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Comparison of the Growth Inhibition by Alpha-Difluoromethylornithine and Hydroxytamoxifen in MCF-7 Human Breast Cancer Cells

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Abstract In estrogen-dependent MCF-7 human breast cancer cells, E_2 at 10 nM stimulated cell proliferation to over 200% compared to the untreated control. EGF and TGFα, which are known as the autocrine/paracrine growth factors induced by E_2, also directly stimulated the cell growth in almost as the same extent as E_2. DFMO which is the specific inhibitor of ODC could inhibit the cell growth even at as low as 0.5 mM. In the treatment with 1 mM DFMO for 4 days, the cell growth was inhibited to 38% of the control. HO-TAM at 1 μM could inhibit the proliferation of MCF-7 cells to 19% of the control. Those inhibitory effects were also found in the cells stimulated with E_2, EGF, and TGFα. The inhibitory effects were found even in 2 days of treatment. However, E_2, EGF, and TGFα did not give any effect in the protein synthesis. Neither DFMO or HO-TAM gave any effect on the total protein synthesis. But the pattern of protein secretion was noticeably influenced by the growth stimulants or inhibitors. Proteins of 160, 52, 42, 36, and 32 kDa belonged to the major secretory troteins. Especially, 42 and 36 kDa proteins were most significantly influenced by the treatment of E2, EGF, or TGFα. DFMO and HO-TAM inhibited the secretion of these major proteins.

Key words: MCF-7 cells, E_2, EGF, TGFα, DFMO, HO-TAM

Introduction

Breast cancer is one of the most prevalent disease in women throughout the world. Human breast cancer has been known to be strongly influenced by the steroid hormones, especially estrogen [1]. Clinically, two-third of human breast cancers are estrogen-dependent tumor, but later they may develope to estrogen-independent cancer. MCF-7 cell line is originally derived from a pleural effusion of a patient with metastatic breast cancer and is known to be dependent on estrogen for cell proliferation [2]. Thus, treatment and prognosis of breast cancer have been improved dramatically during the past decade through the use of the antiestrogen tamoxifen (TAM). TAM is the most commonly used nonsteroidal antiestrogen in breast cancer chemotherapy. It binds to estrogen receptor (ER) and, subsequently, prevents the growth of estrogen-responsive cells. In the uterus and mammary gland, estrogen-induced proliferation is accompanied by the increased mRNA and protein expression of peptide growth factors such as epidermal growth factor (EGF), transforming growth factor α (TGFα), and insulin-like growth factor I (IGF-I). EGF and TGFα promote the growth of both normal mammary epithelium and human breast cancer cells [3]. EGF is a polypeptide originally isolated from the submaxillary gland. EGF has numerous effects, including the stimulation of DNA synthesis on a variety of cells. These effects are thought to be mediated by the interaction of the peptide with a specific plasma membrane receptor. Recent evidence suggest that a variety of polypeptide growth factors such as IGF-I, EGF/TGFα like activity are produced by breast cancer cell and act as local mitogens in an autocrine/paracrine fashion. The synthesis of these growth factors is stimulated by E_2 in hormone-dependent breast cancer cell lines [4-6].

Polyamines (putrescine, spermidine, and spermine) are small aliphatic amines that are essential for cell growth and differentiation [7]. They can interfere with hormones in several key steps regulating breast cancer cell proliferation. It is known that constitutive activation of polyamine pathway enhances cell growth, thus bypassing the need for hormonal presence and leading to the development of the hormone-independent breast cancer phenotype. As a result, the activation of polyamine pathway may contribute to the breast cancer development [8]. In breast cancer cells, growth stimulatory agents including estradiol up-regulate the activity of ornithine decarboxylase (ODC), the key enzyme in polyamine biosynthesis. The catalytic activity of ODC can be blocked specifically and irreversibly by α-difluoromethylornithine (DFMO).

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Since depletion of intra cellular polyamines using DFMO causes a significaant suppression of cell growth and tumor formation *in vivo* as well as *in vitro*, DFMO is under development as a chemopreventive agent.

In the present study, the proliferation of hormone-dependent MCF-7 cells is going to be compared in the presence of E_2, EGF, and TGF α. Also, the antiproliferative effects of DFMO and TAM are going to be studied. Protein synthesis and secretion in the growth stimulants and inhibitors are also studied to find out the major growth regulatory proteins.

