

Effects of Combination Estrogen:Cyclophosphamide Treatment on the Growth of the MXT Transplantable Mammary Tumor in the Mouse¹

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ABSTRACT

The present studies were done to determine if the growth-promoting properties of estrogen on the MXT transplantable mammary tumor in the mouse would enhance the cytotoxicity of cyclophosphamide. Mice bearing these tumors had beeswax pellets implanted which contained 17 β -estradiol (0.01 to 1.0 mg/pellet) and/or injected twice weekly with cyclophosphamide (10 to 40 mg/kg body weight in sesame oil) for 4 to 7 weeks. During this time, tumor size $[(L + W)/2]$ and body weights were monitored every 7 to 14 days. The results show that administration of estradiol (0.01 to 1.0 mg/pellet) by implant slightly stimulated tumor growth, and in no case was tumor regression observed in response to the steroid. Likewise, cyclophosphamide treatment alone (10 to 20 mg/kg) failed to inhibit the tumor; however, combined administration of estradiol plus cyclophosphamide (10 to 40 mg/kg) resulted in significant inhibition of tumor growth. This response was time and dose dependent. These results show that while neither compound alone inhibited tumor growth, estradiol and cyclophosphamide are synergistic and completely block the growth of this transplantable mammary tumor in the mouse. The mechanism for this antagonism of mammary tumor growth remains to be resolved; however, we speculate that estradiol stimulates cellular hypertrophy and hyperplasia in these tumors, and under these conditions the cytotoxic effectiveness of cyclophosphamide is enhanced.

INTRODUCTION

Current therapeutic procedures for management of breast cancer in humans involve endocrine ablation and/or antiestrogen therapy for cancers which are likely to respond to endocrine therapy (3, 12, 17-19). The rationale for these approaches is that estrogens apparently stimulate DNA (5, 6, 13) and RNA (1, 8) synthesis and tumor growth (15, 16) under a variety of experimental conditions. Consequently, removal of endogenous estrogen via ablative procedures, or blocking estrogen effects by "antiestrogen" administration (12, 17-19), may result in tumor regression. Alternatively, chemotherapeutic approaches with cytotoxic drugs are recommended for patients whose tumors are unlikely to respond to this type of endocrine therapy (12, 17).

Although endocrine ablation and/or antiestrogen therapy appear to be a logical approach to the treatment of steroid hormone-responsive breast cancers, we reasoned that perhaps the growth-promoting properties of estrogen could be used to enhance the effectiveness of a cytotoxic agent such as cyclophosphamide. This drug, or a metabolite, apparently causes arrest or prolongation of S and G₂ phases of the cell cycle via DNA

alkylation (4, 9). In addition, it has been shown that cyclophosphamide is most effective on mitotically active tumor cell populations (2). Therefore, if our hypothesis is correct, one would predict that stimulation of tumor cell proliferation with appropriate doses of exogenous estradiol would enhance the cytotoxicity of cyclophosphamide. Under these conditions, combination estradiol:cyclophosphamide therapy would be expected to inhibit tumor growth to a greater extent than drug treatment alone.

The present studies show that in the ovarian-dependent transplantable MXT mammary tumor in the mouse, estradiol and cyclophosphamide are synergistic and inhibit tumor growth.

MATERIALS AND METHODS

Tumor Induction and Transplantation. Tumors were induced by injecting urethan (i.p.; 20 mg/week \times 10 weeks) into 5-week-old female C57BL \times DBA/F₁ mice carrying pituitary isografts under the kidney capsule. Tumors grew in 12 to 15 weeks in the host animals and 1-cu mm pieces were serially transplanted into 10- to 12-week-old syngeneic female mice. This transplantable mammary tumor was previously determined to be ovarian dependent and to contain estrogen and progesterone receptors (24). Animals were maintained throughout the study in a controlled environment with a constant light (6 a.m. to 8 p.m.) and dark cycle. Food and water were provided *ad libitum*.

