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Human Breast Cancer in the Athymic Nude Mouse: Cytostatic Effects of Long-term Antiestrogen Therapy

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Abstract—*We have investigated the effects of long-term estrogen withdrawal or tamoxifen therapy of MCF-7 human breast cancer cells growing in the athymic nude mouse to clarify mechanisms by which endocrine therapy inhibits tumor growth. Estrogen withdrawal with or without tamoxifen inhibited MCF-7 tumor growth, but did not cause significant regression, even after 4 months of treatment. Serial histologic studies of treated tumors revealed a reduction in mitotic rate but no significant gross or ultrastructural cytopathic changes. Treated tumors did show a modest increase in stromal fibrosis as well as occasional cytoplasmic or nuclear vacuolization, perhaps indicating early cytopathic effects. Cell viability was confirmed by cloning tumor cells in soft agar; cloning efficiency in treated tumors was similar to that in controls. Tumor fragments from treated mice were also viable and formed tumors when transplanted into estrogen-supplemented but not estrogen-deprived mice indicating continued hormone dependence. When estrogen-deprived or tamoxifen-treated mice were replenished with estrogen, cell proliferation was reactivated and tumor growth resumed. After 3–4 months of endocrine therapy, tumors began to regrow despite continued treatment suggesting the conversion to hormone independence. These studies suggest that in this model system, estrogen withdrawal and antiestrogen therapy work primarily by cytostatic rather than cytocidal mechanisms.*

INTRODUCTION

ABOUT one-third of human breast cancers are hormone-dependent and require estrogen for maximal growth. Therapy designed to reduce the estrogen concentration or to inhibit the effects of estrogen by competitive blockade of the estrogen receptor results in temporary regression of these tumors. The mechanisms by which endocrine therapy inhibits tumor growth have not been completely defined. Either inhibition of pathways leading to increased cell proliferation and/or stimulation of pathways leading to cell loss could be involved.

In vitro studies using cultured hormone-responsive human breast cancer cells have shown that antiestrogen treatment slows cell proliferation and causes cells to accumulate in early G_1 phase, suggesting that antiestrogens block transit of the cells through the cell cycle [1, 2]. This block is reversible with the addition of 17β-estradiol [3]. Extremely high concentrations of the antiestrogen tamoxifen (five to 10 times the concentration achieved in patients) are lethal to cells by a non-estrogen receptor mediated mechanism [4, 5].

In vivo studies of the effects of estrogens and antiestrogens on human breast cancer growing in the athymic nude mouse demonstrate that ER-positive cells are hormone-dependent in this model system [6–8]. Estrogen is required for tumor growth. Estrogen withdrawal or antiestrogen treatment of mice with established tumors results in inhibition of tumor growth and a reduction in mitotic activity [6]. Furthermore, tumor growth can be restored with estrogen replenishment.

These *in vitro* and *in vivo* studies suggest that a major consequence of estrogen depletion or antiestrogen therapy is a reversible cytostatic block of cell proliferation, and that a lethal cytotoxic effect may be less important. However, the *in vivo* experiments described above employed relatively short term endocrine therapy of only 1–2 months duration. It

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is possible that more prolonged estrogen deprivation or antiestrogen treatment would eventually result in lethal cytotoxicity. In the present studies we have evaluated the effects of long-term (up to 4 months) endocrine therapy in mice carrying tumors derived from the hormone-responsive MCF-7 human breast cancer cell line. Tumor cytotoxicity following endocrine treatment was assessed by histologic studies with light and electron microscopy, by measuring tumor ER and PgR content, by cloning tumor cells *in vitro*, by transplanting tumor fragments into fresh mice, and by the ability of estrogen replenishment to restore tumor growth.

MATERIALS AND METHODS

Breast cancer cells

The MCF-7 cell line (passage 300) used in these experiments was originally obtained from Dr. S. Shafie at the National Cancer Institute in 1981. The cells are free of *Mycoplasma* contamination. Culture methods have been summarized recently [6].

