**In Situ Aromatization Enhances Breast Tumor Estradiol Levels and Cellular Proliferation**

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**ABSTRACT**

The high concentrations of estradiol (E$_2$) found in breast tumors of postmenopausal women could be the result of enhanced uptake from plasma or in situ aromatization of androgens to estrogens. To test the relative importance of these two mechanisms, a model system allowing precise distinction between each is required. Such a model was established using aromatase (A$^+$)- and sham (A$^-$)-transfected MCF-7 cells inoculated into ovariectomized (OVX) nude mice. To validate the model, the confounding effect of peripheral aromatization was first excluded experimentally. A$^-$ cells were inoculated into OVX mice as homoimplants (A$^-$ cells on both flanks) or heteroimplants (A$^-$ cells on one flank and A$^+$ cells on the other), and growth of A$^-$ cells in response to exogenous aromatase substrate, androstenedione (A$_4$A), was evaluated. A$^-$ cells did not grow in either group during the 8 weeks of observation, indicating the lack of peripheral aromatization in OVX mice. The biological effects of in situ aromatization were then directly examined. We found that A$^+$ cells in the heteroimplant group grew rapidly, and that the average weight of A$^+$ tumor was 7.6-fold larger and tissue E$_2$ concentration was 3–4-fold higher than A$^-$ tumors grown in the same animals. These results demonstrate that in situ aromatization rather than uptake can be a determinant of tumor E$_2$ content and growth stimulation.

An additional experiment was then designed to evaluate the relative importance of in situ synthesis versus uptake under conditions reflecting postmenopausal physiology. Groups of OVX mice bearing A$^+$ cells received E$_2$ Silastic implants to clamp plasma levels at 5, 7, 10, and 20 pg/ml or A$_4$A by injection. The highest tumor E$_2$ concentration and growth rate were found in the group receiving A$_4$A. E$_2$ delivered by Silastic implants always produced lower tissue E$_2$ levels and tumor growth rates than resulted from in situ synthesis. These data provide direct evidence that under physiological conditions reflecting those in postmenopausal women, in situ aromatization in breast tumor makes a major contribution to tissue E$_2$ content.

As further validation that our experimental paradigm models the postmenopausal state, we studied OVX animals not given A$_4$A as substrate. A$^+$ cells also grew under these conditions, and the aromatase inhibitor 4-hydroxyandrostenedione reduced both tumor E$_2$ level and growth rate, providing additional evidence of the importance of in situ synthesis. These studies provide the first direct evidence that in situ synthesis of E$_2$ in breast tumors, as opposed to peripheral aromatization and uptake from plasma, can enhance tissue E$_2$ levels and stimulate tumor growth.

**INTRODUCTION**

Estrogen is mitogenic to the breast and plays an important role in the growth of hormone-dependent breast cancer. The source of E$_2$, differs substantially between pre- and postmenopausal women. Before menopause, the majority of E$_2$ is synthesized by the ovaries, secreted into the plasma, and delivered to breast tissue by endocrine mechanisms. After menopause, cessation of ovarian estrogen synthesis leads to a dramatic decrease in plasma estrogen levels. Extraglandular sites in adipose tissue, liver, muscle, brain, and breast then become the primary sources of estrogen production. A parallel decrease in plasma and breast tissue E$_2$ levels would be expected at the time of menopause. However, the concentrations of E$_2$ in malignant breast tissue in postmenopausal patients are much higher than expected and are similar to those in premenopausal patients, despite the large differences in plasma levels. As a consequence, the calculated ratio of tissue:plasma E$_2$ level is 1:1 for premenopausal and 10–50:1 for postmenopausal patients.