Materials and Methods

Chemicals

17 β-estradiol (E_2), 4-hydroxytamoxifen (HO-TAM), EGF, TGF α and Dulbecco's modified Eagle's medium (DMEM, with L-Glutamine and 1,000 mg/L Glucose) were purchased from Sigma Chemical CO. (St. Louis, MO, USA). DFMO was purchased from ILEX oncology INC. (San Antonio, Texas, U.S.A). L-[^{35}S]-methionine (1 mCi/100 μl) were obtained from Amersham (Uppsala, Sweden). All other chemicals were purchased from standard commercial sources.

Cell culture

MCF-7 cell line was supplied by Korean Cell Line Bank (KCLB). Cell were maintained in DMEM containing phenol red, 10,000 units/ml penicillin G, 10 mg/ml streptomycin, and 10% heat-inactivated fetal bovine serum in a humidified atmosphere of 95% air; 5% CO_2 at 37℃. Cells were subcultured at weekly intervals. Two days before each experiment, MCF-7 cells were grown in phenol red-free DMEM supplemented with 2% fetal bovine serum which was pretreated with dextran-coated charcoal (DCC) [9]. All experiments were conducted in phenol red-free medium and DCC-treated serum.

Measurement of Cell Proliferation.

MCF-7 cells were plated in 12 multi-well culture dishes at the density of 1.5×10^4 cells/ml. Two days later, medium was changed with fresh one containing E_2, EGF, TGF α, DFMO, or HO-TAM, (day 1). Cell numbers on day 3 and day 5 were determined by using Coulter Counter Z-1 (Coulter Corporation, Miami, FL, USA).

Analysis of the protein synthesis and secretion

MCF-7 cells were plated into 12 multi-well (4×10^4 cells/ml) in phenol red-free DMEM. Two days later, cells were replaced with fresh medium and treated with E_2, EGF, or TGF α with or without DFMO or HO-TAM (day 1). At day 3, cells were washed once with defined medium (methionine free DMEM) and then replenished with medium-containing 10 μCi/ml/well L-[^{35}S]-methionine, 62.6 mg/L L-cystein in methionine-free DMEM and test drugs. After 8 hrs incubation, the conditioned medium was centrifuged for 5 min to remove cellular debris and the supernatant was dried by using centrifugal evaporator Maxi Dry Plus (Heto, Denmark), then resuspended with a SDS-sample buffer (9% SDS, 6% 2-mercaptoethanol, 15% glycerol, and trace pyronine-Y). Cells were washed twice with 0.15 M KCl and then scrapped off by a rubber policeman and resuspended with a SDS-sample buffer. The radioactivity in the cell pellet or the conditioned media was measured by Liquid Scintillation Counter (LKB 1219 Rackbeta, Pharmacia, Sweden). Proteins were subjected to SDS-PAGE by the method of Laemmli [10]. Radioactivity in total cellular proteins and secreted proteins was determined and the equal amount of cpm was applied to SDS-PAGE. Dried gels were exposed to Imaging Plate (Fuji, Japan) for 2 ~ 3 days and analysed by Bas-1500 Image Analyzer (Fuji Bas, Japan).

Statistical analysis

All experiments were carried out at least three times. Statistical significance of control and treatment group was determined by one-way analysis of variance followed by the Duncan's multiple range test. In all cases, a p value less than 0.05 was considered significant.

Results

Effects of E_2, EGF, and TGF α on cell proliferation.

MCF-7 cells were seeded in phenol red free medium containing charcoal-treated serum for 2 days prior to the experiment. Growth of MCF-7 cells under these conditions was necessary to get optimal sensitivity to E_2 and to avoid the estrogenic effects of phenol red [1]. Cells were seeded at the density of 1.5×10^4 cells /ml, and E_2, EGF, or TGF α was added 48 h after seeding (Fig. 1, day 1). Even though the drug administration did not severely increase in MCF-7 cell proliferation on day 3 of treatment, cell number significantly increased on day 5. The maximum growth stimulatory

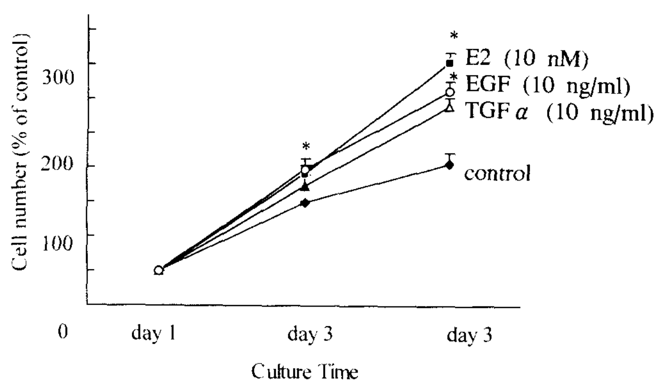

Fig. 1. Effects of E_2, EGF, and TGF α on the proliferation of MCF-7 human breast cancer cells. Cells were seeded at the density of 1.5×10^4 cells/ml of DMEM. After one day of equilibration, cells were treated with each drug on day 1. The data are shown as the mean of at least three experiments. $p<0.05$ vs. control.

