Aromatase within the breast

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Abstract

In situ aromatization and enhanced uptake of estradiol from plasma are two potential mechanisms for maintenance of high concentrations of estradiol found in breast tumors of postmenopausal patients. To test the relative importance of these two mechanisms, a nude mouse model was established by inoculating aromatase (A+) and/or sham (A-) transfected MCF-7 cells into ovariectomized mice. Postmenopausal hormonal status was simulated by providing estradiol Silastic implants which clamped plasma estradiol levels at 5-20 pg/ml. We demonstrated that *in situ* aromatization rather than the uptake mechanism is the key determinant of tumor estradiol levels and tumor growth rate under conditions reflecting the postmenopausal state. The importance of intratumoral aromatase was also suggested by the findings that long-term estrogen deprivation increases sensitivity to estradiol and enhances aromatase activity in MCF-7 cells. The results of our *in vivo* and *in vitro* studies suggest that complete blockade of *in situ* aromatization in the breast would provide added benefit to postmenopausal breast cancer patients, especially those who relapse from antiestrogen therapy.

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Introduction

Estrogen is mitogenic to the breast and plays an important role in the growth of hormone-dependent breast cancer. In premenopausal women, the major source of circulating estradiol (E₂) is the ovaries. Following the menopause, extraglandular sites in adipose tissue, liver, muscle, brain and breast then become the primary sources of estrogen production. Cessation of ovarian estrogen synthesis leads to a significant reduction in plasma estrogen levels. A parallel decrease of E₂ levels in plasma and in the breast would be expected at the time of menopause. However, the concentrations of E₂ in malignant breast tissue in postmenopausal patients are much higher than expected and similar to those in premenopausal patients despite the large differences in plasma levels (van Landeghem *et al.* 1985).

Previous studies have shown that aromatase is present in both normal and malignant breast tissues with varying activities (Perel *et al.* 1982, Lipton *et al.* 1987, Reed *et al.* 1989, 1990, Silva *et al.* 1989, Miller *et al.* 1990, Bulun *et al.* 1993, Koos *et al.* 1993, Lu *et al.* 1996). While breast cancer cells can uptake estrogens from plasma, high tissue E₂ concentrations in postmenopausal breast cancer cannot

be adequately explained by the uptake mechanism. This conclusion is based upon both indirect and direct data.

Indirect data from previous studies suggested that *in situ* aromatization contributes preferentially to the estrogen content of breast tumors. However, no direct biologic data to support this hypothesis are yet available. Direct proof of the importance of *in situ* estrogen production is impossible to obtain in studies of postmenopausal breast cancer patients. Both peripheral and *in situ* aromatization take place, a finding which confounds interpretation. Therefore, an appropriate model system is required. Ideally, this model should be able to distinguish between *in situ* and peripheral aromatization and directly correlate the amount of E₂ produced locally with the biologic response observed. In this study, we chose to use xenografts of aromatase or sham transfected MCF-7 breast cancer cells (Zhou *et al.* 1990) grown in ovariectomized nude mice. Using this model, we demonstrated that *in situ* synthesis predominates over uptake from plasma as a means of maintaining breast tissue E₂ concentrations under the circumstances of the postmenopausal state.

Methods

Cell culture

MCF-7 cells stably transfected with the human placental aromatase gene (A+) or plasmid vector alone (A-) (Zhou *et al.* 1990) were cultured in Eagle's minimum essential medium containing 5% fetal bovine serum (FBS) and neomycin (600 µg/ml; Gibco, Bethesda, MD, USA). Wild type MCF-7 cells were cultured in IMEM with 5% FBS. Long term estrogen deprived (LTED) MCF-7 cells were cultured in phenol red-free IMEM containing 5% dextran-coated charcoal stripped FBS. The culture medium was changed twice weekly.

Athymic mice

Female athymic mice 5 weeks of age were obtained from Harlan Sprague-Dawley (Indianapolis, IN, USA). The animals were housed in a pathogen-free environment under controlled conditions of light and humidity and received food and water *ad libitum*. Ovariectomy was performed under flurothane anesthesia 1-3 days before cell inoculation. Inoculation of A+ or A- cells was carried out according to the procedures described previously (Yue *et al.* 1994, 1998a).