The greater-than-expected tissue E$_2$ concentrations in postmenopausal breast cancer tissues could result either from enhanced uptake of estrogen from plasma or from overproduction of E$_2$ inside the tumor itself. Aromatase, the rate-limiting enzyme catalyzing the conversion of androgen precursors to estrogens, was demonstrated in both normal breast tissue and in the majority of breast carcinomas (2–10). Thus, at least two potential mechanisms could be responsible for the high estrogen content of breast cancers in postmenopausal patients. Based on indirect data from previous studies, we have suggested that in situ production rather than uptake contributes preferentially to the estrogen content of breast tumors. However, no direct biological data to support this hypothesis are as yet available.

Critical proof that in situ estrogen production is predominant requires two elements: (a) direct demonstration of higher tissue E$_2$ level from in situ production than from uptake; and (b) direct correlation between the amount of locally produced E$_2$ and the biological response observed. This bipartite proof is not possible to obtain in studies of postmenopausal women, because the effects of aromatization occurring in nonbreast (peripheral) tissue confounds interpretation (Fig. 1). For that reason, a valid model system is necessary to examine the independent effects of in situ synthesis and uptake.

As an appropriate model system, we chose to use xenografts of human breast cancer capable of synthesizing estrogen in situ and of growing in experimental animals. A critical caveat is that peripheral aromatization does not occur, because this would confound interpretation of results. Our model involves OVX mice implanted with aromatase- or sham-transfected MCF-7 breast cancer cells (11). This experimental paradigm allows direct differentiation of in situ synthesis from tissue uptake via endocrine mechanisms and allows a direct correlation between local synthesis and biological effects. The reported studies demonstrated that in situ synthesis predominates over uptake from plasma as a means of maintaining breast tissue E$_2$ concentrations after menopause.

**MATERIALS AND METHODS**

Reagents and Chemicals. A$_4$A, E$_2$, and cholesterol were obtained from Steraloids (Wilton, NH). Celite was purchased from Sigma (St. Louis, MO). 4-OHA was kindly provided by Dr. Angela Brodie (University of Maryland, Baltimore, MD). Hydroxypropyl cellulose (average M$_w$, 1,000,000; Aldrich Chemical Co., Milwaukee, WI) was dissolved in saline to make a solution of 0.3% and autoclaved. This solution was used to suspend A$_4$A and 4-OHA for injection. RIA kits for estrogen and A$_4$A were obtained from ICN Biomedicals (Costa Mesa, CA).

Athymic Mice. Female athymic mice (5 weeks old) were obtained from Harlan Sprague Dawley (Indianapolis, IN). 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 flurathane anesthesia 1–3 days before cell inoculation.

Cell Culture and Inoculation into Athymic Mice. We used MCF-7 cells stably transfected with the human placental aromatase gene (A+) or plasmid vector alone (A−; Ref. 11). Aromatase activity was measured in cultured A+ and A− cells before cell inoculation. The aromatase activity of A+ cells was at the pmol/mg protein/h level, whereas the activity of A− cells was more than 1000-fold lower. Cell culture and inoculation were carried out as described previously (12). The cells were resuspended in Matrigel (10 mg/ml; kindly provided by Dr. Hynda Kleinman, NIH, Bethesda, MD). A total of 2.5 million cells (0.1 ml/site) were inoculated. Growth rates were determined by measuring the tumors with calipers every week. Tumor volumes were calculated according to the formula 4/3πr12r2 (r1 < r2).

Preparation of Silastic E2 Implants. Silastic E2 implants were prepared using Silastic brand tubing with an inner diameter of 0.078 inch (Dow Coming, Midland, MI) according to the method described previously (13, 14). The complete implant was 0.5 cm in length. E2 doses were adjusted by mixing E2 with cholesterol at ratios of 1:79 to 1:19 in weight. Based on extrapolations from our previous data (14), the predicted plasma E2 concentrations produced by these E2 implants were 5, 7, 10, and 20 pg/ml, respectively.

Measurement of Tissue E2 Levels in Tumors. Tumor samples were maintained at −80°C before 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 an E2 antibody and iodinated E2 trace obtained from ICN. The sensitivity of the assay is 1.0 pg/ml. The inter- and intra-assay coefficients of variation were 12 and 9%, respectively, at a mean concentration of 50 pg/ml.