Athymic nude mice

Four to five-week-old female intact or castrated BALB/c–nu^+/nu^+ mice were purchased from Harlan Sprague–Dawley (Madison, WI). Bilateral ovariectomy was performed at 3 weeks of age. Mice were kept in a facility in the Department of Laboratory Animal Resources in filtered laminar air flow hoods in vinyl cages with air filter tops. Cages, bedding, food and water were autoclaved before use.

Mice were inoculated with log-phase cells harvested by scraping from 150 cm^2 plastic culture flasks in complete tissue culture medium containing 5% calf serum [6]. Five $\times 10^6$ cells in 0.2 ml of culture medium were injected s.c. on the flank just caudal to the forelimb. Castrated mice were supplemented with estrogen in the form of a slow-release 0.25 mg 17β-estradiol pellet placed s.c. in the interscapular region. Estrogen withdrawal was accomplished by piercing the skin with a No. 11 blade and retrieving the pellet with forceps. Tamoxifen citrate (Stuart Pharmaceuticals, Wilmington, DE) was given daily by s.c. injection in peanut oil (50 μg/0.05 ml/mouse). Controls received peanut oil alone.

Tumor diameters were measured at regular intervals and tumor volume in mm^3 was calculated by the formula

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

Histologic studies

Mice were sacrificed by cervical dislocation and tumors were dissected free from s.c. tissues and fixed in 10% formalin. Paraffin-embedded sections were processed and stained with hematoxylin and eosin for light microscopic histologic determinations. The mitotic index was obtained by counting the number of mitoses per 2000 cells. Tumors from duplicate estrogen-treated control mice, estrogen-deprived mice, and tamoxifen-treated mice were analyzed after 1 week, 2 weeks, 2 months and 3 months of treatment.

For transmission electron microscopy, specimens were fixed in phosphate buffered 4% formaldehyde–1% glutaraldehyde and post-fixed in Zetterqvist's osmium fixative. Specimens were dehydrated in ethanol and propylene oxide and embedded in a Poly/bed Araldite resin mixture. Several blocks from each tumor were chosen randomly for thick sectioning. Thin sections were stained with uranyl acetate–Reynold's lead citrate. The grids were examined and photographed with a Joel 100 cx transmission electron microscope. A mean of seven micrographs (range 6–11) were photographed of the entire section surface at the same magnification. The micrographs were analyzed without knowledge of the study groups. A number of histologic features including the number of mitoses, the presence of lipid droplets or secretory granules, and the presence of degenerative changes and necrotic cells were evaluated.

Hormone receptor assays

Tumor ER and PgR were determined by a dual label dextran-coated charcoal assay using [^{125}I]-estradiol and [^{3}H]R5020 as previously described [9].

Clonogenic assay

Mice were sacrificed at the indicated time points and tumors were dissected free from s.c. tissues in a sterile fashion in a laminar air flow biohazard hood. A cell suspension was obtained by teasing apart the tumor with 18 g needles in tissue culture medium supplemented with 5% calf serum and 10 nM estradiol. A single cell suspension was obtained by gentle pipetting. Cells (4000/dish) were then mixed with agar (0.3%) and plated as a top layer over a bottom layer containing the same supplemented medium with 0.5% agar as previously described [10]. Cultures were incubated at 37°C in a 5% CO_2 humidified atmosphere for 14 days. Colonies ($\geq$/50 cell clusters) were then counted using an inverted stage microscope at 30× magnification and the cloning efficiency determined.

Tumor transplant studies

Mice were sacrificed at the indicated time points after endocrine therapy. Tumors were harvested and cut into 2 mm^2 fragments. One fragment was

