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Effect of Estrogens and Antiestrogens on Growth of Human Breast Cancer Cells in Athymic Nude Mice[1]

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ABSTRACT

Endocrine therapy with estrogen deprivation or with antiestrogens results in tumor regression in a subset of patients with advanced breast cancer. To better understand the mechanisms by which estrogens and antiestrogens modulate breast cancer growth *in vivo*, we have studied the effects of endocrine manipulation on the development and growth of tumors derived from cultured human breast cancer cells in the athymic nude mouse. MCF-7 breast cancer cells were inoculated into 6-week-old female BALB/c athymic nude mice. Tumor growth did not occur in ovariectomized mice. Cells remained viable, however, since estrogen supplementation more than 30 days later resulted in tumor formation. Minimal tumor growth was observed in intact female nude mice which have low circulating estrogen levels. Tumor development and growth in ovariectomized or intact mice supplemented with 17β-estradiol in the form of a s.c. pellet were dose dependent; growth rates increased with estrogen doses ranging from 0.01 to 0.5 mg. Antiestrogen treatment with either tamoxifen or LY156758 caused transient stimulation of tumor growth, followed by a prolonged stationary phase. Growth resumed with estrogen supplementation. Treatment of mice bearing established MCF-7 tumors with estrogen withdrawal (removal of estrogen pellet) resulted in cessation of tumor growth, but not in tumor regression. Growth inhibition was also observed with antiestrogens and was dose dependent. However, tumor regression did not occur, even in mice treated with high doses of tamoxifen (serum concentration of 1.0 μM) for as long as 60 days. Tumor growth was restored in these mice with estrogen replenishment. Tumor cells also remained viable histologically despite prolonged (1 month) estrogen deprivation or antiestrogen therapy, although the mitotic index was markedly reduced. Similar observations were made with mice inoculated with the hormone-responsive ZR75-1 human breast cancer cells, but not with hormone-independent MDA-231 cells which were not influenced by estrogen or antiestrogen treatment. In summary, development and growth of MCF-7 and ZR75-1 tumors in nude mice are estrogen dependent. Endocrine therapy by estrogen deprivation or antiestrogen treatment inhibits tumor cell proliferation in nude mice, but does not cause tumor regression or loss of cell viability.

INTRODUCTION

The mechanisms by which steroid hormones regulate growth of human breast cancer have not been totally defined. Most *in vitro* studies have suggested that both estrogens and estrogen antagonists have a direct effect on proliferation of human breast cancer cell lines which contain estrogen receptors. Antiestrogens inhibit a variety of key metabolic pathways, and ultimately, they slow the rate of cell proliferation (4, 6, 11). Although a consistent stimulatory effect of estrogens on cultured human breast cancer cells has been difficult to demonstrate, estrogens consistently restored growth of cells which have been inhibited by antiestrogens (12, 18).

In vitro studies have also focused on the effects of antiestrogens and estrogens on the cell cycle kinetics of cultured human breast cancer cells (14, 15, 25, 26). At serum concentrations of the antiestrogen tamoxifen achieved in breast cancer patients (about 1 μM), cultured human breast cancer cells accumulate in the early to mid-G_1 phase of the cell cycle. This G_1 transition delay can be reversed by the addition of 17β-estradiol (15). Although extremely high concentrations of tamoxifen (10 μM) are clearly cytocidal for cells (25), these data suggest that a predominant effect of antiestrogen therapy may be to slow proliferation by blocking the transit of cells through the cell cycle. These studies have important therapeutic implications in view of the widespread use of combined therapy with tamoxifen and cytotoxic drugs. Theoretically, tamoxifen could alter cell cycle kinetics and thereby either enhance or inhibit the cytocidal effects, depending on the drugs used in combination.

There is a paucity of detailed studies examining the effect of steroid hormones on human breast cancer growth using *in vivo* model systems. Such a system might be better suited for studies of the mechanisms of hormone-induced tumor regression and for studies of the potential interaction between hormonal agents and cytotoxic drugs, since drug pharmacokinetics and metabolism might be more closely related to those observed in patients. Recently, several investigators have shown that cultured human breast cancer cells can be easily propagated as s.c. tumors in the immune-deficient athymic nude mouse (17, 21–24, 27). In the present study, we have used this model system to study the effects of estrogen and antiestrogen on the development and growth of tumors derived from inoculation of human breast cancer cells. We have found that growth of ER[3]-positive tumors is estrogen dependent. However, estrogen deprivation or antiestrogen treatment results only in cessation of tumor growth, but not in tumor regression or loss of cell viability. The data further support the hypothesis that a major *in vivo* effect of antiestrogen therapy is to inhibit cell proliferation rather than to directly kill the cell.

