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Estrogen Modulation of Human Breast Cancer Cell Growth

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To gain further insight into how estrogens modulate cell function, the effects of estrogen on cell proliferation were studied in human breast cancer cells. We examined the effects of estrogen on the proliferation of three human breast cancer cell lines that differed in their estrogen receptor contents. Ten nM estradiol markedly stimulated the proliferation of MCF-7 human breast cancer cells that contained high levels of estrogen receptor (1.15 ± 0.03 pmole/mg protein) over that of control. In T47D cells that contained low levels of estrogen receptor (0.23 ± 0.05 pmole/mg protein), Ten nM estrogen slightly stimulated the proliferation over that of control. MDA-MB-231 cells, that contained no detectable levels of estrogen receptors, had their growth unaffected by estrogen. These results showed their sensitivity to growth stimulation by estrogen correlated well with their estrogen receptor content. Also we examined the effect of estrogen on cellular progesterone receptor level as well as plasminogen activator activity in MCF-7 cells. Ten nM estradiol showed maximal stimulation of progesterone receptor level as well as plasminogen activator activity in MCF-7 cells. It is not clear whether these stimulations of progesterone receptor and plasminogen activator activity by estrogen are related to the estrogen stimulation of cell proliferation of MCF-7 cells. Studies with estrogen in human breast cancer cells in culture indicate that sensitivity to growth stimulation by estrogen correlates well with estrogen receptor contents.

Key words : Estrogen receptor, MCF-7, T47D, MDA-MB-231, Progesterone receptor, Plasminogen activator activity

INTRODUCTION

The determination of the estrogen and progesterone receptor content in human breast cancer tissue has gained an important role in the prediction of the success of an endocrine treatment (Nandi and McGrath, 1973). However, the receptor status of the tumor only allows a general evaluation; patients with a receptor-negative tumor respond rarely to an endocrine treatment; patients with receptor-positive tumors respond more frequently. An individual prediction in a single patient is not possible (Henderson, 1993; Bonadonna, 1993). Consequently, it is necessary to obtain a better understanding of the mechanism of action of estradiol and the antiestrogen tamoxifen, which is the drug most often used in endocrine treatment of advanced breast cancer. It has been suggested that estradiol exerts its effects on estrogen-dependent breast cancer at least in part through the regulation of secreted polypeptide growth factors with growth stimulatory or inhibitory potential (Dickson *et al.*, 1993). Part of the effect of antiestrogens is thought to be mediated through the induction of transforming growth factor β (TGFβ), which has an autocrine growth inhibitory effect on most human breast cancer cells (Knabbe, 1991). While the precise mechanism by which antiestrogens evoke their antitumor effects is still incompletely understood, considerable experimental data are consistent with the hypothesis that antiestrogens exert their effects through the estrogen receptor system of the target cells (Katzellenbogen *et al.*, 1985). In target cells, estrogens appear to exert their biological effects via interaction with an intracellular receptor protein (Welshons *et al.*, 1984; King and Greene, 1984). Ligand-free estrogen receptors are weakly associated with nuclear components. After ligand binding, receptor complexes become tightly associated with specific nuclear components, and this association presumably alters gene expression (Yamamoto, 1985; Katzellenbogen *et al.*, 1985). In the MCF-7 human breast cancer cell line, which contains functional estrogen receptor, estrogen stimulates cell proliferation, pS2 mRNA levels, plasminogen activator activity, thymidin incorporation, DNA synthesis, and progesterone receptor levels (Katzellenbogen *et al.*, 1985). Estrogen treatment of MCF-7 cells also results in the stimulation of two specific secreted proteins (Mr. 160,000 and 52,000) and a cytoplasmic

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protein (Mr. 24,000) (Westley and Rochefort, 1980; Veith *et al.*, 1983; Edwards, 1981). It has been postulated that the 52,000 Mr glycoprotein may be an estrogen-induced growth factor (Westley *et al.*, 1984), although data indicate that the 52,000 Mr protein may not be an autocrine regulator of MCF-7 cell growth (Davidson, 1986). To gain further insight into how estrogens modulate cell function, the effects of estrogen on cell proliferation were studied in MCF-7 cells. We found that sensitivity to growth stimulation by estrogen correlates well with estrogen receptor contents.