Measurement of Serum Δ4A Concentration. Measurement of serum Δ4A concentration was carried out following instructions provided by the supplier of the commercial RIA kit. The sensitivity of the assay was 0.08 ng/ml. Intra-assay coefficients of variation at levels of 0.5, 1.5, and 4 ng/ml were 8.7, 10.3, and 11.2%, respectively. Interassay coefficients of variation at the levels given above were 11.6, 9.7, and 12.7%, respectively.

Detailed Experimental Design

Examination of Peripheral and in Situ Aromatization. Three groups of OVX mice were used. In group 1, A+ cells were inoculated on one flank, and A− cells were inoculated on the other. This design represented the heteroimplant paradigm. The remaining two groups received homoimplants of A+ or A− cells on both sides. All three groups of mice received the aromatase substrate, Δ4A (0.1 mg/mouse/day), s.c. on a daily basis. The mice bearing A− cells tested the effect of peripheral aromatization. Lack of stimulation of tumor growth would demonstrate that no Δ4A was aromatized in peripheral tissues. Heteroimplantation of A+ and A− cells into the same animal allowed a direct comparison of tumor growth with and without the ability to make estrogen in situ. Homoimplants of A+ cells allowed analysis of interactions between two aromatase-positive tumors. It was considered possible that mice with A+ homoimplants would have higher plasma E2 levels than those with heteroimplants or A− homoimplants. This group tested the possible additive effect of the endocrine- and autocrine-mediated actions of estrogen (Fig. 2). Two experiments were carried out separately in which the mice were treated with Δ4A for 4 and 8 weeks, respectively. Results from these two experiments were consistent. Data from the 8-week experiment are shown as representative data.

Comparison of the Endocrine and Autocrine Effect of Estrogen. In this experiment, all groups of mice were OVX and inoculated with A+ cells. To examine the endocrine effects, E2 was provided exclusively by an E2-containing Silastic implant calculated to produce plasma levels of 5, 7, 10, and 20 pg/ml. To examine the autocrine effects, Δ4A (0.1 mg/mouse/day, s.c.) was given.

Utilization of Endogenous Androgen Substrate for in Situ Aromatization. To simulate the physiology operative in the postmenopausal breast cancer patient, OVX mice bearing A+ or A− cells were used to compare tissue E2 concentration and tumor weight without supplementation of aromatase substrate (12). One group of mice with A+ tumors was treated with the aromatase inhibitor 4-OHA from the second week of cell inoculation. All mice were sacrificed at 10 weeks of cell inoculation.

Statistical Analysis. Data were analyzed by one-way ANOVA followed by Duncan’s multiple range test.

RESULTS

Peripheral and in Situ Aromatization. We initially examined whether Δ4A could be converted to estrogen in peripheral tissues and then transported to the tumor. Growth of A− tumors in homoimplant or heteroimplant groups would indicate the presence of peripheral aromatization. Our results showed no growth of A− tumors in the homoimplant group during the 8 weeks of treatment (Figs. 3 and 4). Coinoculation of A+ cells in the heteroimplant group did not significantly accelerate the growth of A− cells (Fig. 3). These data indicate a lack of detectable peripheral aromatization.

As evidence of in situ aromatization, A+ cells, which can convert Δ4A to estrogen, grew rapidly in mice with homoimplants (Fig. 3) when given Δ4A. The average weight of A+ tumors was 7.6-fold larger than A− tumors when compared directly in the heteroimplant group (Fig. 4). In situ aromatization of Δ4A significantly increased tissue E2 to levels 3–4-fold higher than in A− tumors in the heteroimplant group (Fig. 5). As expected, tissue E2 concentrations corresponded to tumor growth. These results directly demonstrate that there is no peripheral synthesis of estrogen in nude mice, and that in situ synthesis is a key determinant of tumor E2 content.