MATERIALS AND METHODS

Breast Cancer Cells. The characteristics and methods of culturing the MCF-7, ZR75-1, and MDA-231 human breast cancer cell lines have

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[3] The abbreviations used are: ER, estrogen receptor; PGR, progesterone receptor.

been reported previously (16). The MCF-7 cell line used in these experiments was obtained from Dr. Samir Shafie at the National Cancer Institute. All cell lines were routinely screened for *Mycoplasma* contamination. Cell lines were maintained in 75- or 150-sq cm plastic culture flasks in Richter's improved Eagle's minimal essential medium-ZO (Grand Island Biological Co., Grand Island, NY) supplemented with glutamine (0.6 g/liter), penicillin (62 mg/liter), streptomycin (130 mg/liter), and 10% fetal bovine serum (MDA-231 cells) or 5% calf serum with 1.0 nM insulin (MCF-7 and ZR75-1 cells). Cells were subcultured every 5 to 7 days by suspension in 1 mM EDTA in Dulbecco's phosphate-buffered saline.

Animals. Four- to 6-week-old female intact or ovariectomized, homozygous inbred BALB/c-*nu*+/*nu*+ athymic mice were purchased from Harlan Sprague-Dawley (Madison, WI). Bilateral ovariectomy was performed at 3 weeks of age. Mice were housed in filtered laminar air flow hoods in standard vinyl cages with air filter tops. Cages, bedding, and food were autoclaved before use. Water was autoclaved, acidified to pH 2.8, and provided *ad libitum*.

Cell Inoculation and Hormone Treatments. Logarithmically growing cells were harvested by scraping in complete culture medium. Five × 10^6 cells in 0.2 ml of culture medium were inoculated s.c. on the flank immediately caudal to the axilla through a 22-gauge needle tunneled 1 to 2 cm to prevent leakage of cell inoculum.

Mice were supplemented with 17β-estradiol in the form of slow-release s.c. pellets purchased from Innovative Research (Rockville, MD). The pellets were fused and compressed individually with filler material including cholesterol, microcrystalline cellulose, α-lactose, di- and tricalcium phosphate, calcium and magnesium stearate, and stearic acid. The pellet was placed with the aid of a 14-gauge needle in the interscapular region. Pellets could be easily removed by puncturing the skin with a No. 11 scalpel blade and retrieving the pellet with a forceps. Progesterone was also given in the form of a s.c. pellet. Tamoxifen citrate was generously provided by Stuart Pharmaceuticals (Wilmington, DE). The antiestrogen LY156758 was the gift of Lilly Research Laboratories (Indianapolis, IN). Mice were treated with antiestrogens by s.c. injection of the drug in peanut oil (0.05 ml). Controls received peanut oil alone. In some experiments, tamoxifen (5 mg/mouse) was given in the form of a s.c. pellet purchased from Innovative Research (Rockville, MD).

Tumor Measurement. Tumor diameters were measured at least twice weekly, and tumor volume in cumm was calculated by the formula

$$\text{Tumor vol} = \frac{(\text{width})^2 \times \text{length}}{2}$$

Hormone Receptor Assays. Tumor ERs were measured by the dextran-coated charcoal assay described previously (13). PGR was measured by calculating the 8S fraction of [^{3}H]R5020 binding on sucrose density gradients (19).

Histology of Tumors. Mice were sacrificed by ether anesthesia, and tumors were dissected free from s.c. tissues and fixed in 10% formalin. Paraffin-embedded tumors were processed for histological determination and stained with hematoxylin-eosin.

The mitotic index was measured by counting a total of 2000 cells/tumor (×40 objective) and determining the fraction of mitotic cells. Data were expressed as number of mitoses/1000 cells.

Assay for Tamoxifen and Metabolites. Serum samples were assayed for tamoxifen by the high-pressure liquid chromatography method described in detail previously (3).

Statistical Methods. Measurements made on a continuous scale (tumor diameter, tumor volume, uterine weight, steroid hormone receptor levels) were analyzed by analysis of variance followed by application of the Newman-Keuls multiple-comparison procedure (28). Receptor values were expressed as logarithms before analyses were performed. Categorical data (number of mice developing tumors, mitotic rate) were analyzed by χ^2 tests or Fisher's exact tests, depending on the magnitude of the expected frequencies (7). All statistical tests were performed at the 5% level of significance.

RESULTS

Effect of Hormones on Tumor Development. The effect of estrogen and progesterone on the development of MCF-7 cell tumors in nude mice is shown in Table 1. Four weeks after cell inoculation, less than 50% of intact mice and only 10% of ovariectomized mice had tumors greater than 3 mm in diameter. Frequently, a small palpable nongrowing nodule would persist at the injection site in these groups, but growth did not occur. Nearly all intact or ovariectomized mice supplemented with estrogen developed large tumors approaching 8 to 10 mm in diameter at 1 month. The addition of progesterone to estrogen had no significant effect on tumor development or size, and progesterone supplementation was not used in subsequent experiments.

