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Peroxidase Activity in Mammary Tumors—Effect of Tamoxifen

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Abstract—*Dimethylbenz(a)anthracen (DMBA)-induced tumors in rats were studied for changes in size, cytosolic estrogen (ER) and progesterone (PgR) receptors, and for peroxidase activity before treatment, after 15 days of treatment with the anti-estrogen tamoxifen, and 15 days following cessation of the treatment. Mean size of the tumors decreased significantly during the treatment period to 1/3 the original size and ER and PgR decreased significantly to 1/5 their original levels. Peroxidase activity increased 30-fold. All the changes reversed significantly 15 days after cessation of tamoxifen treatment. These results are consistent with a previous study in which regression of the tumors was induced by castration and recovery was brought about by treatment with estradiol. In DMBA-induced mammary tumors in rats, peroxidase activity in regressing and growing tumors is in inverse relation to changes in tumor size, ER and PgR, which are accepted indicators of estrogenic activity.*

INTRODUCTION

EVEN THOUGH most human breast cancers, which contain receptors for estradiol, regress after endocrine therapy, about one-third do not react to this treatment [1]. Yet, there is no satisfactory way to assess in advance which of the tumors will not react to endocrine treatment. It was recently found that estrogens induce the production of progesterone receptors both in uterine tissue [2] and in human breast cancer [3], which suggested the possible use of progesterone receptors as makers for estrogenic activity [3]. Indeed, it was shown that the presence of progesterone receptors in human breast cancer significantly improved the chance that the tumors would respond to endocrine treatment [1].

The enzyme peroxidase was recently shown to be dependant on estrogenic activity in uterine tissue of various species [4–6] and was suggested as a possible additional marker for tumor response to endocrine treatment. In a previous study from our laboratory, we examined in a model of estrogen sensitive dimethylbenz(a)anthracen(DMBA)-induced mammary tumors in rats [7] the relations between peroxidase activity and well-established estrogenic markers such as growth, cytosolic estradiol and progesterone receptors in estrogen sensitive tissues. We found that, in contrast to the situation in uterine tissue, there was an inverse relation between the changes in peroxidase activity and the changes in size of the tumors and their estradiol and progesterone receptor content. The study model used castrated rats which were initially given no treatment and were subsequently injected with estradiol.

In the present study we extend our observations and present our results on the relations between peroxidase activity and the cytosolic steroid hormone receptors in the DMBA-induced tumors in rats on antiestrogen treatment with tamoxifen. The results confirm our previous observation that tumors in regression show high peroxidase activity that reverses after cessation of the anti-estrogenic insult.

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MATERIALS AND METHODS

Materials

Guaiacol was obtained from Sigma Chemical Company, St. Louis, MO. 17β-estradiol (2,4,6,7,(*n*))^{3}H, 85 Ci/mmole, was obtained from Radiochemical Centre, Amersham,

England. R-5020 (17,21-dimethyl-19-norpregna-4, 9-diene-3, 20-dione), 17, 21-dimethyl ^3H, 80–90 Ci/mmole, was obtained from New-England Nuclear, Boston, MA, and estrogen and progesterone from Ikapharm, Ramat Gan, Israel.

Animals and DMBA-induced tumors

Fifty-day-old female Sprague–Dawley rats were given 20 mg DMBA in soya oil by gastric intubation. Palpable tumors, which appeared 1–4 months after induction, were measured weekly by calipers and introduced into the study when their surface area approximated 1–2 sq. cm. Only 1–3 tumors per rat were allowed to grow, the others being surgically excised.

Study protocol

Rats bearing tumors of appropriate size were biopsied (with removal of between 50–150 mg of tissue) and subsequently separated into a tamoxifen-treated group and a control group. Tamoxifen was dissolved in an aliquot of absolute alcohol and propylene glycol; 200 μg were injected subcutaneously daily. Control rats were injected with the vehicle alone. Tumors were measured at 3-day intervals. The end point for this phase of the study (the tamoxifen treatment phase) was defined at the end of 15 days. At this end point a second biopsy was obtained. Twenty-four hours after the last injection, the tumors were followed for another 15 days (the 'no treatment phase').

Biopsy material

Biopsies were obtained surgically under light ether anesthesia and were immediately frozen and kept at −70°C. On the day of the assay, biopsies were weighed, homogenized and examined for peroxidase activity, cytosolic estradiol and progesterone receptors. All biopsies from the same tumors were assayed on the same day.

