Antiestrogenic Action of Toremifene on Hormone-dependent, -independent, and Heterogeneous Breast Tumor Growth in the Athymic Mouse

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ABSTRACT

The antiestrogen toremifene has been used to study the growth control of hormone-dependent (MCF-7), -independent (MDA-MB-231), or mixed tumor cell populations in athymic mice. Maximal MCF-7 tumor growth was produced in ovariectomized athymic mice by circulating estradiol levels of approximately 200 pg/ml (produced by 0.5-cm silastic capsules implanted s.c.). The antiestrogen toremifene (77 ± 4 mg/day from a 2-cm silastic capsule) inhibited estradiol (0.5-cm capsule)-stimulated growth by more than 70%. No tumor growth was observed in mice treated with toremifene alone, although toremifene acted as a weak partial agonist on the mouse uterus.

The growth of hormone-independent MDA-MB-231 breast tumors implanted in athymic mice was not influenced by either estradiol (0.5-cm capsule) or toremifene (2-cm capsule) when administered alone or in combination. Furthermore, even very large doses of toremifene (5 mg/day p.o.) did not alter the rate of MDA-MB-231 tumor growth.

Mixtures of MCF-7 and MDA-MB-231 cells in 9:1 and 99:1 ratios inoculated into athymic mice produced tumors which grew in the absence of estradiol but responded to estradiol supplementation (0.5-cm capsule) with a more rapid rate of tumor growth. Tumors grown from inoculated MCF-7:MDA-MB-231 cells (99:1 ratio) in the presence of estradiol had estrogen receptor levels of 33.2 ± 9.2 fmol/mg of protein at Day 44 compared to 84.8 ± 4.8 fmol/mg of protein in pure MCF-7 tumors. Toremifene (2-cm capsule) treatment inhibited the estrogen stimulation of these mixed tumors (99:1 starting ratio) to that of toremifene alone. However, toremifene-alone treatment produced a more rapid rate of tumor growth than control or tumors grown from irradiated MCF-7 cells mixed with viable MDA-MB-231 cells. Increasing the ratio of MCF-7:MDA-MB-231 cells (999:1) initially inoculated resulted in tumors which developed less rapidly than the lower ratio (99:1). Toremifene (2-cm capsule) again produced partial inhibition of 17β-estradiol-stimulated tumor growth while increasing tumor growth above control when the antiestrogen was administered alone.

These results demonstrate that toremifene is effective in inhibiting estrogen stimulation of hormone-dependent tumors and partially successful at controlling mixed hormone-dependent/independent tumors; however, the antiestrogen cannot control the growth of a hormone-independent tumor in this model.

INTRODUCTION

The hormone receptor content of breast tumor biopsies is currently used as a predictive marker; the presence of both ER and PgR indicates about a 77% chance of a tumor responding to endocrine therapy, whereas a tumor containing neither receptor type has only about an 11% chance of a response to similar therapy (1). The antiestrogen, tamoxifen, has now become the endocrine therapy of choice in the postmenopausal breast cancer patient. Tamoxifen therapy produces objective remission in about 30% of unselected cases (2). However, even in cases where tumor remission is initially observed, therapy ultimately fails.

Immunocytochemical techniques with monoclonal antibodies to the ER and PgR have been used to stain receptor-containing cells in frozen breast tumor sections. These studies indicate that a heterogeneous distribution of both ER (3, 4) and PgR-containing cells (5) is a frequent occurrence and support the findings that repeat microsamples from the same tumor can contain markedly differing receptor levels (6). An antiestrogen would be expected to control the ER-positive cell population. However, if the drug cannot exert control over the ER-negative cells, this population might be expected to continue to proliferate so that the antiestrogen would eventually fail. The timing of this failure would depend upon the relative proportion of cell types. The control of both hormone-dependent and -independent tumor cells in heterogeneous breast tumors would be a major advantage in breast cancer therapy. Recently, a new antiestrogen, toremifene, has been developed and is currently being studied in clinical trials (7). Interestingly, toremifene has been reported to have some activity against a hormone-independent mouse uterine sarcoma (8).