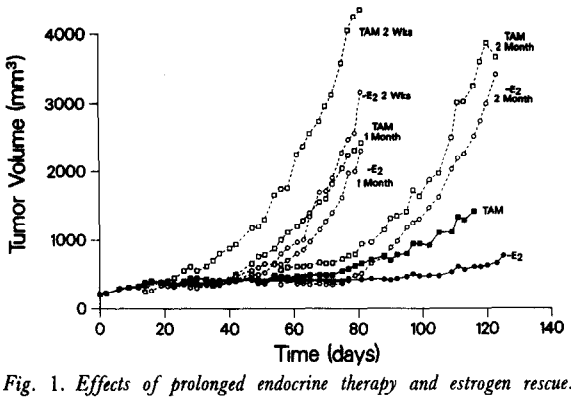

Fig. 1. Effects of prolonged endocrine therapy and estrogen rescue. Ovariectomized nude mice supplemented with a 0.25 mg E_2 *pellet were inoculated with MCF-7 cells. Two weeks later when palpable growing tumors had formed (Day* 0 *in Fig.* 1), 40 *mice were randomly divided into two groups: tam, removal of the* E_2 *pellet plus daily injections of tamoxifen* 50 μg/*mouse;* -E_2, *removal of the* E_2 *pellet only. At* 2 *weeks,* 1 *month and* 2 *months of treatment, five mice from each group were replenished with estrogen in the form of a* 0.25 mg *pellet placed s.c. (dashed lines). Tumor volume was measured at the indicated times. Each point represents the mean of five tumors. Standard error of the mean ranged from* 10 *to* 20%.

then placed s.c. on the flank just caudal to the forelimb of a 4–6 week-old recipient nude mouse by piercing the skin with a No. 11 blade and inserting with forceps. One group of recipient mice had been ovariectomized and the other was ovariectomized and was supplemented with a 0.25 mg estradiol pellet. The volume of the transplanted tumors was determined weekly.

Statistical methods

Measurements made on a continuous scale (tumor volume, cloning efficiency) were analyzed by analysis of variance followed by application of the Newman–Keuls multiple comparison procedure [11].

RESULTS

Effects of prolonged endocrine therapy and estrogen rescue on tumor growth

Mice with palpable growing MCF-7 tumors were treated either by estrogen deprivation (removal of the E_2 pellet) or by removal of the pellet plus daily tamoxifen injections. With both treatments, tumors stopped growing and tumor volumes remained stable despite prolonged observation for 120 days (Fig. 1). Significant tumor regression did not occur in either group. After 80 days of treatment, tumors in the tamoxifen-treated group began to grow even with continued daily injections of the drug. Later, tumors in the estrogen-deprived group also began to regrow spontaneously. This pattern of acquired resistance to endocrine therapy was reproducible in several experiments and was observed with another MCF-7 cell line obtained from a different laboratory (data not shown). Tamoxifen-treated tumors invariably began to regrow several weeks earlier than tumors treated by estrogen deprivation only.

To determine whether the inhibitory effects of prolonged estrogen withdrawal or tamoxifen treatment were reversible, groups of mice were retreated with estrogen. After 2 weeks, 1 month and 2 months of treatment, subsets of mice from both treated groups were replenished with estrogen (estrogen rescue) by placement of an estrogen pellet. After a 1–3-week lag period, growth was restored and tumor volumes increased rapidly indicating that cells remained viable and that they retained their hormone dependence. Tamoxifen-treated tumors began to grow earlier after estrogen replenishment than did the estrogen-deprived group.

Histologic changes with prolonged endocrine therapy

Tumor histology and ultrastructure were also studied at various times during endocrine therapy. When compared to estrogen-treated specimens (Fig. 2a), histologic studies using light microscopy showed no evidence of tumor necrosis or cell death after prolonged estrogen deprivation or tamoxifen treatment (Fig. 2b, c). A slight increase in cell size, a modest increase in stromal fibrosis, occasional cytoplasmic and nuclear vacuolization and subtle nuclear clearing with less prominent nucleoli were observed with both treatments compared to control tumors from estrogen-supplemented mice, but no increase in pyknotic cells or other significant cytopathic changes were observed at 1, 2 or 3 months of treatment. The most obvious change was a 50% reduction in mitotic index with tamoxifen during the first 2 months of treatment (data not shown). Thereafter the mitotic index in tamoxifen treated tumors increased as resistance to tamoxifen and regrowth of tumors occurred.

Transmission electron microscopy confirmed the absence of significant cytopathic changes from antiestrogen treatment. When compared to estrogen-treated controls (Fig. 3a), photomicrographs of tumors from tamoxifen-treated mice (Fig. 3b, c), showed no increased edema or abnormal vacuolization. The majority of cells from all tumors had well-formed compact mitochondria with no dissolution of christie, well defined nucleoli and finely dispersed chromatin with no dissolution of the nuclear membrane.

