[CANCER RESEARCH 48, 2734–2739, May 15, 1988]

# Regulation of Epidermal Growth Factor Receptor Levels by 1,25-Dihydroxyvitamin $D_3$ in Human Breast Cancer Cells[1]

**Masafumi Koga,[2] John A. Eisman, and Robert L. Sutherland[3]**

*Garvan Institute of Medical Research, St. Vincent's Hospital, Sydney, NSW, 2010, Australia*

## ABSTRACT

**Specific, high affinity receptors for 1,25-dihydroxyvitamin $D_3$ [1,25-$(OH)_2D_3$] have been demonstrated in human breast cancer cells. In addition, 1,25-$(OH)_2D_3$ has been shown to inhibit replication in some human breast cancer cell lines, although the mechanism(s) of this antitumor activity remain undefined. There is currently considerable interest in the role of autocrine growth factors in the control of breast cancer cell proliferation and the effects of steroid hormones on their production, receptor binding, and action. Since the epidermal growth factor (EGF) receptor mediates the effects of both EGF and the autocrine growth factor, α-transforming growth factor, we investigated the effect of 1,25-$(OH)_2D_3$ on EGF receptor levels in several human breast cancer cell lines.**

**Preincubation of T-47D cells with 1,25-$(OH)_2D_3$ for 24 h resulted in a significant concentration-dependent decline in the specific binding of [$^{125}$I]EGF. The effect was observed when EGF binding was assayed at either 0 or 37°C, both before and after treatment with acid to remove receptor bound endogenous ligand. This indicated that the effect on [$^{125}$I]-EGF binding was not due to effects of 1,25-$(OH)_2D_3$ on receptor internalization and degradation or receptor occupancy. The half-maximal inhibitory concentration of 1,25-$(OH)_2D_3$ was approximately 2 nM. The decrease in EGF binding was due to a decrease in receptor number from 2,900 sites/cell in control cultures to 2,330 and 1,730 sites/cell in cells treated for 24 h with $10^{-8}$ and $10^{-6}$ M 1,25-$(OH)_2D_3$, respectively. There was no change in the affinity of the receptor for EGF following treatment with 1,25-$(OH)_2D_3$ [$K_d$ = 0.075 ± 0.006 nM (± SEM) for control and $K_d$ = 0.083 ± 0.004 nM for treated cells]. Decreased EGF receptor levels were also achieved with a number of analogues of 1,25-$(OH)_2D_3$ in accordance with their affinities for the 1,25-$(OH)_2D_3$ receptor, *i.e.*, potencies for decreasing EGF binding in T-47D cells were in the order: 1,25-$(OH)_2D_3$ > 1,24,25-trihydroxyvitamin $D_3$ > 1,25,26-trihydroxyvitamin $D_3$ > 24,25-dihydroxyvitamin $D_3$ ≥ 25-hydroxyvitamin $D_3$. Specific, saturable EGF binding to MCF-7 cells was also reduced by 1,25-$(OH)_2D_3$ while binding to BT-20 and HBL-100 cells was unaffected by this treatment.**

**These results demonstrate that 1,25-$(OH)_2D_3$ treatment reduces EGF receptor levels in two breast cancer cell lines known to be growth inhibited by this hormone and suggest that one potential mechanism through which 1,25-$(OH)_2D_3$ could exert its known inhibitory effects on these cells is by reducing their sensitivity to autocrine growth factors that act via the EGF receptor.**

## INTRODUCTION

It is now well established that the biologically active metabolite of vitamin $D_3$, 1,25-dihydroxyvitamin $D_3$, 1,25-$(OH)_2D_3$,[4] has a mode of action analogous to that of other steroid hormones (1, 2). Although 1,25-$(OH)_2D_3$ has a major physiological role as a calcium-regulating hormone it is becoming increasingly apparent that it also plays fundamental roles in the control of replication and differentiation (3–6). Recently, 1,25-$(OH)_2D_3$ has been shown to have inhibitory effects on the replication of some human breast cancer cell lines *in vitro* (5, 6) and other tumors *in vivo* (7). Although these cancer cell lines possess specific high affinity receptors for 1,25-$(OH)_2D_3$ (8–12), the molecular mechanisms by which the inhibitory effects on replication are mediated are not well understood.

It has recently been demonstrated that human breast cancer cells secrete a number of growth factors including; α-TGF, β-TGF, IGF-I and platelet-derived growth factor (13–19). Furthermore, the effects of several steroid hormones, including estrogens, antiestrogens, and progestins, on breast cancer cell proliferation have been suggested to be due, at least in part, to effects on these autocrine pathways. In particular estrogens and antiestrogens have been shown to regulate the production and/or release of α-TGF, β-TGF, and IGF-I (15–18), while progestins have been shown to regulate the concentration of EGF receptors in some breast cancer cell lines (20, 21). Epidermal growth factor receptors are responsible for the binding and action of α-TGF as well as EGF. The intimate involvement of steroids with α-TGF production and EGF receptor levels led us to investigate the effects of 1,25-$(OH)_2D_3$ on EGF receptor levels in some human breast cancer cell lines with known differential growth responses to 1,25-$(OH)_2D_3$.

## MATERIALS AND METHODS

**Materials.** Vitamin $D_3$ and its metabolites were the generous gift of Dr. M. Uskokovic (Hoffmann-La Roche Inc., Nutley, NJ). Receptor grade murine EGF was purchased from Collaborative Research Inc., Lexington, MA. MPA was obtained from Upjohn Pty. Ltd., Sydney, Australia, through the courtesy of Dr. Dudley Jacobs. ORG 2058 was purchased from Amersham Australia, Sydney, and R5020 from Du Pont (Australia) Ltd., Sydney. Progesterone was obtained from the Sigma Chemical Co., St. Louis, MO. All other reagents were of analytical grade.

The preparation of highly purified human growth hormone used in these studies was supplied by Dr. G. Chapman and had been purified as previously described (22).