Interactions between Tumors. Tumors developed from the same type of cells (A+ or A−) showed similar growth rates (Fig. 3) and tissue E2 levels (Fig. 5) in homoimplant and heteroimplant groups.
tumor volume of each group. Three groups of mice were injected with A4A (0.1 mg/mouse, s.c.) once a day. Tumor on one flank (two inoculation sites) and A + cells on the other (two inoculation sites). All receiving homoimplants and hctcrnimplums. Twenty-five OVX nude mice were divided into three groups. Group I (n = 8) was inoculated with A- cells (2.5 X 10^6 cells/site: 4 sites/mouse). Group 2 (n = 8) was inoculated with A+ cells at same cell concentration on one flank (two inoculation sites) and A + cells on the other (two inoculation sites). All three groups of mice were injected with Δ⁴A (0.1 mg/mouse, s.c.) once a day. Tumor volume was measured weekly. Tumor growth was expressed as a percentage of initial total volume. The difference in tumor volume between tumors grown in the same animal.

These results suggested that there were no significant interactions between tumors grown in the same animal.

**Predominance of the in Situ Synthesis over the Uptake Mechanism.** These studies compared in situ synthesis to the uptake mechanism under physiological conditions reflecting those of postmenopausal women. We implanted E₂-containing Silastic implants to clamp plasma E₂ levels at 5–20 pg/ml to evaluate the uptake mechanism and administered Δ⁴A to test in situ E₂ synthesis. We initially assessed the levels of E₂ resulting from the Silastic implants. Limited availability of mouse serum and assay sensitivity precluded measurement of serum E₂ by RIA. Consequently, uterine weight measurements were used as a bioassay for serum E₂ levels. As shown in Fig. 6, dose-dependent increases in uterine weights in the mice treated with E₂ Silastic implants were observed as a reflection of increased serum E₂ levels. Uterine weights from all of the mice receiving E₂ Silastic implants were similar to or higher than those of intact mice. In animals receiving Δ⁴A alone, the E₂ made in situ in A+ tumors seemed to reenter plasma and stimulate uterus. The degree of stimulation (64.3 ± 4.6 mg), however, indicated lower circulating E₂ levels than in intact mice and in mice with E₂ Silastic implants.

We next compared tumor tissue E₂ levels in Δ⁴A- and E₂ Silastic implant-treated animals. Δ⁴A administration resulted in higher tissue E₂ levels through in situ synthesis than E₂ did through uptake (Fig. 7). Only the highest dose of E₂ implant could provide a tissue E₂ level comparable to that resulting from in situ synthesis. However, the predicted plasma E₂ level produced by this dose of E₂ (20 pg/ml) is higher than the level in postmenopausal women.

Finally, we assessed tumor growth rate as a means of determining the biological effects of E₂ found in the tumor. Tumor growth rate was highest in the mice receiving Δ⁴A with a mean tumor weight of 449 ± 70.2 mg, which is 5-fold as large as that with 5 pg/ml E₂ implant (84.3 ± 10.2 mg; Fig. 8). These data demonstrate that the autocrine mechanism provides higher tissue E₂ levels and greater stimulation of tumor growth than an endocrine-mediated (uptake) action.

**Utilization of Endogenous Androgen Substrate for in Situ Aromatization.** These experiments used a model more closely mimicking the physiology of postmenopausal women, because exogenous Δ⁴A was not given. In these animals, A+ tumors grew more slowly than in those given Δ⁴A in prior experiments (see Figs. 4 and 8). However, in the absence of exogenous Δ⁴A, the growth rate of A+ tumors was impaired.
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Difficult to obtain direct evidence for this in postmenopausal women, because uptake and in situ synthesis take place concurrently and confound interpretation of the relative importance of individual effects. The present study is the first to establish a model system allowing precise distinction between in situ estrogen synthesis and uptake mechanisms. Tumor tissue E₂ concentrations, growth rates, and tumor weights at sacrifice provided end points of the biological effect of E₂. The results demonstrated that in situ production of estrogen via aromatase resulted in higher levels of tissue E₂ and tumor growth rates than did E₂ taken up from plasma. These data suggest that the in situ synthesis mechanism may predominate in postmenopausal women, but uptake may also contribute to tumor tissue estrogen levels.

