might result from loss of receptor under culture conditions and impairment of receptor-catalytic unit coupling. The high content both of calcitonin and of 1,25-$(OH)_2D_3$ receptors in T 47D cells is of interest in view of the report (19) that T 47D cells have a much higher prolactin receptor content than any other breast cell line examined.

Whenever cancer cells metastasize to organs other than those of their origin, their establishment and flourishing in the new environment probably requires special properties to provide for this (17). The possession of specific receptors for calcium-regulating hormones might be relevant to the propensity for breast cancer cells to maintain themselves as metastases in bone. In testing this possibility, studies of receptors in bony metastases of breast cancer will be necessary.

The discovery of receptors for 1,25-$(OH)_2D_3$ in human breast cancer cell lines provides the first readily available abundant source of human receptors for study. Their coexistence in some lines with receptors for calcitonin, another calcium-regulating hormone, will bear further investigation, particularly in relation to the bone-eroding properties of breast cancer cells.

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