**Cell Culture.** MCF-7 cells were supplied by Dr. C. M. McGrath, Meyer L. Prentis Cancer Center, Detroit, MI, while T-47D, BT-20, MDA-MB-231, and HBL-100 cells were provided by E. G. and G. Mason Research Institute, Worcester, MA, for the National Cancer Institute Breast Cancer Program Cell Culture Bank. Cells were maintained in RPMI 1640 medium supplemented with 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, 14 mM sodium bicarbonate, 6 mM L-glutamine, 20 μg/ml gentamicin, 10 μg/ml porcine insulin, 6 μg/ml phenol red, and 10% FCS as described previously (23).

**Polypeptide Binding Assays.** EGF and growth hormone were iodinated using a modified chloramine T method as previously described (24). The specific activities of these peptides varied from 50 to 270 μCi/μg as determined by the tracer dilution technique (25).

For the EGF binding studies, cells were placed into 24-well tissue culture trays and grown to confluence (21). Twenty-four h prior to assay, medium was removed and replaced with new medium supple-

Received 9/3/87; revised 1/15/88; accepted 2/12/88.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported by the New South Wales State Cancer Council and the National Health and Medical Research Council of Australia.

[2] Visiting Research Fellow from the Third Department of Internal Medicine, Osaka University Hospital, Osaka, Japan.

[3] To whom requests for reprints should be addressed, at the Garvan Institute of Medical Research, St. Vincent's Hospital, Sydney, NSW, 2010, Australia.

[4] The abbreviations used are: 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin $D_3$; TGF, transforming growth factor; IGF-I, insulin-like growth factor-I; EGF, epidermal growth factor; MPA, medroxyprogesterone acetate (6α-methyl-17α-hydroxy-4-pregnene-3,20-dione acetate); FCS, fetal calf serum; hGH, human growth hormones; 1,24,25-$(OH)_3D_3$, 1,24,25-trihydroxyvitamin $D_3$; 1,25,26-$(OH)_3D_3$, 1,25,26-trihydroxyvitamin $D_3$; 24,25-$(OH)_2D_3$, 24,25-dihydroxyvitamin $D_3$; 25-OH $D_3$, 25-hydroxyvitamin $D_3$; ORG 2058, 16α-ethyl-21-hydroxy-19-norpregn-4-en-3,20-dione; R5020, 17α,21-dimethyl-19-norpregn-4,9-diene-3,20-dione; ER, estrogen receptor.

mented with 1% charcoal-treated FCS and various concentrations of 1,25-$(OH)_2D_3$ or its analogues in ethanol, to give a final ethanol concentration of 0.1%. The incubation was continued at 37°C for 24 h except for the time course study. Monolayers were then washed twice with binding buffer (RPMI 1640 medium supplemented with 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid and 0.1% bovine serum albumin, pH 7.4). Specifically bound [$^{125}$I]EGF was determined by incubating approximately 40,000 cpm of [$^{125}$I]EGF with or without 50 nM of unlabeled EGF per well at 37°C for 1 h (21).

In some experiments T-47D cells were treated for 24 h with 10 nM progestin (ORG 2058, MPA, R5020, or progesterone) in the presence of either 100 nM 1,25-$(OH)_2D_3$ or vehicle (0.1% ethanol) in medium supplemented with 1% charcoal-treated FCS.

A similar assay procedure was employed for Scatchard analysis of EGF binding except that 5,000 cpm [$^{125}$I]EGF was added and unlabeled EGF covered the range of 0.02 to 30 ng/ml (0.003–5.0 nM). Apparent $K_d$ and binding site concentrations were calculated by the method of Scatchard (26). Specific binding of [$^{125}$I]hGH was determined as previously reported (24, 27).

Following incubation with [$^{125}$I]EGF or [$^{125}$I]hGH the monolayers were washed and solubilized as previously described (24). Specific binding was calculated from the difference between total ([$^{125}$I]hormone alone) and nonspecific ([$^{125}$I]hormone + unlabeled hormone) binding.

Cells in parallel wells were trypsinized and counted in a Coulter Counter. Where the treatment resulted in a significant change in cell number, specific [$^{125}$I]EGF binding was corrected for cell number unless otherwise stated.

In an attempt to assess the contribution of changes in EGF receptor occupancy with 1,25-$(OH)_2D_3$ treatment, EGF binding activity was assessed following dissociation of ligand by treatment of the monolayers with 50 mM ammonium acetate buffer, pH 5.0, for 1 min at 0°C.

**Statistics.** Student's *t* test was used to test the difference between the means of experimental groups.

## RESULTS

When T-47D cells were incubated with increasing concentrations ($10^{-12}$–$10^{-6}$ M) of 1,25-$(OH)_2D_3$, there was a concentration-dependent decline in the level of specific binding of [$^{125}$I]-EGF at 24 h as illustrated in Fig. 1. The half-maximal inhibitory concentration of 1,25-$(OH)_2D_3$ was approximately 2 nM. When cells were treated for 24 h, under these experimental conditions, cell numbers were not changed by treatment with 1,25-$(OH)_2D_3$ nor was there any evidence of a decrease in cell viability. Thus the measured effect was not due to an effect of the drug on cell growth. However, with more prolonged periods of exposure to 1,25-$(OH)_2D_3$ cell numbers declined at concentrations >$10^{-9}$ M as previously reported (5, 6).[5]

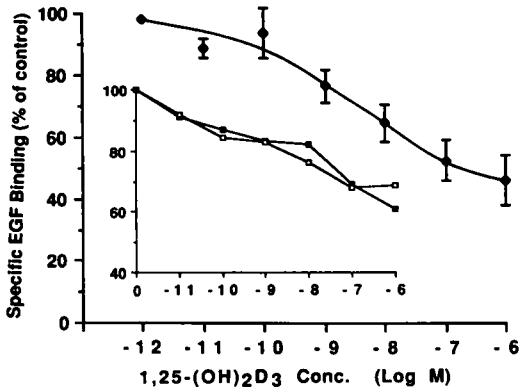


Fig. 1. Effect of various concentrations of 1,25-$(OH)_2D_3$ on the specific binding of [$^{125}$I]EGF by T-47D human breast cancer cell monolayers. Specifically bound [$^{125}$I]EGF was determined at 37°C for 1 h as described in "Materials and Methods" in cultures exposed to various concentrations of 1,25-$(OH)_2D_3$ for 24 h. Specifically bound [$^{125}$I]EGF has been expressed as a percentage of that measured in control cultures. *Data*, mean ± SEM for three separate experiments, where each point was determined in triplicate; *inset*, comparison of the effects of assaying for specifically bound [$^{125}$I]EGF at 37°C for 1 h (■) or 0°C for 18 h (□) following treatment with 1,25-$(OH)_2D_3$ as described above. *Points*, mean of triplicate observations in one experiment.

