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Original Article

Effect of 17-β Estradiol on the Expression of Inducible Nitric Oxide Synthase in Parent and Tamoxifen Resistant T47D Breast Cancer Cells

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Abstract

Indirect evidence suggests that estrogen is involved in the etiology of breast cancer. Estrogen is also thought to modulate nitric oxide (NO) in human breast tumor tissue via regulation of inducible nitric oxide synthase (iNOS). Objectives of this study were to determine whether estradiol (E2) affects iNOS expression level in breast cancer cells and to study the effect of various concentrations of E2 on cell proliferation. Immunocytochemical technique was employed to assess iNOS expression level. Proliferation of parent and 10^{-6} M tamoxifen resistant cells (T47D/TAMR-6) were assessed by MTT assay in the presence of E2.

Addition of E2 (10^{-12} to 10^{-8} M) increases the expression of iNOS in parent cells, but not T47D/TAMR-6, Further increase in concentrations of E2 (10^{-8} to 10^{-4} M) again decreases the expression of iNOS in parent cells, but increase that of the T47D/TAMR-6 cells. Expression of iNOS in parent cells in a medium containing 1% serum (low serum) is less than the cells grown in a medium containing 10% FBS (normal serum). This trend was not seen in T47D/TAMR-6 cells. The results of these experiments may indicate that increasing of iNOS expression decreases the viability of parent cells whilst increasing the number of T47D/TAMR-6.

Keywords: Breast cancer; Nitric oxide synthase; Tamoxifen; Estrogen; Estradiol.

Introduction

Breast cancer is the second most common malignancy in pregnancy, with an estimated incidence of 10-30 women/100000 pregnancies (1). It has been reported that mortality rate from breast cancer has been significantly greater in women whose cancer was first diagnosed during pregnancy compared with those who had never been pregnant (2). Whether estrogen plays a key role in the risk of breast cancer remains uncertain, though the early stages of breast cancer are known to be estrogen-dependent and responsive to treatment with tamoxifen.

Nitric oxide (NO), a short-lived inorganic free radical, has many functions in different tissues (3, 4). It is produced by nitric oxide synthase (NOS), by converting L-arginine to L-citrulline and NO (5). NO acts as an intracellular second messenger in most mammalian organs and participates in vascular homeostasis, neurotransmission, antimicrobial defense, and angiogenesis. The exact role of nitric oxide in tumor biology has not yet been fully elucidated. This is due to the fact that NO has been implicated in various aspects of cancer biology, including both pro and anti- tumor functions

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(6). It has been shown that NO may affect tumor growth, differentiation, metastatic capability, chemosensitivity and radiosensitivity (7). Estrogen can increase the expression of NO synthesis in a variety of tissues. Furthermore, estrogen-induced stimulation of growth of primary human tumors in athymic mice has often been used as evidence for their dependence on hormones (8, 9). It has been suggested that, because of its free radical nature, NO may have a role in carcinogenesis by inducing DNA strand breaks (10) and impairing the tumor suppressor function of p53 (11). NO has also been implicated as a part of a signaling cascade for neovascularization (12), which can increase tumor blood flow (13). The presence of inducible NOS (iNOS) has been correlated with metastatic disease (14) and a NOS inhibitor significantly reduces bone metastasis (15). Although NO plays a role in tumor biology, so far its role is poorly understood. It has been reported that many cancer cell lines have been shown to express nitric oxide synthases (16, 17) and the production of NO by a melanoma cell line has been shown to correlate inversely with the production of metastases (18). NOS activity has also been reported in human gynecological (19) and breast (7) tumor tissue where its presence correlated inversely with tumor grade. Interestingly, inflammatory mediators stimulated NO production in the EMT-6 murine breast cancer cell line (20) and relaxin was shown to stimulate iNOS activity and NO production in MCF-7 cells (19). With regards to its anti-tumor role, NO has a cytostatic/cytotoxic role towards tumor cells (21, 22). Cytotoxic effects are mediated via interference with DNA replication and several enzymes, including aconitase and ubiquinone oxireductase (23). NO has also been shown to induce apoptosis in tumor cells (24, 25). The balance between these opposing roles may depend upon the local concentration of NO, with high concentrations of NO exerting anti-proliferative effects and low concentrations facilitating tumor growth (3). Furthermore, recent evidence suggest that E2 modulates the levels of nitric oxide via regulation of inducible nitric oxide synthase in a variety of tissues, including rat uterus (26), rabbit uterus and vagina (24), human ovarian follicular tissue (27) and human ovarian tumor tissue (3). Since initial reports described NO as an endogenously generated molecule (28), extensive investigations have revealed a diversity of physiological roles for NO (29, 30). Although tamoxifen is still the treatment of choice for breast cancer patients with estrogen receptor (ER) positive tumors (31), nearly all patients will eventually become resistant to this treatment (32). Treatment failure may be associated with a variety of changes in tumor characteristics, leading to a more malignant phenotype, including the development of anti-estrogen resistance and progression to estrogen independence.

