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Combined Endocrine Therapy and Chemotherapy of Mouse Mammary Tumors

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Abstract—*Hormone-responsive mammary tumors of GR mice were treated with tamoxifen, cyclophosphamide, or with both drugs combined. Tamoxifen alone or cyclophosphamide alone caused inhibition of tumor growth, but more growth inhibition was obtained with the combined therapy.*

INTRODUCTION

HORMONE-RESPONSIVE mammary tumors of GR mice can be used as a model system to study the relationship between hormone receptor levels and hormone responsiveness of tumors [1, 2]. The tumors consist of heterogeneous populations of hormone-dependent and independent cancer cells (for a review see ref. 3). Previous studies in our laboratory have shown that the cytostatic drug cyclophosphamide inhibits growth of mouse mammary tumors [4]. Since tamoxifen is known to be effective against breast cancer [5, 6] it was of interest to see whether this drug could be investigated in mouse tumor studies. We now report on the effects of tamoxifen on the growth of hormone-responsive and independent mammary tumors of GR mice. Growth inhibition by tamoxifen was compared to that by cyclophosphamide, and the result of combined treatment with these drugs was investigated.

MATERIALS AND METHODS

Induction and serial transplantation of tumors

Mammary tumors were induced by estrone and progesterone treatment in overiectomized mice of the GR inbred strain as described previously [1]. The tumors were serially transplanted in hormone-treated or untreated castrated $(020 \times GR)F_1$ hybrid mice [1]. The terms 'hormone-dependent', 'hormone-responsive' and 'hormone-independent' for the tumors in this system have have been defined [1] and are used in the same meaning in the present study.

Tamoxifen and cyclophosphamide treatments

Tamoxifen base, ICI 46,474, *trans* 1-(*p*-*β*-dimethyl-amino-ethoxy-phenyl)-1,2-diphenyl but-1-ene, was a gift from Imperial Chemical Industries Ltd., Macclesfield, Cheshire, U.K. The cyclophosphamide used was NSC-26271; CAS reg. No. 6055-19-2; 2H-1,3,2-oxazaphosphorine,2-[bis(2-chloroethyl)amino]tetrahydro-2-oxide, monohydrate.

Response to treatment with cyclophosphamide and tamoxifen was measured in 8 groups (designated A–H), each consisting of 12 castrated $(020 \times GR)F_1$ hybrid mice which were treated continuously with estrone and progesterone. Mice of groups A–D each received 10^7 cells derived from a combined batch of hormone-responsive tumors, and mice of groups E–H each received 10^6 cells derived from a combined batch of hormone-independent tumors. The single-cell suspensions were prepared according to the method of Wiepjes and Prop [7], and were administered in 0.5 ml physiological saline by s.c. grafting in the right flank.

Cyclophosphamide was injected i.p. in portions of 1 mg/0.5 ml physiological saline on days 10, 17 and 24 after tumor cell inoculation. Tamofixen (portions of 0.5 mg) was mixed with progesterone prior to the insertion of the latter as a pellet [1]; the pellets were administered on days 10, 17 and 24 after tumor cell inoculation.

Tumor size was estimated by measuring length (l), width (w) and height (h) of the tumor, and calculating the volume (V) from

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the formula: $V=0.5235\times l\times w\times h$ [8]. Statistical analysis of the data obtained was carried out with the Wilcoxon test [9, 10].

Other materials

[2,4,6,7-^{3}H]Estradiol (96 Ci/mmol) was obtained from the Radiochemical Centre, Amersham, U.K. Nafoxidine hydrochloride (U 11100A) was a gift from the Upjohn Co., Kalamazoo, Mich., U.S.A.

RESULTS

Hormone-responsive tumors

Growth inhibition of hormone-responsive mouse mammary tumors was investigated in 4 groups (designated A–D) consisting of 12 tumor-bearing mice each. Group A was given physiological saline, Group B was given cyclophosphamide, Group C was given tamoxifen and Group D was given both cyclophosphamide and tamoxifen. Treatments were given on days 10, 17 and 24 after tumor cell grafting. Figure 1 (a) presents the growth curves of these tumor groups, and Table 1 the tumor weight yields at the end of the experiment.

Figure 1 (a) shows that the combined treatment of hormone-responsive mouse mammary tumors with tamoxifen and cyclophosphamide caused more inhibition than tamoxifen alone or cyclophosphamide alone. On day 31 after the start of the experiment, tamoxifen alone or cyclophosphamide alone each gave 82% inhibition of tumor growth, whereas the combined treatment gave 96% inhibition. After 31 and 36 days of growth, tumors treated either with tamoxifen alone, cyclophosphamide alone, or the combined treatment, had statistically lower average size ($P<0.01$) than untreated tumors. The combined treatment caused a significantly larger reduction in tumor size than tamoxifen alone on days 27, 31, 36, 42 and 48. Combined treatment had a larger effect than cyclophosphamide alone on days 31, 36 and 42.