Homogenization, cytosol preparation for receptor assay and extraction of peroxidase activity

All procedures were performed at 0–4°C. Frozen tissue was weighed, thawed at 0–4°C and homogenized in an all-glass homogenizer in 2.5 ml of TED buffer (0.01 M Tris–HCl, 1.5 mM EDTA, 0.5 mM dithiothreital, pH 7.4). Cytosol was obtained by centrifugation for 35 min at 35,000 *g* in a Sorvall RC-2 centrifuge. Prior to the receptor assay the cytosol was treated for 10 min with a pellet of 1 vol. of Dextran Coated Charcoal (DCC), 250 mg Norit A and 2.5 mg Dextran in 100 ml buffer (10 mM) Tris buffer, pH 8.0). The supernatant obtained after centrigugation at 2000 *g* was used for estradiol and progesterone receptor assay. The precipitate from the 35,000 *g* centrifugation was examined for peroxidase activity. The enzyme activity was extracted by homogenization in an all-glass homogenizer in 1.5 ml buffer (0.5 M $CaCl_2$ in 10 mM Tris buffer, pH 7.2). After centrifugation at 35,000 *g* for 35 min, the supernatant was used for the guaiacol assay.

Cytosolic Estradiol and Progesterone Receptors

Cytosolic receptors were measured by the DCC technique, as previously described [8, 9]. For the measurement of ER, cytosol was incubated with 2 nM radioactive estradiol in the presence and absence of an excess of unlabelled estradiol (40 nM) at 0°C for 18 hr. This technique measures the available estrogen receptor sites, and results were expressed as pmol estrogen bound per g tumor.

Cytosolic progesterone receptors were also measured by the DCC technique [8, 9]. Forty percent glycerol in TED buffer was added to 150 μl of the above cytosol to obtain a final concentration of 10% glycerol in TED buffer. The cytosol was incubated with 12 nM of radioactive R-5020 for 18 hr. Parallel incubations with an excess of unlabelled hormone (1.2 μM) were also performed. The specific binding was calculated by subtraction. This technique measures the total progesterone binding sites as pmol progesterone bound per g tumor. A single saturating dose assay for both ER and PgR gave results similar to those obtained with a 5–7 point Scatchard plot analysis [7].

Peroxidase assay

Peroxidase activity was assayed by measuring the increase in absorbance at 470 nm due to the oxidation of guaiacol [5, 10]. The assay mixture contained 0.3 mM H_2O_2 and 13 nM guaiacol in a total volume of 3.0 ml of the extraction buffer (10 mM Tris–HCl, pH 7.2, containing 0.5 M $CaCl_2$). The optimal concentration of H_2O_2 and guaiacol were validated again for the tumor tissue. The assays were performed at 25°C and were started by the addition of the enzyme. Initial rates were measured over the first 2 min of the reaction. A unit of peroxidase activity was defined as the amount giving an initial rate of 1 absorbance unit 1 min under the assay conditions.

Dithiothreitol, at the concentration used in the TED buffer for the cytosol preparation for receptor study, did not affect the assay

significantly. Addition of 2 mM *N*-ethylmaleimide to the extraction reaction buffer did not affect the peroxidase activity found in the tissue [8]. Peroxidase activity in the biopsy tissue was stable for at least one month at −70°C (results not shown).

Statistics

The statistical significance of difference between various experimental values was assessed by use of the paired *t* analysis.

RESULTS

Tamoxifen treatment was followed by a significant decrease in tumor size from 2.4 ± 0.5 cm^2 (mean ± S.E.M.) before treatment to 0.8 ± 0.16 cm^2 ($P < 0.005$) after 15 days of treatment (Fig. 1). There was a concomitant decrease in ER from 2.5 ± 0.4 pmole/g tumor to 0.5 ± 0.04 pmole/g tumor ($P < 0.001$) and a decrease in PgR from 9.4 ± 2.4 pmole/g tumor to 1.85 ± 0.5 pmole/g tumor ($P < 0.01$). Peroxidase activity increased from 2.2 ± 1.1 units/g tumor to 86.4 ± 16.9 units/g tumor ($P < 0.001$).

Fifteen days after cessation of tamoxifen treatment, tumor size increased to 1.5 ± 0.3 cm^2, which represents a significant change in respect to mean size at the end of tamoxifen treatment ($P < 0.05$). ER increased significantly to 1.6 ± 0.6 ($P < 0.05$) and PgR increased significantly to 4.9 ± 1.0 pmole/g tumor ($P < 0.02$). Peroxidase activity decreased significantly to 21.5 ± 7.8 units/g tumor at the end of the recovery period. The data concerning the control tumors are shown in Table 1. A slight decrease in ER and PgR was noted comparing the first and the third biopsies. However, only the change of ER was statistically significant ($P < 0.05$). A modest increase in peroxidase activity was noted as well ($P < 0.05$).