Since intact athymic mice of this age have low circulating estradiol concentrations (21, 24), and since these mice are unable to optimally support the growth of MCF-7 tumors, we next asked whether tumor development was dependent upon the dose of estradiol administered (Chart 1). Mice were inoculated with MCF-7 cells and divided into 5 groups. Tumor growth was not observed in ovariectomized mice. Progressive tumor growth was

Table 1

Effect of estrogen and progesterone on tumor development

Four- to 6-week-old nude mice were inoculated with 5 × 10^6 MCF-7 cells. Intact mice or mice ovariectomized at 3 weeks of age were used. Some mice were supplemented with 17β-estradiol (0.25 mg/pellet) or progesterone (0.5 mg/pellet) 1 day before cell inoculation. The number of mice developing growing tumors >3 mm in diameter was recorded 4 weeks after inoculation. Each group contained 20 mice.

Group	% with tumors	Tumor diameter (mm)
Intact	45[a]	3.2 ± 0.5[b]
Ovariectomized	10	2.4 ± 0.2
Intact + estradiol	90[a, c]	7.4 ± 0.6[a, c]
Ovariectomized + estradiol	95[a, c]	9.0 ± 0.6[a, c]
Intact + estradiol + progesterone	100[a, c]	9.7 ± 0.5[a, c]
Ovariectomized + estradiol + progesterone	90[a, c]	8.8 ± 0.7[a, c]

[a] Significantly different from the ovariectomized group.
[b] Mean ± S.E.
[c] Significantly different from intact group.

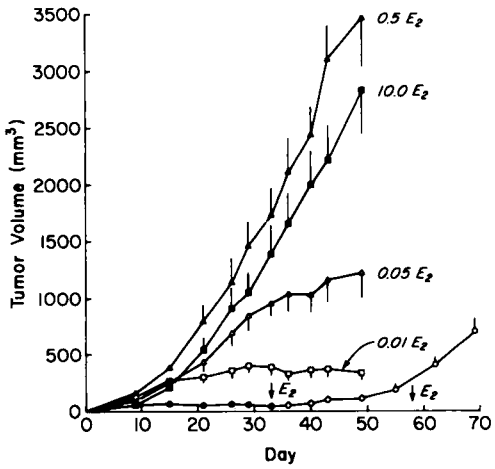

Chart 1. Effect of estrogen dose on growth of MCF-7 tumors in nude mice. Ovariectomized mice (●) or ovariectomized mice supplemented with 0.01-mg (□), 0.05-mg (△), 0.5-mg (▲), or 10.0-mg (■) estradiol (E_2) pellets were inoculated with MCF-7 cells on Day 0. Tumor volume was measured on the days shown. On Days 33 and 58 (*arrows*), ovariectomized mice only were supplemented with an estrogen pellet (0.5 mg, ○). *Points*, mean of 8 mice; *bars*, S.E.

noted as the dose of estradiol was increased from 0.01 mg/pellet to 0.5 mg/pellet. By Day 50, tumor volume had reached 3500 cu mm in mice supplemented with 0.5-mg estradiol pellets. Supplementation of mice with a 10-mg pellet did not result in more rapid growth. When ovariectomized mice were replenished with estrogen in the form of a pellet (0.25 mg estradiol) placed on Day 33 and again on Day 58 after initial cell inoculation, rapid tumor growth ensued after a lag period of about 20 days.

The effect of antiestrogen treatment on tumor development is shown in Chart 2. Again in this experiment, sustained tumor growth did not occur in ovariectomized mice but did occur in mice supplemented with estrogen. Treatment with tamoxifen by a s.c. pellet or by daily injections or treatment with the antiestrogen LY156758 by injection consistently showed an initial stimulation of tumor growth above that seen in ovariectomized mice. A prolonged plateau phase was observed after the early stimulatory effect. When an estradiol pellet was placed in animals that had been given injections of tamoxifen for 30 days, tumor growth was restored, again after a lag period of 10 to 15 days.

Effect of Hormonal Manipulation on Growth of Established Tumors. The effect of antiestrogen dose on growth of established tumors is shown in Chart 3. Tumors were grown for 20 days in estrogen-supplemented mice. Daily s.c. injections of tamoxifen or LY156758 were then begun. Antiestrogen treatment antagonized the effect of estrogen in a dose-dependent manner. Tumors continued to grow in control mice (estrogen treated only), whereas growth slowed with the addition of antiestrogens. After 33 days, tumor volume in mice treated with 50 μg of antiestrogen per day was half that in control mice. Actual tumor regression was not observed at any antiestrogen dose.