Previously, two such variants of the T47D human breast cancer cell lines have been characterized. In T47D/TAMR-6 cells (33) which have acquired tamoxifen resistance, there is a loss of detectable ERs and progesterone receptors and an increase in epidermal growth factor receptor (EGFR) expression (34).

Recently, the presence of iNOS in the T47D human breast cancer cell line have been reported (4). Since a recent report has suggested that iNOS may play a significant role in breast cancer progression (28). We decided to investigate the relationship between E2 and iNOS expression in the viability of parent T47D and T47D/TAMR-6 cells.

Experimental

Cell cultures and reagents

The parent T47D cell line, a progesterone receptor (PR) rich and estrogen receptor (ER) positive breast cancer cell line, were obtained from Pasteur Institute (Tehran) and T47D/TAMR-6 was prepared in the molecular research laboratory of TUMS.

All the cultures were maintained at 37°C in a humidified incubator with 5% CO_2, using RPMI-1640 medium (PAN, Germany) supplemented with 10% heat-inactivated fetal bovine serum (FBS Gibco BRL, USA), 1% pyruvate, 2 mM L-glutamine, 0.1 mM non-essential amino acids, penicillin (100 u/mL) and streptomycin (100 µg/mL) (PEN/STREP; PAN, Germany). For T47D/TAMR-6 cells, the medium contained

10^{-6} M Tamoxifen.

Chemicals

17 β estradiol (E2) and MTT were obtained from Sigma Chemical Company (St.louis, MO, USA; Sigma E4389). Tamoxifen was purchased from Iran Hormone Co, Tehran, Iran.

Cell growth assay

Cells growing in log phase were harvested by trypsinization, seeded in 96-well, flat-bottomed plates (NUNC, Denmark) at a density of 10^5 cells per mL in RPMI-1640, for parent T47D cells and with RPMI-1640 containing tamoxifen 10^{-6} M, for T47D/TAMR-6 cells, supplemented with 10% FBS and incubated at 37°C in a humidified incubator with 5% CO_2. After 24 h, the medium was removed and cells were washed with phosphate-buffer saline (PBS) and maintained in RPMI-1640 with 1% pyruvate, 2 mM L-glutamine, 0.1 mM non-essential amino acids, penicillin (100 u/mL), streptomycin (100 µg/mL), insulin (10 µg/mL) and supplemented with, 1% FBS to deplete or reduce medium concentration of steroid hormones and estrogenic compounds for the remainder of the assay period. Prior to experiments, the cultures were maintained for 24 h in this medium. After 24 h, the medium was removed from the sub-confluent cells and cultures were replenished with fresh 1% FBS-medium containing different concentrations of E2 (10^{-12} M to 10^{-4} M) and treated for three days (72 h). The control sample was treated with normal serum condition. A minimum of triplicate wells were examined for each concentration of estradiol in each experiment. The relative cell viability was measured using indirect MTT assay (Mossman, 1983). Briefly, 100 µl of MTT solution (5 mg/mL in PBS) was added to each well at the end of experiment. After 4 h of incubation, the formazan crystals were dissolved in 300 µl isopropanol containing 0.4 M HCl and the extinctions were measured by a microplate reader at 570 nm (reference 690 nm). Before the final experiment, the range of linearity for the MTT assay (extinction vs. cell number) was defined. In a single experiment, 6 wells were used for each concentration and each experiment was repeated two to three times.

