Influence of 1α,25-dihydroxyvitamin D₃ on growth regulation through epidermal growth factor receptor in human breast cancer cell lines

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Abstract. We investigated the relationship between epidermal growth factor (EGF) dependent cell growth and anti-proliferative effects of 1α,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) in hormone responsive breast cancer cell lines in vitro. MCF-7 breast cancer cells and GMC-M, which is a serum-independent, hormone receptor-positive subtype derived from MCF-7, were used in this study. EGF stimulated the growth of both cell lines, and 1,25(OH)₂D₃ inhibited the EGF-stimulated cell growth in a dose dependent fashion. But treatment with 1,25(OH)₂D₃ did not change the EGF receptor (EGFR) level significantly in either cell line. GMC-M had a higher level of EGFR and was more sensitive to EGF than MCF-7. These results suggest that other mechanisms of action, which are different from EGFR modulation, concern with the growth inhibitory effect of 1,25(OH)₂D₃, and that 1,25(OH)₂D₃ will be a new effective treatment for breast cancer irrespective of EGFR.

Introduction

Endocrine therapy is widely accepted for breast cancer because of its effectiveness and low toxicity (1-3). However, breast cancer cells consistently lose their hormone sensitivity and change from hormone-responsive to hormone-resistant after long-term use of antiestrogens or progestins (1-4). Many reports have recently demonstrated the importance of autocrine and/or paracrine growth factors and their receptors in relation to the loss of hormone sensitivity in breast cancer cells (1-5). Among many autocrine growth factors, epidermal growth factor (EGF) and transforming growth factor α (TGF-α) are potent mitogens of breast cancer cells, and they bind to the EGF receptor (EGFR) (6,7). There is an inverse relationship between EGFR and estrogen receptor (ER) in both breast cancer patients and breast cancer cell lines (8-10). The over-expression of EGFR correlates with poor prognosis in breast cancer (8).

It has been demonstrated that 1α,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) suppresses proliferation and induces differentiation of cancer cells including breast cancer cells (11,12). 1,25(OH)₂D₃ and its analogues are known to express their antitumor effects by binding 1,25(OH)₂D₃ receptor, but the actual mechanism involved in this action is not clear (13-15). Among these analogues, 1,25(OH)₂D₃ has been studied most and its calcium metabolism and antitumor effects in breast cancer are known.

In the present study, we investigated as a first step the relationship between EGF-dependent breast cancer cell growth and anti-proliferative effects of 1,25(OH)₂D₃ using ER-positive MCF-7 breast cancer cell line and GMC-M, which was derived from MCF-7 in our institute.

Materials and methods

Cell lines. The MCF-7 breast cancer cell line (ER-positive and progesterone receptor (PgR)-positive) was generously donated by Dr. S. Kobayashi (Nagoya City University, Nagoya) and stored in our institute. GMC-M (ER-positive and PgR-positive) was obtained by culturing of MCF-7 in serum-free RPMI-1640-S (described later).

Materials. Phosphate buffered solution (PBS), RPMI-1640 medium and fetal bovine serum (FBS) were purchased from Gibco BRL (New York, NY). Mouse EGF was from Takara Shuzo Co., Ltd. (Tokyo). [³²P]-EGF was from NEN Research Products (Boston, MA). Bovine serum albumine (BSA) was from Sigma Chemical Co. (St. Louis, MO). 1,25(OH)₂D₃ was supplied by Chugai Pharmaceutical Co., Ltd. (Tokyo). Serum-free RPMI-1640-S was made as described (16).

Cell culture. MCF-7 cells were routinely maintained in RPMI-1640 with 10% FBS, 100 U/ml penicillin G (Meiji Seika Kaisha Ltd., Tokyo), 0.1 mg/ml streptomycin (Meiji Seika Kaisha Ltd., Tokyo) and 1.5 mg/ml sodium bicarbonate at 37°C in humidified 5% CO₂, 95% air. For GMC-M cells, RPMI-1640-S was routinely used. For cell growth experiments, RPMI-1640 (without phenol red) with 5% FBS was used. For
subculture, cells were trypsized, centrifuged and resuspended in the medium.