To determine whether more prolonged antiestrogen treatment might result in tumor regression, another dose-response study was performed (Chart 4). MCF-7 cells were inoculated into estrogen-treated mice on Day 0. On Day 9, mice were divided randomly into 7 groups. Daily tamoxifen injections at doses ranging from 0.5 to 100 μg were given, and tumor volumes were

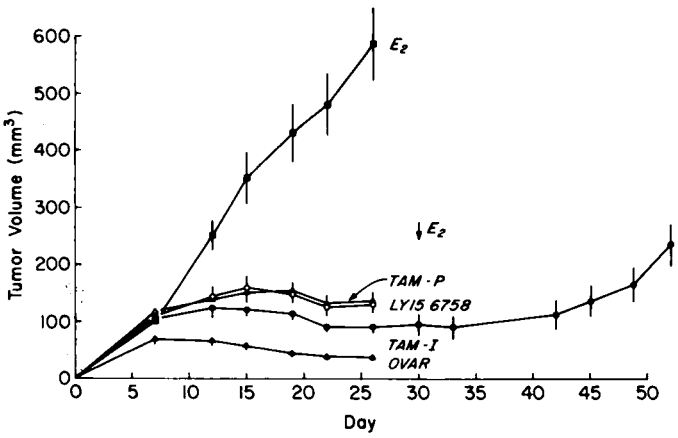

Chart 2. Effect of antiestrogen therapy on MCF-7 tumor development. Ovariectomized (*OVAR*) nude mice or ovariectomized nude mice supplemented with tamoxifen (25 μg/mouse) by daily injections in oil (*TAM-I*), with tamoxifen in the form of a 5-mg s.c. pellet (*TAM-P*), with LY156758 (25 μg/mouse) by daily injections, or with 0.25-mg estradiol (E_2) pellets were inoculated with MCF-7 cells on Day 0, and tumor volume was determined with time. On Day 30, 0.25-mg estrogen pellets were placed in mice treated with tamoxifen by injection, and the tamoxifen injections were discontinued. *Points*, mean of 13 mice; *bars*, S.E. By Day 12, tumors from estradiol, TAM-P, TAM-I, and LY156758 were all significantly larger than those in the ovariectomized group.

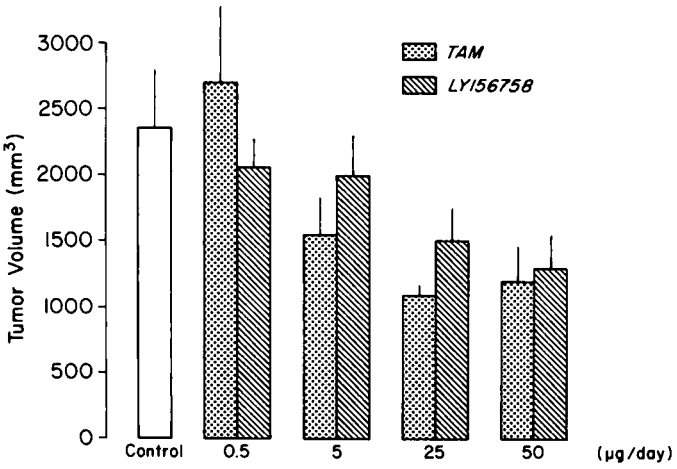

Chart 3. Effect of antiestrogens on established tumors. Estrogen-supplemented ovariectomized nude mice (0.25-mg pellet) were inoculated with MCF-7 cells, and tumor growth was measured. On Day 20, when tumor volume measured about 300 cu mm, mice were randomly assigned to a control group or to groups receiving increasing doses of tamoxifen (*TAM*) or LY156758 by daily injections in oil. Tumor volume was measured twice weekly. Data shown are tumor volumes measured after 33 days of antiestrogen therapy; *columns*, mean of 6 mice; *bars*, S.E.

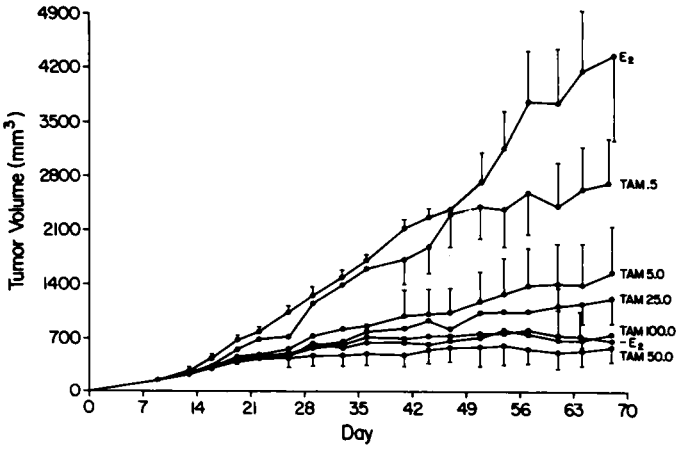

Chart 4. Effect of prolonged tamoxifen treatment on tumor volume. Estrogen-treated ovariectomized nude mice were inoculated with MCF-7 cells on Day 0. On Day 9, mice were randomly divided into 7 groups: E_2, 17β-estradiol (0.25-mg pellet); $-E_2$, removal of estrogen pellet; *TAM*, tamoxifen at the doses indicated (μg/day). Tumor volume was measured on the days shown. *Points*, mean of 7 mice/group; *bars*, S.E.

measured for 60 days. A dose-response effect was again evident with maximal inhibition of growth observed with 50 μg of tamoxifen per day. However, neither estrogen deprivation nor tamoxifen treatment resulted in actual reduction in tumor volume despite prolonged treatment. When these treated mice were again supplemented with estrogen, tumor volume increased after a lag time of 2 to 3 weeks (not shown).