Preparation of Silastic estradiol implants

Silastic E₂ implants were prepared using Silastic brand tubing with an inner diameter of 0.078" (Dow Corning, Midland, MI, USA) according to the method previously described (Masamura *et al.* 1995, Smith *et al.* 1977). The complete implant was 0.5 cm in length. E₂ doses were adjusted by mixing E₂ with cholesterol at ratios of 1:79-1:19 in weight. Based on extrapolations from our previous data (Masamura *et al.* 1995), predicted plasma E₂ concentrations produced by these E₂ implants were 5, 7, 10 and 20 pg/ml respectively.

Measurement of tissue estradiol levels in tumors

Tumor samples were maintained at -80 °C before radioimmunoassay (RIA) of E₂. Tumors were homogenized in PBS with a Polytron homogenizer at 4°C at a tissue concentration of 50 mg/ml. The tissue homogenates were extracted with diethyl ether, and the E₂ was isolated using celite column chromatography. E₂ was measured using a high titer E₂ antibody and iodinated E₂ trace obtained from ICN (Costa Mesa, CA, USA). The inter- and intra-assay coefficients of variation were 12 and 9% respectively at a mean concentration of 50 pg/ml.

Growth assay

Cells were plated in six-well plates in corresponding culture medium. The medium was replaced with phenol

red-free IMEM with or without charcoal stripped serum 2 days after plating. At 4 days after plating, medium containing vehicle or treatment compound was applied. The final concentration of vehicle was 0.1-0.2%. The media were changed every 3 days. At the end of treatment, cells were rinsed twice with saline. Nuclei were prepared by sequential addition of 1 ml Hepes-MgCl₂ solution (Hepes 10 mM and MgCl₂ 1.5 mM) and 0.1 ml ZAP solution (ethylhexadecyldimethylammonium bromide 0.13 M and glacial acetic acid 3 ml/100 ml), and counted using a Coulter Counter.

Radiometric aromatase assay (³H₂O release assay)

Confluent wild type MCF-7 or LTED cells grown in 60-mm dishes were rinsed with Hanks' solution and incubated at 37 °C for 6 h with 1.5 ml serum-free IMEM containing approximately 1.5 µCi [1β-³H]androstenedione (specific activity 24.5 Ci/mmol; DuPont NEN, Boston, MA, USA). After incubation, the medium was transferred to a test tube and 3 ml chloroform were added to each tube to extract unconverted substrate and other steroids. An aliquot of 0.7 ml aqueous phase was treated with 2.5% activated charcoal suspension to remove residual steroids. Tritiated water (³H₂O) formed during aromatization of [1β-³H]androstenedione to estrogen was measured in a scintillation counter.

Statistical analysis

Data were analyzed by one-way ANOVA followed by Duncan's multiple range test.

Results

Detection of peripheral aromatization in ovariectomized nude mice

To validate our animal model, two issues needed to be clarified: whether there is peripheral aromatization in ovariectomized nude mouse and, if so, could this peripheral aromatization produce a sufficient amount of estrogen to stimulate the growth of hormone-dependent breast cancer cells? To answer these questions, we evaluated the growth of aromatase transfected MCF-7 cells (A+) and sham transfected cells (A-) in response to aromatase substrate, androstenedione (Δ⁴A), in ovariectomized mice. Mice bearing either A+ or A- cells on both flanks were called homoimplants. These two groups were tested *in situ* and peripheral aromatization respectively. Animals with A+ cells on one flank and A- cells on the other were called heteroimplants. This group minimized individual differences between the animals when comparing the effect of E₂ from different sources.

During the 49-day treatment with $\Delta^4\text{A}$, A- cells did not grow in either the homoimplant or the heteroimplant groups. In contrast, A+ cells grew rapidly. The total volume of A+ tumor increased 5-6-fold. The growth rate

Figure 1 Growth curves of A+ and A- tumors in ovariectomized nude mice receiving $\Delta^4\text{A}$. A total of 25 ovariectomized mice were divided into three groups. Group 1 ($n=8$) was inoculated with A- cells (2.5×10^6 cells/site; four sites/mouse). Group 2 ($n=8$) was inoculated with A+ cells at the same cell concentration and number of sites as group 1. Group 3 ($n=9$) was inoculated with A- cells on one flank (two inoculation sites) and A+ cells on the other (two inoculation sites). All three groups of mice were injected with $\Delta^4\text{A}$ (0.1 mg/mouse, s.c.) once a day. Tumor volume was measured weekly. Tumor growth was expressed as percentage of initial total tumor volume of each group (Yue *et al.* 1998a).

