A New Nude Mouse Model for Postmenopausal Breast Cancer Using MCF-7 Cells Transfected with the Human Aromatase Gene

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ABSTRACT

MCF-7 cells transfected with human placental aromatase gene (MCF-7CA cells) or cells transfected with plasmid vector only (MCF-7Cc cells) were inoculated into nude mice with Matrigel. Tumors formed from both MCF-7CA and MCF-7Cc cells grew faster in intact mice than in ovariec-
tomized mice, suggesting that the tumors maintained their responsiveness to estrogen stimulation and that their growth was supported by ovarian estrogen. Injections of androstenedione (0.1 mg/mouse/day) to provide the substrate for aromatization to ovariectomized mice bearing MCF-7CA tumors accelerated their growth but did not affect growth of MCF-7Cc tumors. This result indicates that local production of estrogen by intra-
tumoral aromatase was sufficient to stimulate tumor growth. When ovari-
ectomized mice with MCF-7CA tumors supplemented with androstenedi-
one were treated with aromatase inhibitors 4-hydroxyandrostenedione (1 mg/mouse/day, s.c.) or CGS 16949A (0.5 mg/mouse/day, s.c.), or with the antiestrogen tamoxifen (10 μg/mouse/day, s.c.), tumor growth was signif-
ificantly inhibited. Tumor aromatase activity measured at the end of treat-
ment was also inhibited by 4-hydroxyandrostenedione when the mice were sacrificed 4 h after the last injection. The tumors of this mouse model are dependent for their growth on estrogens from an endogenous nonovarian source. Thus, it simulates the situation in the postmenopausal breast cancer patient and could be used to evaluate the effect of aromatase inhibitors and antiestrogens.

INTRODUCTION

Athymic mice have been used as a model to study behavior of human tumors in vivo since 1969, when heterotransplantation of malignant human tumors to nude mice was first reported by Rygaard and Povlsen (1). The growth rate among human tumors was variable. Hormone-dependent tumors, such as breast tumors and prostate cancers, are among the most difficult to grow in the nude mice (2). The success rate in establishing tumors was very low, and tumor growth from transplants of either primary breast carcinomas or breast cancer cell lines was slow. Estrogen supplementation was found to increase the growth rate of these tumors (3–5). This model has subsequently proved useful for studying the effects of estrogen receptor antagonists. However, since estrogen was supplied from an exogenous source, this model was unsuitable for the study of inhibitors of estrogen synthesis.

Matrigel, a mixture of basement membrane proteins, has been reported to enhance tumorigenicity of small cell lung, renal, prostatic, and MCF-7 breast carcinoma cells in nude mice (6–9). Our previous studies have indicated that the tumors retained their responsiveness to ovarian estrogen (9, 10). Thus, this model appears to be useful for studies of aromatase inhibitors.

In postmenopausal women, estrogens are synthesized in extraver-
ian tissues, such as adipose tissue, by aromatization of androgens from the adrenals. A number of reports have demonstrated the presence of aromatase enzyme in some, but not all, breast tumors (11–14). Recently, we used transcription-polymerase chain reaction to detect aromatase mRNA in breast tumors and found that 12 of 15 primary breast tumors investigated contained aromatase mRNA (15).

In the present study, we have attempted to simulate the postmeno-
pausal breast cancer patient in the OVX nude mouse by using MCF-7 cells which have been transfected with the human placental aromatase gene (MCF-7CA cells). The presence of aromatase provides an endogenous nonovarian source of estrogen to stimulate tumor growth. Also, we compared the growth of tumors formed from these cells in intact mice with tumors formed from control transplanted cells. Further, we have determined the effects on tumor growth of 4-OHA, the first selective aromatase inhibitor to be used in breast cancer patients (16, 17), and the antiestrogen tamoxifen. Tamoxifen has been studied by several investigators in nude mice supplemented with estradiol (5, 18). The new nonsteroidal inhibitor fadrozole (CGS 16949A) (19), currently in clinical trials, was also compared.

MATERIALS AND METHODS

Reagents and Chemicals. Androstenedione, d-glucose 6-phosphate, NADP, glucose 6-phosphate dehydrogenase, and tamoxifen were purchased from Sigma (St. Louis, MO). [1B-3H]Androstenedione (24.2 Ci/mmol) was purchased from NEN Dupont (Boston, MA).

4-Hydroxyandrostenedione was synthesized in our laboratory as described previously (16). CGS 16949A (fadrozole) was kindly provided by Dr. Ajay Bhatnagar, Ciba-Geigy Pharmaceutical Co., Basel, Switzerland (19). Hy-
droxypropyl cellulose, average Mr, 1,000,000 (Aldrich Chemical Company, Milwaukee, WI), was dissolved in saline to make a solution of 0.3% and autoclaved. This solution was used to suspend the injected compounds androstenedione, 4-OHA, CGS 16949A, and tamoxifen.