MATERIALS AND METHODS

Chemicals and Materials

[^{3}H]Estradiol (106 Ci/mmol) was obtained from Amersham (Arlington Heights, IL, USA). The synthetic progestin [^{3}H]R5020 (17,21-dimethyl-19-nor-4,9-pregnadiene-3,20-dione) (89 Ci/mmol) was obtained from New England Nuclear (Boston, MA, USA). All media, sera and antibiotics used to culture the MCF-7 cells were obtained from Grand Island Biological Co. (Grand Island, NY, USA). Insulin, hydrocortisone and estradiol were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Bis (Cbz-Iso-Pro-Arg)-rhodamine was synthesized and purified according to the procedure of Leytus *et al.* (1984). Plasminogen was purified from fresh dog plasma by modification of the method of Castellino and Sodetz as described in Leytus *et al.* (1984). The toluene-based scintillation fluid was 0.5% 2,5-diphenyloxazole and 0.03% p-bis-[2-(5-phenyloxazoyl)]-benzene in toluene. The Triton-xylene-based scintillation fluid was 0.3% 2,5-diphenyloxazole, 0.02% p-bis[2-(5-phenyloxazoyl)]-benzene, and 25% Triton X-114 in xylene.

Cell Culture

MCF-7 human breast cancer cells were originally obtained from Dr. Charles McGrath of the Michigan Cancer Foundation (Detroit, MI, USA) and were grown in 60mm plastic culture dishes in Eagles Minimal Essential medium (MEM) without phenol red supplemented with 10 mM HEPES buffer, gentamycin (50 g/mL), penicillin (100 U/mL), streptomycin (1 mg/mL), bovine insulin (6 ng/mL), hydrocortisone (3.75 ng/mL), and 5% calf serum that had been treated with dextran-coated charcoal for 45 min at 55°C to remove endogenous hormones.

Estrogen Receptor Binding Analysis

Cells from 20 near-confluent 100 mm culture dishes were suspended in 2.0 mL of PTG buffer (5 mM sodium phosphate, pH 7.4 at 4°C, 10 mM thioglycerol, and 10% glycerol) and homogenized in a Dounce homogenizer using the B-pestle. The homogenate was centrifuged (800×g, 10 min) and the supernatant was collected. The crude nuclear pellet was washed twice at 0~4°C with buffer and the nuclear washes combined with the supernatant fraction. This was centrifuged at 180,000×g (30 minutes) to yield the cytosol which was diluted to 15.4 mL with PTG buffer. Aliquots of cytosol (200 µL) were incubated at 0~4°C for 20 h with [^{3}H]estradiol at concentrations ranging from 5×10^{-11} M to 5×10^{-9} M. Parallel tubes contained the radioactive ligand plus a 100-fold excess of radioinert estradiol to assess non-specific binding. An aliquot was withdrawn for determination of total radioactivity, and unbound ligand was then removed by incubating one part charcoal-dextran slurry (5% Norit A, 0.5% dextran in buffer) with nine parts extract for 8 min at 0~4°C. The charcoal was pelleted by a 3 minute centrifugation at 12,800×g, and an aliquot of the supernatant was withdrawn for counting.

Cell Proliferation Experiments

The effect of etradiol on cell proliferation was studied in MCF-7 ,T47D and MDA-MB-231 cells. MCF-7, T47D and MDA-MB-231 cells were seeded into 35 mm culture dishes (20×10^3 cells/dish) and grown for two days in the MEM medium described in the Cell Culture section above. After this time, cells from two flasks were harvested and counted with a Coulter Counter (Day 0). The medium was changed to MEM supplemented as described above except containing 2% charcoal-dextran treated calf serum and various concentrations of estradiol or ethanol vehicle (0.1%). Triplicate dishes of cells were counted at several points throughout the 13 day growth period.

Hydroxylapatite Assay of Progesterone Receptor

Cytosol was incubated for 4 h at 0~4°C with 10 nM [^{3}H]R5020 in the presence or absence of 1 µM radioinert R5020, a synthetic progestin. Pretreatment of cytosol with 10^{-6} M cortisol, prior to incubation with [^{3}H]R5020 had no effect on the level of progestin binding suggesting no contribution from glucocorticoid receptor. After incubation, samples were assayed for bound [^{3}H]R5020 using hydroxylapatite.