iNOS immunocytochemistry

Cultured T47D cells were fixed in methanol-acetone (8:2 v/v) for 10 min at 4°C, washed with PBS and hydrogen peroxide (3% in methanol for 30 min) and then used to inhibit endogenous peroxidase activity. Cells were then incubated with normal horse serum (10% for 30 min) to block the non-specific binding sites. Immunocytochemical staining was performed, using the mouse monoclonal inducible nitric oxide synthase primary antibody (10% BSA, Santa Cruz Biotechnology, CA) at 1:200 dilution, incubated overnight at 4°C. After extensive washing in tris buffer at room temperature, the cells were incubated with a biotinylated goat anti-mouse IgG (1:600) for 60 min at room temperature as the secondary antibody (Santa Cruz Biotechnology, CA). Subsequently, the amplification of avidin-biotin-horseradish peroxidase complex was carried out (ABC complex/HRP; Santa Cruz Biotechnology, CA) at 1:100 dilntion for 30 min at room temperature and 3, 3-diaminobenzidine tetrachloride dihydrate (Santa Cruz Biotechnology, CA) was used as substrate. Cells were then rinsed after each step with tris buffer saline (0.05M Tris–HCl plus 0.15 M NaCl, pH 7.6) containing 0.05% Triton-X100 (TBS-T). In control experiments, cells were processed by replacing the primary antibody with mouse serum (Dako, UK). The digital images were then taken and analyzed using an OLYSIA image program (Olympus, Japan). Saturation density was chosen from several descriptors provided by the software. The saturation of iNOS staining were calculated at 40x power microscopic fields, measuring more than 600 points of cells per image taken from 4 random area from each slide. All the data obtained were expressed as mean ± standard error of mean (SEM) of three independent experiments.

Statistical analysis

Statistical significance was determined using the Student's t-test and the differences were regarded as significant for values of $p<0.05$.

Results

The effects of E2 on proliferation rate of

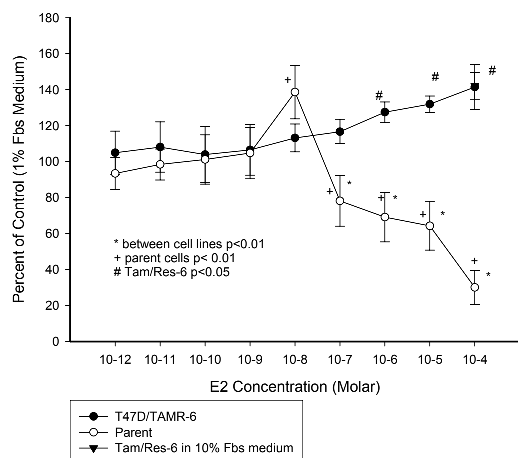

Figure 1. The effect of ß estradiol on the viability of parent and Tam/Res-6 T47D cancer cells. (1% FBS medium, n=15, MTT).

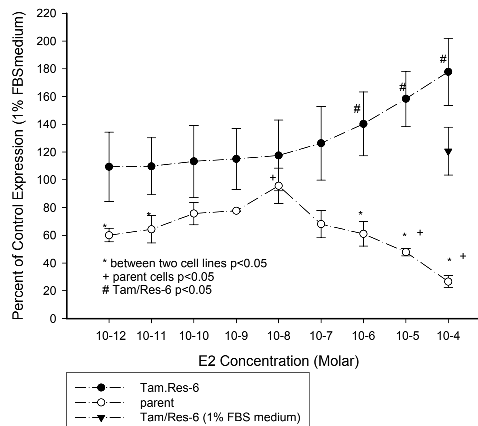

Figure 2. The effect of ß estradiol on the iNOS expression in the parent and T47D/TAMR-6 cells. Note the tamoxifen result was obtained in the medium containing 10% FBS. (n=15).

