Aromatase inhibitors and their antitumor effects in model systems

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Abstract

The potential of aromatase (estrogen synthetase) within the breast to provide a significant source of estrogen mediating tumor proliferation is suggested by studies reporting 4- to 6-fold higher estrogen levels in tumors than in plasma of postmenopausal patients with breast cancer. Recent studies in our laboratory have identified aromatase and its mRNA in tumor epithelial cells using immunocytochemistry and in situ hybridization. In addition, significant aromatase activity, which was stimulated 7-fold by dexamethasone, was measured in metastatic cells isolated from a breast cancer patient. Increase in proliferation, as measured by proliferating cell nuclear antigen immunostaining in tumor sections and by thymidine incorporation into DNA in response to testosterone, was observed in histocultures of breast cancer samples. This latter effect could be inhibited by 4-hydroxy-androstenedione. These results imply that intratumoral aromatase has functional significance and may be an important target for successful inhibitor treatment of breast cancer patients. To investigate treatment strategies with aromatase inhibitors and antiestrogens, we developed an intratumoral aromatase model to simulate the hormone responsive postmenopausal breast cancer patient. Tumors of estrogen receptor positive human breast carcinoma cells (MCF-7) transfected with the human aromatase gene are grown in ovariectomized nude mice. These cells synthesize sufficient estrogen to stimulate tumor formation. We have utilized this model to investigate the effects on tumor growth of the antiestrogens, tamoxifen and ICI 182780, and the aromatase inhibitors, letrozole and anastrozole (arimidex), alone and in combination. Both the aromatase inhibitors and the antiestrogens were effective in suppressing tumor growth. However, letrozole was significantly more effective than the antiestrogens. When the aromatase inhibitors were combined with the antiestrogens, tamoxifen, tumor growth was suppressed to about the same extent as with the aromatase inhibitors alone. Furthermore, the results do not suggest any benefit from combining tamoxifen with the pure antiestrogen, ICI 182780. Thus sequential use of these agents is likely to be more advantageous to the patient in terms of longer duration of effective treatment.

Introduction

The incidence of breast cancer increases with age and is more common among postmenopausal women than younger women. However, the sensitivity of breast cancer to estrogen also increases with the age of the patient. Based on measurements of estrogen receptor concentrations in tumors, two-thirds of postmenopausal patients have tumors that are likely to be responsive to hormone ablative therapy (McGuire 1980).

After menopause, ovarian production of estrogen and progesterone declines. Although estrogen synthesis increases in peripheral tissues, circulating levels of estrogen are usually low. A number of studies have reported that concentrations of estrogen are 4- to 6-fold higher in breast tissue and similar to those in premenopausal patients (Szyczak et al. 1998). Furthermore, estrogen concentrations in tumors are higher than in breast fat (van Landegham et al. 1985, Blankenstein et al. 1998). Thus, estrogen production within the breast and by breast cancers may be critical in stimulating tumor proliferation in postmenopausal patients. Effective inhibition of breast aromatase might therefore be an important determinant of the outcome of aromatase inhibitor treatment. Recent studies in our laboratory investigated aromatase expression in the breast using immunocytochemistry and
in situ hybridization with sequence-specific probes (Lu et al. 1996). Aromatase was identified mainly in the tumor epithelial cells of human breast cancers. In addition, enzyme activity was detected in metastatic tumor cells isolated from the ascites fluid of a patient with advanced breast cancer. The level of activity was increased 7-fold on incubation of the cells with dexamethasone. In cryosections of primary tumors, aromatase activity correlated with a marker of proliferation, proliferating cell nuclear antigen score, suggesting that locally produced estrogens may stimulate the growth of these tumors. Proliferation of some tumors in histoculture was also found to be enhanced by testosterone as well as estrogens. The stimulation by testosterone but not by estrogen could be blocked by aromatase inhibitors (Lu et al. 1996). This suggests that androgens are aromatized to estrogens by the tumors and that the tumor could produce sufficient estrogen to stimulate tumor proliferation.