Soft agar cloning after endocrine therapy

To further assess the effect of prolonged endocrine therapy on MCR-7 breast cancer, tumor cells from treated or control mice were cloned in soft agar to determine their viability. With estrogen deprivation, cloning efficiency showed no consistent change and ranged from 0.4% to 0.9% over the 4-month treatment period (Table 1). With tamoxifen, cloning efficiency remained stable during the first 2 months of treatment during the static phase of

tumor growth in the mouse. However, concomitant with the development of tamoxifen resistance and the regrowth of tumors *in vivo*, cloning efficiency markedly increased to 3% at 3 months and to 15.7% at 4 months when tumors were growing rapidly. Antiestrogen treatment did not result in a significant reduction in cloning efficiency at any time point indicating continued viability of clonogenic cells despite prolonged treatment.

Transplantation of tumors after endocrine therapy

Viability of treated tumors was also assessed by the transplantation of tumor fragments into fresh mice. Both estrogen-deprived and estrogen-supplemented recipient mice were used to evaluate the hormone dependence of the transplanted fragments. Tumor fragments taken from control mice before endocrine therapy (day 0) grew in estrogen-supplemented but not in ovariectomized mice (Fig. 4). Tumor fragments from mice treated with estrogen deprivation without or with tamoxifen for periods up to 4 months invariably formed tumors after a lag period of about 50 days in recipient mice that were supplemented with estrogen. Thus, tumor cells in these fragments remained viable and responsive to estrogen for growth. Interestingly, fragments taken from mice after 1–4 months of treatment also eventually formed tumors even in estrogen-deprived recipient mice indicating the development of hormone independence. The longer the treatment prior to transplantation, the shorter the lag time for tumor growth in estrogen-deprived recipient mice.

Estrogen and progesterone receptors

To determine whether the development of *in vivo* hormone independence and regrowth of tumors after prolonged endocrine therapy was due to loss of receptor, ER and PgR were measured in growing tumors from animals treated for 4 months (Table 2). Tumors from estrogen-deprived mice had a high ER concentration but low PgR. Those from tamoxifen-treated mice had no detectable cytoplasmic ER but had high PgR. Thus resistance to endocrine treatment in these mice was not due to receptor loss or to the selection of a receptor-negative clone. Interestingly, although tumor cell proliferation was no longer suppressed by tamoxifen, another pathway regulated by tamoxifen, induction of PgR, remained intact.

DISCUSSION

These experiments demonstrate that with the conditions employed, reduction of serum estrogen to castrate levels in the nude mouse without or with tamoxifen treatment results in a cessation of MCF-7 tumor growth but not in significant tumor regression even when endocrine therapy is continued for up to 4 months. Since net tumor volume represents a balance between cell production and cell loss factors, endocrine treatment could cause a reduction in cell production factors such as the rate of proliferation, an increase in cell loss factors such as cell death, or both. Other investigators have reported partial MCF-7 tumor regression with antiestrogen therapy [7]. Differences among laboratories regarding the effects of antiestrogen therapy on MCF-7 tumor growth may relate to differences in the cell lines or in the strains of mice used which may affect the cell loss compartment. We have recently studied another MCF-7 cell line (provided by Dr. C. Benz) in which partial tumor regression followed by the development of resistance and regrowth of the tumor were observed with long-term tamoxifen therapy (unpublished observations). In any event, in the present studies, tumor volume remained constant with treatment indicating an equilibrium between cell production and cell loss. The histologic studies exclude the possibility that treated tumors are comprised predominantly of stromal tissue with a major loss of the epithelial component. It could be argued that the lack of tumor regression in these mice is due to the partial estrogen agonist activity of tamoxifen in this species. This seems unlikely since the breast cancer cells themselves are of human origin, and since we previously observed similar results with another antiestrogen (LY 156758) that is an antagonist in mice [6].