We next asked whether combined endocrine therapy with estrogen deprivation and antiestrogens would result in tumor regression. The effect of estrogen withdrawal (removal of the estrogen pellet) on tumor growth, without or with antiestrogen therapy, is shown in Chart 5. On Day 0, ovariectomized mice supplemented with an estrogen pellet were inoculated with MCF-7 cells. On Day 7, when tumor volume was approximately 150 cu mm, mice were divided randomly into 5 groups. Tumors continued to grow rapidly in the estrogen-supplemented group;

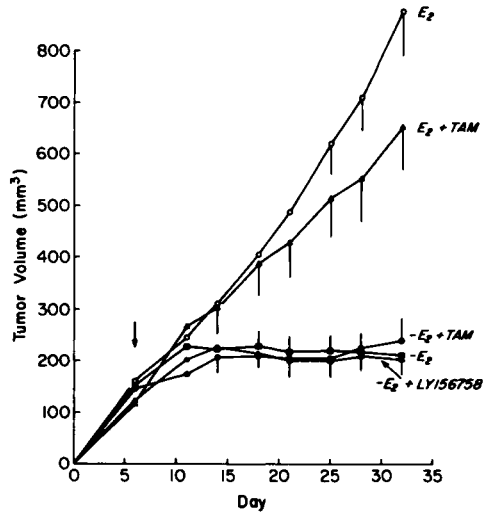

Chart 5. Effect of estrogen withdrawal and antiestrogen therapy on established tumors. Estrogen-treated, ovariectomized nude mice were inoculated with MCF-7 cells on Day 0. On Day 7, mice were randomly assigned to 5 groups: placement of a second estrogen pellet (E_2); placement of a second estrogen pellet plus daily 25-μg injections of tamoxifen (E_2 + TAM); removal of the estrogen pellet ($-E_2$); or removal of the estrogen pellet plus 25-μg injections of tamoxifen ($-E_2$ + *TAM*) or LY156758 ($-E_2$ + *LY156758*). *Points*, mean of 10 mice; *bars*, S.E.

Table 2

Effect of estrogen and tamoxifen on tumor mitotic rate

Histological sections were prepared as described in Chart 1. Slides were scanned (×40), and a total of 2000 cells was examined for each group.

Group	No. of mitoses/ 1000 cells
Estrogen supplemented	25
Estrogen + tamoxifen	19
Estrogen pellet removed	5[a]
Estrogen pellet removed + tamoxifen	4[a]

[a] Significantly different from estrogen supplemented ($p < 0.001$).

Table 3

Effects of estrogen and antiestrogen on tumor hormone receptors and mouse uterine growth

Mice with MCF-7 tumors were treated as described in Chart 5. After 40 days, tumors were harvested for cytoplasmic receptor status, and mouse uterine weights were obtained.

Group	Tumor receptor (fmol/mg protein)		Uterine wt (mg)
	ER	PGR	
Estrogen pellet removed	535 ± 63[a]	16 ± 8	18 ± 1.7
Estrogen supplemented	18 ± 6[b]	128 ± 30[b]	69 ± 6.9[b]
Estrogen supplemented + tamoxifen	10 ± 1[b]	229 ± 61[b]	59 ± 5.8[b]
Estrogen supplemented + LY156758	3 ± 2[b, c]	210 ± 14[b]	70 ± 7.3[b]
Estrogen pellet removed + tamoxifen	30 ± 7[b]	89 ± 32[b]	36 ± 2.5[b, c]
Estrogen pellet removed + LY156758	19 ± 6[b]	10 ± 3	21 ± 2.1

[a] Mean ± S.E. of 5 mice/group.
[b] Significantly different from estrogen pellet-removed group.
[c] Significantly different from all other groups.

by Day 35, mean tumor volume approached 900 cu mm. As in the previous experiment, the addition of tamoxifen to estrogen-supplemented mice antagonized the estrogen effect on tumor growth. In the other 3 groups, the estrogen pellet was removed, or the pellet was removed, and mice were given daily injections of tamoxifen or LY156758. By 1 week, tumor growth ceased in all 3 of these groups. Thereafter, the tumor remained static at an approximate volume of 200 cu mm.

The failure of endocrine therapy to cause tumor regression and the ability of estrogen replenishment to restore growth suggested that estrogen deprivation or antiestrogen therapy was not lethal to the majority of cells, but simply slowed or blocked cell proliferation. The viability of tumor cells after prolonged estrogen deprivation or antiestrogen therapy was suggested by 2 other studies. (*a*) When tumor cells from the groups of mice described in Chart 4 were dispersed and placed in monolayer tissue culture in medium containing 5% bovine serum, all proliferated rapidly (data not shown). (*b*) Histological sections of tumors from estrogen-deprived or antiestrogen-treated mice showed no tumor necrosis or other cytological evidence of cell death (not shown). Necrosis was observed only in large tumors from estrogen-supplemented mice. An increase in mouse supporting tissue elements was not apparent in any group. Tumor cells from both estrogen- and tamoxifen-treated mice were histologically intact and appeared viable, although the mitotic rate was significantly reduced by tamoxifen (Table 2). Removal of the estrogen pellet without or with tamoxifen resulted in a marked reduction in the number of mitoses.