Recent laboratory studies suggest that ER-positive breast cancer may be capable of influencing the proliferation of ER-negative breast cancer cells (9, 10). Antiestrogens can cause an increased secretion of transforming growth factor β from ER-positive cells (9), and this factor is inhibitory to the proliferation of the ER-negative breast cancer cell line, MDA-MB-231.

The aim of this study is to examine the activity of toremifene on hormone-dependent, -independent, and mixed tumors using breast cancer cell lines grown into solid tumors in the athymic mouse (11–13).

MATERIALS AND METHODS

Tumors. MCF-7 cells (American Type Culture Collection passage 144) were karyotyped and maintained in minimal essential medium (Gibco, Grand Island, NY) with phenol red, containing nonessential amino acids, 10 mM, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, insulin (6 ng/ml), penicillin (100 units/ml), and 10% complete calf serum (Gibco, Grand Island, NY). MDA-MB-231 cells (American Type Culture Collection passage 25) were maintained in the same medium with 5% calf serum that was charcoal stripped.

Cells were grown in 150- x 25-mm dishes (Falcon, Cockeysville, MD), harvested at log phase with a disposable cell scraper (Costar, Cambridge, MA), and diluted in serum-free medium. When required, cell mixtures were made, and 10^7 cells in 0.1 ml of medium were inoculated into the axillary mammary fat pads of 4- to 5-wk-old ovariectomized BALB/c athymic mice (Harlan Sprague-Dawley, Indianapolis, IN). Transplantation procedures for solid tumors, animal housing conditions, and tumor size calculations were all as previously described (11).

Drug Administration. Estradiol administration was by silastic capsules implanted s.c. on the back of the animals using a trochar. Toremifene was administered either in a similar manner or, when very large doses were required, by intubation (p.o.) of a crystalline suspension in 0.1 ml of peanut oil.

Silastic capsules were formed by plugging one end of lengths of medical grade silastic tubing (0.078 in inner diameter by 0.125 in outer
RESULTS

The sensitivity of MCF-7 tumor pieces implanted in the athymic mouse to 17β-estradiol-stimulated growth was examined using different sized 17β-estradiol capsules (Fig. 1). The capsules produced a constant release of 17β-estradiol in the tumor-bearing mice (Table 1). The smallest capsule capable of producing maximal tumor growth was 0.5 cm in length and produced circulating levels of approximately 200 pg/ml of 17β-estradiol. 

ER tended to be higher in tumors grown with lower 17β-estradiol levels (Table 1).

To examine the antitumor action of toremifene in hormone-dependent MCF-7 tumors, the 0.5-cm 17β-estradiol capsule was used to stimulate tumor growth. These capsules released an average (±SEM) 4 ± 0.4 μg/day of 17β-estradiol. Toremifene produced more than a 70% reduction in the 17β-estradiol-stimulated growth regardless of whether 0.5-, 1-, or 2-cm toremifene capsules were used [silastic capsules released on average (±SEM) 28 ± 0.2 μg, 45 ± 1.0 μg, or 77 ± 4.0 μg/day over the treatment period, respectively]. However, even with 77 μg/day of toremifene, the tumor growth was not inhibited to control or toremifene-treated alone (Fig. 2).

Long-term (27 days) treatment of athymic mice with toremifene silastic capsules (2 cm) produced some uterine stimulation. However, a marked inhibition of 17β-estradiol (0.5-cm silastic capsule)-stimulated uterine weight was produced by toremifene (Fig. 3).

The influence of estrogen and toremifene on the hormone receptor-negative breast cancer cell line, MDA-MB-231 (15), was determined using the 0.5-cm 17β-estradiol capsule and 2-
cm toremifene capsule. The growth rate of MDA-MB-231 tumors was not changed by 17β-estradiol or toremifene either alone or in combination (Fig. 4). Furthermore, the p.o. administration of 5 mg of toremifene per day for 10 days did not change the rate of MDA-MB-231 tumor growth compared to controls (Fig. 5). This large dose of toremifene did, however, produce a loss in body weight and 50% fatality.

Having established the influence of estrogen and toremifene on MCF-7 and MDA-MB-231 cells when grown alone as solid tumors, the effect of these agents was examined on tumors grown from mixtures of these cell lines.