both parent T47D and T47D/TAMR-6 resistant cells were assessed after 72 h of hormonal treatment. Results presented in Figure 1 show that increasing the concentration of E2 from 10^{-12} to 10^{-8} M enhances the viability of T47D cells to 140 percent of control. However, increasing the hormone concentrations from 10^{-8} to 10^{-4} M, decreases viability to less than 25% of control at dose of 10^{-4} M. T47D/TAMR-6 cells show a noticeable growth at the range of 10^{-12} to 10^{-8} M however by increasing the dose to $10^{-4,}$ M, the cells grow significantly more compared to the control. At the dose of 10^{-4} M, the number of viable cells is comparable to the cells growing in the medium containing 10% serum and 10^{-6} M tamoxifen. Figure 2 shows the expression of iNOS in parent and TAMR-6 T47D cells at the concentrations of E2 indicated in Figure 1. The expression of iNOS in parent cells was much lower than the control group at dose of 10^{-12} M E2, indicating that expression of iNOS is directly dependent on the amount of E2 medium. By increasing the amount of E2, the same trend was observed to that seen in the proliferation assay, but in a lower percentage than the control group. The expression of iNOS in T47D/TAMR-6 is similar to its viability, with the same percentage of control group being observed. When the ratio of expression quantity (measured in Figure 2) to viability of cells (Figure 1) was calculated, as shown in Figure 3, the highest viability of parent cells was obtained at the lowest degree of this ratio (i.e. 10^{-8}). As a result of increasing this ratio, the viability of cells significantly ($p<0.001$) decreased at a dose of 10^{-4} M. The ratio of expression to viability for T47D/TAMR-6 cells does not significantly change, up to 10^{-7} M estradiol concentration. Above this concentration, this ratio increases to the maximum level at 10^{-4} M. This increase observed is parallel to the increase in the viability of cells. This ratio is similar to the ratio observed for growing cells in a normal medium containing 10% serum and 10^{-6} M tamoxifen. Parent cells, at a concentration of 10^{-4} M, showed apoptotic bodies (Figure 3).

Discussion

The role of nitric oxide (NO) in cancer is ubiquitous (35). NO has been reported in order to inhibit cell proliferation, to induce differentiation and decrease the metastatic spread of different tumor cell lines (36). This effect seems to be related to the type and origin of the cancer cells studied (36), as well as the oxidative status of the cells (37). The role of the NO/NOS system in breast cancer is controversial (36), inhibition of NOS activity

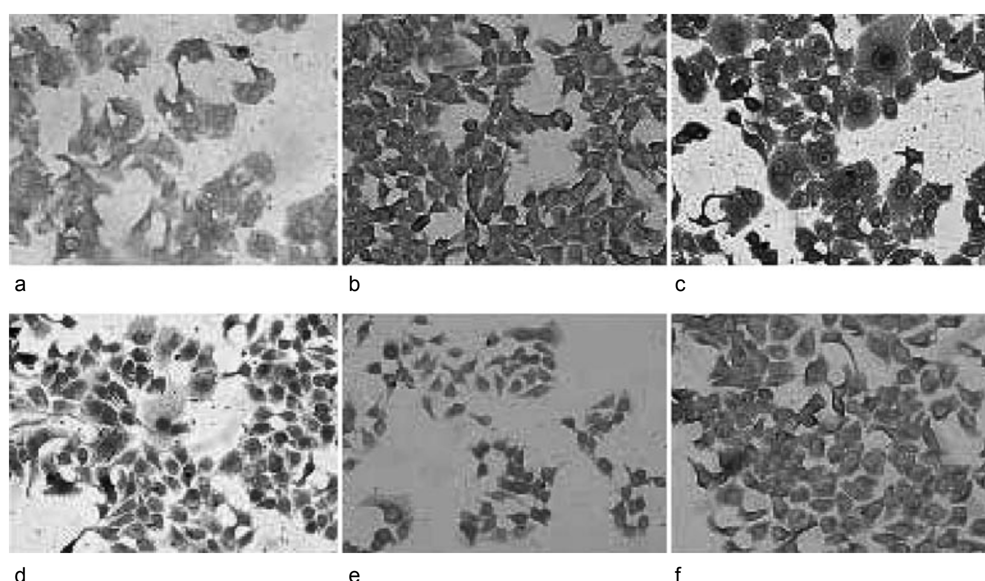

Figure 3. Immunocytochemistry of iNOS in parent and T47D/TAMR-6 cell treated with different concentration of E2: a) T47D/TAMR-6 at 10^{-8} M E2 and counterstained with haematoxylene; b) parent at 10^{-8} M E2; c) parent at 10^{-8} M E2 and counter stained with haematoxylene; d) parent without primary iNOS antibody and counter stained; e)T47D/TAMR-6 at 10^{-4} M E2; f) parent at 10^{-10} M E2.