To determine whether the dose of antiestrogen was sufficient to elicit other expected biological effects, we next examined the effects of estrogen and antiestrogen on MCF-7 tumor estrogen and progesterone receptor status, and on mouse uterine weight (Table 3). After 40 days of estrogen withdrawal, as expected, tumor cytoplasmic estrogen receptor was high, progesterone receptor was low, and mice had small uteri indicating estrogen deficiency. Estrogen supplementation resulted in depletion of tumor cytoplasmic estrogen receptor, increased progesterone receptor content, and large uteri. Antiestrogen therapy of estrogen-supplemented mice further depleted cytoplasmic ER and had no significant effect on the other parameters. Antiestrogen therapy with LY156758 in estrogen-deprived mice depleted tumor cytoplasmic estrogen receptor but had no stimulatory effect on progesterone receptor or uterine weight, perhaps because of its relatively pure antiestrogenic properties (2). However, treatment with tamoxifen, a partial estrogen agonist in the mouse, increased progesterone receptor content and uterine weight. Thus, the doses of antiestrogen used in these experiments were sufficient to induce the expected biological effects on tumor receptor content and mouse uterine growth. Furthermore, we also measured the concentration of tamoxifen in mouse serum. Concentrations of tamoxifen in mice receiving 25 μg/day for 30 days ranged from 162 to 434 ng/ml. These concentrations are similar to those observed in patients treated with the drug (5).

Effects of Hormones on *In Vivo* Growth of Other Breast Cancer Cell Lines. To determine whether the *in vivo* effects of estrogens and antiestrogens were restricted to MCF-7 cells, we also examined their effects on 2 other human breast cancer cell lines, the estrogen receptor-positive ZR75-1 cells and the receptor-negative MDA-231 (Charts 6 and 7). Like MCF-7 cells, ZR75-1 cells required estrogen supplementation for optimal growth in nude mide (Chart 6). In general, these tumors grew more rapidly and reached a larger volume than did MCF-7 tumors. Interestingly, significant initial tumor growth followed by a plateau phase was observed even in estrogen-deprived mice. Slightly larger tumors were observed in ovariectomized mice treated with the antiestrogen LY156758, compared to ovariectomized mice without or with tamoxifen. When mice with established ZR75-1 tumors were treated with antiestrogens or with estrogen deprivation by removal of the estrogen pellet, antagonism of estrogen

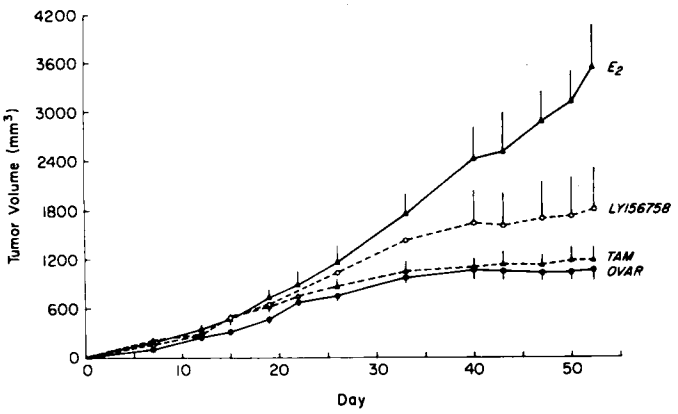

Chart 6. Effects of estrogen and antiestrogen on ZR75-1 tumor growth. ZR75-1 cells were inoculated into 4 groups of nude mice: ovariectomized (*OVAR*); ovariectomized plus 25 μg tamoxifen s.c. daily (*TAM*); ovariectomized plus 25 μg LY156758 s.c. daily (*LY156758*); or ovariectomized mice supplemented with a 0.25-mg 17β-estradiol pellet (E_2). *Points*, mean of 5 mice; *bars*, S.E.

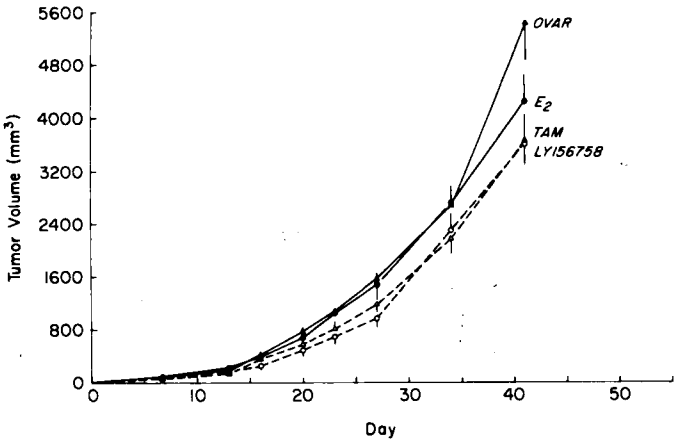

Chart 7. Effects of estrogen and antiestrogen on MDA-231 tumor growth. MDA-231 cells were inoculated into nude mice as described in Chart 6.

stimulation occurred, and tumor growth ceased. Like the MCF-7 cells, tumor regression did not occur even after 40 days of therapy (data not shown).