Mixing MCF-7 cells with MDA-MB-231 cells in 9:1 and 99:1 ratios and inoculating 10 million cells produced tumors which grew in the absence of 17β-estradiol but responded to 17β-estradiol (0.5-cm capsule) with a more rapid rate of growth (Fig. 6). When 1 million MDA-MB-231 cells were inoculated alone, tumor developed less rapidly than the mixed inoculates containing the same number of MDA-MB-231 cells (9:1 ratio). ER were not detected in MDA-MB-231 tumors, whereas MCF-7 tumors contained on average 84.8 ± 4.8 fmol/mg of protein after 44 days of growth. At 44 days, little ER was present in mixed tumors not treated with estrogen or tumors starting with a 9:1 ratio of MCF-7 cells to MDA-MB-231 and supplemented with 17β-estradiol. However, tumors grown in the presence of 17β-estradiol for 44 days from a 99:1 ratio of MCF-7:MDA-MB-231 cells were still ER positive with more than a third of the receptor content measured in pure MCF-7 tumors (Fig. 6). On the basis of the tumor responses and ER content, a tumor grown from mixtures of cell (total of 10 x 10^6 cells) were inoculated into athymic mice with or without estradiol supplementation (0.5-cm capsule). After 44 days tumors were dissected, and ER levels were measured by ER-EIA (see "Materials and Methods"). As a control, 1 x 10^6 MDA-MB-231 cells alone were inoculated. At 44 days, insufficient tumor was available for ER-EIA, so continued growth was followed and ER measured at Day 70.
growth than control (Fig. 8). The combination of irradiated MCF-7 (10,000 rads) cells with viable MDA-MB-231 cells at 99:1 ratio produced a tumor growth pattern very similar to control. This suggests the control tumor is resulting from predominantly MDA-MB-231 cell growth. Increasing further the ratio of MCF-7:MDA-MB-231 cells (999:1) initially inoculated resulted in tumors which took approximately 10 to 15 days longer to reach a size comparable to tumors inoculated at a 99:1 cell ratio. This difference was seen whether 17β-estradiol-treated or control tumors were compared (compare Figs. 8 and 9). Toremifene (2-cm capsule) produced a marked inhibition of the 17β-estradiol-stimulated portion of the growth of these tumors which started with 999 hormone-dependent cells to every hormone-independent cell. However, even with this large starting ratio of MCF-7:MDA-MB-231 cells, toremifene still stimulated tumor growth above control when administered alone (see Fig. 9). The combination of irradiated (10,000 rads) MCF-7 cells with viable MDA-MB-231 cells at a 999:1 ratio resulted in a long period to tumor development. However, as in the study with 99:1 ratio of MCF-7:MDA-MB-231 cells, the growth of tumors from these cells is similar although slightly slower than control.

**DISCUSSION**

The growth of MCF-7 tumors in the athymic mouse has previously been demonstrated to require estrogen stimulation (11–13). Our present findings indicate that maximal MCF-7 tumor growth is produced by circulating levels of 17β-estradiol in the athymic mouse comparable to the levels observed in the premenopausal woman (14) and suggest that estrogen levels in the postmenopausal woman (29 to 60 pg/ml of estrone, 10 to 21 pg/ml of 17β-estradiol; Ref. 14) are sufficient to cause partial stimulation. The trend of an increased ER content of MCF-7 tumors grown with low circulating levels of 17β-estradiol is consistent with findings in culture where estrogen deprivation results in a marked rise in the ER content of MCF-7 cells (15, 16).

The present finding that toremifene can inhibit the growth of MCF-7 tumors *in vivo* is in keeping with the report that toremifene is inhibitory to this cell line in culture (8) and is consistent with the antiestrogenic action reported for this agent (8, 17). Inhibition of MCF-7 and other estrogen-dependent breast tumors has been described for tamoxifen (11–13) and metabolites (18). Tamoxifen has been reported to be ineffective in preventing the growth of hormone-independent tumors such as MDA-MB-231 (13, 18) or T60 (19) in the athymic mouse. Our findings for toremifene on the MDA-MB-231 tumor are consistent with the hypothesis that antiestrogens are ineffective in preventing the growth of hormone-independent tumors. The lack of antitumor action of massive doses of toremifene in the MDA-MB-231 tumor contrasts with the reported reduced growth rate produced by similar toremifene treatment of a mouse uterine sarcoma shown to be estrogen receptor negative (8). This difference may, however, be related to the difference in tumor type or species origin.