Hormone and Drug Administration. The transplanted mammary tumors were allowed to grow until they reached a size of approximately 0.5 \times 0.5 cm (20 to 30 days) prior to the initiation of hormone and/or drug therapy. Animals with uniform size tumors were divided into different groups (4 to 6 animals/group) and were treated as follows. Controls had beeswax pellets implanted on Day 0 and were given injections of sesame oil (0.2 ml) 2 times per week (Monday a.m.; Thursday p.m.) for 4 to 7 weeks. Estradiol-treated animals had beeswax pellets (10 mg) containing the indicated doses (0.01 to 1.0 mg) of 17 β -estradiol implanted s.c. (Day 0) and were injected twice weekly either with sesame oil (estradiol treatment group) or cyclophosphamide (10 to 40 mg/kg; estradiol:cyclophosphamide treatment group). Our rationale for administering estradiol by pellet implant is that the hormone can be delivered continuously at very low levels over prolonged periods of time (>365 days). Under these conditions, we hoped to very slightly, but continuously, stimulate tumor cell proliferation without causing tumor regression due to high levels of exogenous steroid (7, 10, 11, 20, 22). According to earlier studies concerning estrogen release from paraffin pellets (21), we calculated that these implants were releasing approximately 0.3% (w/w) of the estrogen per day. These values would be 0.03, 0.3, and 3.0 μ g/day for the 0.01-, 0.1-, and 1.0-mg estradiol implants, respectively.

Cyclophosphamide (Mead Johnson Drug Development Branch; National Cancer Institute, Bethesda, Md.) was injected in sesame oil to facilitate a slower rate of absorption of the drug. Although cyclophosphamide is usually given in aqueous vehicles, we felt a slower absorption rate would indirectly increase its half-life and ensure a more continuous exposure of tumor cells to the drug.

Body weights and tumor size $[(L + W)/2]$ were monitored every 7 to 14 days throughout the course of these studies. At the termination of the experiments, the mice were sacrificed by cervical dislocation, and tumors were dissected free of extraneous tissues and weighed. Histo-

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logical sections were fixed in Bouins solution and stained with hematoxylin:eosin. This histological examination revealed that following 28 days of treatment, these tumors in control, as well as estradiol- and/or cyclophosphamide-treated animals, were poorly differentiated adenocarcinomas. No significant treatment effects were observed with respect to the numbers of mitotic figures, and only slightly more necrosis was observed in the estradiol- or estradiol:cyclophosphamide-treated animals.

RESULTS

Effect of 17β-Estradiol Implants on the Growth of the MXT Mammary Tumor. Although the ovarian dependence of this tumor has been well established (24), we examined the growth response of the MXT tumor to the beeswax implants of estradiol. This study was extremely important to establish a dose level of exogenous estrogen which would marginally stimulate tumor growth without causing tumor regression as is usually observed when high levels of estrogen are administered (7, 10, 11, 20, 22). The data presented in Chart 1 clearly demonstrate that none of the doses of estradiol used here (0.01- to 1.0-mg implants) caused tumor regression. In addition, all doses provided a slight level of tumor growth stimulation; however, the response appeared more consistent with the 0.1- and 1.0-mg estradiol implants. For this reason, we chose to use 1-mg estradiol implants for subsequent experiments with combination estradiol: cyclophosphamide treatment.

Effect of Combined Estrogen:Cyclophosphamide Treatment on Tumor Growth. To determine whether the cytotoxicity of cyclophosphamide on mammary tumor growth could be enhanced by coadministration of estrogen, tumor-bearing mice had 1 mg estradiol implanted or had estradiol implants and cyclophosphamide injections (10 to 40 mg/kg) 2 times/week for 4 weeks. Control animals had blank beeswax pellets implanted and were given injections 2 times/week with sesame oil. The data in Chart 2 show that administration of either estradiol or cyclophosphamide (20 mg/kg) alone failed to inhibit tumor growth and, if anything, these levels of either compound were slightly stimulatory. In contrast, combined administration of 1 mg estradiol plus cyclophosphamide (10 to 40 mg/kg) resulted in a dose-dependent inhibition of tumor growth, suggesting that these compounds are synergistic. Following 4 weeks of treatment, combined administration of 1-mg estradiol plus cyclophosphamide