Quite different effects were observed with the MDA-231 cells (Chart 7). Tumor development in nude mice was not dependent on estrogen. In fact, tumors grew extremely rapidly even in ovariectomized animals. Treatment with either tamoxifen or LY156758 had no significant effect on tumor growth compared to estrogen-treated animals.

DISCUSSION

In this study, we have examined the effects of estrogens and antiestrogens on the development and growth in nude mice of tumors derived from cultured human breast cancer cells. We have shown that development of MCF-7 tumors requires estrogen, confirming other reports (21–24, 27). We have extended these observations and demonstrated clearly that the growth of MCF-7 tumors is dependent upon the dose of 17β-estradiol administered in the form of a s.c. pellet. The failure of intact 6-week-old female athymic nude mice to support optimal tumor growth is probably related to the low circulating estradiol concentrations in these mice, which are about 5-fold lower than in premenopausal women (21, 24). Progesterone supplementation in the dose used has no effect on the latency period, frequency of tumor development, or tumor growth rate. This result is similar to the work of Shafie and Grantham (23) but differs from that of Soule and McGrath (24), who found a shorter latency period for growth of a cloned MCF-7 subline (MCF-7ED) with progesterone supplementation in athymic Swiss mice. These differences may be related to the progesterone dose used. Soule's study used a 1.0-mg progesterone pellet compared to 0.2- and 0.5-mg pellets used by Shafie and us, respectively. These pellets result in physiological progesterone concentrations (23).

We also examined the effects of estrogens and antiestrogens on 2 other human breast cancer cell lines, the ZR75-1 and the MDA-231. The ER- and PGR-positive ZR75-1 cells are similar to the MCF-7 cells and require estrogen for optimal growth. As predicted, the receptor-negative MDA-231 cells, which do not respond to estrogen or antiestrogen *in vitro* (12), grow rapidly even in ovariectomized nude mice. These data suggest that the major growth effect of *in vivo* administration of estrogens and antiestrogens is a direct effect on cell proliferation mediated through the ER, rather than an indirect effect mediated by estrogen regulation of some other growth factor or "estromedin."

Antiestrogens have a biphasic effect on tumor formation following the inoculation of ovariectomized nude mice with MCF-7 cells. During the initial 7 to 10 days, stimulation of tumor growth is observed with both tamoxifen and LY156758. Thereafter, growth stops, and tumor volume remains constant. It could be argued that early stimulation with tamoxifen could somehow be related to its estrogenicity in the mouse. This seems unlikely since LY156758, an antiestrogen with no estrogenic activity in the mouse (2), also induces a transient growth-stimulatory effect. A more likely explanation is that these antiestrogens, after binding to ER and translocating it to the nucleus, can then induce a brief burst of estrogenic activity prior to the onset of the antiestrogen inhibitory effect. A similar mechanism may be operative in breast cancer patients, whose disease "flares" on beginning tamoxifen therapy.

An important observation in our study is that human breast cancer cells growing in the nude mouse remain viable for prolonged periods despite estrogen deprivation or antiestrogen treatment. MCF-7 and ZR75-1 cells remain viable for long periods even when inoculated into ovariectomized or antiestrogen-treated mice. Furthermore, established tumors do not regress when mice are treated with estrogen withdrawal or antiestrogen therapy; rather, tumor volumes remain constant for up to 60 days with such therapy. Continued viability of these cells is confirmed by several observations. (*a*) Cells derived from these tumors can be propagated in tissue culture, where they retain their *in vitro* sensitivity to estrogens and antiestrogens. (*b*) The cells are viable by histological criteria; stationary-phase tumors from treated mice show no necrosis or cytological evidence for cell death. (*c*) Most importantly, cell proliferation and tumor growth can be restored by supplementation with 17β-estradiol. The cessation of tumor growth in estrogen-deprived or antiestrogen-treated mice, the low mitotic index, and the ability of estrogen to restore growth suggest that regulation of cell cycle transit is an important mechanism by which these hormones control breast cancer growth. This conclusion is further supported by *in vitro* data showing that estrogens and antiestrogens regulate the

transit of cells through the G_1 phase of the cell cycle (14, 15, 25, 26). We cannot exclude the possibility that continued viability of these cells is due to low levels of estrogen present even in ovariectomized animals and that total estrogen lack, which cannot be achieved *in vivo*, might result in lethal cellular damage. It is also possible that changes in levels of other hormones such as growth hormone or prolactin induced by estrogen withdrawal or antiestrogen therapy might be sufficient to maintain cell viability. We also cannot exclude the possibility that some cells are in fact killed by the hormone treatment. However, our data suggest that the majority remain viable despite prolonged treatment.

