Reduction of Epidermal Growth Factor Binding in Human Breast Cancer Cell Lines by an Alkyl-Lyosphospholipid

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ABSTRACT

The effects of 1-O-octadeyl-2-O-methyl-sn-glycero-3-phosphocholine (ET-18-OCH₃), an alkyl lyosphospholipid derivative, on the binding of epidermal growth factor (EGF) to human breast cancer cell lines (MCF-7, ZR-75-1, and BT-20), the human epidermoid cancer cell line (A431), and the rat fibroblast cell line (NIH3T3) were investigated. The addition of 10 µg/ml ET-18-OCH₃ to the growth medium reduced the binding of EGF to hormone-dependent breast cancer cell lines (MCF-7 and ZR-75-1) and A431 but did not change that to the hormone-independent breast cancer cell line (BT-20). ET-18-OCH₃ suppressed the EGF-binding prior to the onset of its inhibitory action on cell growth in MCF-7 and ZR-75-1. Scatchard plot analysis demonstrated that ET-18-OCH₃ reduced the number of EGF receptor sites without affecting the affinity of EGF receptors in MCF-7 and ZR-75-1. Both EGF-binding and cell growth in NIH3T3 were not changed by treatment with 10 µg/ml ET-18-OCH₃.

These results suggest that ET-18-OCH₃ inhibits the growth of hormone-dependent breast cancer cell lines (MCF-7 and ZR-75-1) by reducing the binding capacity of EGF receptors and consequently by disturbing the transfer of a variety of growth-promoting signals.

INTRODUCTION

The growth of hormone-dependent breast cancer has long been known to be under endocrine control by estradiol. Recent preliminary evidence suggested that the estrogen-dependent growth of human breast cancer cell lines is mediated by TGFs which are produced and secreted in a large quantity under the influence of estradiol (1–3).

On the other hand, it has been demonstrated in cultured cells that a tumor promoter such as TPA decreases the binding affinity of EGF receptors and reduces the activity of EGF receptor-linked protein kinase (4, 5).

It has been known that ALPs are highly cytotoxic to certain tumor cells than to normal cells (6–12). Antitumoral phospholipids of this type are structurally related to a recently identified group of novel and native bioactive phospholipids known as PAF (13).

In the present study, we have investigated the effect of ET-18-OCH₃, an ALP derivative with a potent and selective cytotoxic activity to tumor cells, on EGF binding to human breast cancer cells.

MATERIALS AND METHODS

Chemicals. ¹²⁵I-EGF (168 µCi/µg) was purchased from New England Nuclear (Boston, MA). ET-18-OCH₃, lyso-PAF, and PAF were purchased from Bachem Feinchemikalien AG (Bubendorf, Switzerland), and LPC (from egg yolk) was from Sigma Chemical Company (St. Louis, MO). LPC, lyso-PAF, PAF, and ET-18-OCH₃ were dissolved in the culture medium to the desired concentrations from stock solutions of 0.5 mg/ml. Unlabeled mouse submaxillary EGF was purchased from Takara Shuzou (Kyoto, Japan), and all culture media used were obtained from Flow Laboratories (McLean, VA). Other chemicals used were obtained from Wako Pure Chemical Industries (Osaka, Japan).

Cell Lines. The breast cancer cell line MCF-7 was supplied by Dr. Nomura of the Kyushu Cancer Center. The breast cancer cell lines ZR-75-1 and BT-20, rat fibroblast cell line NIH3T3, and human epidermoid cancer cell line A431 were obtained from ATCC (Rockville, MD). MCF-7 was subcultured in RPMI 1640 supplemented with 10% (v/v) FBS (Filtron, Altona, Australia). ZR-75-1 (RPMI 1640 plus 10% FBS), BT-20 (minimal essential medium plus nonessential amino acids plus 10% FBS), NIH3T3 (Dulbecco's modified Eagle's medium plus 10% calf serum (Flow Laboratories)), and A431 (Dulbecco's modified Eagle's medium plus 10% FBS) were subcultured according to ATCC manuals. Penicillin (100 U/ml) and streptomycin (100 µg/ml) were added to all the media used. All the cell lines used were passaged in T-150 flasks (Falcon No. 3024; Becton-Dickinson and Company, Lincoln Park, NJ) at 37°C in a humidified atmosphere containing 5% CO₂.