Table 1. Weights of mammary tumor outgrowths

Group*	Growth time (days)	Tumor weight† (g)
A	37	1.4±0.7
B	45	1.0±0.3
C	48	0.9±0.3
D	48	0.5±0.1
E	34	1.6±0.6
F	34	0.9±0.3
G	34	0.9±0.3
H	34	0.6±0.2

*Groups A–D, hormone-responsive tumors; groups E–H, hormone-independent tumors. Groups A, E, untreated; groups B, F, cyclophosphamide-treated; groups C, G, tamoxifen-treated; groups D, H, tamoxifen + cyclophosphamide treated.
†Groups of 12 mice each. Mean ± S.D.

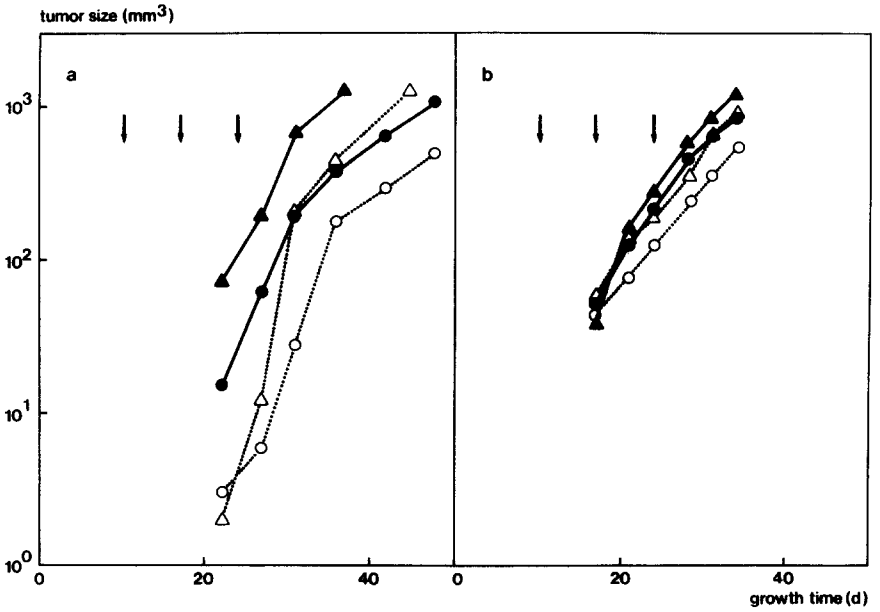

Fig. 1. Effects of tamoxifen and cyclophosphamide treatment on the growth of mouse mammary tumor transplants. (a) Hormone-responsive tumors; (b) hormone-independent tumors. Treatment with cyclophosphamide (△----△), tamoxifen (●—●), cyclophosphamide plus tamoxifen (○----○). Arrows indicate days on which treatment was given. Untreated tumors (▲—▲). Each point denotes the average size of 12 tumors.

The data presented in Table 1 show that in the untreated control group (Group A) the outgrowths had to be harvested after only 37 days because the tumors had already attained a significant size by that time. In contrast, outgrowths from the treated groups B, C and D could be harvested 8–11 days later. Statistical analysis revealed that the tumor weights from the tamoxifen plus cyclophosphamide treated group were significantly lower than those from the groups treated with tamoxifen alone ($P=0.002$) or cyclophosphamide alone ($P<0.001$).

Hormone-independent tumors

Similar studies were carried out on hormone-independent mouse mammary tumors. Average growth curves of untreated tumors (Group E) were compared with those of tumors treated with cyclophosphamide (Group F), tamoxifen (Group G), and tamoxifen plus cyclophosphamide (Group H).

After 31 days of growth, tumors treated with cyclophosphamide, tamoxifen, and the combined treatment had 27, 33 and 57% inhibition, respectively, compared to untreated controls (Fig. 1b). Statistical analysis of the growth curves revealed that combined treatment with tamoxifen and cyclophosphamide caused significant growth inhibition compared to untreated controls on days 21, 24, 28, 31 and 34 of tumor growth.

All the tumors were harvested on day 34; the weights of the outgrowths are listed in Table 1. Treatment of the tumors with tamoxifen alone or cyclophosphamide alone caused only a slight growth inhibition compared to untreated controls ($P=0.006$ in both cases). The weights of tumors submitted to combined treatment were only slightly lower than those of untreated controls ($P<0.001$). The combined treatment was slightly more effective than tamoxifen alone ($P=0.025$) or cyclophosphamide alone ($P=0.018$).