The failure of estrogen withdrawal and/or antiestrogen treatment to induce tumor regression in our studies differs somewhat from results reported previously. Two groups reported a rapid reduction in MCF-7 tumor volume in nude mice treated by estrogen withdrawal plus tamoxifen (9, 23). One of them also reported that estrogen withdrawal alone resulted in transient stabilization followed by a resumption of tumor growth, suggesting that the tumor cells themselves may have been synthesizing sufficient estrogens to stimulate their own proliferation, or that an autonomous, estrogen-independent clone had developed (23). Another group using a slightly different technique in castrated male nude mice supplemented with estrogen found a lower rate of tumor development, a slower growth rate, and a gradual reduction in tumor volume with estrogen withdrawal (8). However, viable tumor cells with mitotic figures were still evident in regressing tumors, and regression eventually stopped and was followed by stable tumor volume despite 7 months of observation. Still another group reported only a minimal reduction in tumor volume, preservation of architecture histologically, and a low mitotic index in mice treated with estrogen withdrawal plus tamoxifen, data very similar to our own (20). The explanation for these discrepant results is not clear. It is possible that the results reflect differences in the cell lines used. Although the MCF-7 cells used in our studies were obtained from Shafie's laboratory just prior to experimentation, it is possible that passage of the cells in our laboratory resulted in the selection of a variant cell population. However, we find identical results with another unrelated cell line, ZR75-1. It is unlikely that the lack of tumor regression observed in our study is due to insufficient antiestrogen concentrations. (*a*) We used tamoxifen doses ranging up to 10 times those used in one other study (23) without any evidence of tumor regression. (*b*) Serum concentrations of tamoxifen and LY156758 were sufficient to induce the expected alterations in tumor cytoplasmic ER and mouse uterine weight. (*c*) The concentrations were also sufficient to antagonize the tumor growth-promoting effects of estrogen supplementation. (*d*) Serum tamoxifen concentrations measured by high-pressure liquid chromatography were in the range of those measured in patients on tamoxifen therapy (5).

It is also unlikely that the failure of tamoxifen to induce tumor regression is related to its estrogen agonist properties in the mouse. Identical effects are observed with LY156758, an antiestrogen without estrogen agonist activity. The discrepancy between studies could be due to the different sites of tumor cell inoculation or to the nude mice themselves. We inoculated cells into the axillary region, whereas the thoracic region or mammary fat pads were used by other groups. It is possible that these areas differ regarding local hormone concentrations or other unknown factors. The immune status of the mice could also be important. Immune stimulation from subclinical infection or subtle genetic differences in mouse immunity could alter tumor growth or regression.

A consistent finding in our study was the prolonged lag time of 10 to 20 days required for restoration of tumor growth after estrogen replenishment in ovariectomized or anti-estrogen-treated mice. This lag time is considerably longer than the initial lag time for tumor development after cell inoculation in estrogen-supplemented mice. Soule and McGrath (24) also found that exponential growth was delayed for 3 to 4 weeks following estrogen replenishment. It is possible that part of the lag time is simply related to the relative insensitivity of early measurable changes in tumor volume. Measurement of other biochemical or cell kinetic parameters might have shown an earlier effect of estrogen replenishment. Part of the lag time could also be explained by the prolonged retention of tamoxifen *in vivo*, although this would not account for the latent period in ovariectomized mice. A more plausible explanation is that cells maintained in a prolonged nonproliferative state (G_0G_1) due to estrogen lack or antiestrogen therapy require additional time after estrogen replenishment to initiate the metabolic machinery necessary for reentry into the proliferative cell pool. The observation that the initial lag time for tumor development is longer in mice inoculated with late-stationary-phase (non-log-phase) cultured MCF-7 cells supports this conclusion (23). Tumor cell heterogeneity could provide another possible explanation for the prolonged lag phase. If tumors are comprised predominantly of endocrine-independent cells after prolonged estrogen deprivation or antiestrogen therapy, then estrogen replenishment would not be reflected in an increase in tumor volume until a significant endocrine-dependent cell population had been restored. The presence of high levels of ER and PGR in these tumors argues against this possibility, as does our observation that cultured cells derived from these tumors retain their hormone dependence *in vitro*. Furthermore, one would expect endocrine-independent tumors to start growing without estrogen replenishment. This was never observed in our studies. This apparent lag phase has potentially important therapeutic implications. Several ongoing clinical trials have been designed to take advantage of the ability of estrogen replenishment to stimulate cells into S phase, where they may be more susceptible to cytotoxic drugs (1, 10). A short (1 to 4 days) duration of "estrogen rescue" has been chosen empirically. Based on our studies in the nude mouse, this duration of the estrogen rescue may be too short, emphasizing the need for detailed cell kinetic studies in patients treated with this new therapeutic strategy.

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