To determine whether endocrine therapy influenced the viability of the hormone-dependent cells within the tumor, we performed histologic studies, determined *in vitro* cloning efficiency, assessed the transplantability of tumor fragments and studied the reversibility of growth inhibition by estrogen replenishment as a function of the duration of endocrine therapy. Histologic studies by light microscopy and ultrastructural studies by electron microscopy revealed no necrosis or increase in pyknotic cells with treatment. Stromal fibrosis was only slightly increased in estrogen-deprived and tamoxifen-treated tumors. Treated tumors did demonstrate occasional cytoplasmic and/or nuclear vacuolization or clearing. These changes could be indicative of early cytopathic effects of treatment. Cell proliferation was inhibited as evidenced by the drop in mitotic index as we have previously reported [6]. In the present study, the drop in mitotic index was greatest in the tamoxifen-treated group. More striking histologic changes have been reported to occur in another human breast carcinoma that is inhibited by estrogen therapy in the nude mouse [12]. Estrogen treatment of this tumor caused a reduction of mitoses and an accumulation of cells in S phase. Serial histologic studies revealed gradual loss of the epithelial tumor cells with a concomitant increase in stromal connective tissue and the

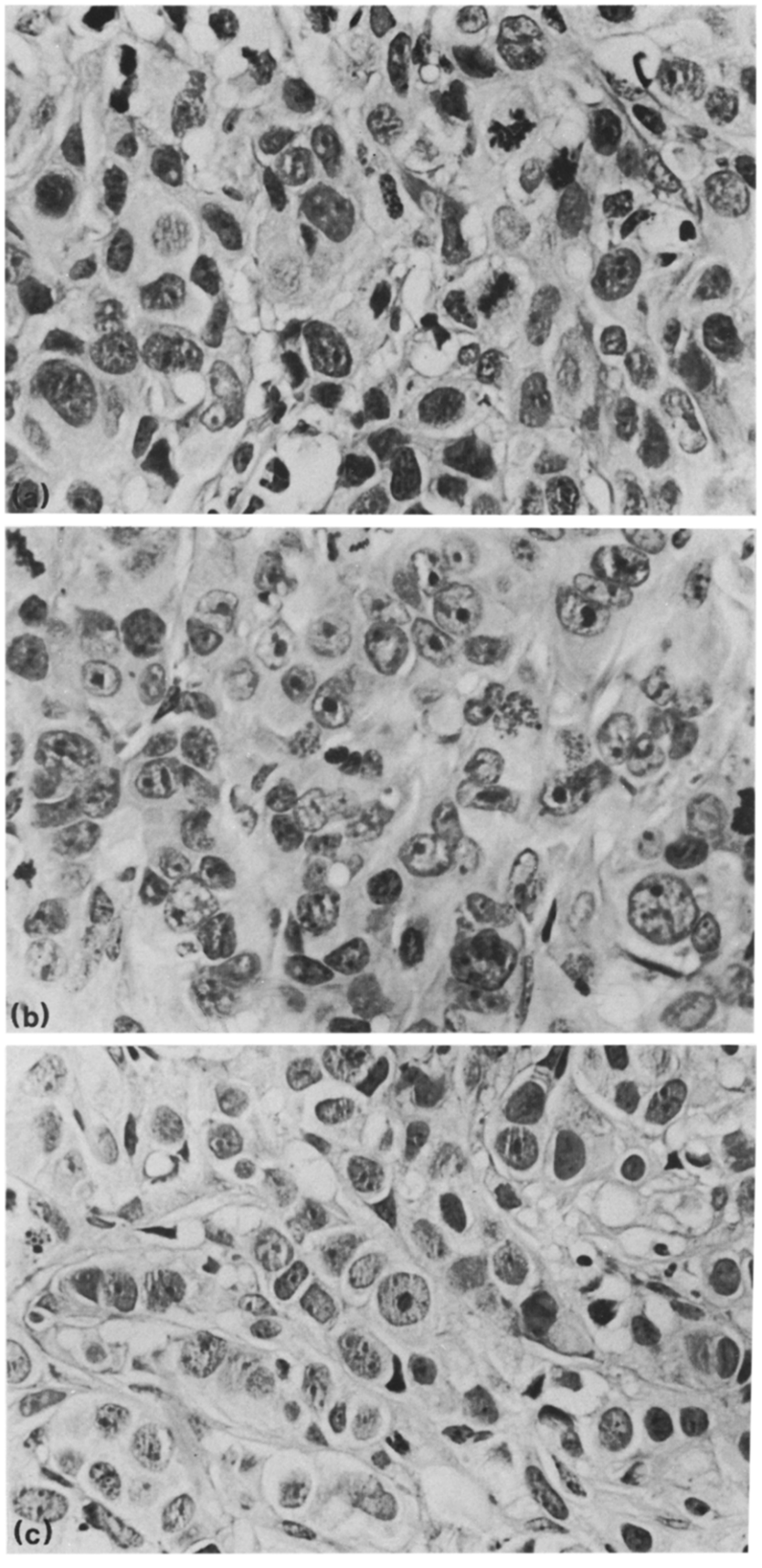

Fig. 2. Histology of treated tumors. Mice with MCF-7 tumors were treated with estrogen (Control, Fig. 2a), estrogen withdrawal (Fig. 2b) orestrogen withdrawal plus amoxifen (Fig. 2c) for 2 months. Tumors were harvested and processed as described in Materials and Methods for light microscopy. Slightly increased stromal fibrosis is evident with both estrogen withdrawal and with tamoxifen. Figure 2c also demonstrates an area of increased cytoplasmic vacuolization that was occasionally observed with tamoxifen. Hematoxylin and eosin; × 190.