Table 1 *Effect of acid washing on the binding of epidermal growth factor to T-47D cells following treatment with 1,25-$(OH)_2D_3$*

Cells were treated with vehicle or 1,25-$(OH)_2D_3$ for 24 h and then washed with 50 mM ammonium acetate buffer (pH 5.0) or saline. Specifically bound EGF was then determined as described in "Materials and Methods" following incubation at 37°C for 1 h or 0°C for 4 h.

| Treatment | Assay condition | Specifically bound [$^{125}$I]-EGF (cpm/well) | |
|---|---|---|---|
| | | Saline wash | Acid wash |
| Control | 37°C, 1 h | 2220 ± 95 | 2414 ± 61 |
| $10^{-8}$ M 1,25-$(OH)_2D_3$ | | 1815 ± 65 | 1854 ± 43 |
| $10^{-6}$ M 1,25-$(OH)_2D_3$ | | 1315 ± 22 | 1238 ± 15 |
| Control | 0°C, 4 h | 1013 ± 45 | 881 ± 45 |
| $10^{-8}$ M 1,25-$(OH)_2D_3$ | | 648 ± 13 | 603 ± 16 |
| $10^{-6}$ M 1,25-$(OH)_2D_3$ | | 427 ± 48 | 444 ± 19 |

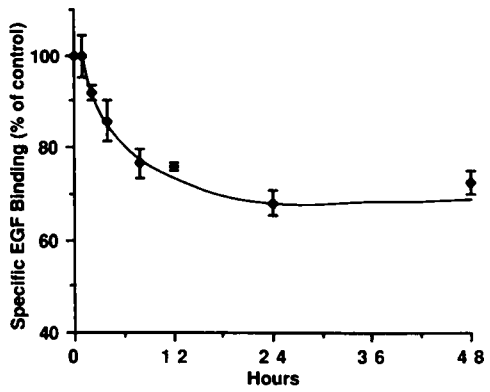


Fig. 2. Time course of the 1,25-$(OH)_2D_3$ induced decline of EGF receptors in T-47D cells. Specific binding of [$^{125}$I]EGF was determined as described in Fig. 1, *legend*, after exposure to 100 nM 1,25-$(OH)_2D_3$ for the time periods indicated. *Data*, mean of triplicate wells.

To ensure that the observed effect of 1,25-$(OH)_2D_3$ on EGF binding was not due to effects on internalization and degradation of [$^{125}$I]EGF during the assay at 37°C an experiment comparing the effects of assay at 37°C for 1 h with assay at 0°C for 18 h was undertaken following pretreatment of cells with 1,25-$(OH)_2D_3$. The data are presented in the inset to Fig. 1 and demonstrate that identical effects were apparent under the two different assay procedures.

Potential effects of 1,25-$(OH)_2D_3$ on EGF receptor occupancy were studied by assaying for [$^{125}$I]EGF binding following acid washing (pH 5.0 for approximately 1 min) of the T-47D cell monolayer to dissociate receptor bound endogenous ligand. The data presented in Table 1 show that 1,25-$(OH)_2D_3$ treatment resulted in a similar decline in specific EGF binding in cells treated with acid and in saline treated control cells. Thus the effect of 1,25-$(OH)_2D_3$ could not be accounted for by hormone-induced changes in EGF receptor occupancy. The binding measured at 0°C for 4 h was 32–45% of that assayed at 37°C for 1 h (Table 1) and this is consistent with previously published data on temperature effects on binding kinetics (28, 29).

The time dependence of this decline in EGF receptor was next investigated. A decline in [$^{125}$I]EGF binding to T-47D cell monolayers was seen after 2 h incubation with 100 nM 1,25-$(OH)_2D_3$. The maximal effect was apparent by 24 h and was maintained until at least 48 h (Fig. 2).

[5] J. A. Eisman, R. L. Sutherland, M. L. McMenemy, J-C. Fragonas, and G. Y. N. Pang. Effect of 1,25-dihydroxyvitamin $D_3$ on cell cycle kinetics of T47D human breast cancer cells. Submitted for publication.

Saturation analysis of competitive binding data obtained by incubating control and 1,25-$(OH)_2D_3$ pretreated T-47D cells with increasing concentrations of EGF is shown in Fig. 3. The data presented in Fig. 3, *upper*, illustrate that treatment with 1,25-$(OH)_2D_3$ results in decreased levels of saturable binding indicating an effect of the hormone on the number of saturable binding sites. Scatchard analysis (Fig. 3, *lower*) revealed no major difference in the apparent $K_d$ value for the EGF receptor between control and 1,25-$(OH)_2D_3$ treated cells [$K_d = 0.075 \pm 0.006$ nM (± SEM) for control and $K_d = 0.083 \pm 0.004$ nM for treated cells]. However, the total number of receptors was decreased in a concentration-dependent manner from 2,900 sites/cell in control cultures to 2,330 and 1,730 sites/cell in cells treated with $10^{-8}$ and $10^{-6}$ M 1,25-$(OH)_2D_3$, respectively, indicating that the 1,25-$(OH)_2D_3$ induced decline in [$^{125}$I]EGF binding was a consequence of a decrease in the number of EGF receptors.