effect was found in 10 nM E_2 with 207% of the control in the present study, and its effect was not increased at the higher concentration (Fig. 1). EGF or TGF α also stimulated cell proliferation to 165% or 168% of untreated control respectively. Bott EGF and TGF α exerted growth stimulation on a dose- dependent fashion (data not shown).

Inhibitory effects of DFMO and HO-TAM on cell proliferation

DFMO, a specific inhibitor of ODC, was used to study the involvement of polyamines in the proliferation of MCF-7 cells. The administration of 1 mM DFMO inhibited cell growth to 38% of the control on day 5 (Fig. 2). The significant growth inhibition could be detected even at 0.5 mM DFMO (data not shown). HO-TAM is an active metabolite of TAM which is most commonly used antiestrogen. At 1 μM HO-TAM, the growth of MCF-7 cells was blocked to 20% of the control on day 5. There were no difference between DFMO and HO-TAM in the growth inhibitory effect on day 3 culture.

Antiproliferative effects of DFMO in E_2-, EGF-, TGF α-treated cells

To investigate the importance of polyamines in estrogen- or growth factor-stimulated proliferation of MCF-7 cells, DFMO of 1 mM was simultaneously added into the culture with each treatment (Fig. 3). DFMO blocked cell proliferation in all three growth stimulated cultures. The inhibitory effects were found from the culture of day 3 (data not shown). On day 5, E_2-stimulated cell proliferation was blocked to 54% of the control by 1 mM DFMO (Fig. 3,A). DFMO also significantly inhibited EGF or TGF α-stimulated cell proliferation to 46% and 57% of the control, respectively. HO-TAM also significantly inhibited proliferation of the cells treated with E_2, EGF, or TGF α (Fig. 3,B).

Effects of Growth Stimulants and Inhibitors on the protein synthesis and secretion.

In order to determine the total protein synthesis and secretion,

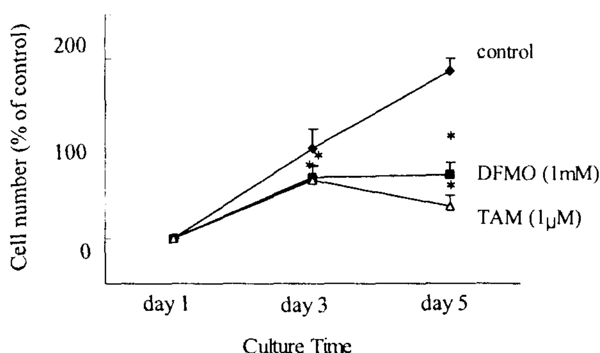

Fig. 2. Effects of DFMO and Ho-TAM on the proliferation of MCF-7 cells. Culture conditions are described on Fig. 1. The data are shown as the mean of at least three separate experiments. $p<0.05$ vs. control.

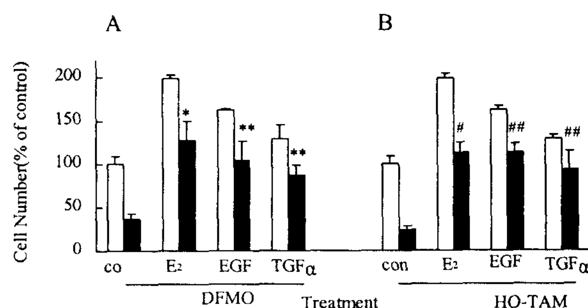

Fig. 3. Effects of DFMO and HO-TAM on the proliferation of MCF-7 cells stimulated with E_2, EGF, and TGF α for 4 days. Cells were treated with each drug on day 1 and cell numbers were counted on day 5. The culture conditions are described in Fig. 1. □, without inhibitors; ■, with 1 mM DFMO (A) or 1 μM HO-TAM (B). $p<0.05$ vs. each control without inhibitor.