Assay for Plasminogen Activator Activity

The plasminogen activator activity of the cells was measured by a two-step assay using the rhodamine-based compound Bis (Cbz-Ile-Pro-Arg)-rhodamine, abbreviated BZIPAR, as a substrate for plasmin. The substrate is non-fluorescent but cleavage by plasmin of an amide bond between arginine and rhodamine yields

a produce mono (Cbz-Ile-Pro-Arg)-rhodamine that is highly fluorescent. In brief, cells were incubated with plasminogen for 2 h at 37°C and the amount of plasmin formed was then measured by incubating an aliquot of the reaction mixture with BZIPAR and determining the rate of increase in fluorescence. The rate of activation of plasminogen during the 2 h incubation of plasminogen with cells was found to be constant and, therefore, proportional to the amount of plasminogen activator. Conversion of relative fluorescence units per minute to molar concentrations of plasmin was accomplished by using a standard curve.

RESULTS AND DISCUSSION

Effects of estrogen on proliferation of breast cancer cells *in vitro*

We examined the effects of estrogen on the proliferation of three human breast cancer cell lines that differed in their estrogen receptor content. As shown in Fig. 1, 10 nM estradiol markedly stimulated the proliferation of MCF-7 human breast cancer cells that contained high levels of estrogen receptors over that of control. In T47D cells that contained low levels of estrogen receptors, 10 nM estradiol slightly stimulated the proliferation over that of control (Table I, Fig. 2). MDA-MB-231 cells, that contained no detectable levels of estrogen receptor, had their growth unaffected by estradiol (Fig. 3). These results showed their sensitivity to growth stimulation by estrogen correlated well with their estrogen receptor content. These findings are mirrored by the results with human breast cancer patients indicating that estrogen receptor-containing breast cancers are most sensitive to antiestrogen treatment (McGuire, 1979). Studies with estrogen in human breast cancer cells in culture indicate that estrogen selectively stimulate the proliferation of estrogen receptor-containing breast cancer cells.

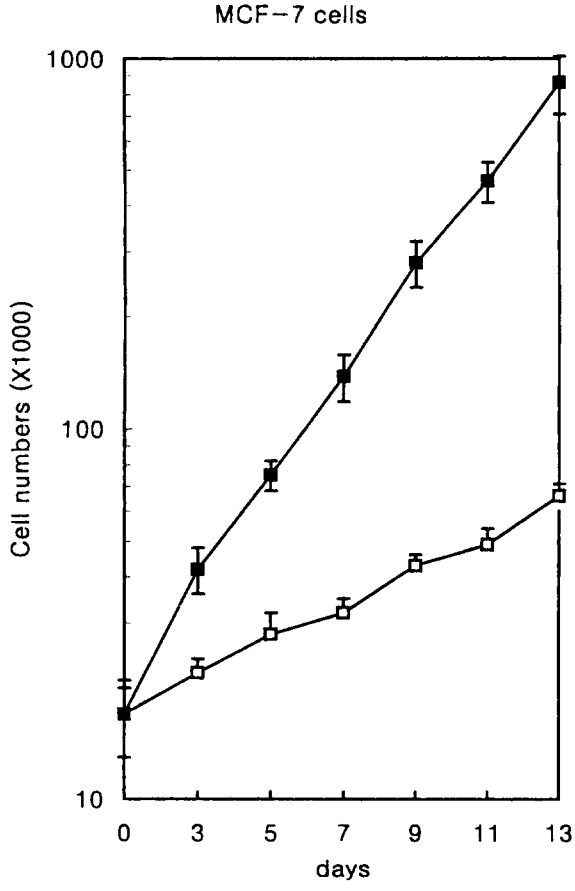

Fig. 1. Effect of estrogen on the growth of MCF-7 cells. Cells were grown in the continuous presence of 10 nM estradiol, and media with fresh estradiol were renewed every other day. On the days indicated, triplicate dishes of cells were counted. Values are the means of the triplicate determinations. Bars represent S.E. ■; 10 nM Estradiol, □; control.