To ascertain the specificity of the response to 1,25-$(OH)_2D_3$ we next evaluated the potency of 1,25-$(OH)_2D_3$ as compared to four other vitamin $D_3$ analogues. A decline in [$^{125}$I]EGF binding was also achieved with all four analogues of 1,25-$(OH)_2D_3$ in accordance with their affinities for the 1,25-$(OH)_2D_3$ receptor, *i.e.*, potencies for decreasing [$^{125}$I]EGF binding were in the order: 1,25-$(OH)_2D_3$ > 1,24,25-$(OH)_3D_3$ > 1,25,26-$(OH)_3D_3$ > 24,25-$(OH)_2D_3$ ≥ 25-OH $D_3$ (Fig. 4).

It has previously been reported that treatment of T-47D cells with progestins results in elevation of EGF receptor levels (20, 21). In the next series of experiments we tested the effect of 1,25-$(OH)_2D_3$ on EGF receptor levels in the presence or absence of several progestins. When T-47D cells were incubated for 24 h in medium containing 1% charcoal-treated FCS and 10 nM progestin, stimulation of [$^{125}$I]EGF binding to 266 ± 7.0%, 212 ± 8.4%, 202 ± 4.9%, and 198 ± 9.4% of the control level was observed with ORG 2058, R5020, MPA, and progesterone, respectively ($p < 0.001$, *versus* control; Fig. 5). In parallel experiments 1,25-$(OH)_2D_3$ decreased [$^{125}$I]EGF binding in progestin treated cells to a similar degree as it did in control cells, *i.e.*, [$^{125}$I]EGF specific binding in 1,25-$(OH)_2D_3$-treated cells was 67.1 ± 2.4% ($p < 0.001$), 68.8 ± 1.9% ($p < 0.001$), 78.1 ± 1.0% ($p < 0.01$), 69.9 ± 0.2% ($p < 0.001$), and 68.8 ± 2.0% ($p < 0.01$; *versus* progestin treatment alone, respectively) of control, ORG 2058-, R5020-, MPA-, and progesterone-treated cells, respectively (Fig. 5).

Since the concentration of 1,25-$(OH)_2D_3$ receptors was reported to be significantly higher in T-47D cells than in MCF-7 cells (10), the dose responsiveness of changes in EGF binding by 1,25-$(OH)_2D_3$ was examined in MCF-7 cells. Preincubation with 1,25-$(OH)_2D_3$ resulted in a significant decline in the specific binding of [$^{125}$I]EGF by MCF-7 cell monolayers to 80.2 ± 4.5 and 73.3 ± 1.0% of control at $10^{-9}$ and $10^{-7}$ M, respectively [$p < 0.05$ and $p < 0.001$ for control *versus* $10^{-9}$ and $10^{-7}$ M 1,25-$(OH)_2D_3$], but the maximal inhibitory effect was considerably less marked than with T-47D cells (Fig. 6). EGF receptor levels in BT-20 and HBL-100 cells were unaffected by treatment

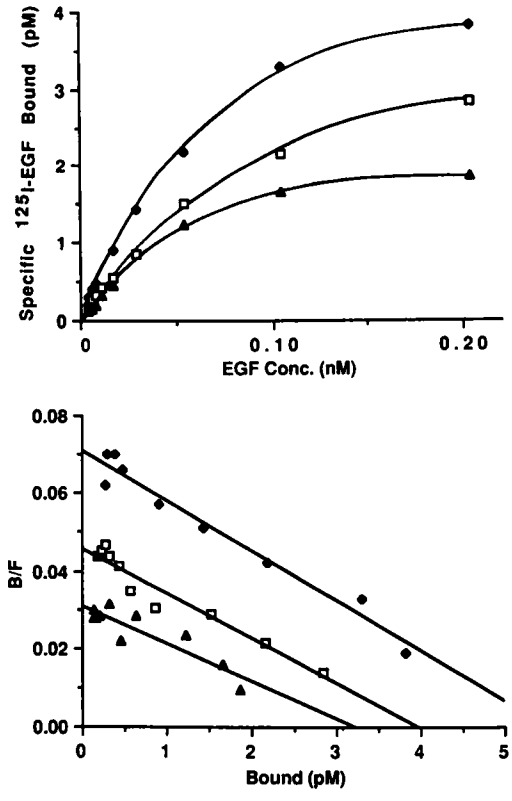


Fig. 3. The effect of 1,25-$(OH)_2D_3$ on the affinity and concentration of EGF receptors in T-47D cells. [$^{125}$I]EGF binding data for T-47D cell monolayers preincubated for 24 h in the presence of 0 (◆), $10^{-9}$ M (□), or $10^{-6}$ M (▲) 1,25-$(OH)_2D_3$, are presented as saturation curves, *upper*, and Scatchard plots, *lower*. [$^{125}$I]EGF binding was determined in the presence of increasing concentrations of unlabeled EGF as described in "Materials and Methods." *Data*, follow correction for nonspecific binding.

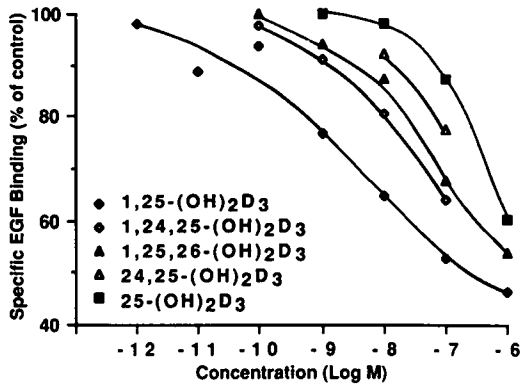


Fig. 4. Influence of various concentrations of 1,25-$(OH)_2D_3$ and analogues on the specific binding of [$^{125}$I]EGF by T-47D cells. Various concentrations of 1,25-$(OH)_2D_3$ and analogues were incubated with T-47D cells for 24 h, and specifically bound [$^{125}$I]EGF was measured as described in "Materials and Methods." *Points*, mean of triplicate wells.