cells were grown in the media with or without the experimental treatments containing [^{35}S]-methionine. For the assay of radioisotope labeled total intracellular protein synthesis, the whole cell protein extracts were separated by SDS-PAGE. In the cells treated with E_2, EGF, or TGF α, the pattern of proteins was very similar to that of the untreated control (data not shown). The protein synthesis was not even influenced by either DFMO or HO-TAM. However, E_2 and growth factors gave significant influence on the protein secretion in MCF-7 cells (Fig. 4). proteins of 160, 52, 42, 36, and 32 kDa belonged to the major group (Table 1). Among those, the secretion of 46, and 36 kDa proteins was most significantly stimulated by the growth stimulants. Especially, the secretion of 36 kDa protein was increased to 239% of the untreated control in the presence of EGF. E_2 and EGF stimulated the secretion of 32 kDa protein to 142 and 161%, respectively, but its secretion was dropped to 52% of the control by TGF α. The treatment of HO-TAM inhibited the secretion of most major secretory proteins, but DFMO inhibited the secretion of 42, 36, and 32 kDa proteins. Strangely, DFMO stimulated the secretion of 160 kDa protein to 130% of the control.

Discussion

In ER-positive MCF-7 cell, estrogen has been found to stimulate cell proliferation. E_2 showed growth promoting effect on MCF-7 cells at the concentration range of 0.1 ~ 50 nM in a dose-dependent manner (unpublished results). E_2 treatment stimulated cell growth to 2-fold compared to the untreated control. Similar results were obtained in the treatment of EGF and TGF α, but the growth stimulatory effects of those drugs were a little lower than that of E_2. Berthois *et al* [9] also reported that E_2 at 10 nM increased the cell number to the maximun level in phenol red free media. Freter *et al* [11] even showed that E_2 treatment of MCF-7 cells resulted in an approximate 4-fold increase in the pro-

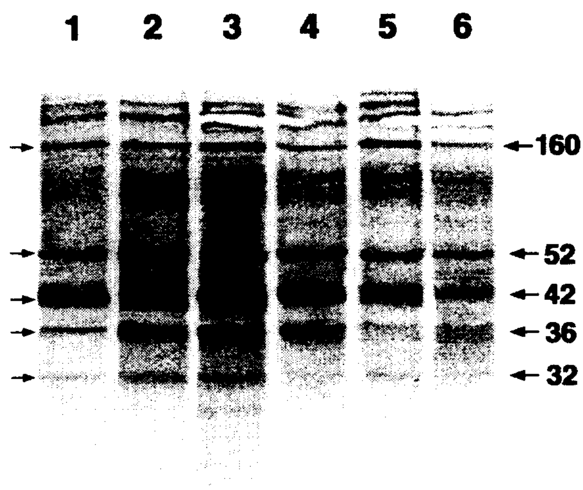

Fig. 4. Autoradiogram of ^{35}S-methionine labeled proteins secreted from MCF-7 cells. Equal amount of cpm in the media was applied on each lane of the gel. 1. control, 2. +E_2(10 nM), 3. +EGF(10 ng/ml), 4. +TGF α (10 ng/ml), 5. DFMO(1 mM), 6. HO-TAM(1 μM). The gel presented here is representative of at least 3 separate experiments.

liferation. These discrepancy might be resulted from the difference of culture conditions and cell passage numbers. Lima *et al* [12] observed that other ER-positive T-47D and ZR-75-1 human breast cancer cells also showed a 2- to 3-fold increase in both ODC activity and cell number in response to E_2.

Estrogens has been known to stimulate cell growth by the production of autocrine/paracrine growth factors, such as EGF and TGF α, which directly induce cell proliferation. Some *in vivo* studies indicate that EGF might replace estrogen in an autocrine and/or paracrine way to mimic estrogen-induced effects [13]. The above results suggest that E_2, EGF or TGF α stimulates proliferation of cells. Especially, E_2 is the most effective in stimulating cell proliferation.