Figure 2 E₂ levels in A+ and A- tumors in ovariectomized nude mice receiving $\Delta^4\text{A}$. One tumor from each mouse mentioned in Fig. 1 was homogenized and E₂ concentration was measured as described in 'Materials and Methods'. ^a $P < 0.01$ compared with A- tumor from the homoimplant group; ^b $P < 0.01$ compared with A- tumor from the heteroimplant group (Yue *et al.* 1998a).

however, was similar in both homoimplant and heteroimplant groups (Fig. 1). As expected, tissue E₂ concentrations corresponded to tumor growth. *In situ* aromatization of Δ^4 A significantly increased tissue E₂ levels that were 3-4-fold higher than A- tumors in the heteroimplant group (Fig. 2). These results directly demonstrate that there is no detectable peripheral aromatization in ovariectomized nude mice and that *in situ* aromatization is a key determinant of tumor E₂ content and growth stimulation.

Predominance of the *in situ* synthesis over the uptake mechanism

To determine the relative importance of *in situ* aromatization versus the estrogen uptake mechanism under the conditions reflecting the estrogen hormonal status in postmenopausal women, we implanted E₂-containing Silastic tubing to ovariectomized mice to 'clamp' plasma E₂ levels at approximately 5-20 pg/ml. Growth of A+ cells and tumor E₂ levels in these animals were compared with those animals receiving Δ^4 A for *in situ* aromatization.

To monitor plasma levels of E₂ resulting from the Silastic implants, uterine weight measurements were used as a bioassay. A dose-dependent increase in uterine weight in the mice treated with E₂ implants was observed as a reflection of increased serum E₂ levels. Uterine weights from all the mice receiving E₂ implants were similar to or higher than that of intact mice (Table 1). These data suggest that serum E₂ levels achieved by Silastic implants are either at or above the physiologic concentrations normally found in the mouse. In animals receiving the substrate Δ^4 A, uterine weights were also increased, indicating that the E₂ made *in situ* in A+ tumors re-entered plasma and stimulated the uterus. The degree of

stimulation, however, was consistent with lower circulating levels of E₂ when compared with intact mice and the mice with E₂ implants.

The concentration of E₂ in the tumor tissue was significantly increased by *in situ* aromatization. In contrast, the three lower E₂ doses of the Silastic implants did not enhance the levels of E₂ in the tumor even though plasma levels were increased (Table 1). Only the highest dose of the E₂ implant produced a tissue E₂ level comparable to that resulting from *in situ* aromatization. However, the predicted plasma E₂ level achieved by this dose of E₂ is higher than the level of postmenopausal women.

Finally, we assessed tumor growth rate as a means of determining the biologic effects of E₂ found in the tumor. The weight of the tumor was the highest in the mice receiving Δ^4 A. These tumors were 5-fold larger than those with 5 pg/ml E₂ implant (Table 1). These data demonstrate that the *in situ* aromatization provides higher local tissue E₂ levels and thus greater stimulation of tumor growth than that observed with the estrogen uptake mechanism.

Utilization of endogenous androgen substrate for *in situ* aromatization

To mimic postmenopausal breast cancer more closely, studies using ovariectomized nude mice without supplementation of exogenous Δ^4 A were carried out. In these animals, A+ tumors grew more slowly than in those given exogenous Δ^4 A, as noted in prior experiments. However, in the absence of exogenous Δ^4 A, the growth rate of A+ tumors was higher than that of the A- tumors. The average weight was 88±9.7 and 35.8±2.8 mg for A+ and A- tumor respectively. Tissue E₂ concentration was 3.8-fold higher in A+ tumors than in A- tumors. Treatment of the mice bearing A+ tumors with the aromatase inhibitor,

Table 1 Effect of E₂ from different sources on uterine weight, tumor E₂ concentration and growth. Ovariectomized (OVX) mice were inoculated with A+ cells and treated with Δ^4 A or E₂ Silastic implants for 8 weeks. At killing, uteri and tumors were weighed and tumor tissue E₂ concentration measured. Values are means±S.E.M.

Source of E ₂	Treatment	Estimated plasma E ₂ (pg/ml)	Uterine weight (mg)	Tumor E ₂ concentration (pg/g)	Tumor weight (mg)
	OVX control		9.7±1.9	461.4±4.4	30.4±4.0
<i>In situ</i> aromatization	Androstenedione		64.4±4.6 ^a	1132.6±221.3 ^a	449.4±70.2 ^a
Uptake	E ₂ implant	5	90.6±5.4 ^a	548.6±19.0	84.3±10.2
		7	107.1±4.2 ^a	638.0±54.6	87.4±7.7
		10	114.7±4.9 ^a	629.9±74.1	159.6±18.9 ^a
		20	126.0±14.1 ^a	1060.7±120.3 ^a	231.4±30.2 ^a

^aP<0.05 compared with OVX control.