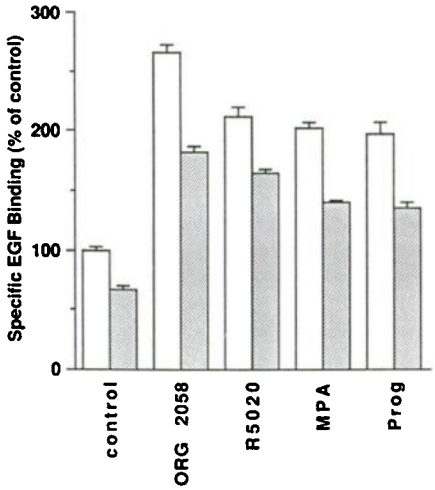


Fig. 5. Effect of 1,25-$(OH)_2D_3$ on specifically bound [$^{125}$I]EGF in control and progestin treated cells. T-47D cell monolayers were preincubated with 10 nM progestin (ORG 2058, MPA, R5020, or progesterone) alone (□) or progestin plus 100 nM 1,25-$(OH)_2D_3$ (■) for 24 h. Specifically bound [$^{125}$I]EGF was determined as described in "Materials and Methods." *Points*, are the means of triplicate wells.

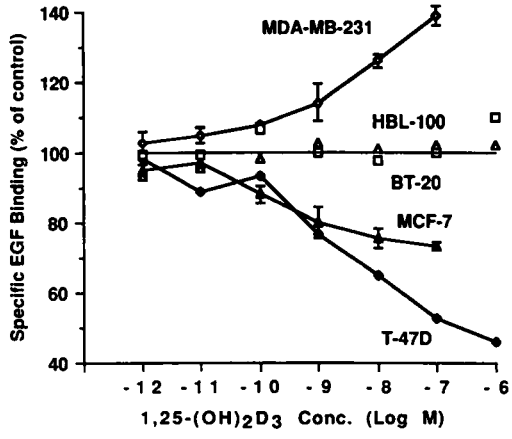


Fig. 6. The effect of various concentrations of 1,25-(OH)$_2$D$_3$ on [$^{125}$I]EGF binding to five human breast cell lines. Approximately 5 × 10$^5$ cells of the T-47D (◆), MCF-7 (▲), MDA-MB-231 (◇), BT-20 (□), or HBL-100 (△) lines were preincubated for 24 h with increasing concentrations of 1,25-(OH)$_2$D$_3$. Specific binding of [$^{125}$I]EGF was determined as described in "Materials and Methods." *Points*, mean ± SEM of three to six wells.

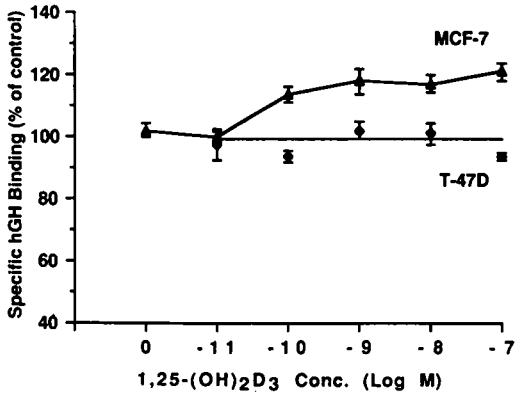


Fig. 7. The effect of various concentrations of 1,25-(OH)$_2$D$_3$ on the specific binding of [$^{125}$I]human growth hormone to human breast cancer cells. T-47D (◆) and MCF-7 (▲) cells were preincubated for 24 h with increasing concentrations of 1,25-(OH)$_2$D$_3$. Specific binding of [$^{125}$I]hGH was determined as described in "Materials and Methods." *Points*, mean ± SEM of triplicate wells.

with 1,25-(OH)$_2$D$_3$, although these cells have previously been reported to possess 1,25-(OH)$_2$D$_3$ receptors (10). On the other hand, specific EGF binding to MDA-MB-231 cells was increased following treatment with 1,25-(OH)$_2$D$_3$ (Fig. 6).

To determine if the effect of 1,25-(OH)$_2$D$_3$ on EGF receptor concentration in T-47D and MCF-7 cells was specific for this receptor or was also shared by the lactogenic receptor which is known to be controlled in a similar manner to EGF receptor by progestins (20, 21), we next examined the binding of [$^{125}$I]-hGH to cell monolayers that had been preincubated with increasing concentrations of 1,25-(OH)$_2$D$_3$. No significant changes in the binding of [$^{125}$I]hGH to T-47D cells was observed, while [$^{125}$I]hGH binding to MCF-7 cells increased slightly following treatment with 1,25-(OH)$_2$D$_3$ to 121 ± 2.9% of control at $10^{-7}$ M ($p < 0.001$; Fig. 7). These results indicate that the effect of 1,25-(OH)$_2$D$_3$ on EGF receptor in T-47D and MCF-7 cells was not accompanied by similar changes in the lactogenic receptor.

## DISCUSSION

It has recently been demonstrated that 1,25-(OH)$_2$D$_3$ has a number of physiological functions in addition to its classical role as a regulator of calcium and bone metabolism. Of particular relevance to the present study are the recently described effects of 1,25-(OH)$_2$D$_3$ on replication and differentiation which have been described in greatest detail for lymphoid cells (4, 30, 31).

Eisman and his colleagues were the first to demonstrate that human breast cancer cells possess high affinity receptors for 1,25-(OH)$_2$D$_3$ (8–10, 12). They were subsequently able to show the presence of these receptors in normal mammary tissue of the rabbit favoring the hypothesis that expression of this receptor in tumor tissue is a carry-over of normal physiological function (32). Furthermore, these workers demonstrated that 1,25-(OH)$_2$D$_3$ regulated the proliferation of the T-47D human breast cancer cell line in a biphasic manner. At low concentrations ($10^{-11}$–$10^{-9}$ M) 1,25-(OH)$_2$D$_3$ stimulated cell replication while at higher concentrations (>$10^{-9}$ M) the hormone caused marked inhibition of T-47D cell proliferation (5). The inhibitory effect of 1,25-(OH)$_2$D$_3$ on breast cancer cell growth is of potential therapeutic importance and has prompted a more detailed investigation of its effects on breast cancer cell proliferation. Since autocrine growth factors have recently been implicated in the control of breast cancer cell growth, and one class of these factors, *i.e.*, α-TGF, act upon cells via interaction with the EGF receptor the studies described here involved the effect of 1,25-(OH)$_2$D$_3$ on EGF binding by several breast cancer cell lines.