Growth Experiment. Each cell line was plated at a density of 1 × 10⁴ cells/well into 16-mm multiwell dishes (Falcon No. 3047), and the incubation was started after 1 day by addition of various concentrations of ET-18-OCH₃. Cell proliferation was monitored at various stages by measuring DNA by the method of Burton (14) as modified by Taylor et al. (15).

EGF Receptor Assay. EGF receptor assay was carried out as described previously (4) with minor modifications. Briefly, every cell line was grown for 3 days in 35-mm 6-well dishes (Falcon No. 3046) to a density of 2–4 × 10⁴ cells/well and then incubated with ET-18-OCH₃ for a given period. Then, the cells were rinsed once with 2 ml of HBSS/albumin. For the binding assay, the cells were incubated for 4 h at 20°C with 1 ml of HBSS/albumin containing 0.1 ng/ml of ¹²⁵I-EGF. The nonspecific binding was detected by adding a 100-fold excess of unlabeled EGF. After the binding incubation, the medium was removed and the cells were rinsed three times each with 1 ml of ice-cold HBSS/albumin. The cells were dissolved in 1 N NaOH/0.5% Triton X-100 for overnight, and radioactivity was counted using the gamma counter (ARC-605; Aloka, Tokyo, Japan).

RESULTS

The EGF-binding capacities of MCF-7 and ZR-75-1 were dose dependently decreased by 10–25 µg/ml of ET-18-OCH₃ (Fig. 1) but unaffected by 0.01, 0.1, or 1 µg/ml of ET-18-OCH₃ in 12-h incubation (data not shown). The time courses of the suppressive effects of ET-18-OCH₃ on EGF binding to MCF-7 and ZR-75-1 are shown in Fig. 1. In both cell lines, the EGF binding began to decrease within 3 h after the addition of 10–25 µg/ml of ET-18-OCH₃. The binding to MCF-7 was more greatly decreased by ET-18-OCH₃ than that to ZR-75-1 (Fig. 1). Fig. 2 shows the time course of cell growth monitored by DNA content. The growth of MCF-7 was not depressed by 3–12 h incubation with 1–25 µg/ml ET-18-OCH₃ (Fig. 2A). The proliferation of ZR-75-1 was also unaffected by 1–25 µg/ml ET-18-OCH₃ in 3–18-h incubation (Fig. 2B).

Scatchard plots obtained by saturation binding experiments are shown in Fig. 3. By treatment with 10 µg/ml of ET-18-
REDUCTION OF EGF BINDING IN BREAST CANCER BY ALP

Time (hours)

Fig. 1. Time dependence of the inhibitory effect of ET-18-OCH₃ on EGF binding to human breast cancer cell lines MCF-7 (O) and ZR-75-1 (■). 10 µg/ml ET-18-OCH₃, 25 µg/ml ET-18-OCH₃. The EGF concentration used was 0.1 ng/ml. The values shown are those normalized to the control (100%) measured in the absence of ET-18-OCH₃ and are the means of triplicate determinations.

Fig. 2. Effect of ET-18-OCH₃ on the growth of human breast cancer cell lines MCF-7 (A) and ZR-75-1 (B) monitored by the cell content of DNA. ET-18-OCH₃ concentrations were (ng/ml): 0 (■, control); 1 (O); 10 (A); 25 (■). The data shown are the means of quadruplicate determinations.

The effects of ET-18-OCH₃ and their analogues on EGF binding to various cell lines are shown in Table 1. In a 12-h incubation, 10 µg/ml ET-18-OCH₃ decreased EGF binding to MCF-7, ZR-75-1, and A431, but it did not affect that to either BT-20 or NIH3T3. Moreover, LPC, lyso-PAF, or PAF, all at 10 µg/ml, in 48 h of incubation, but the growth of A431 was inhibited significantly by incubation for 48 h with 10 µg/ml of either PAF or lyso-PAF (Table 2).