Systemic treatment of breast cancer patients with aromatase inhibitors should effectively block synthesis of estrogen in all tissue sites. Clinical trials of aromatase inhibitors carried out to date have demonstrated the benefits of these agents for treating patients with advanced disease. However, most of the patients had received tamoxifen first or other forms of hormone therapy and then later relapsed before receiving aromatase inhibitor treatment. Thus, the low response rates in these patients compared with the marked reduction in serum estrogen levels during aromatase inhibitor treatment are likely to be influenced by the insensitivity of some tumors to estrogens or other forms of drug resistance that may have developed, rather than to lack of efficacy of the inhibitors.

In order to investigate the antitumor activity of aromatase inhibitors as first-line agents and to compare them in strategies with antiestrogens, we recently developed an intratumoral aromatase model in nude mice to simulate the postmenopausal breast cancer patient. As the rodent has no significant production of estrogen from androgens, the tumors were inoculated with castrated Balb/c mice (aged 4-6 weeks) were inoculated s.c. in four groups with the human aromatase gene (MCF-7CA) as the source of estrogen to stimulate tumor formation in ovariectomized nude mice (Yue et al. 1994, 1995). Thus tumors in mice inoculated with MCF-7CA cells grow faster than those in the same animal without the aromatase transfection (Yue et al. 1994), or which depend on circulating estrogen from neighboring MCF-7CA tumors (Yue et al. 1998).

In a series of recent studies, this intratumoral aromatase model was used to investigate the effects of the aromatase inhibitors, letrozole and anastrozole, on tumor growth, as well as on the uterus. Letrozole and anastrozole are triazole derivatives and are competitive and reversible aromatase inhibitors, which are highly potent and selective for aromatase. Both compounds were recently approved by the FDA for the treatment of advanced breast cancer. Recent clinical studies that compared these inhibitors with other second-line agents found that letrozole caused more complete and partial tumor responses than aminoglutethimide (Marty et al. 1997) and that Arimidex (anastrozole) increased survival of patients to a significantly greater extent than Megaace (Buzdar et al. 1997). However, the optimal use of these well-tolerated agents remains to be determined.

The antiestrogen, tamoxifen, is known to be significantly more effective than cytotoxic chemotherapy in the treatment of postmenopausal hormone-responsive breast cancer (Early Breast Cancer Trials Collaborative Groups 1992). However, it has been reported that tamoxifen treatment increases the risk of developing endometrial carcinoma, and the incidence is correlated with the duration of treatment (Killackey et al. 1985). This effect is thought to be due to the partial agonistic action of tamoxifen. The steroidal antiestrogen, ICI 182780, was developed in the late 1980s. This compound is a more potent antiestrogen than tamoxifen and is without agonistic effects (Wakeling & Bowler 1987, Wakeling et al. 1991). Clinical trials with ICI 182780 in breast cancer patients are in progress. We have used our mouse tumor model to study the effects of the aromatase inhibitors and to compare them with those of antiestrogens, as single agents and in combination treatment (Lu et al. 1998, 1999).

**Intratumoral aromatase model**

As previously described (Yue et al. 1994, 1995), subconfluent MCF-7 cells transfected with the human aromatase gene (MCF-7CA) were scraped into Hanks’ solution and centrifuged at 1000 g for 10 min at 4°C. The cells were then resuspended in Matrigel (10 mg/ml) to a concentration of 3x10^6/ml. Ovariectomized female Balb/c mice (aged 4-6 weeks) were inoculated s.c. in four sites each with 0.1 ml of the cell suspension. Animals were injected s.c. throughout the experiment with 0.1 mg/mouse per day androstenedione, the substrate for aromatization to estrogens. Tumor growth was measured with calipers weekly and tumor volumes were calculated according to the formula (4/3)πr1²r2 (r1 < r2). The animals were housed in a pathogen-free environment under controlled conditions of light and humidity and received food and water ad libitum.

Animals were assigned to groups of four or five mice when all tumors had reached a measurable size (~500 mm^3), usually 28-35 days after androstenedione injections were started. They were then injected s.c. daily with the following aromatase inhibitors, antiestrogens, or a combination of these agents. Letrozole (CGS 20267; kindly provided by Dr Ajay Bhatnagar, Novartis, Basel, and approved by the FDA for the treatment of advanced breast cancer. Recent clinical studies that compared these inhibitors with other second-line agents found that letrozole caused more complete and partial tumor responses than aminoglutethimide (Marty et al. 1997) and that Arimidex (anastrozole) increased survival of patients to a significantly greater extent than Megaace (Buzdar et al. 1997). However, the optimal use of these well-tolerated agents remains to be determined.