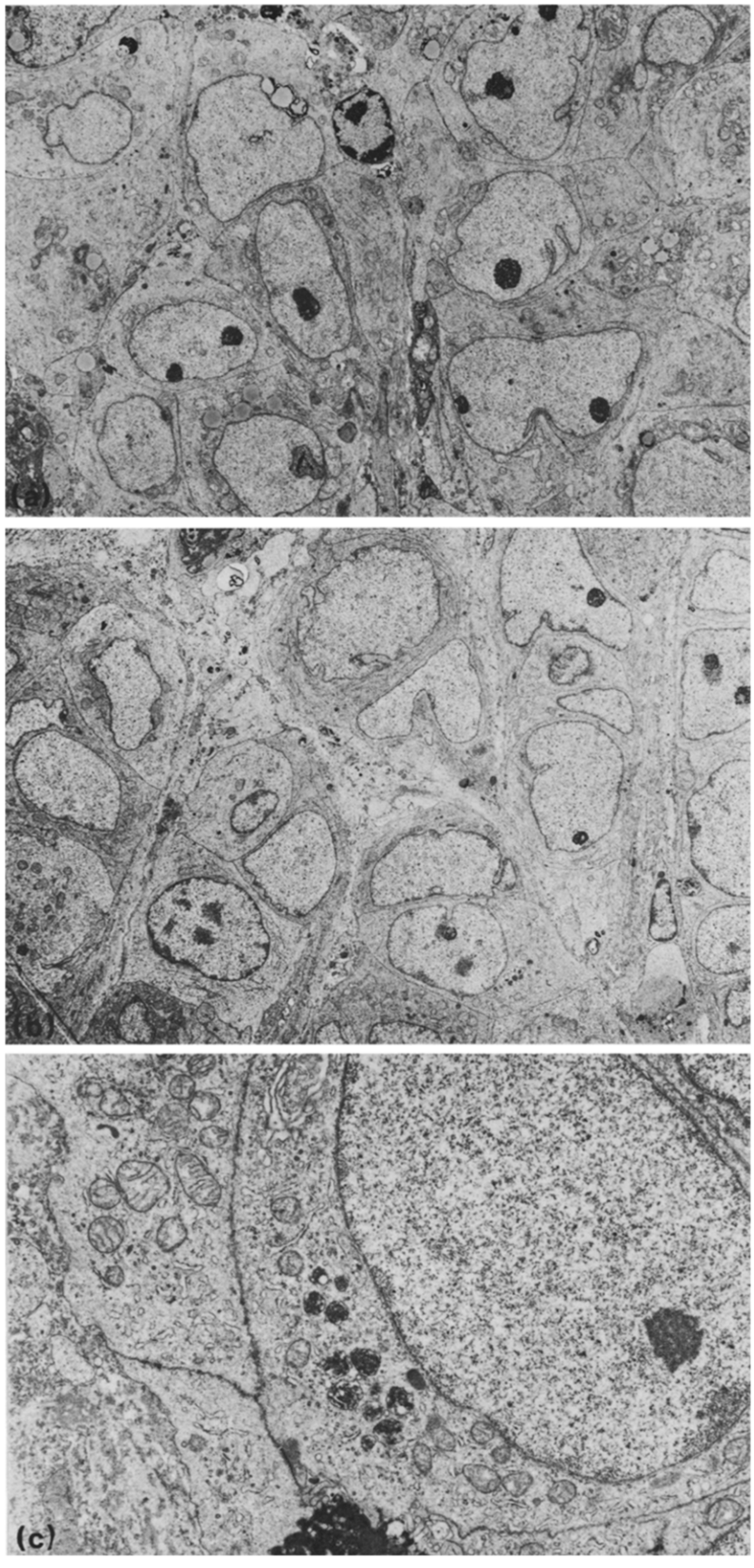

Fig. 3. Electron microscopy of treated tumors. Mice were treated as described in Fig. 2 and tumors were harvested and processed for transmission electron microscopy. Figure 3a, estrogen-treated control, × 4408; Fig. 3b, tamoxifen treatment for 8 weeks, × 4408; Fig. 3c, tamoxifen treatment for 8 weeks, × 18,183.

Table 1. *Effect of tamoxifen and estrogen deprivation on tumor cloning*

Time	Cloning efficiency (%) Estrogen deprivation	Tamoxifen
Day 0 (control)	0.9 ± 0.1*	0.9 ± 0.1
1 month	0.7 ± 0.2	0.6 ± 0.05
2 months	0.5 ± 0.2	1.0 ± 0.3
3 months	0.8 ± 0.02	3.2 ± 0.2†
4 months	0.4 ± 0.05	15.7 ± 1.0†

Estrogen-supplemented ovariectomized female nude mice with growing MCF-7 tumors were treated by removal of the estrogen pellet or by removal of the pellet plus injections of tamoxifen. At the times shown, tumor cells from triplicate mice were cloned in soft agar as described in Materials and Methods and the cloning efficiency determined.

*Mean ± S.E.

†Significantly different from others in group ($P < 0.01$).

appearance of polyploid tumor giant cells. This study suggests that initial cytostatic effects were followed by a significant cell loss component in this model system. Histologic studies of other heterotransplanted breast cancers treated with endocrine therapy have not been reported.