has been considered as a possible target for anticancer treatment. In MCF7 breast cancer cells, inhibitors of NO synthesis (NG-nitro-L-arginine methyl ester) and NO scavengers have induced apoptosis (38), via a p53- associated pathway, while in T47D cells, suppression of NO production triggers an induction of apoptosis via a FKHRL1 (FOXO3a) kinase pathway, independent of phosphoinositide 3-kinase-Akt and caspase 3 activation (36). NO is a labile substance with a short half-life, and its direct measurement has proven to be difficult. Several studies have suggested that activation of inducible nitric oxide synthase is important in NO production, especially after any cellular stress (39). Thus, in this study we have focused our investigation on the effect of E2 on iNOS expression in two breast cancer cell lines; namely the parent T47D and T47D/TAMR-6, employing the elevated estradiol concentrations thought to be present within the ovary during peak ovarian steroid hormone synthesis. Performing the assays under low serum (1%) conditions allowed us to assess the effects of exogenously introduced hormone within our in vitro assay system, without interfering with the hormonal component of FBS.

Tamoxifen has become the agent most commonly used in the hormonal manipulation of breast cancer. While its entire mechanism of action has yet to be elucidated, the proposed mode of action of tamoxifen is through competitive binding to estrogen receptor (ER) found in breast cancer cells, which inhibits estrogen binding and stimulation of tumor growth (40). Like L-NMMA, tamoxifen completely inhibited the stimulatory effect of estradiol, progesterone, and the combination of estrogen and progesterone on NO production, indicating that the effect of estradiol on NO production is mediated by interaction with the ER. There was no inhibitory effect on NO production, when the cells were exposed to tamoxifen alone, suggesting that tamoxifen does not exert a direct effect on NO production. An identical finding was reported in ZR-75-1 human breast cancer cells. Moreover, tamoxifen significantly down-regulated phorbol ester increased NO production in this cell line (41). Several investigations have reported that the amount of iNOS and NO production changed in different breast cancer cell lines,

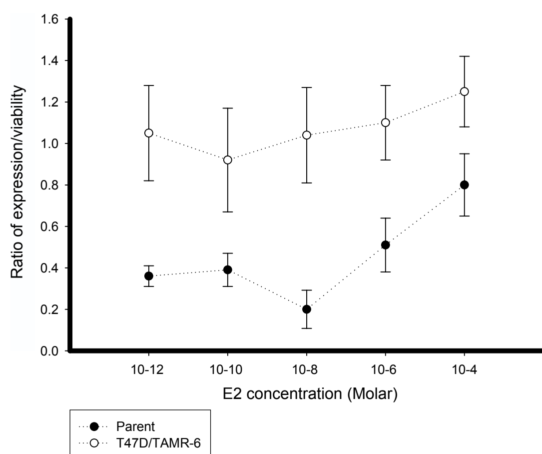

Figure 4. The ratio of expression to viability of parent and T47D/TAMR-6 cells at different concentration of E2 (n=15, mean ± SEM).