As described in the “Introduction,” many breast tumors contain both hormone-dependent and hormone-independent elements. Our studies on MCF-7:MDA-MB-231 mixed tumors demonstrate, when sufficient hormone-dependent cells are present, that estrogen can stimulate heterogeneous tumors to grow more rapidly [i.e., inoculated cells in the ratio of 9:1, 99:1, or 999:1 MCF-7:MDA-MB-231 were stimulated by 17β-estradiol, whereas the transplanted initially heterogeneous tumor (ultimately ER negative) was not]. The inhibitory action of toremifene on estrogen-stimulated growth may be analogous to the inhibition of endogenous and environmental estrogens by antiestrogens in the postmenopausal breast cancer patient. Since only partial inhibition of the estrogen-stimulated effect was produced by toremifene, this may be a consequence of incomplete suppression of 17β-estradiol-stimulated MCF-7 growth as seen in pure MCF-7 tumors. However, the increased rate of tumor growth produced by toremifene alone over control in heterogeneous tumors is inconsistent with a partial inhibition of estrogen stimulation alone. Tamoxifen has estrogenic actions in the mouse (20) and has been reported to stimulate the growth of the EnCa101 endometrial tumor (11, 21) when implanted in this species. Similarly, toremifene has an estrogen-like action on the normal mouse uterus (17), and we have demonstrated a partial agonist action on the uterus of the athymic mouse uterus. The stimulatory action of toremifene in the heterogeneous tumor may be related to these effects. Whether this represents partial stimulation of MCF-7 cells by toremifene, as observed for tamoxifen in culture (22), or a host-mediated action such as natural killer cell suppression (23) is unclear. There have been some reports that antiestrogens are ineffective in preventing growth factor-stimulated T47D (24) and MCF-7 (25, 26) cell
growth in culture. If toremifene promotes increased growth factor production in the mammary fat pads of the athymic mouse, then this may influence the growth of both the implanted hormone-dependent and independent breast cancer cells.

The principle of inoculating the same number of cells when making comparisons of tumor growth is obviously important. This is seen in the present study where inoculated MDA-MB-231 cells alone grew into tumors less rapidly than when mixed with viable MCF-7 cells. The mixtures of MDA-MB-231 cells with irradiated MCF-7 cells formed tumor at a similar rate to mixtures with viable MCF-7 tumors. Clearly the MCF-7 cells act as either a “feeder layer” or buffer the MDA-MB-231 cells against the host immune system.

The increased time to tumor development observed when the ratio of MCF-7:MDA-MB-231 cells was increased (and consequently the number of MDA-MB-231 cells in each inoculation reduced) is consistent with the MDA-MB-231 tumor cells being the predominant factor in mixed tumor growth. The dominance of the hormone-independent cells was indicated by the ER differences seen between pure MCF-7 tumors and heterogeneous tumors when measured at 44 days by ER-EIA. Since the completion of these studies, a report using DNA flow cytometry has indicated that the MDA-MB-231 cell is the predominant cell type at 3 wk after inoculation of either a 2:1 or 10:1 mixture of MCF-7:MDA-MB-231 cells into the athymic mouse. MDA-MB-231 cells are not promoting MCF-7 cell growth through a paracrine mechanism, and MDA-MB-231 cells do not substantially stimulate MCF-7 cell growth (27).

The present study now indicates that the control of hormone-independent elements of heterogeneous tumors by hormone-dependent cells is not achieved in this model even with a 999:1 ratio of MCF-7:MDA-MB-231 cells, as untreated or antiestrogen-treated heterogeneous tumors grew at a similar rate or more rapidly than tumors grown from inactivated (by radiation) MCF-7 cells mixed with viable MDA-MB-231 cells. This is in contrast to findings made in culture at a similar ratio (9) but consistent with clinical findings that tamoxifen therapy cannot control the growth of all receptor-positive tumors, and even when this is achieved, the therapy ultimately fails (25). Nevertheless, questions regarding the suitability of the athymic mouse to study the therapy of breast cancer must be considered, and the limitations of the model need to be further characterized and defined.

REFERENCES

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