Table I. Estrogen receptor levels in human breast cancer cell lines. Measurement of estrogen receptor was carried out as described in materials and methods

cells	estrogen receptor (pmole/1 mg protein)
MCF-7	1.15±0.03
T47D	0.23±0.05
MDA-MB-231	not detected

mean±S.E. n=6

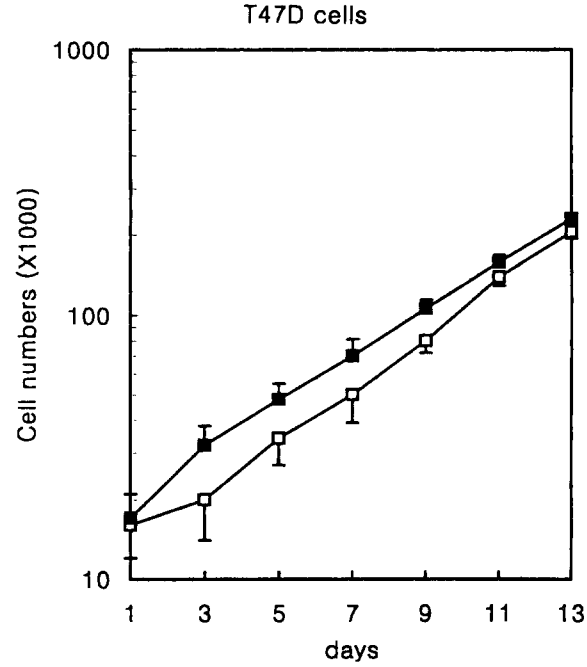

Fig. 2. Effect of estrogen on the growth of T47D cells. Cells were grown in the continuous presence of 10 nM estradiol, and media with fresh estradiol were renewed every other day. On the days indicated, triplicate dishes of cells were counted. Values are the means of the triplicate determinations. Bars represent S.E. ■; 10 nM Estradiol, □; control.

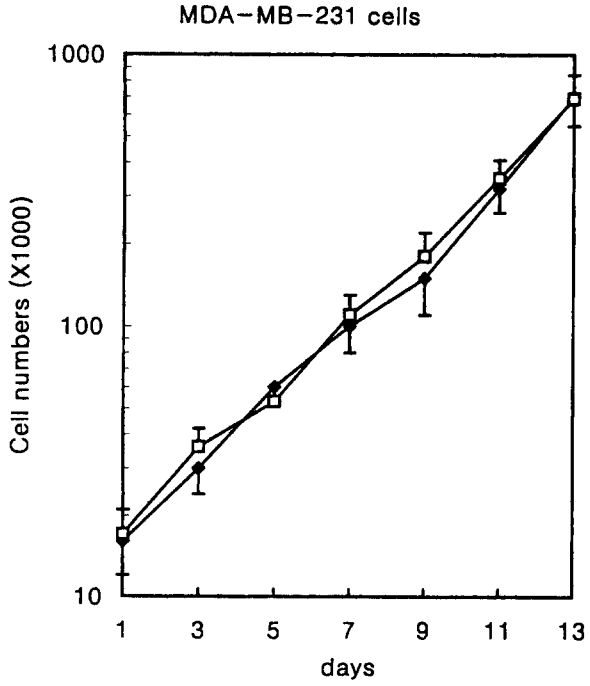

Fig. 3. Effect of estrogen on the growth of MDA-MB-231 cells. Cells were grown in the continuous presence of 10 nM estradiol, and media with fresh estradiol were renewed every other day. On the days indicated, triplicate dishes of cells were counted. Values are the means of the triplicate determinations. Bars represent S.E. ■: 10 nM Estradiol, □: control.

Analysis of estrogen receptor in breast cancer cells

Saturation binding analysis for estradiol in MCF-7 cells is shown in Fig. 4. Data indicate the presence of a single class of high affinity binding site in MCF-7 cell cytosol with equilibrium dissociation constants (Kd) of 0.25 nM for estradiol. Estrogen receptor levels in human breast cancer cell lines were compared. As shown in table 1, MCF-7 cell contained high level of estrogen receptors and T47D cell contained low level of estrogen receptors. However, MDA-MB-231 cell contained no detectable estrogen receptor.

Effect of estrogen on cellular progesterone receptor content

Increase in progesterone receptor content have been used as an index of estrogen action in MCF-7 cells (Eckert and Katzellenbogen, 1982). In time course studies of progesterone receptor stimulation by 10 nM estrodiol, we found maximal levels of progesterone receptor at 3 to 6 days of exposure to estradiol (Fig. 5A). Hence, 5-day time point was selected for evaluation of progesterone receptor stimulation by estrogen. Fig. 5B shows a dose dependent increase in progesterone receptor by estradiol treatment and 10 nM estradiol treatment shows 6-fold increase in progesterone receptor level.