Polyamines are well known to be involved in normal and neoplastic cell proliferation in numerous experimental data and these compounds can be important mediator of hormonal proliferative effects in human breast cancers [6,12]. High activity of ODC and increased the levels of intracellular polyamines are known to occur in rapid proliferating cells or cells undergoing differentiation and transformation. DFMO which is the specific inhibitors of the key enzyme ODC, has been used extensively not only to investigate the roles of polyamines in cell growth and differentiation but also in a variety of ways to understanding of the regulation of polyamine metabolism [7]. It has been suggested that the mitogenic effect of estrogen may require the synthesis of polyamines in breast cancer cells and consequently DFMO could effectively block the mitogenic effect of estrogen in nitrosomethylurea-induced rat mammary tumors growth in soft agar [14]. In the present experiment, DFMO was able to significantly inhibit cell proliferation. The growth inhibition DFMO could be detected even at as low as 0.5 mM. These results support that polyamines are fundamentally associated with cell growth. When E_2 and DFMO were cotreated to the cells to investigate the involvement of polyamines in the E_2-induced cell growth, DFMO inhibited the E_2-induced cell proliferation. At the same time, DFMO also significantly inhibited EGF or TGF α-stimulated cell proliferation. Therefore, DFMO can significantly inhibit the basal growth as well as the stimulatory effect of E_2, EGF, or TGF α. TAM is the most frequently used endocrine therapeutic agent for all stages of breast cancer. However, long-term treatment of TAM leads to development of resistance and even stimulation of mammary tumor. This may be due in part to the estrogen agonistic activity of TAM. TAM mediates its antitumor activity mainly due to competitive inhibition of estrogen binding at the estrogen receptor which is observed at a concentration of 10^{-6} M or lower. Above 10^{-6} M, the antitumor activity of TAM is known to include appreciable cytotoxicity which is not ER-mediated [15]. The suppressive effect of TAM on cell proliferation was observed and stronger than that of DFMO.

It has been found that MCF-7 cells produce and secrete both known growth factors as well as proteins of unknown function for growth [16,17]. These proteins may act as autocrine or paracrine mediators of estrogen-induced mitogenesis. In the present study, no significant difference was found in the intracellular protein synthesis between the control and the treated cells. However, the patterns of protein secretion were varied at the different treatment. These findings indicate that the cell growth is more likely to be regulated by the secretory proteins. Among the many secretory proteins, proteins of 160, 52, 42, 36, and 32 kDa showed noticeable changes. Interestingly, 52 kDa protein is known to be glycosylated and have cathepsin D-like proteolytic

Table 1. Comparison of the synthesis of major secretory proteins in MCF-7 cells treated with various growth stimulants or inhibitors. The concentration of each treatment is shown in Fig. 4. The data presented here is the mean of at least 3 separate experiments.

M. W (kD)	E_2(10 nm)	EGF (10 ng/ml)	(10 ng/ml)	DFMO (1 mM)	TAM (1 μM)
160	105 ± 3.08	120 ± 15	96 ± 9.9	95 ± 19.23	63 ± 22.16
52	122 ± 13.8	144 ± 18.5	104 ± 23.32	79 ± 29.4	61 ± 22.09
42	158 ± 10.3	147 ± 5.1	123 ± 21.98	70 ± 9.67	57 ± 11.42
36	99 ± 16.87	169 ± 18.26	122 ± 18.32	77 ± 37.33	90 ± 26.88
32	122 ± 7.96	131 ± 10.37	120 ± 19.6	78 ± 25.86	74 ± 22.82

activity. It was suggested that its mitogenic effects may be linked to cell-surface proteolysis [17]. In the present experiment, the secretion of 52 kDa protein was incresed in all three growth stimulants. But DFMO did not blocked the secretion of this protein at all, indicating DFMO can inhibit cell growth by other pathway. HO-TAM also gave minor influence in the secretion of 52 kDa protein. Other unknown secretory proteins of 160, 24 and 7 kDa were previously reported in MCF-7 cells [18]. However, the importance of those proteins are not known yet. In our results, the secretory proteins of 52, 42, and 36 kDa are seems like to be the most important ones in the proliferation of MCF-7 cells. Especially, the secretion of 36 kDa protein was most significantly influenced by growth stimulants and DFMO. It can be speculated that DFMO and HO-TAM exerted the growth inhibition in different pathway. In case of 160 kDa protein, TAM inhibited its secretion to 63% but DFMO stimulated to 130% of the untreated control. However, at some points, they inhibit the cell growth in co-related manner because they both inhibited the secretion of 42, 36, and 32 kDa proteins. In other words, the secretion of these three proteins can be more important for the cell growth. The significance of all these secretory proteins for cell proliferation seems very likely that the regulation of cell proliferation occurs via a complex cooperation as well as competition between stimulatory and inhibitory factors produced by the tumor cells themselves or stimulants from the supporting media. In conclusion, the present data indicate that E_2, EGF or TGFα induce the extent of cell proliferation, and polyamines influence cell proliferation, perhaps by modulating the secretion of various protein in MCF-7 cells.

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