Effect of tamoxifen on ER in vitro

In order to investigate whether tamoxifen might act on mouse mammary tumor growth by blocking the estrogen receptor in the tumors, we looked whether this blocking effect could be demonstrated *in vitro*. Portions of mouse mammary tumor cytosols were incubated for 16 hr in the cold with 2 nM [^{3}H]estradiol in the presence or absence of 0.02, 0.2 or 2 μM tamoxifen. Unbound [^{3}H]estradiol was removed with Dextran-coated charcoal, and the radioactivity of the supernatant assayed. The binding of [^{3}H]estradiol under these conditions was compared to that obtained in the presence of 2 μM nafoxidine. Table 2 shows the results of this experiment. Increasing amounts of tamoxifen caused increased inhibition of [^{3}H]estradiol binding in the cytosols of hormone-responsive mouse mammary tumors. Of interest was the finding that tamoxifen also blocked the residual amounts of estrogen receptor present in the cytosols of hormone-independent mouse mammary tumors.

Table 2. Inhibition of [^{3}H]*estradiol binding in mouse mammary tumor cytosols by tamoxifen* in vitro

Tumor type	N*	ER†	Tamoxifen concentration (μM)	Percentage inhibition‡
Hormone-responsive	8	21.7	0.02	26.9±0.7
			0.2	67.9±3.7
			2.0	75.9±4.8
Hormone-independent	7	8.2	0.02	66.7±4.9
			0.2	145.2±12.9
			2.0	65.5±3.4

*Number of tumors assayed.

†Average estrogen receptor content of the tumors (fmoles per mg cytosol protein).

‡Percentage inhibition of that obtained with 2 μM nafoxidine. Average values±S.D. Assay in 10 mM Tris–HCl, 1.5 mM EDTA buffer, pH 7.4.

DISCUSSION

That tamoxifen treatment inhibited the growth of hormone-responsive mammary tumors in mice was surprising in view of the report that tamoxifen is estrogenic in the mouse [11], and therefore might be expected to stimulate tumor growth in these animals rather than inhibit it. However, our results show that tamoxifen can block the estrogen receptor of mouse mammary tumor cytosol *in vitro* (Table 2), and reports from other laboratories state that tamoxifen also suppresses the binding of [^{3}H]estradiol in the mouse uterus and vagina [11]. That tamoxifen also inhibited hormone-independent tumors in our present study, albeit to a lesser extent than it did hormone-responsive tumors, was unexpected in view of data in the literature indicating that tamoxifen acts on mammary tumors by blocking the estrogen receptor [5, 12–14]. The data presented in Table 2 show that the low amounts of estrogen receptor present in hormone-independent mammary tumors of GR mice could be blocked by adding tamoxifen *in vitro*. We therefore think it likely that the hormone-independent mouse mammary tumors used by us in the present study, still contained low amounts of hormone-dependent cells, and that these were blocked by the tamoxifen treatment. In subsequent studies (not reported here) in which tamoxifen treatment was given to hormone-independent tumors from later transplant generations (which had lower ER contents), the drug did not give any growth inhibition at all.

The hormone-responsive mouse mammary tumors investigated in the present study were more sensitive to tamoxifen and cyclophosphamide than the hormone-independent tumors. This result cannot, however, be taken to imply that *all* hormone-responsive mammary tumors are more sensitive to these drugs than *all* hormone-independent mammary tumors. In fact we have found previously that there is no relationship between estrogen receptor content of mouse mammary tumors and their growth inhibition by cyclophosphamide treatment [4]. In general, rapidly growing mouse mammary tumors appear to be inhibited by cyclophosphamide administration to a larger extent than slowly growing tumors [4]. Recent studies indicate that the concentrations of certain susceptible subpopulations of cells in the tumors influence the sensitivity of these tumors to chemotherapy [15]. These subpopulations change during serial transplantation and outgrowth of the transplants. The sensitivity of mouse mammary tumors towards tamoxifen may also depend on these factors.

Our results with mouse mammary tumors might be of use in explaining discrepancies between some published data on the effects of chemotherapy on human breast cancer. Lippman *et al.* [16] reported that hormone-independent breast tumors in women generally respond better to chemotherapy than hormone-responsive tumors, but this has been refuted by other groups [17]. A recent consensus at the National Institutes of Health concluded that there was no clear evidence that responses to chemotherapy correlate with the presence or absence of estrogen receptor [18].

In view of the heterogeneity of mammary tumors we have proposed that combined endocrine treatment and chemotherapy of breast cancer patients might be more effective than either treatment alone [4]. In this way, not only the hormone-dependent, but also the independent cells in the tumor might be prevented from growing out. Our present results on mouse mammary tumors lend support to this notion. In the low-risk indolent breast cancers where sufficient time exists to analyse the various endocrine and cytotoxic sensitivities of the tumor, the appropriate time to apply chemotherapy would be at the stage where hormone-independent cells first start to emerge in the tumors. Perhaps low-dose chemotherapy would suffice to destroy the small amount of autonomous cells present at this stage. In women with high-risk life-threatening metastatic patterns, it might be advisable to add chemotherapy to endocrine treatment at the most early stage possible, before the hormone-independent cells can grow out to form an autonomous tumor mass.

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