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Switzerland), Arimidex, anastrozole (ZD1033; kindly provided by Dr Michael Dukes, Zeneca Pharmaceuticals, Macclesfield, Cheshire, UK) and the antiestrogen, tamoxifen, were prepared for injection in 0.3% hydroxypropylcellulose (HPC). The pure antiestrogen, faslodex (ICI 182780) (kindly provided by Dr A Wakeling, Zeneca Pharmaceuticals), was injected in oil once a week. Control animals received vehicle (0.3% HPC, 0.1 ml/mouse per day) s.c. daily. Groups of mice were also injected with a combination of an aromatase inhibitor and an antiestrogen, or a combination of tamoxifen and ICI 182780. The doses administered in combination were the same as for each agent used alone. The treatments lasted 5-6 weeks, as indicated in each figure. Animals were autopsied 4-6 h after the last injection. Tumors and uteri were removed from the mice, cleaned, weighed, and stored at −80 °C until analyzed.

Figure 1 Effect of the antiestrogen, ICI 182780, and the aromatase inhibitor, letrozole, on tumor and uterine wet weight in the intratumoral aromatase mouse model. Groups of four mice were injected s.c. with ICI 182780 (ICI) 0.7 mg or 0.07 mg in oil once per week, letrozole (CGS) 10 µg in 0.3% HPC/mouse per day, or 0.3% HPC vehicle (control). (A) Tumors were measured weekly and the percentage change in volume calculated. (B) After 35 days of treatment, mice were killed, and tumors and uteri were removed and weighed. Values are mean±S.E. *P<0.05, **P<0.01 compared with controls. Taken with permission from Lu et al. (1998), published by Kluwer.

Figure 2 Effect of the antiestrogen, tamoxifen, and the aromatase inhibitor, Arimidex (anastrozole), on tumor and uterine weight in the intratumoral aromatase mouse model. Groups of five mice were injected s.c. daily with tamoxifen (TAM; 3 µg/mouse per day), Arimidex (5 µg/mouse per day) or vehicle (control). (A) Tumors were measured weekly and the percentage change in volume calculated. (B) After 36 days of treatment, mice were killed, and tumors and uteri were removed and weighed. Values are mean±S.E. *P<0.05, **P<0.001 compared to tamoxifen treatment. Taken with permission from Lu et al. (1998), published by Kluwer. *P<0.05, **P<0.001 compared to tamoxifen treatment.
Aromatase inhibitors and antiestrogens in the tumor model

In the control mice, all tumors continued to increase in volume throughout the course of the experiments. Both the aromatase inhibitors and the antiestrogens were effective treatments and reduced the extent of tumor growth (Lu et al. 1998). Dose-response effects were evident with the antiestrogens. Tamoxifen at 60 µg/day almost completely suppressed tumor growth, whereas 3 µg/day reduced tumor growth to 60% of control. The effect of faslodex (ICI 182780) (70 µg and 700 µg/day) is shown in Fig. 1 (Lu et al. 1998). The aromatase inhibitor, letrozole (10 µg/day) was more potent in reducing tumor growth than the pure antiestrogen, ICI 182780 (70 µg/week) (Fig. 1) (Lu et al. 1999). Arimidex (anastrozole; 5 µg/day), in contrast with tamoxifen (3 µg/day), caused significant inhibition of tumor growth compared with tumor growth in the controls ($P<0.05$) (Fig. 2) (Lu et al. 1998). Letrozole (10 µg/day) has been found to be more potent than tamoxifen (60 µg/day) and ICI 182780 (5 mg/week), although both ICI 182780 and letrozole showed regression of established tumors (Lu et al. 1998). Letrozole (5 µg/day) was also able to cause marked regression of large tumors (Fig. 3). Treatment with letrozole (5 µg/day) resulted in regression of tumor growth for up to approximately 15 weeks of continuous treatment. Thereafter, the tumors gradually resumed growth and almost reached their initial volume by 19 weeks of treatment. This suggests that resistance to the treatment may have developed.