Table 2 shows the effects of ET-18-OCH₃ and their analogues on the growth of various cell lines. The growth of MCF-7, ZR-75-1, and A431 was inhibited but that of BT-20 and NIH3T3 was not depressed by 10 µg/ml of ET-18-OCH₃ in incubation for 48 h. The growth of MCF-7, ZR-75-1, BT-20, and NIH3T3 was not affected by LPC, lyso-PAF, or PAF, all at 10 µg/ml, in 48 h of incubation, but the growth of A431 was inhibited significantly by incubation for 48 h with 10 µg/ml of either PAF or lyso-PAF (Table 2).

There was a significant positive correlation between the percentage of control of EGF binding in 12 h incubation with 10 µg/ml of ET-18-OCH₃ and the percentage of control of DNA content in 48 h incubation with 10 µg/ml of ET-18-OCH₃ in five cell lines tested (Fig. 4).

DISCUSSION

The ethers with (a) a long aliphatic side chain at position sn-1, (b) an unmetabolizable group at sn-2 (e.g., amide or ether), and (c) a quaternary phospho base at sn-3 are known to be cytotoxic to animal tumors and possibly to human tumors as well, but not to normal cells and tissues (6-11, 16). The mechanisms proposed for the antitumoral activity of ALPs are: (a) stimulation of macrophages in vivo (17, 18) and (b) expression of a direct and specific cytotoxic action to tumor cells in the absence of macrophage (7-11, 16). Recently, Hoffman et al. (12) have demonstrated that the unnatural alkyl phospholipid derivatives accumulate on the surface membrane of “sensitive” cancer cells and also pointed out that the difference in the cellular activities of alkyl cleavage enzyme is not responsible for the differential cytotoxic activities of ET-18-OCH₃ on normal cells and the specific types of neoplastic cells.

Induction by estrogen of a TGF-α-like activity was reported in 3 species of hormonally responsive breast cancer cells (19). This previous report supports the hypothesis that estrogen stimulates the growth of breast cancer via TGF-α acting as autocrine growth factors.

In this study, we found that the addition of ET-18-OCH₃ to the growth medium reduced the binding of EGF to hormone-dependent breast cancer cell lines MCF-7 and ZR-75-1. Although 10-25 µg/ml ET-18-OCH₃ began suppressing EGF binding within 3 h, it did not depress the growth of MCF-7 in 3-12 h incubation. The proliferation of ZR-75-1 was also unaffected by 1-25 µg/ml ET-18-OCH₃ in 3-12 h incubation. A similar result was obtained from Meth A cells by the trypan blue exclusion assay (20). Moreover, the inhibition of [³H] thymidine uptake induced by ET-18-OCH₃ in more than 24 h incubation have been reported in various cells (8-10). In our results, [³H] thymidine uptake of MCF-7 and ZR-75-1 was not
inhibited by incubation for 6–12 h but began to be inhibited after 18 h incubation with 10 μg/ml of ET-18-OCH3 (data not shown). These findings indicate that ET-18-OCH3-induced blockage of EGF binding is not the result of the growth suppression induced by ET-18-OCH3. Therefore we think that the cell growth and [3H]thymidine uptake in the early stage after the addition of ET-18-OCH3 is important to elucidate the mode of action of ET-18-OCH3.

On the other hand, the EGF binding and cell growth of the hormone-independent breast cancer cell line BT-20 and the rat fibroblast NIH3T3 cell line remained unchanged by treatment with 10 μg/ml ET-18-OCH3. Furthermore, both EGF binding and cell growth in MCF-7, ZR-75-1, BT-20, and NIH3T3 were also not changed by treatment with 10 μg/ml of PAF, lyso-PAF, or LPC. Therefore, we suspected that the growth-inhibitory activity of ET-18-OCH3 may be at least partly due to the inhibition of EGF binding by ET-18-OCH3 in hormone-dependent breast cancer cell lines and that the growth-inhibitory action induced by this reduction in EGF binding may be one of the modes of antitumoral action of ET-18-OCH3 in hormone-dependent breast cancer cells. The reduction of EGF binding by ET-18-OCH3 was closely related to the inhibition of cell growth because there was a significant correlation between the percentage of control of EGF binding and the percentage of control of DNA content in five cell lines tested.