Our data clearly demonstrate that treatment of T-47D cells with 1,25-(OH)$_2$D$_3$ results in a decrease in the ability of these cells to bind [$^{125}$I]EGF. This effect was apparent when specifically bound [$^{125}$I]EGF was assayed either by a 1-h incubation at 37°C or for 18 h at 0°C. Since under the latter assay conditions the result is unlikely to be complicated by changes in the rate of internalization and degradation of [$^{125}$I]EGF it appears likely that the decline in [$^{125}$I]EGF specific binding is due to a decrease in the number of EGF receptor molecules at the cell surface. In accordance with such a conclusion Scatchard analysis of competitive binding data showed that 1,25-(OH)$_2$D$_3$ caused a concentration-dependent decrease in the number of EGF receptor sites without a change in receptor affinity. Further experiments, involving acid washing of the cells, illustrated that the observed changes in EGF receptor levels were unrelated to changes in the degree of receptor occupancy by autocrine ligands.

Demonstration that the decrease in EGF receptor levels by several analogues of 1,25-(OH)$_2$D$_3$ was in accordance with their previously reported affinities for the 1,25-(OH)$_2$D$_3$ receptor (5, 6, 33) provided strong support for the view that this effect of 1,25-(OH)$_2$D$_3$ is mediated via its high affinity, intracellular receptor. Furthermore, when the effects of these analogues on EGF binding and cell proliferation in T-47D cells were compared with their affinities for the 1,25-(OH)$_2$D$_3$ receptor (Table 2) it was apparent that in both cases the biological response was related to receptor affinity. This not only provides further support for the effects of 1,25-(OH)$_2$D$_3$ on EGF binding being 1,25-(OH)$_2$D$_3$ receptor mediated but also suggests a relation-

Table 2 *Relationship between affinity of 1,25-(OH)$_2$D$_3$ analogues for the 1,25-(OH)$_2$D$_3$ receptor and their ability to inhibit EGF binding and growth in T-47D cells at a concentration of $10^{-8}$ M*

| Ligand | Receptor affinity[a] | Percent of Control | |
|---|---|---|---|
| | | EGF binding[b] | Cell growth[c] |
| 1,25-(OH)$_2$D$_3$ | 100 | 64.9 | 32 |
| 1,24,25-(OH)$_3$D$_3$ | 42 | 80.6 | 43 |
| 1,25,26-(OH)$_3$D$_3$ | 2[c] | 87.4 | 58 |
| 25-(OH)D$_3$ | 0.5 | 98.1 | 77 |
| 24,25-(OH)$_2$D$_3$ | 0.2 | 92.2 | 84 |

[a] Data are from Ref. 33.
[b] Data are from Fig. 4.
[c] Data are from Ref. 5.

ship between the decline in EGF receptor levels and decreased growth rates.

Progestins, like 1,25-$(OH)_2D_3$, are potent inhibitors of T-47D cell growth[6] (34, 35) and this effect is associated with a 2–3-fold increase in EGF receptor levels (20, 21). Since, as evidence by the data presented in Fig. 5, 1,25-$(OH)_2D_3$ can decrease [$^{125}$I]EGF binding to T-47D cells in the presence of progestins, it seems likely that these two different classes of steroids regulate EGF receptors by independent mechanisms. This study has not addressed in any detail the mechanisms involved in the steroidal regulation of EGF receptor levels in breast cancer cells which potentially could involve changes in receptor internalization and recycling as well as direct or indirect effects, via changes in autocrine growth factor production, on receptor synthesis and turnover. We are currently investigating these possibilities.

The differential sensitivity of the five breast cell lines to decreases in EGF receptor levels induced by 1,25-$(OH)_2D_3$ is to a major degree paralleled by their sensitivity to the growth inhibitory effects of 1,25-$(OH)_2D_3$. Both T-47D and MCF-7 cells are growth inhibited by $10^{-9}$–$10^{-7}$ M concentrations of 1,25-$(OH)_2D_3$ (5, 6) while the MDA-MB-231 and HBL 100 cell lines appear to be insensitive to concentrations of 1,25-$(OH)_2D_3$ up to $10^{-7}$ M.[7] Such data in conjunction with the 1,25-$(OH)_2D_3$ analogue data presented in Table 2 lend further support to the hypothesis that 1,25-$(OH)_2D_3$-induced down-regulation of EGF receptor and growth inhibition may be interrelated. However, the observation that in MDA-MB-231 cells, 1,25-$(OH)_2D_3$ treatment increases the level of EGF receptor, without affecting growth rate, would indicate that if such a relationship occurred, it does not apply to all breast cancer cell lines.

Interpretation of these data is further complicated by the differential expression and regulation of EGF receptors in human breast cancer cell lines (28, 29, 36–38). Epidermal growth factor receptors are present in significantly lower concentrations in ER-positive breast cells than in ER-negative cells (28, 29, 36, 37) and are differentially regulated by progestins and glucocorticoids in these subclasses of breast cancer cells[8] (20, 21). Such data indicate that it is perhaps not surprising to find differential regulation of EGF receptor levels by 1,25-$(OH)_2D_3$ in ER-positive (T-47D and MCF-7) and ER-negative (MDA-MB-231, BT-20, and HBL-100) cell lines.

In previous studies from this laboratory we demonstrated that progestin treatment of MCF-7 and T-47D cells leads to an increase in the concentration of both the EGF receptor and lactogenic receptors while insulin, transferrin, and calcitonin receptor levels were unaffected (19, 20, 26). Despite this parallelism of effects of progestins on EGF and lactogenic receptors, the decline induced by 1,25-$(OH)_2D_3$ in these two cell lines is confined to the EGF receptor while lactogenic receptor levels remain constant (T-47D) or are slightly increased (MCF-7). These data illustrate that the EGF and lactogenic receptor concentrations are not necessarily controlled in parallel by steroid hormones.