Athymic Mice. Female BALB/c athymic mice 4–6 weeks of age were obtained from Charles River Breeding Laboratories (Boston, MA). The ani-
mals were housed in a pathogen-free environment under controlled conditions of light and humidity and received food and water ad libitum. Ovariectomy was carried out as required under flurothane anesthesia 1–3 days before cell inoculation.

Cell Culture and Inoculation to Athymic Mice. We used MCF-7 cells stably transfected with the human placental aromatase gene (MCF-7CA) or plasmid vector alone (MCF-7Cc) (20). The cells were cultured in Eagle’s minimum essential medium containing 5% fetal bovine serum and neomycin (600 μg/ml); Gibco, Bethesda, MD). The culture medium was changed twice weekly. Subconfluent MCF-7Ca or Cc cells were scraped into Hanks’ solution and centrifuged at 1,000 rpm for 2 min at 4°C. The cells were then resuspended in Matrigel (10 mg/ml; kindly provided by Dr. Hynda Kleinman, NIH, Bethesda, MD (7)) to make a cell suspension of 2–5 × 10⁶ cells/ml. Intact mice were inoculated with MCF-7Ca or Cc cells in Matrigel. Each mouse was given in both flanks an s.c. injection of 0.1 ml of the cell suspension. OVX mice were inoculated with MCF-7Ca or Cc cells as above into 2 sites in each flank (2.3–10⁶ cells/site). Growth rates were determined by measuring the tumors.

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1 This work was supported by NIH Grants CA-61206 and CA-44735.
2 To whom requests for reprints should be addressed.
with calipers every week. Tumor volumes were calculated according to the formula:

\[ \frac{4}{3} \pi r^3 \]

**Treatments.** As indicated in the Figures and Tables, beginning on the day following inoculation, some animals were given injections of 0.1 mg/mouse/day androstenedione. Mice treated with aromatase inhibitors received s.c. injections of 4-OHA (1 mg/mouse/day) or CGS 16949A (0.5 mg/mouse/day). Mice treated with the antiestrogen received s.c. injections of tamoxifen (10 \( \mu g/mouse/day \)). Control animals were given injections of vehicle (0.3% hydroxypropyl cellulose, 0.1 ml/mouse/day) s.c. daily. Treatments began 10–12 days after androstenedione injections. The treatment lasted for 6–7 weeks.

**Measurement of Aromatase Activity in Tumors Grown In Nude Mice.** Animals were autopsied 4 or 6 h after the last injection. Tumors were removed from the mice and homogenized in phosphate buffer with a Polytron homogenizer at 4°C. Aromatase activity in the tumor homogenates was measured as described previously (16). Briefly, 0.9 ml homogenate was incubated with 1 \( \mu Ci \) of [\( ^{1}H \)-androstenedione] and a NADPH-generating system (NADP, 6.7 mM; glucose 6-phosphate, 70 mM; and glucose 6-phosphate dehydrogenase, 25 I.U.) at 37°C under an atmosphere of oxygen. After 2 h, 2 ml of chloroform were added to extract the steroids. The aqueous phase was removed and treated with 2.5% charcoal suspension to eliminate residual steroids. The tritium released to form \( ^{3}H_2O \) during aromatization of [\( ^{1}H \)-androstenedione] to estrogens was measured in a liquid scintillation counter. Aromatase activity was expressed as fmol estrogen produced/mg protein/h. The protein concentration of the homogenate was measured by the method of Lowry et al. (21).

**RESULTS**

**Growth of Tumors from MCF-7Cc Cells In Intact and OVX Mice.** Human breast carcinoma cells transfected with plasmid vector alone (MCF-7Cc) were inoculated in Matrigel into one group of intact mice and one group of ovariectomized mice. One month after inoculation, the first measurement of the tumor volume was carried out (initial volume). After 110 days, the mean tumor volume in intact mice was 364.9 ± 54.0 (SE) mm³, whereas the tumor volume in ovariectomized mice was only 171.5 ± 27.3 mm³ (Fig. 1). The mean weight of tumors from the intact mice was 2.13-fold greater than that of tumors from the OVX mice [178.5 ± 26.2 mg versus 69.9 ± 13.2 mg (P < 0.05)]. These results suggest that the ovary synthesizes sufficient estrogen to support the growth of tumors formed from the MCF-7Cc cells.