In the present study, MCF-7 tumor cells remained viable despite prolonged estrogen deprivation or antiestrogen therapy on the basis of three other parameters. *In vitro* cloning efficiency was not significantly reduced by the *in vivo* treatment, suggesting that at least the clonogenic fraction of cells was not lethally effected and was capable of proliferating *in vitro* to form colonies. Tumor fragments were also capable of proliferating and forming tumors when transplanted into fresh recipient mice. Furthermore, prior treatment did not select out a hormone-independent cell clone, since the tumor fragments were still dependent on estrogen for growth in recipient mice. Finally, hormone-dependent tumor cells from treated mice remained viable on the basis that estrogen replenishment was able to restore cell proliferation and tumor growth. These cumulative data suggest that hormone-dependent MCF-7 cells are capable of surviving prolonged periods of estrogen deficiency or antiestrogen therapy, and that a major effect of such therapy is to reversibly inhibit cell proliferation rather than to directly kill the cell. We cannot exclude the possibility that increased cell loss (death) does occur with treatment of the MCF-7 or other human breast cancers *in vivo*. More direct measures of cell loss in this model system are required to more definitely answer this question. Tumor regression seen in patients treated with endocrine therapy does not necessarily indicate that the treatment itself caused increased cell loss or death. A treatment-induced reduction in tumor cell proliferation coupled with stable cell loss from ongoing host defense pathways would result in a net reduction in tumor volume.