The MCF-7CA tumors in the mouse model synthesize sufficient amounts of estrogens to support estrogen-dependent tumor growth and also to maintain the uterus of these ovariectomized animals at a weight similar to that of intact mice during metestrus. The uterine weights of mice treated with tamoxifen were not significantly different from those of the control mice, consistent with previous findings of specific effects reported for the agonist/antagonist actions of tamoxifen (Jordan et al. 1978). In contrast, ICI 182780, considered to be a pure antiestrogen, blocked the actions of estrogen on the uterus, suggesting a difference in the sensitivity of the two antiestrogens with regard to their effects on the tumor and the uterus. The two aromatase inhibitors also caused a decrease in the mean uterine weight compared with that of the control mice ($P<0.01$).

As both antiestrogens and aromatase inhibitors are effective in treating breast cancer patients, combining these agents with these different modes of action might result in greater antitumor efficacy than either alone. We used the intratumoral aromatase model to investigate this question. In these experiments, we used low doses of the compounds, which resulted in partial tumor suppression, in order to determine whether greater reduction in tumor growth could be achieved by combining the two types of agent. Based on our previous studies and those above (Fig. 1), which determined that 10 µg/mouse per day letrozole caused almost complete regression of tumors, a dose of 5 µg/day letrozole was used in the combined treatments. This was compared with the same dose of 300

\[ \text{% Change in Total Tumor Volume} \]

\[ \begin{align*}
\text{Androstenedione (0.1 mg/day)} & \quad \text{Letrozole (5 µg/day)} \\
\text{Letrozole (5 µg/day)} & \quad \text{Letrozole (5 µg/day)}
\end{align*} \]

Figure 3 Effect of letrozole on tumor growth in the intratumoral aromatase mouse model. Letrozole (5 µg/mouse per day) was injected s.c. for 16 weeks (●). Mice were injected s.c. with vehicle from day 1, for 7 weeks until the tumors reached a large volume. Letrozole (5 µg/mouse per day) injections s.c. began on week 7 (■).
Arimidex and 3 µg/day tamoxifen. All compounds alone, or in combination at these doses, were effective in suppressing tumor growth in comparison with that of the control mice (Fig. 4). Weights of tumors removed at the end of treatment were significantly reduced by treatment with the aromatase inhibitors, letrozole and Arimidex (anastrozole), compared with tamoxifen (Lu et al. 1999). Treatment with either Arimidex or letrozole together with tamoxifen did not produce greater reductions in tumor growth, as measured by tumor weight, than either aromatase inhibitor treatment alone. Estrogen concentrations measured in tumor tissue of the letrozole-treated mice were markedly reduced, from 460 pg/mg tissue to 20 pg/mg tissue. The combination of aromatase inhibitor and tamoxifen tended to be less effective than either aromatase inhibitor alone, but this was not statistically significant for either letrozole or anastrozole (Lu et al. 1999). We have previously observed a similar result in this model, when 4-hydroxyandrostenedione was used in combination with tamoxifen to treat the mice (Yue et al. 1995). When ICI 182780 was combined with tamoxifen, the mean tumor weight was also slightly greater than after treatment with ICI 182780 alone (Lu et al. 1999). Taken together, these results suggest that tamoxifen may have a partial agonistic action on the tumors, which overrides the reduction in estrogen concentrations caused by the aromatase inhibitors and which counteracts the effect of the pure antiestrogen at the low dose used. This effect was also evident on the uterus, as treatment with the combination of tamoxifen and ICI 182780 was less effective in reducing uterine weight than the pure antiestrogen alone (P<0.05).

In conclusion, our findings indicate that combining aromatase inhibitors with antiestrogens does not result in any additional antitumor efficacy over the use of these agents alone. Letrozole is the most potent agent of those studied in the mouse model. The studies reveal the agonist effects of tamoxifen on the tumors, particularly when estrogen levels are reduced. Although these agents are used in higher doses and may have different pharmacokinetic properties in patients, the results do not suggest any benefit in combining aromatase inhibitors with antiestrogens. Rather, their sequential use, as currently applied, is likely to be more advantageous for treating breast cancer patients by extending effective treatment with agents that act by different mechanisms.

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