In lymphoid cells the predominant role of 1,25-$(OH)_2D_3$ appears to be the induction of differentiation (5, 30, 31) a process that is also induced by phorbol esters and retinoic acid (39, 40). Whether the effects of 1,25-$(OH)_2D_3$ on breast cancer cell proliferation and EGF receptor levels are due to differentiation is unclear at this time. However, it is of considerable interest to note that 12-*O*-tetradecanoylphorbol-13-acetate and retinoic acid both reduce the rate of proliferation of some breast cancer cells and these effects are also associated with a decline in EGF receptor levels in T-47D cells.[9] More detailed studies are clearly required to further delineate the molecular basis of 1,25-$(OH)_2D_3$ effects on human breast cancer cell proliferation and differentiation. Such studies are being actively pursued in our laboratories.

## ACKNOWLEDGMENTS

The authors wish to thank Dr. Christine Clarke for her invaluable comments during the preparation of the manuscript.

## REFERENCES

1. Kream, B. E., Jose, M., Yamada, S., and DeLuca, H. F. A specific high-affinity binding macromolecule for 1,25-dihydroxyvitamin $D_3$ in fetal bone. Science (Wash. DC), *197:* 1086–1088, 1977.
2. Norman, A. W., Roth, J., and Orci, L. The vitamin D endocrine system: steroid metabolism, hormone receptors, and biological response (calcium binding proteins). Endocrinol. Rev., *3:* 331–366, 1982.
3. Colston, K., Colston, M. J., and Feldman, D. 1,25-dihydroxyvitamin $D_3$ and malignant melanoma: the presence of receptors and inhibition of cell growth in culture. Endocrinology, *108:* 1083–1086, 1981.
4. Abe, E., Miyaura, C., Sakagami, H., Takeda, M., Konno, K., Yamazaki, T., Yoshiki, S., and Suda, T. Differentiation of mouse myeloid leukemia cells induced by 1$\alpha$,25-dihydroxyvitamin $D_3$. Proc. Natl. Acad. Sci. USA, *78:* 4990–4994, 1981.
5. Frampton, R. J., Omond, S. A., and Eisman, J. A. Inhibition of human cancer cell growth by 1,25-dihydroxyvitamin $D_3$ metabolites. Cancer Res., *43:* 4443–4447, 1983.
6. Hausseler, C. A., Marion, S. L., Pike, J. W., and Haussler, M. R. 1,25-dihydroxyvitamin $D_3$ inhibits the clonogenic growth of transformed cells via its receptor. Biochem. Biophys. Res. Commun., *139:* 136–143, 1986.
7. Eisman, J. A., Barkla, D. H., and Tutton, P. J. M. Suppression of the *in vivo* growth of human cancer solid tumor xenografts by 1,25-dihydroxyvitamin $D_3$. Cancer Res., *47:* 21–25, 1987.
8. Eisman, J. A., Martin, T. J., MacIntyre, I., and Moseley, J. M. 1,25-Dihydroxyvitamin D receptor in breast cancer cells. Lancet, *2:* 1335–1336, 1979.
9. Eisman, J. A., Martin, T. J., MacIntyre, I., Frampton, R. J., Moseley, J. M., and Whitehead, R. 1,25-dihydroxyvitamin $D_3$ receptor in a cultured human breast cancer cell line (MCF-7). Biochem. Biophys. Res. Commun., *93:* 9–15, 1980.
10. Findlay, D. M., Michelangeli, V. P., Eisman, J. A., Frampton, R. J., Moseley, J. M., MacIntyre, I., Whitehead, R., and Martin, T. J. Calcitonin and 1,25-dihydroxyvitamin $D_3$ receptors in human breast cancer cell lines. Cancer Res., *40:* 4764–4767, 1980.
11. Freake, H. C., Marcocci, C., Iwasaki, J., and MacIntyre, I. 1,25-Dihydroxyvitamin $D_3$ specifically binds to human breast cancer cell line (T-47D) and stimulates growth. Biochem. Biophys. Res. Commun., *101:* 1131–1138, 1981.
12. Frampton, R. J., Suva, L. J., Eisman, J. A., Findlay, D. M., Moore, G. E., Moseley, J. M., and Martin, T. J. Presence of 1,25-dihydroxyvitamin $D_3$ receptors in established human cancer cell lines in culture. Cancer Res., *42:* 1116–1119, 1982.
13. Baxter, R. C., Maitland, J. E., Raison, R. L., Reddel, R. R., and Sutherland, R. L. High molecular weight somatomedin-C/IGF-I from T-47D human mammary carcinoma cells: immunoreactivity and bioactivity. *In*: E. M. Spencer (ed.), Insulin-like Growth Factors/Somatomedins, pp. 615–618. Berlin: Walter de Gruyter Co., 1983.
14. Salomon, D. S., Zwiebel, J. A., Bano, M., Losonczy, I., Felnel, P., and Kidwell, W. R. Presence of transforming growth factors in human breast cancer cells. Cancer Res., *44:* 4069–4077, 1984.
15. Dickson, R. B., Huff, K. K., Spencer, E. M., and Lippman, M. E. Induction of epidermal growth factor-related polypeptides by 17$\beta$-estradiol in MCF-7 human breast cancer cells. Endocrinology, *118:* 138–142, 1985.
16. Dickson, R. B., Bates, S. E., McManaway, M. E., and Lippman, M. E. Characterization of estrogen responsive transforming activity in human breast cancer cell lines. Cancer Res., *46:* 1707–1713, 1986.
17. Huff, K. K., Kaufman, D., Gabbay, K. H., Spencer, E. M., Lippman, M. E., and Dickson, R. B. Secretion of an insulin-like growth factor-I-related protein by human breast cancer cells. Cancer Res., *46:* 4613–4619, 1986.
18. Dickson, R. B., and Lippman, M. E. Estrogenic regulation of growth and

[6] R. L. Sutherland, R. E. Hall, G. Y. N. Pang, E. A. Musgrove, and C. L. Clarke. Effect of medroxyprogesterone acetate on proliferation and cell cycle kinetics of human mammary carcinoma cells, submitted for publication.