Figure 3 Growth of wild type and estrogen deprived MCF-7 cells in culture. Wild type and LTED MCF-7 cells were plated in six-well plates at the density of 30 000 cells/well in corresponding culture medium. At 2 days, wild type MCF-7 cells were re-fed with phenol red-free IMEM containing 5% charcoal stripped FBS. At 4 days after plating, both types of cells were treated with vehicle (0.1% ethanol), E₂ (10 nM), or ICI 182,780 (1 nM) in IMEM-5% DCC-FBS. At 9 days, nuclei prepared from each well were counted using a Coulter Counter.

4-hydroxyandrostenedione (OHA), reduced tissue E₂ to the levels found in A- tumors and tumor growth was inhibited. These results suggest that endogenous steroids provided sufficient substrate to allow biologically meaningful amounts of estrogen to be produced *in situ* which could locally stimulate tumor growth.

Growth characteristics of MCF-7 cells after long-term estrogen deprivation

Our laboratory and others have established a subline of MCF-7 cells by culturing the cells in phenol red-free medium supplemented with 5% charcoal stripped FBS (Masamura *et al.* 1995). In this medium, MCF-7 cells stop growing initially and re-gain their growth ability without the addition of E₂. At 6 months, the growth rate is as high as that of wild type MCF-7 cells maximally stimulated with E₂. The basal growth rate of these cells can be inhibited by the antiestrogen, ICI 182,780 (Fig. 3). Rescue experiments showed that, in the presence of ICI 182,780 (10⁻⁹ M), the growth curve of deprived cells in response to E₂ was shifted to the left by three orders of magnitude compared with that of wild type MCF-7 cells (Fig. 4). This demonstrated that long-term estrogen deprivation causes the development of hypersensitivity to E₂ in MCF-7 cells. These cells adapt themselves to a low estrogen environment and require much lower amounts of E₂ for growth stimulation.

Aromatase activity in long-term estrogen deprived MCF-7 cells

We measured aromatase activity in wild type and long-term estrogen deprived cells using the tritiated water release assay. We observed that aromatase activity was 4-5-fold higher in deprived cells when compared with wild type cells (Fig. 5). Treatment with the phorbol ester phorbol 12-myristate 13-acetate (PMA) (10 ng/ml) for 24 h stimulated aromatase activity in both types of cells with higher stimulation observed in the deprived cells (Fig. 6).

Discussion

We have established an animal model to examine the relative importance of *in situ* aromatization in the maintenance of high tissue E₂ concentrations in the breast of postmenopausal women. The key advantage of our animal model is the lack of appreciable peripheral aromatization in nude mice. Therefore, it can distinguish the effect of estrogen from different origins. To validate the model, exogenous Δ^4 A was given to ovariectomized mice to provide sufficient substrate to determine whether peripheral aromatization was present. Under this condition, aromatase negative cells (A-) did not grow, demonstrating that peripheral aromatization in ovariectomized mice, if any, is negligible. Aromatase positive cells (A+), in contrast, aromatized exogenous Δ^4 A, which led to higher tissue E₂ levels and an enhanced

Figure 4 Rescue of ICI 182,780-induced growth inhibition by E₂. Wild type (WT) and LTED MCF-7 cells were plated in six-well plates at the density of 60 000 cells/well in corresponding culture medium. At 2 days, the cells were re-fed with phenol red- and serum-free IMEM and cultured in this medium for another 2 days before treatment with ICI 182,780 (10⁻⁹ M) in combination of various concentrations of E₂. At 5 days, nuclei prepared from each well were counted using a Coulter Counter.

Figure 5 Aromatase activity in wild type and estrogen deprived MCF-7 cells. Confluent wild type and estrogen deprived MCF-7 cells in 60-mm dishes were incubated at 37°C with [1β-³H]androstenedione plus or minus aromatase inhibitor 4-hydroxyandrostenedione (4-OHA) (10 μM) for 6 h. Radioactivity of tritiated water formed in the culture media during aromatization of [1β-³H]androstenedione was measured after chloroform extraction and charcoal treatment. Aromatase activity was expressed as fmol/mg protein/h (Yue *et al.* 1998b).

tumor growth rate. These data suggest that *in situ* aromatization is a key determinant of tissue E₂ levels and tumor growth.