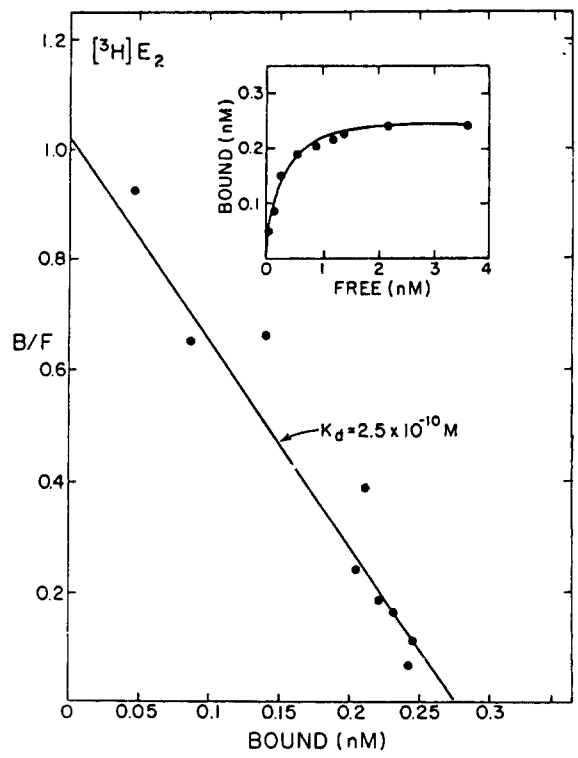

Fig. 4. Analysis of the binding of [^{3}H] estradiol to MCF-7 estrogen receptor. Cytosol was incubated with varying concentrations of radiolabeled estradiol from 5×10^{-11} M to 5×10^{-9}, in the presence or absence of a 100-fold excess of radioinert estradiol for 16 hours at 0~4°C. An aliquot was removed to assess total binding, and bound radioactivity was determined following charcoal-dextran treatment. Specific binding is plotted according to the equation of Scatchard (Scatchard, 1949) and as direct plot (insert). Protein concentration in the cytosol incubation was 1.0 mg/ml.

Effect of estrogen on plasminogen activator activity

Estradiol stimulates plasminogen activator activity with dose dependent manner (Fig. 6). It has been demonstrated previously that plasminogen activator activity serve as a useful marker for the biological activity of estrogen in human breast cancer cells (Butler *et al.*, 1983; Kneifel *et al.*, 1982). Plasminogen activator activity is stimulated by low, physiological concentrations of estradiol in MCF-7 cells, while antiestrogen such as tamoxifen and trans-hydroxytamoxifen failed to stimulate plasminogen activity (Butler *et al.*, 1983). Plasminogen activator activity is under hormonal control in many tissues, and increased activity is found during rapid tissue growth and remodeling of the uterus and mammary gland (Kneifel *et al.*, 1982).

Estrogens are physiological mitogens for mammary cells, playing a fundamental role in the ductal and lobulo-alveolar development of the mammary gland. During the first steps of malignant transformation, cells exploit this mitogenic action, which accompanies the progressive accumulation of genetic alterations,