[7] M. Koga, A. D. Semler, and R. L. Sutherland, unpublished observations.

[8] T. E. Ewing, L. J. Murphy, M.-L. Ng, G. Y. N. Pang, and R. L. Sutherland. Differential regulation of epidermal growth factor receptor by progestins and glucocorticoids in human breast cancer cell lines. Manuscript in preparation.

[9] M. Koga, and R. L. Sutherland, unpublished observations.

polypeptide growth factor secretion in human breast carcinoma. Endocrinol. Rev., *8:* 29–43, 1987.
19. Knabbe, C., Lippman, M. E., Makefield, L. M., Flanders, K. C., Kasid, A., Derynck, R., and Dickson, R. B. Evidence that transforming growth factor-$\beta$ is a hormonally regulated negative growth factor in human breast cancer cells. Cell, *48:* 417–428, 1987.
20. Murphy, L. J., Sutherland, R. L., and Lazarus, L. Regulation of growth hormone and epidermal growth factor receptors by progestins in breast cancer cells. Biochem. Biophys. Res. Commun., *131:* 767–773, 1985.
21. Murphy, L. J., Sutherland, R. L., Stead, B., Murphy, L. C., and Lazarus, L. Progestin regulation of epidermal growth factor receptor in human mammary carcinoma cells. Cancer Res., *46:* 728–734, 1986.
22. Chapman, R. E., Renwick, A. G. C., and Liversey, J. H. The isolation of human pituitary hormones from frozen gland. J. Clin. Endocrinol. Metab., *53:* 1008–1013, 1981.
23. Reddel, R. R., Murphy, L. C., and Sutherland, R. L. Differential sensitivity of human breast cancer cell lines to the growth-inhibitory effects of tamoxifen. Cancer Res., *45:* 1525–1531, 1985.
24. Murphy, L. J., Vrhovsek, E., Sutherland, R. L., and Lazarus, L. Growth hormone binding to cultured human breast cancer cells. J. Clin. Endocrinol. Metab., *58:* 149–156, 1984.
25. Ketelalegers, J. M., Knott, G. D., and Catt, K. J. Kinetics of gonadotrophin binding by receptors of the rat testis. Analysis by a nonlinear curve-fitting method. Biochemistry, *14:* 3075–3081, 1975.
26. Scatchard, G. The attractions of proteins for small molecules and ions. Ann. NY Acad. Sci., *51:* 660–665, 1949.
27. Murphy, L. J., Murphy, L. C., Stead, B., Sutherland, R. L., and Lazarus, L. Modulation of lactogenic receptors by progestins in cultured human breast cancer cells. J. Clin. Endocrinol. Metab., *62:* 280–287, 1986.
28. Imai, Y., Leung, C. K. H., Friesen, H. G., and Shiu, R. P. C. Epidermal growth factor receptors and effect of epidermal growth factor on growth of human breast cancer cells in long-term tissue culture. Cancer Res., *42:* 4394–4398, 1982.
29. Fitzpatrick, S. L., La Chance, M. P., and Schultz, G. S. Characterization of epidermal growth factor receptor and action on human breast cancer cells in culture. Cancer Res., *44:* 3442–3447, 1984.
30. Tsoukas, C. D., Provvedini, D. M., and Manolagas, S. C. 1,25-Dihydroxyvitamin $D_3$: a novel immunoregulatory hormone. Science (Wash. DC), *224:* 1438–1440, 1984.
31. Bar-Shavit, Z., Teitelbaum, S. L., Reitrsma, P., Hall, A., Pegg, L. E., Trial, J., and Kahn, A. J. Induction of monocytic differentiation and bone resorption by 1,25-dihydroxyvitamin $D_3$. Proc. Natl. Acad. Sci. USA, *80:* 5907–5911, 1983.
32. Eisman, J. E., MacIntyre, I., Martin, T. J., and Frampton, R. J. Normal and malignant breast tissue is a target for 1,25-dihydroxyvitamin $D_3$. Clin. endocrinol., *13:* 267–272, 1980.
33. Sher, E., Eisman, J. E., Moseley, J. M., and Martin, T. J. Whole-cell uptake and nuclear localization of 1,25-dihydroxycholecalciferol by breast cancer cells (T47 D) in culture. Biochem. J., *200:* 315–320, 1981.
34. Vignon, F., Bardon, S., Chalbos, D., and Rochefort, H. Antiestrogenic effect of R5020, a synthetic progestin in human breast cancer cells in culture. J. Clin. Endocr. Metab., *56:* 1124–1130, 1983.
35. Horwitz, K. B., and Freidenberg, G. R. Growth inhibition and increase of insulin receptors in antiestrogen-resistant T47 $D_{co}$ human breast cancer cells by progestins: implications for endocrine therapies. Cancer Res., *45:* 167–173, 1985.
36. Sainsbury, J. R. C., Farndon, J. R., Sherbet, G. V., and Harris, A. L. Epidermal-growth-factor receptors and oestrogen receptors in human breast cancer. Lancet, *1:* 364–366, 1985.
37. Davidson, N. E., Gelmann, E. P., Lippman, M. E., and Dickson, R. B. Epidermal growth factor receptor gene expression in estrogen receptor-positive and -negative human breast cancer cell lines. Mol. Endocrinol., *1:* 216–223, 1987.
38. Filmus, J., Pollak, M. N., Cailleau, R., and Bruick, R. N. MDA-468, a human breast cancer cell line with a high number of epidermal growth factor (EGF) receptors, has an amplified EGF receptor gene and is growth inhibited by EGF. Biochem. Biophys. Res. Commun., *128:* 898–905, 1985.
39. Huberman, E., and Callahan, M. Induction of terminal differentiation in human promyelocytic leukemia cells by tumor-promoting agents. Proc. Natl. Acad. Sci. USA, *76:* 1293–1297, 1979.
40. Breitmen, T. R., Selonick, S. E., and Collins, S. J. Induction of differentiation of the human promyelocytic leukemia cell line (HL-60) by retinoic acid. Proc. Natl. Acad. Sci. USA, *77:* 2936–2940, 1980.