**Cell growth experiments.** To determine cell growth rates, a DNA fluorometric assay was performed according to the method of Hinegardner (17). Cells were plated into a 96-well culture plate at 1000 cells/well. Seventy-two hours after inoculation, each well was replaced with the medium supplemented with EGF and/or 1,25(OH)₂D₃, and the medium was changed every 48 h. At two, four and six days after the addition of EGF and/or 1,25(OH)₂D₃, cells were fixed by 0.2 ml/well of ethanol. Each well was drained, dried and 8 mg/100 μl/well of 3,5-diaminobenzoic acid•2HCl was added. Plates were heated at 60°C for 90 min. Fluorescence of 520 nm excited by 450 nm was measured with MTP-32 microplate reader (Corona Electric Co., Ltd., Katsuta, Japan). Calf thymus DNA (Sigma Chemical Co., St. Louis, MO) was used as the standard.

**EGFR binding assay.** Cells were plated into a 24-well culture plate at 5x10⁴ cells/ml/well in RPMI-1640 with 5% FBS. When the cells had reached confluence, the medium was replaced with RPMI-1640 with 0.1% BSA to wash the cells for an hour. After the wash medium was aspirated, 0.5 ml of RPMI-1640 with 0.1% BSA containing [¹²⁵I]-EGF (0.5 to 5 ng/ml) with or without an excessive unlabeled EGF (200 ng/ml) was added to each well and the cells were incubated for 3 h at 20°C. After incubation, the cells were washed twice with 1 ml/well of PBS with 0.1% BSA. The cells were then dissolved in 0.5 ml/well of 1N NaOH. Radioactivity was measured with Cobra 5020. Specific binding was calculated as a difference between wells in the presence and in the absence of the excessive unlabeled EGF. Cell numbers of comparably treated parallel wells were counted with Coulter Counter (Coulter Electronics Ltd., Luton, UK). EGFR binding sites/cell and dissociation constant (kd) were calculated with the Scatchard analysis (18).

**Statistical analysis.** Statistical analysis of the differences between groups was conducted using one-way factorial analysis of variance (ANOVA) and Fisher's PLSD method to determine significant differences between group means. Values were expressed as the mean ± standard error of the mean. Differences resulting in a p-value less than 0.05 was considered to be significant.

**Results**

**Effects of EGF on the cell growth of MCF-7 or GMC-M.** Ten nM of EGF significantly (p<0.01) stimulated the proliferation of MCF-7 cells compared to the MCF-7 untreated group (control) on day 6, while 1 nM of EGF did not significantly stimulate the cell growth of MCF-7 cells. Both 1 and 10 nM of EGF significantly (p<0.05) stimulated the GMC-M cell growth dose-dependently, and GMC-M cells responded more sensitively to EGF than MCF-7 cells (Fig. 1).

**Effect of 1,25(OH)₂D₃ on the cell growth of MCF-7 or GMC-M.** Both 0.1 and 10 nM of 1,25(OH)₂D₃ significantly (p<0.001) inhibited the cell growth of MCF-7 cells in a dose dependent manner. 1,25(OH)₂D₃ inhibited cell growth of GMC-M in the same manner, but the response of GMC-M cells to 1,25(OH)₂D₃ was not as sensitive as MCF-7 cells (Fig. 2).

In GMC-M cells, about ten times higher concentration of 1,25(OH)₂D₃ was needed to obtain the same grade of the growth inhibitory effect as shown in MCF-7 cells.

**Effects of EGF and 1,25(OH)₂D₃ on the cell growth of MCF-7 or GMC-M.** Ten nM of EGF increased the cell growth of MCF-7 to 105% of the control, but an addition of 0.1 and 10 nM of 1,25(OH)₂D₃ decreased it to 87.3% and 47.1% of the control, respectively. 1,25(OH)₂D₃ suppressed the growth stimulatory effects of EGF in MCF-7 cells dose-dependently (Fig. 3). In GMC-M cells, 10 nM of EGF increased the cell growth to 154.6% of the control, but an addition of 10 and 100 nM of 1,25(OH)₂D₃ decreased it to 110.2% and 33.9% of
Figure 3. Effect of EGF and 1,25(OH)_{2}D_{3} on the cell growth of MCF-7. EGF stimulated the cell growth and 1,25(OH)_{2}D_{3} inhibited the stimulatory effect of EGF dose-dependently. Bars represent the mean with S.E.M.

Figure 4. Effect of EGF and 1,25(OH)_{2}D_{3} on the cell growth of GMC-M. EGF stimulated the cell growth and 1,25(OH)_{2}D_{3} inhibited the stimulatory effect of EGF dose-dependently. Bars represent the mean with S.E.M.