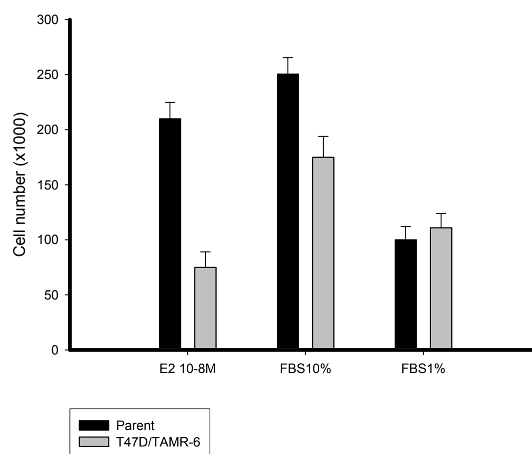

Figure 5. The proliferation of parent and Tam/Res-6 T47D after 72 h in different medium condition (n=15, mean ±SEM).

indicating that NO may play a role in resistance to the drug or it is just one of the components which is produced during the transformation of cell to a resistant form (35). Proliferation of parent cells shows a significant increase at concentration of 10^{-8} M of ß estradiol and decreases at higher concentrations (Figure 1). Expression of iNOS in the parent T47D cells, at a concentration of 10^{-12} M E2, is about 60% of the normal control in 10% serum indicating the role of E2 for synthesis of iNOS in this cell lines. This reduction did not affect the viability of cell suggesting that the iNOS expression is not necessary for viability of the parent T47D cells. The amount of expression increased, when reaching E2 concentration to 100% of 10^{-8} M. However the increase is not as much as the increase seen in proliferation measured in Figure 1, making the ratio of expression/viability as its lowest (as observed in Figure 4). By increasing the amount of E2 to 10^{-4} M, the expression and viability decreased in a dose related manner but not parallel to each other, resulting in the increase noted in the ratio of expression/viability. This ratio shows that increasing the iNOS expression may result in a reduction of viability due to producing NO radicals in the cells. Several studies show that at some stage the NO radical may cause cell death or apoptosis in the cancer cell line, but the present study cannot prove this cause-effect relationship (35). It needs to measure NO production directly, using NO donor and inhibitor agents to study this hypothesis. As shown in Figure 5, the trend of E2 effect in T47D/TAMR-6 is different from that of the parent cells. As previously reported, this cell line is less dependent on estradiol, showing good viability at very low estradiol concentrations. At a 10^{-12} M E2 concentration, the viability of cells is equal to the culture of cells in normal medium containing 10^{-6} M tamoxifen. Increasing the E2 concentration to 10^{-9} M, did not change the proliferation of cells. Further increases in the amount of E2 beyond this point, enhanced proliferation in a dose dependent manner, reaching a maximum at 10^{-4} M. This could be resulted from a competitive effect on E2 receptor, or be due to a slow response of drug-receptor complex in this cell line. The proliferation effect in T47D/TAMR-6 is not as much as the parent cells, as previously reported, and may result in a lower less dependency of cells on the action of E2 would make them more resistant to estrogen inhibitors. When we look at Figure 2, a similar pattern can be observed. In fact, the increase in expression of iNOS is similar to the increase in parent cells, indicating that estrdiol enhances the iNOS expression through another

previously reported (35) mechanism. Another difference observed in the parent cell line, is in the ratio of expression/viability (Figure 5). Up to a 10^{-7} M concentration of E2, the ratio of expression/viability is around 1, showing that the change in iNOS expression is parallel to viability. This may be related to the estrogen resistant nature of this cell line. By increasing E2 concentration from 10^{-7} M to 10^{-4} M, the ratio gradually increases, which means that the expression of iNOS increases more rapidly than proliferation in these cells. This could also be related to an enhancement of iNOS synthesis through a non-estrogen receptor effect. This figure if compared to Figure 4 may also indicate that the increase in proliferation is not related to iNOS expression. However, in the parent cells the increase of iNOS may increase the susceptibility of cells to the overdose of estradiol in order to decrease the viability of cells. Based on the results obtained from these two cell lines, the controversial effect of E2 on the proliferation of cancer cells may be explained. Therefore, an increase of E2 concentration up to 10^{-8} M increases the iNOS expression and may also increase the amount of NO production, resulting in an enhanced apoptosis and cell death in the parent cell. On the other hand, the increase in E2 concentration up to 10^{-8} M, not only did not affects the viability of T47D/TAMR-6 cells but also may increase the growth of cells, compared to the control. This trend has been observed in vivo in several investigations and may indicate that E2, in high concentrations, enhances NO production and increases cell proliferation in tumor resistant cells.

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