A marked reduction in EGF binding and strong inhibition of cell growth were induced by treatment with 10 μg/ml ET-18-OCH3 in EGF receptor-rich A431 cell lines. These findings suggest that the growth inhibition of A431 cells also took some part in the reduction of EGF binding. We surmise, therefore, that ET-18-OCH3 may cause the growth inhibition of not only hormone-dependent breast cancer cells but also other cancer cells possessing EGF receptors through the same mechanism. The mechanism of the growth inhibition of A431 treated by lyso-PAF and PAF has not been clarified.

Herrmann (20) has reported that, within 2 h incubation, ET-18-OCH3 decreases the incorporation of radiolabeled fatty acid into phosphatidylcholine. Furthermore, Bolscher et al. (21) reported the influence of ALP on the expression of the carbohydrate moiety of cell surface glycoproteins. These biochemical events may be related to the biochemical effect of ET-18-OCH3 on hormone-dependent breast cancer cells and A431 cells, i.e., the reduction of EGF binding.

Roos et al. (4) have investigated the inhibitory effect of TPA on EGF binding in breast cancer cells and presented preliminary evidence that TPA not only decreases the affinity of EGF receptor but also reduces the number of EGF receptor sites in hormone-dependent human breast cancer cell lines (MCF-7 and ZR-75-1). In our experiment, it was demonstrated by Scatchard plots that ET-18-OCH3 caused an apparent reduction in the number of EGF receptor sites but did not affect the affinity of the EGF receptor in hormone-dependent breast cancer cells. Thus, the action of ET-18-OCH3 is distinct from that of TPA as far as the EGF binding to MCF-7 and ZR-75-1 cell lines is concerned. Since we used only one cell line of hormone-independent breast cancer in the present study, we
could not show a clear difference in the mechanism of action between hormone-dependent and hormone-independent cells. ET-18-OCH₃, however, will be useful for elucidation of signal transduction in hormone-dependent breast cancer cells in future research.

It was reported that PKC phosphorylates the EGF receptor and reduces the activity of EGF receptor-stimulated tyrosine protein kinase (22). Moreover, PKC is activated by high affinity binding with tumor-promoting phorbol ester (TPA) (23). Therefore, it is inferable that the inhibitory effect of TPA on EGF binding may be mediated by PKC (4). On the other hand, since PAF analogues cannot activate PKC (24) and alkyl-linked diglycerides dose dependently inhibit the PKC activity stimulated by the synthetic diacylglycerol, 1-O-oleoyl-2-acetyl-sn-glycerol (25), it is likely that the reduction of EGF binding caused by ET-18-OCH₃ may not be mediated by stimulation of the PKC activity but probably by inhibition of the activity of PKC or other kinase species.

Although 10–25 μg/ml (18-45 μM) ET-18-OCH₃ began to suppress the EGF binding of MCF-7 andZR-75-1 within 3 h, a sufficient decrease was attained 12 h after the addition of ET-18-OCH₃, while 10 μM TPA was reported to begin depressing the EGF binding within 2 h (4). We think that this is due to the weaker cytotoxicity of ET-18-OCH₃ compared with a potent tumor promoter, TPA.

Possible mechanisms of action for ET-18-OCH₃ and the antitumor activities of its analogues have been well documented (7–12, 16–18, 20, 21, 26), and the biological activities of alkyl-linked glycerides may be expressed though PKC inhibition (25). However, the effects of these compounds on intracellular processes such as those mediated by PKC or other kinase species and the EGF-receptor interaction as described here must be investigated further for deeper understanding of the mechanism of the antitumoral action of ET-18-OCH₃.

From a clinical point of view, the activity of EGF receptors has been attracting much attention as one of the most important indexes for predicting relapse-free primary breast cancer and the overall survival of patients with breast cancer (27). ET-18-OCH₃ is fairly promising in breast cancer therapy as the drug capable of inhibiting the binding of EGF to the receptor without showing any serious adverse effect.

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