Discussion

Both EGF and TGF-α are known as growth factors of breast cancer. Their growth stimulatory effects are expressed by binding to EGFR (6,7,19). EGF affects the differentiation in the developmental stage and stimulates the epithelial cell proliferation including mammary glands (20,21). It is also known that EGF stimulates the cell growth of several breast cancer cell lines including MCF-7 (6,7,10,12,19). In our study, EGF stimulated the cell proliferation of both MCF-7 and GMC-M cells, but the response of these two cell lines to EGF was different. GMC-M responded to lower concentrations of EGF than MCF-7. GMC-M contained higher levels of EGFR than MCF-7. Increased sensitivity of GMC-M to EGF may accompany the changes in EGFR levels and the growth regulatory mechanisms through EGFR.

1,25(OH)_{2}D_{3} receptors were found not only in normal and malignant breast tissues but also cultured human breast cancer cells (13-15,22-24). 1,25(OH)_{2}D_{3}, in addition to its role in bone and calcium metabolism, has growth inhibitory effects on many types of cancer cells including breast cancer cells, but the mechanism of growth inhibitory effect of 1,25(OH)_{2}D_{3} is obscure (11-15). In the present study, 1,25(OH)_{2}D_{3} inhibited the cell growth of both MCF-7 and GMC-M, but in GMC-M, higher concentrations of 1,25(OH)_{2}D_{3} were needed to obtain the same degree of growth inhibitory effects as MCF-7. Shabahang et al demonstrated that 1,25(OH)_{2}D_{3} receptor levels correlated with the grade of differentiation in colon cancer cell lines (25). The difference of sensitivity to 1,25(OH)_{2}D_{3} between MCF-7 and GMC-M suggests the difference of 1,25(OH)_{2}D_{3} receptor levels of these cell lines, which may correlate with the grade of differentiation.

Growth stimulatory effects of EGF were blocked by 1,25(OH)_{2}D_{3} in a dose dependent manner in both MCF-7 and GMC-M.
GMC-M. At high concentrations, 1,25(OH)2D3 suppressed the proliferation of both cell lines under control levels. Although there are some evidence that 1,25(OH)2D3 modulates EGFR of some breast cancer cell lines (11,12,26-28), the changes in EGFR levels of MCF-7 after the incubation of 1,25(OH)2D3 were not significant in our experimental study. It is possible that mechanisms which are different from EGFR modulation are involved in regulation of breast cancer cell growth by 1,25(OH)2D3.

Due to its hypercalceamic side effects, the clinical utility of 1,25(OH)2D3 is limited. Recently, chemically modified forms of 1,25(OH)2D3, such as 22-oxa-calcitriol (OCT) and EB1089, have been developed which retain the antiproliferative and differentiating effect of the 1,25(OH)2D3 and minimize its hypercalceamic activity (13-15). These agents express the effects in ER-positive and ER-negative breast cancer cells by binding the same 1,25(OH)2D3 receptor (14). 1,25(OH)2D3 and its analogues can inhibit tumor growth by a variety of mechanisms, including regulation of angiogenesis, apoptotic cell death, and tumor invasiveness (14,29). Induction of the autocrine and paracrine growth inhibitor TGF-β by 1,25(OH)2D3 is also likely to be one of mechanisms which regulates tumor cell growth (30). In ER-positive breast cancer cells, anti-proliferative effect of OCT was enhanced by combined treatment with the antitestrogen tamoxifen in vitro (31,32). Patients with locally advanced or metastatic breast cancer were treated with OCT and the tumors containing 1,25(OH)2D3 receptors responded to the treatment (33).

Recently, bisphosphonates such as clodronate and alendronate was effectively used to control hypercalceemia in breast cancer patients with bone metastasis (34,35). In addition to their anti-hypercalceemic effects, bisphosphonates improved osteolytic lesions and reduced pain of bone metastasis from breast cancer (36-38). Combined usage of 1,25(OH)2D3 with bisphosphonates may be a new effective treatment for breast cancer.

In conclusion, 1,25(OH)2D3 inhibited EGFR-stimulated breast cancer cell growth without altering EGFR levels. These findings suggest that 1,25(OH)2D3 and its analogues can be useful in new clinical treatments for advanced or metastatic breast cancer irrespective of ER or EGFR.

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References