Demonstrating the importance of *in situ* aromatization does not exclude the role of an estrogen uptake mechanism to maintain high tissue E₂ levels. To critically compare the biologic effects of E₂ made *in situ* with that made available through an uptake mechanism in postmenopausal women, it was necessary to develop a model that

reflected the postmenopausal hormone environment. To that end, we used Silastic E₂ implants to clamp plasma E₂ levels in ovariectomized nude mice at 5-20 pg/ml. These concentrations reflect free E₂ levels in female plasma, because sex hormone binding globulin is not present in the mouse. As shown by uterine weight changes, the Silastic E₂ implants caused dose-dependent increases in plasma E₂ concentrations. Tumor E₂ levels can also be enhanced through an estrogen uptake mechanism. However, the

Figure 6 Stimulation of aromatase activity by phorbol 12-myristate 13-acetate (PMA) in wild type and estrogen deprived MCF-7 cells. Confluent wild type and estrogen deprived MCF-7 cells in 60-mm dishes were treated with vehicle (0.1% DMSO) or PMA (10 ng/ml) for 24 h and aromatase activity was measured as described in Fig. 5.

plasma levels of E_2 required to increase the tissue E_2 to that achieved by *in situ* aromatization was considerably higher than the estrogen levels usually observed in the postmenopausal individuals (Bonney *et al.* 1983, Vermeulen *et al.* 1986, Mehta *et al.* 1987). Consistent with the higher tissue E_2 concentration observed with *in situ* aromatization, tumor growth was maximally stimulated by estrogen synthesized *in situ* when compared with that taken up from clamped levels of plasma E_2 produced by the Silastic implants. This study thus quantitatively demonstrates that *in situ* aromatization can effectively increase tissue E_2 levels to a greater extent than that produced by the uptake of estrogen from the circulation under physiologic conditions that reflect the postmenopausal female.

Our studies have shown that growth of hormone-dependent breast cancer cells in a nude mouse model correlates with the levels of E_2 found in the tumor. Interestingly, a tendency towards an inverse correlation between tumor tissue E_2 concentration and aromatase activity has been observed. We found that aromatase activity in A+ tumors varied substantially, depending on whether the substrate Δ^4A was provided or not. In ovariectomized animals not supplemented with Δ^4A , tumor E_2 levels were relatively low and the tumor growth rate was also low even though the aromatase activity in the tumor was as high as 2 pmol/mg/h. When Δ^4A was provided, tumor E_2 levels and tumor growth rate were enhanced, although the tumor aromatase activity dropped

to levels of 0.1-0.4 pmol/mg/h. These results suggest that the amount of E_2 synthesized *in situ* is determined by both intratumoral aromatase activity and availability of its substrate. Previous studies have shown that aromatase activities in breast tumors of postmenopausal patients are lower than in our model system. High concentrations of circulating Δ^4A , however, could allow sufficient amounts of estrogen to be produced *in situ* for growth stimulation.

The inverse correlation between tumor E_2 concentration and aromatase activity suggests that, in addition to well-known factors, aromatase activity might be subjected to regulation by E_2 and/or aromatase substrate through other unknown mechanism(s). Several lines of evidence support this hypothesis. We observed that aromatase activity in MCF-7 human breast cancer cells increased 4-8-fold after more than 6 months of culture under estrogen deprived conditions. Treatment of A+ cells in culture with E_2 caused a dose-dependent reduction of aromatase activity within a certain range (data not shown). Rubin *et al.* (1998) have shown that estrogen treatment of adipose stromal cells reduces aromatase activity due to differentiation of stromal cells to adipocytes.

This kind of regulation of aromatase activity would also be expected to take place *in vivo* in postmenopausal patients. Reduction of circulating E_2 levels following the menopause might result in an up regulation of aromatase activity in the breast. High concentration of circulating Δ^4A in postmenopausal women would then provide

sufficient substrate for *in situ* aromatization. Further elevation of aromatase activity could occur following antiestrogen therapy, which causes estrogen deprivation. On the other hand, we have shown that breast cancer cells become more sensitive to the mitogenic effect of E₂ after long-term estrogen deprivation. Once hypersensitivity develops, these cells will need much smaller amounts of E₂ to grow. If these concepts are indeed correct, utilization of potent aromatase inhibitors to completely block *in situ* rather than peripheral aromatization, might provide added benefit to postmenopausal patients, especially those who relapse from previous antiestrogen therapy.

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