An interesting observation in the present studies is the eventual development of hormone-resistance and regrowth of tumors despite continued therapy, a situation which invariably develops in patients

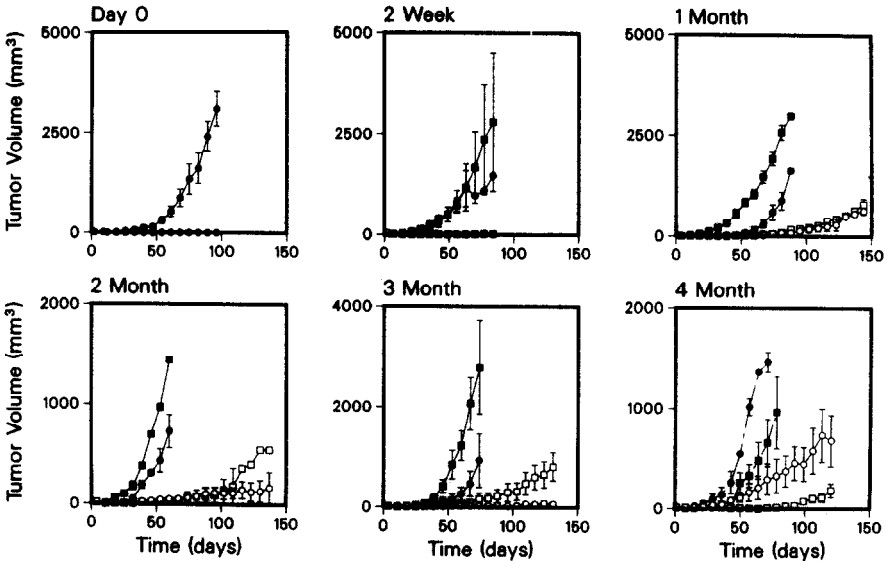

Fig. 4. *Effect of endocrine therapy on transplantability of tumor fragments. Tumor fragments* (2 mm^2) *were taken from control mice* (*Day* 0) *before treatment and from mice after* 2 *weeks*, 1 *month*, 2 *months*, 3 *months and* 4 *months of treatment with estrogen deprivation* (*circles*) *without or with tamoxifen* (*squares*). *Fragments were transplanted s.c. in recipient ovariectomized mice* (*open circles or squares*) *or in recipient mice supplemented with an estradiol pellet* (*closed circles or squares*). *Points represent the mean ± S.E. of three mice.*

Table 2. Effect of prolonged endocrine therapy on tumor hormone receptor status

Group	ER*	PgR*
Estrogen-deprived	615 ± 61	4 ± 2
Tamoxifen	< 3	529 ± 339

Ovariectomized, estrogen-supplemented mice were inoculated with MCF-7 cells. When growing tumors had formed, the estrogen pellets were removed and half of the mice also were treated with daily tamoxifen injections. After 4 months, when tumors were resistant to treatment and growing rapidly, mice were sacrificed and tumors excised for measurement of E and PgR.

*Fmol per mg protein; mean ± S.E. of five tumors.

treated with endocrine therapy. Regrowth of MCF-7 tumors was also observed by Shafie and Grantham [7] in nude mice treated with estrogen deprivation but not with tamoxifen, although the duration of observation was much shorter than in the present study. The mechanism for this acquired resistance is not clear. Although tumor ER and PgR levels in patients tend to fall with endocrine therapy, patients frequently respond to second- or third-line endocrine manipulation indicating that loss of hormonal sensitivity is not total. Resistant MCF-7 tumors growing in the nude mouse continued to express high concentrations of functional ER and PgR eliminating receptor loss as a possible mechanism. Furthermore, preliminary data show no change over time in the endocrine profile of the mouse; estrone and estradiol levels remain in the castrate range and prolactin levels are unchanged in mice treated with estrogen deprivation and/or tamoxifen (unpublished observations). Preliminary data from our laboratory also show that increasing the tamoxifen dose 100-fold does not recapture growth inhibition. In our model system endocrine resistance invariably develops faster in antiestrogen-treated tumors, and tumor growth is restored earlier in these tumors after estrogen replenishment. It is interesting to speculate that tamoxifen itself may eventually stimulate the cells through its partial estrogen agonist activity. We have previously reported a temporary stimulation of tumor growth in tamoxifen treated nude mice inoculated with MCF-7 cells [6]. Furthermore, tamoxifen resistant MCF-7 variants developed by continuous *in vitro* exposure have been reported to show estrogenic response to tamoxifen [13]. This model provides a unique system for further studies of acquired hormone resistance in breast cancer.

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