**Growth of Tumors from MCF-7Ca Cells In Intact and OVX Mice.** To determine whether production of estrogen by the tumor could stimulate its growth in absence of ovarian estrogen, MCF-7Ca cells were inoculated into ovariectomized mice. The growth rate of these tumors from MCF-7Ca cells was found to be similar to that of MCF-7Cc tumors in intact mice. Thus, in 110 days, the MCF-7Ca tumors in intact mice had reached a mean volume of 564.3 ± 94.9 mm³, whereas in ovariectomized mice the mean tumor volume was 365.6 ± 78.3 mm³ (Fig. 1). However, there was considerable variation in the size of the MCF-7Ca tumor of OVX mice, as indicated in Fig. 2. Thus, of the 5 OVX animals shown in Fig. 1, tumors grew slowly in 3 mice, whereas in 2 mice, tumors grew at rates comparable to those of intact mice.

**Nitrogen Mouse Model for Breast Cancer**
NUDE MOUSE MODEL FOR BREAST CANCER

Effect of Aromatase Inhibitors and Antiestrogen on Tumor Growth. 4-OHA was effective against tumors of ovariectomized mice given inoculations of MCF-7Ca cells and supplemented with androstenedione (0.1 mg/mouse/day) from the second day of inoculation. In mice treated with 4-OHA injections (50 mg/kg/day) for 42 days, tumor aromatase activity was significantly inhibited in animals autopsied 4 h after the last injection but not 6 h after injection (Table 1). In another experiment, the effects of treatment with 4-OHA, CGS16949A, and tamoxifen were compared. Mean tumor weights were significantly less in the aromatase inhibitor and antiestrogen-treated groups than in controls (P < 0.01) (Fig. 5). However, a greater reduction in tumor growth occurred in both groups of animals treated with aromatase inhibitors, 4-OHA or CGS16949A, and tamoxifen were compared. Mean tumor weights in OVX mice supplemented with androstenedione. Seventeen OVX mice were given injections of 4-OHA (1 mg/mouse/day) beginning 11–13 days after cell inoculation. Androstenedione (0.1 mg/mouse/day) was administered from the day after cell inoculation. Androstenedione (0.1 mg/mouse) injections s.c. daily significantly and consistently accelerated the growth of MCF-7Ca tumors in OVX mice, whereas the growth of MCF-7Cc tumors was not affected. Thus, the growth rate of the MCF-7Ca tumors in OVX mice supplemented with aromatizable substrate was similar to or greater than that in intact mice. As seen in Figs. 1 and 3, tumors in intact mice reached a volume of 380 mm³ in 70 days, whereas tumors in OVX mice supplemented with androstenedione reached the same size in 61 days. The result suggests that the aromatase activity of these tumors is sufficient to synthesize enough estrogen to support tumor growth as long as there is substrate available.

DISCUSSION

Matrigel is composed of laminin, collagen IV, heparin sulfate, proteoglycan, and entactin, and has been demonstrated to enhance tumor cell adhesion, migration, growth, collagenase IV activity (invasiveness), and resistance to cytotoxic drugs in vitro (22–24). It has also been reported to increase tumorigenicity and growth of some otherwise poorly growing human cancer cells or primary tumor tissue in athymic mice. Our preliminary study (10) and other studies (9) demonstrated that Matrigel also enhances tumorigenicity of wild type MCF-7 cells in athymic mice. In the present study, we inoculated MCF-7Ca transfected with the human aromatase gene or the control MCF-7Cc cells transfected with the plasmid vector suspended in Matrigel into nude mice. Tumor formation was 100% in both intact and ovariectomized mice. In previous reports, tumor incidence from breast tumor cell lines inoculated without Matrigel was 0% if exogenous estradiol was not provided (25). Similar results were obtained with prostatic carcinoma tumor grown in nude mice (8). It is apparent that Matrigel provides a suitable microenvironment for cell-matrix interaction which facilitate development of tumors in nude mice.

Tumors from both MCF-7Ca and MCF-7Cc cells grew faster in intact than in ovariectomized mice as shown in Fig. 1. The data indicate that MCF-7Ca and MCF-7Cc tumors grown in vivo maintain their responsiveness to estrogens and that the ovary is the major source of estrogen for tumor growth in these animals. However, estrogen synthesized by the tumor may contribute to stimulating the growth of the MCF-7Ca tumors, as these grew more rapidly than the MCF-7Cc tumors in both ovariectomized and intact mice.

In ovariectomized mice (without androstenedione supplement), MCF-7Ca tumors of 2 mice had comparable values to those of intact mice (Fig. 2). In the other 3 mice, all tumors grew very slowly, although the aromatase activity in these tumors was similar to that in the more rapidly growing tumors. This suggests that some of these tumors could synthesize sufficient estrogen to stimulate their growth. In contrast, all tumors from MCF-7Cc cells grew very slowly in OVX mice. Although we did not measure the serum concentration of androgens, reduced serum androgen levels have been reported in nude mice 30 days of age or older compared to normal mice (26). Androgen substrate insufficiency might account for the slow growth of MCF-7Ca tumors in some OVX mice. Androstenedione (0.1 mg/mouse) injections s.c. daily significantly and consistently accelerated the growth of MCF-7Ca tumors in OVX mice, whereas the growth of MCF-7Cc tumors was not affected. Thus, the growth rate of the MCF-7Ca tumors in OVX mice supplemented with aromatizable substrate was similar to or greater than that in intact mice. As seen in Figs. 1 and 3, tumors in intact mice reached a volume of 380 mm³ in 70 days, whereas tumors in OVX mice supplemented with androstenedione reached the same size in 61 days. The result suggests that the aromatase activity of these tumors is sufficient to synthesize enough estrogen to support tumor growth as long as there is substrate available.