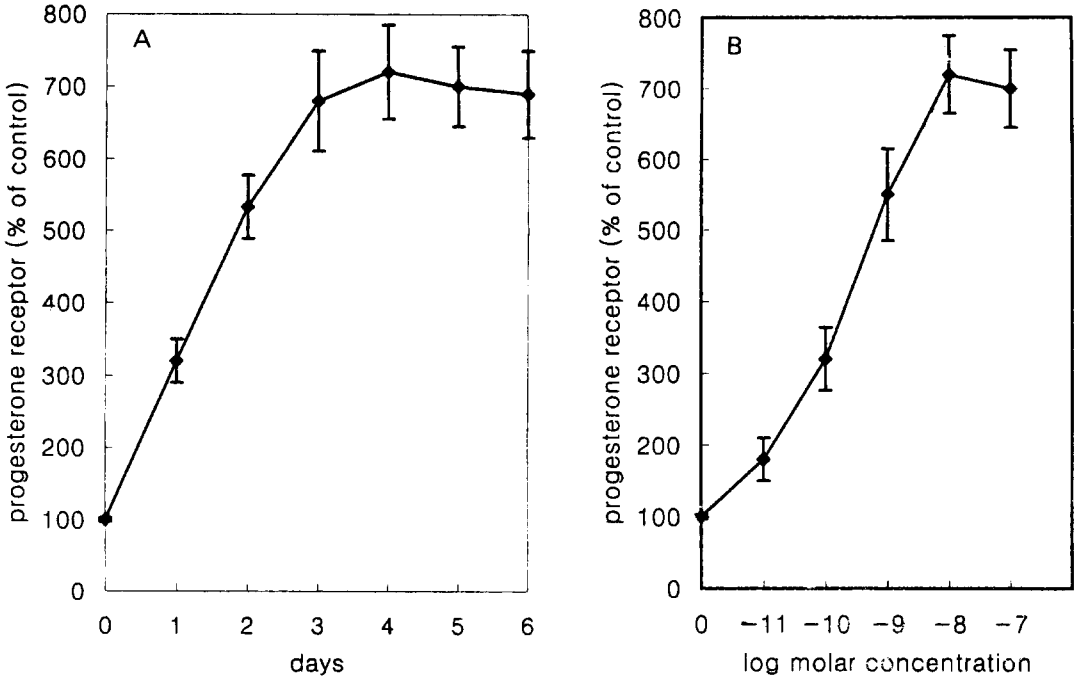

Fig. 5. Effect of estrogen on progesterone receptor levels in MCF-7 cells. Cells were incubated for 5 days with estradiol (E_2) at the concentrations indicated. Fresh media and estradiol were added daily during 5-days period. The cells were then harvested, fractionated, and the cytosol was assayed for progesterone receptor using hydroxylapatite, as described in "Materials and Method". A;estrogen time course study, B; estrogen dose response study. Control progesterone receptor level was 330±32 fmol/mg DNA.

such as erbB-2 amplification and p53 deletion (Barnes, 1993; Poller *et al.*, 1993). Homogenous expression of estrogen receptors is always seen in nonmalignant atypic hyperproliferative disease of the breast, and three out of four in situ ductal carcinomas possess estrogen receptor (Barnes, 1993). Invasive cancers can thereafter become independent from estrogens, likely through the aquisition of further genetic changes (van de Vijver, 1993), yet about one-third of invasive breast carcinomas are responsive to antiestrogenic therapy, testifying that even fully neoplastic cells can require an estrogenic stimulus to proliferate and invade. Estrogen stimulates mammary cell growth by diverse and complementary mechanisms. DNA synthesis occurs early after treatment of the cells with estrogen, preceeded by transcriptional activation of several "early genes", including transcriptional regulators such as fos, jun (AP-1), myc, and myb. In addition, estrogen profoundly influences cell-cell communication and stromal epithelial interaction, by increasing autocrine and paracrine positive growth factor (e.g., TGFα, IGFs) as well as repressing negative growth factor (e.g., TGF-β) secretion (Dickson *et al.*, 1990). Hormone-dependent breast cancer represents a paradigm for the role of hormones in cancer development. The primary role of estrogens in breast cancer development and progression is firmly established by a wide collection of clinical, experimental, and biological data.

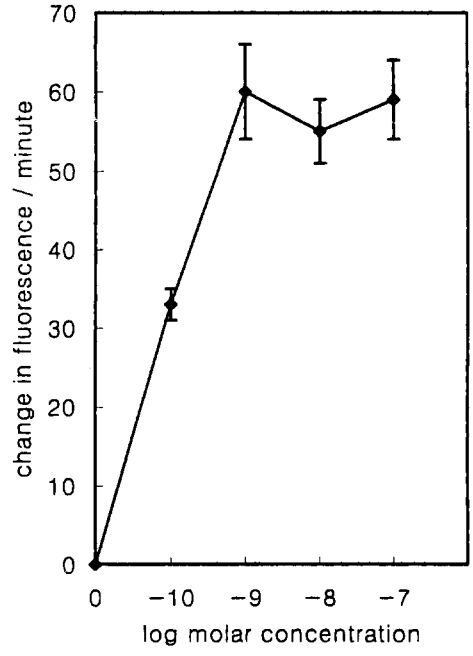

Fig. 6. Effect of estradiol (E_2) on the plasminogen activator activity of MCF-7 cells. Cells were incubated with the indicated concentration of estradiol for 4 days, with fresh media and estradiol renewed every 48 hours. Cells were then harvested, distributed to microwells, and assayed for plasminogen activator activity, was measured by the change in fluorescence (△F) per minute, representing plasmin formed per minute; bars, S.E.

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