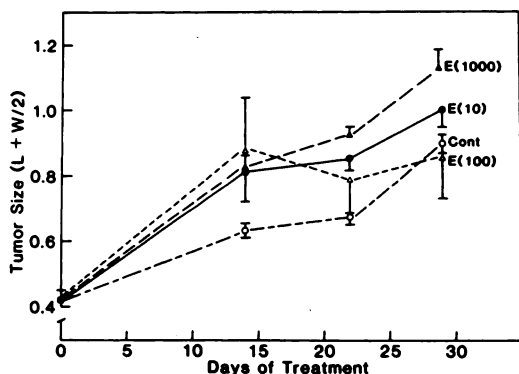


Chart 1. Effects of estradiol on the growth of the MXT transplantable mammary tumor of the mouse. Adult cycling female C57BL × DBA/F₁ mice bearing mammary tumors (0.5 × 0.5 cm) were implanted with beeswax pellets (controls (Cont), ○) containing 10 (●), 100 (△), or 1000 µg (▲) of 17β-estradiol (E) on Day 0. Bars, S.D. Tumor size [(L + W)/2] was monitored throughout the study by measurement with vernier calipers.

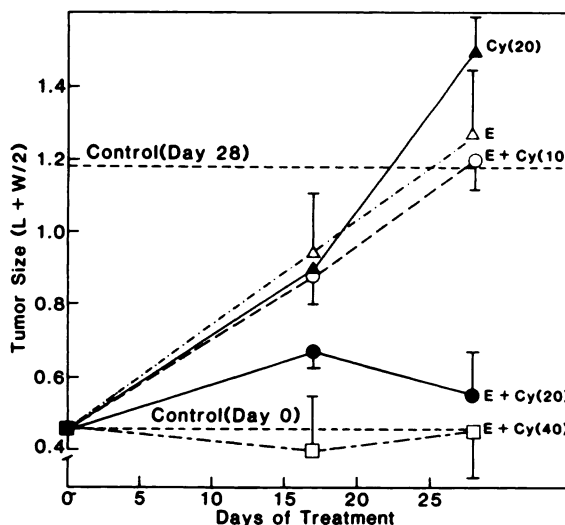


Chart 2. Effects of combination estradiol:cyclophosphamide treatment on the growth of the MXT-transplantable mammary tumor. Estradiol (E; 1 mg) was administered by pellet implant (Chart 1: "Materials and Methods"), and cyclophosphamide (CY) was injected twice weekly in sesame oil vehicle (0.5 ml). All treatments were started on Day 0. Animals were treated with estradiol (△) or cyclophosphamide (20 mg/kg; ▲) alone or given a combination of estradiol (1 mg) plus a 10-mg/kg [E + CY(10); ○], 20-mg/kg [E + CY(20); ●], or 40-mg/kg [E + CY(40); □] dose of cyclophosphamide. Controls had blank beeswax pellets implanted and were given injections of sesame oil vehicle. Tumor size [(L + W)/2] was monitored as described in Chart 1 and "Materials and Methods."

Table 1
Effects of estradiol and cyclophosphamide on the growth of the MXT mammary tumor

Treatment ^a	Tumor wt (g)	Body wt (g)
17β-Estradiol control	2.35 ± 0.85 ^b	28.0 ± 3.2
17β-Estradiol + cyclophosphamide (10 mg/kg)	1.59 ± 0.17	29.0 ± 1.7
17β-Estradiol + cyclophosphamide (20 mg/kg)	0.63 ± 0.24 ^c	23.8 ± 1.4
17β-Estradiol + cyclophosphamide (40 mg/kg)	0.21 ± 0.08 ^c	24.8 ± 1.3

^a Intact cycling mice (4 per group) bearing MXT transplantable mammary tumors (0.5 × 0.5 cm on Day 0) were implanted with beeswax pellets containing 1 mg 17β-estradiol on Day 0 and given injections (2 times per week) with sesame oil vehicle (estradiol control) or the indicated doses of cyclophosphamide (10 to 40 mg/kg). Following 7 weeks of treatment, animals were sacrificed, tumors were dissected free of extraneous tissue and weighed, and body weights were determined.

^b Mean ± S.D.

^c Significantly different from estradiol control (p < 0.01) and estradiol: cyclophosphamide (10 mg/kg; p < 0.05).

mid (20 or 40 mg/kg) resulted in 77 and 100% inhibition of tumor growth, respectively, whereas no inhibition was observed with estradiol plus cyclophosphamide (10 mg/kg) (Chart 2). By 7 weeks, however, significantly greater inhibition (33%) was observed in the animals treated with estradiol plus cyclophosphamide (10-mg/kg) (Table 1) than observed on Day 28. These results suggest that duration of treatment also affects response. Following 7 weeks of treatment, no significant effects of cyclophosphamide were observed on the body weights of these animals (Table 1), suggesting that general systemic toxicity was minimal.