Table 1 Serum Δ⁴A concentration in tumor-free nude mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean ± SE</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact</td>
<td>0.12 ± 0.11</td>
<td>0.017-0.74</td>
</tr>
<tr>
<td>OVX</td>
<td>0.1 ± 0.002</td>
<td>0.006-0.16</td>
</tr>
<tr>
<td>OVX + Δ⁴A</td>
<td>53.4 ± 22.7</td>
<td>2.16-127.7</td>
</tr>
</tbody>
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DISCUSSION

Breast tumors from postmenopausal women contain levels of E₂ similar to those found in premenopausal women, although plasma E₂ levels are 10-50-fold lower after menopause. This unexpected finding could be due to in situ production of E₂ in the tumor, enhanced uptake from plasma, or a combination of effects. A number of studies have suggested the importance of in situ aromatization. However, it is
Demonstration that aromatization can only occur in tumors and not in peripheral tissues in our model system is a necessity to draw valid conclusions. Use of aromatase-negative cells (A−), both as homoimplants and heteroimplants, provided the means to directly assess the presence or absence of peripheral aromatization. Notably, we found that the A− cells did not grow in response to exogenous Δ4A during the 8-week experiment. These data demonstrated that peripheral aromatization in OVX nude mice is negligible, even with supplementation of very high levels of exogenous substrate.

Heteroimplantation of A+ and A− cells into the same animal enabled us to directly demonstrate the importance of in situ-produced E2. This method eliminates differences between host animals because two different cell lines were implanted into the same animal. Because A+ and A− cells showed identical growth responsiveness in vitro and in vivo to E2 (data not shown), the only difference between A+ and A− tumors is the presence or absence of aromatase. These experiments clearly demonstrate that aromatase-positive tumors exhibited higher tissue E2 levels and growth rates than aromatase-negative tumors growing in the same animal.

Our goal in these studies was to determine whether E2 produced in situ is relatively more important than uptake from plasma under conditions of low circulating E2 (i.e., as in postmenopausal women). There is no doubt that the stimulatory effect of endocrine E2 (i.e., from uptake) becomes dominant when plasma E2 concentration reaches the levels found in premenopausal women (15, 16). However, the relative importance of in situ synthesis versus the endocrine mechanism on tumor growth under the circumstance of low plasma E2 concentration has never been directly demonstrated. Using a Silastic E2 clamp technique, we were able to determine what level of tissue E2 could be reached through uptake under various physiological concentrations of E2. Whereas uptake of E2 into tumors always occurred, the levels achieved were consistently lower than those produced by in situ synthesis. The highest dose of E2 implant (20 pg/ml) did provide a tissue E2 concentration similar to in situ synthesis. The predicted plasma E2 level with this dose, however, was higher than that of postmenopausal women. Consistent with tissue E2 concentration, tumor growth was maximally stimulated by estrogen synthesized in situ compared with that taken up from clamped levels of plasma E2. This study is the first to demonstrate quantitatively that the autocrine mechanism can effectively increase local E2 production to a biologically greater extent than the endocrine mechanism under physiological conditions reflecting those in postmenopausal women.

The validity of the E2 delivery system is critical to the interpretation of our data. According to our previous studies, predicted serum E2 concentrations produced by four different doses of E2 Silastic implants were 5, 7, 10, and 20 pg/ml, respectively (14). These predicted serum E2 concentrations are equivalent to or higher than the E2 levels of postmenopausal women (17–19). In humans, about 40% of circulating E2 is tightly bound to sex hormone-binding globulin. If the average total plasma E2 concentration in postmenopausal women is 8 pg/ml, biologically available E2 in the circulation is only about 5 pg/ml. Sex hormone-binding globulin is not present in mouse plasma. Thus, the plasma concentrations of E2 in the mice receiving E2 Silastic implants represent biologically available concentrations. Based on this calculation, the lowest dose of E2 (5 pg/ml) in our experiment is equivalent to the physiological E2 concentration of postmenopausal women.