Table 1  Effects of 4-OHA on tumor growth and aromatase activity in OVX nude mice supplemented with androstenedione.

<table>
<thead>
<tr>
<th>No. of</th>
<th>Treatment</th>
<th>Time (h) after</th>
<th>Tumor vol.</th>
<th>Tumor wt</th>
<th>Tumor aromatase activity</th>
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<td>injection</td>
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<td>6</td>
<td>42.3</td>
<td>41.5</td>
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<td>42</td>
<td>4</td>
<td>41.3</td>
<td>34.4</td>
<td>15.5</td>
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</tbody>
</table>

* Mice were autopsied at the times indicated after the last injection of 4-OHA; tumors were removed and weighed, and aromatase activity measured.

Fig. 4. Mean weights and aromatase activity in MCF-7Ca and MCF-7Cc tumors grown in OVX mice supplemented with androstenedione. Data are from the same experiment as shown in Fig. 3. Tumors were removed and weighed, and aromatase activity was measured as described in "Materials and Methods" (mean ± SE; **P < 0.005).

Fig. 5. Effect of aromatase inhibitors and antiestrogen on growth of MCF-7Ca tumors in OVX mice supplemented with androstenedione. Seventeen OVX mice were given injections of MCF-7Ca cells suspended in Matrigel (2.89 × 10⁶ cells/site, 4 sites/mouse). Beginning on the following day, androstenedione (0.1 mg/day, s.c.) was injected. Treatment with 4-OHA (1 mg/day s.c., n = 5), CGS 16949A (0.5 mg/day s.c., n = 4), tamoxifen (TAM) (10 μg/day s.c., n = 4), or vehicle (0.1 ml/day s.c., n = 4) started 13 days after inoculation. Seven weeks later, the mice were sacrificed, and tumors were removed and weighed (mean ± SE; **P < 0.01 compared to control mice; +P < 0.01, aromatase inhibitors compared to tamoxifen).
Both aromatase inhibitors 4-OHA and CGS 16949A, and the antiestrogen tamoxifen, were effective in reducing tumor growth, indicating that the tumors depend on the stimulatory effects of estrogens. Tumor aromatase activity was inhibited by 4-OHA after the mice were sacrificed 4 h after the last injection, although no effect was observed in animals killed more than 6 h after the last injection of 4-OHA. This short duration of inhibition is in contrast to previous findings with this inhibitor. We have reported that 4-OHA causes enzyme inactivation in vitro and has lasting effects in vivo in rats (27).

Tumor aromatase activity was inhibited by 4-OHA when the mice were killed 24 h after the last injection of the inhibitor, whereas CGS 16949A is currently in clinical trials. It is difficult to quantify that the tumors depend on the stimulatory effects of estrogens. Aromatase in the ovary, but not in the peripheral tissues, is subject to feedback regulation by gonadotropins. Thus, aromatase inhibitors may aid in improving the treatment of postmenopausal breast cancer patients. On the other hand, 4-OHA has only recently been evaluated for its antitumor effects in women, whereas CGS 16949A is currently in clinical trials. It is difficult to compare treatments in breast cancer patients since most receive tamoxifen as first line therapy. To date, treatment with aromatase inhibitors has focused on postmenopausal patients. Following the menopause, estrogen production is from nonovarian sources, such as adipose tissue. While breast tumors may also synthesize estrogens, the level of breast and tumor aromatase is quite low and variable (11, 14, 15). In the model described here, we have utilized the tumor as the nonovarian source of estrogen. Aromatase in the ovary, but not in the peripheral tissues, is subject to feedback regulation by gonadotropins. Thus, compounds such as aminoglutethimide, which reduce ovarian estrogen synthesis incompletely, can have a reflex rise in gonadotropins leading to enlarged ovaries and stimulation of estrogen production (29). Alternatively, some aromatase inhibitors may reduce ovarian estrogen production indirectly by inhibiting gonadotropins. We have shown that the MCF-7Ca tumors in OVX mice are dependent on estrogens from a nonovarian source. The model appears suitable for studying aromatase inhibitors, estrogen receptor antagonists, and other hormonally active agents and may aid in improving the treatment of postmenopausal breast cancer.

REFERENCES

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