DISCUSSION

These results demonstrate that combination therapy with estrogens and cytotoxic drugs may be a feasible approach in the treatment of breast or mammary cancer. Combination estro-

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diol:cyclophosphamide therapy completely blocked the growth of the ovarian-dependent MXT transplantable mammary tumor in the mouse. This observation substantiates our hypothesis that very low, continuous doses of estradiol which stimulate tumor cell proliferation (Chart 1) will also enhance the cytotoxic effects of drugs such as cyclophosphamide. The effective doses of cyclophosphamide used in this study (20 to 40 mg/kg) are 10- to 40-fold lower than the 50% lethal dose for this drug in the mouse (9) and are 2.5- to 10-fold lower than those usually reported to inhibit tumor growth in this species (14, 23). As clearly demonstrated in Chart 2, cyclophosphamide alone (20-mg/kg) did not inhibit tumor growth; however, in combination with estradiol, this level of the drug inhibited tumor growth by 77 and 83% following 4 (Chart 2) and 7 weeks (Table 1) of treatment, respectively. Combination estradiol plus cyclophosphamide (40 mg/kg) completely blocked tumor growth (Chart 2; Table 1). On the basis of body weight measurements, we observed no generalized drug toxicity with these levels of estradiol and/or cyclophosphamide. Therefore, when combined with estradiol, the therapeutic effectiveness of cyclophosphamide is increased. Consequently, perhaps drug dosage and the resulting generalized systemic toxicity can be reduced by combination estrogen:cyclophosphamide therapy without a significant decrease in tumor growth inhibition.

The present studies were not designed to examine the mechanism of this synergistic interaction between estradiol and cyclophosphamide on mammary tumor growth. However, we speculate that this interaction involves the mitogenic properties of low levels of estradiol on tumor cells, and consequently the DNA-alkylating capacity of cell cycle agents such as cyclophosphamide is increased. Estrogens have been demonstrated to stimulate tumor cell proliferation under a variety of experimental conditions (1, 5, 6, 8, 13, 15, 16) and rapidly dividing mammary cancer cells appear to be more susceptible to inhibition by cyclophosphamide (2). Therefore, it would appear that the synergistic effects of estradiol and cyclophosphamide reside in the capacity of the estrogen to slightly increase the rate and/or proportion of tumor cells undergoing mitosis, thereby indirectly increasing the overall sensitivity of the tumor to cyclophosphamide.

One must keep in mind that the level of estradiol administered with cytotoxic agents is an important consideration when evaluating the effectiveness of combination estrogen chemotherapy. We were very careful to utilize doses of the steroid which would very slightly stimulate tumor growth (Chart 1) without causing tumor regression (7, 10, 11, 20-22). Following administration of massive doses of estrogen, one might predict that the rate of tumor cell division would be reduced or inhibited by the estrogen and consequently that the cell cycle agents such as cyclophosphamide would be less effective. A similar response was observed by Kiang and Kennedy (11) who reported that cyclophosphamide actually blocked the diethylstilbestrol-induced regression of dimethylbenzanthracene-induced mammary tumors in the rat.

In light of the above considerations, we feel that continuous administration of very low levels of estrogen (0.03 to 3.0 μ g/day) to these animals was very effective in slightly stimulating the growth of ovarian dependent mammary tumors (Chart 1), and this level of estrogen enhanced the effectiveness of cyclophosphamide (Chart 2; Table 1). Therefore, these results demonstrate that combination estrogen chemotherapy is more effective in

inhibiting the growth of the ovarian-dependent mammary tumor in the mouse than treatment with the drug alone. Whether this treatment will cause similar responses in ovarian-independent mouse mammary tumors is currently under investigation in our laboratory. However, recent studies have shown that estrogens are capable of stimulating tumor cell proliferation regardless of the cellular content of cytoplasmic estrogen receptors (5), so one might predict that this combination estrogen:cyclophosphamide therapy would also regress ovarian-independent or estrogen receptor-negative mouse mammary tumors. Certainly the concept of combination steroid hormone chemotherapy may be applicable not only to the treatment of breast cancer, but also to a variety of endocrine-related tumors which grow in response to sex steroids.

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