Having segregated the effects of in situ synthesis and uptake in a model system, we next sought to more closely mimic the postmenopausal state. In these studies, we inoculated tumors into OVX mice but did not provide Δ4A as substrate. Under these conditions, we also observed an elevated growth rate and tissue E2 concentration in A+ tumors compared with A− tumors. As evidence of the biological importance of aromatase, the inhibitor 4-OHA reduced both tissue E2 concentration and tumor growth. We believe that the difference in tissue E2 concentrations between A+ and A− tumors is not due to different abilities of Δ4A uptake, because the uptake of steroids by cells is a passive process, and the uptake of E2, a steroid, was similar in these two kinds of tumors grown in nude mice, as indicated by tissue E2 concentrations.

In OVX mice, aromatase substrate Δ4A originates from the adrenals. To further characterize our model, we measured serum Δ4A concentrations and found levels of 0.1 ng/ml, similar to that reported by Brünnner et al. (20). This level is only about one-tenth of that in postmenopausal women. Even at such a low circulating Δ4A level, significantly higher tissue E2 concentration and growth rate were observed in A+ tumors compared to A− tumors. This provides further support for our hypothesis that the in situ synthesis mechanism is more important than the uptake from plasma.

The situation in postmenopausal breast cancer patients is more complicated than in our nude mouse model. Both in situ and periph-
eral aromatization take place in postmenopausal patients. The amount of aromatase in peripheral tissue of postmenopausal women varies widely and increases as a function of adipose mass. Besides peripheral aromatization, at least two factors are considered to be determinants of the amount of estrogen synthesized in situ: (a) aromatase activity in the tumor; and (b) availability of aromatase substrate. Our prior data demonstrated a wide range of aromatase activity in breast carcinomas from <5 to >80 pmol/g/h (6). This variation might be due to dier-
gency of cellular aromatase activity in the tumor or uneven distribution of aromatase-positive cells. Lu et al. (10) recently suggested that aromatase activities obtained from the studies using tissue homoge-
nates could be underestimated. They found greater aromatase activi-
ties in cryosections of human breast tumor tissue than in homogenates of the same samples (10). The levels of aromatase activity they reported were comparable to those in A+ tumors developed in our nude mouse model (21). On the other hand, plasma Δ4A concentra-
tions in postmenopausal women are much higher than in OVX mice. This would provide a relatively higher tissue E2 level in the area where aromatase-positive cells reside. The study conducted by Reed et al. (3) also suggested the importance of in situ synthesis of estrogen in breast tumors. Using an elegant double-isotope infusion technique, this group showed that 75–97% of estrone found in breast tumor tissues was contributed by in situ aromatization (3). Their studies, however, could not provide direct correlation between the amount of estrogen made in tumor tissue and tumor growth rates. Whereas we demonstrate the role of aromatase in situ, sulfatase could also contribute to the tissue estrogen concentration, depending on the levels of plasma estrogen sulfite and tumor sulfatase content (22).

Considering the above-mentioned results and our findings, we suggest that a biologically significant level of E2 is synthesized in postmenopausal breast cancer tissue through the aromatase enzy-
me. de Jong et al. (23) recently reported that a short period of treatment with the third-generation aromatase inhibitor vorozole significantly inhibited tumor aromatase activity and reduced tumor tissue estrogen contents (23). These results suggest that more attention be directed toward measurement of tissue E2 concentra-
tions rather than plasma levels, which represent peripheral aromatization. Local synthesis alters the relationship between plasma and tissue E2 concentrations. However, it is the level of E2 in tissue that is biologically important.

REFERENCES

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