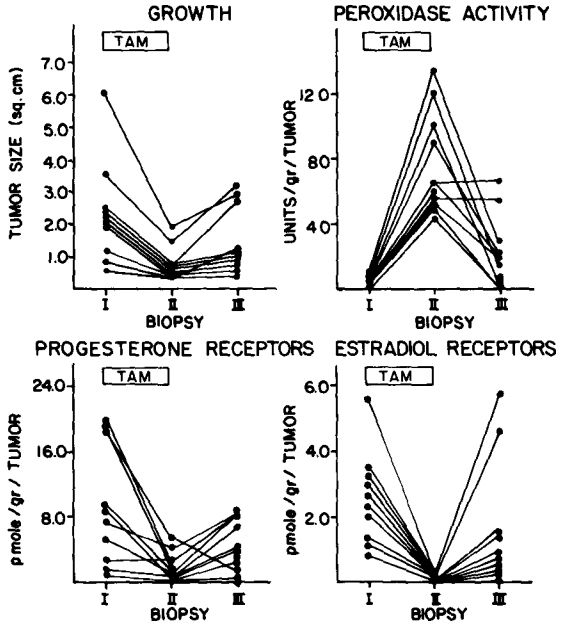

Fig. 1. Tumor growth, cytosolic estradiol and progesterone receptors and peroxidase activity in DMBA-induced tumors. Biopsy I indicates observations just before the beginning of treatment with tamoxifen. Biopsy II indicates observations after 15 days on tamoxifen and biopsy III indicates observations 15 days following cessation of tamoxifen treatment.

DISCUSSION

Tamoxifen is a well established and effective anti-estrogenic drug. Previous studies demonstrated its ability to induce regression in both DMBA-induced mammary tumors in rats [11] and in human breast cancer [12]. The anti-esterogenic property is attributed to a decrease in estrogen-binding sites [11, 13], possibly due to 'defective' processing of the receptor–estradiol complex, which is required for continuous esterogenic stimulation [14]. Tamoxifen is known to induce a prolonged retention of estradiol receptors on the nucleus, as well as to reduce estrogen-induced prolactin secretion. Prolactin itself is a potent inducer of mammary tumors in the rat and is important for their maintenance [15].

In the present study we extend previous observations [7] on peroxidase activity in DMBA-induced mammary tumors in rats and its relation to growth pattern and to estradiol and progesterone receptors. The induction of tumor regression, either by castration or anti-estrogen treatment, induces a marked increase in peroxidase activity, which establishes an

Table 1. Sequential biopsies in control rats

	Size (cm^2)	Peroxidase (units/g tumor)	PgR (pmole/g tumor)	ER (pmole/g tumor)
1st Biopsy	1.94 ± 0.23	0.7 ± 0.5*	10.1 ± 1.6	3.5 ± 0.4* $n = 15$
2nd Biopsy	1.94 ± 0.23	5.0 ± 3.2	10.3 ± 1.7	3.0 ± 0.4 $n = 15$
3rd Biopsy	1.64 ± 0.26	7.9 ± 3.4*	7.8 ± 1.2	2.2 ± 0.4* $n = 11$

DMBA-induced tumors in intact rats were sequentially biopsied at 15 day intervals. Pattern of change in growth, peroxidase activity ER and PgR are depicted.

*$P < 0.05$.

inverse relationship between the changes in peroxidase activity and the changes in the classical markers of estrogenic activity: tumor growth and the receptors for estradiol and progesterone. It should be noted that the apparent lack of growth of the control tumors between the first and second biopsies is in part related to the actual biopsy process, which removed up to 15% of the tumor. Indeed, it is possible that recurrent surgical interventions in themselves affected the tumors, bringing about the decrease in estradiol receptors and evaluation of peroxidase activity. However, these changes (peroxidase activity increasing to 7.9 units/g tumor and estradiol receptors falling to 2.2 fmole/g tumor) are negligible compared to the study results (peroxidase activity up to 86.4 units/g tumor and estradiol receptors down to 0.5 fmole/g tumor) following tamoxifen treatment. The study results cannot be explained by the influence of the surgical procedure alone (Table 1).

A similar situation was found in our study of peroxidase activity and cytosolic estrogen and progesterone receptors in human breast cancer [16]. In this study, too, we were able to demonstrate an inverse relationship between the tumor content of estrogen receptors and peroxidase activity.

The difference in peroxidase reaction to estrogenic stimulation in the rat uterus, where it acts as a marker for estrogenicity, and in the tumors remains to be explained. It is possible that peroxidase-like activity in the uterus and in the mammary tumors differ both in the nature of the enzyme moiety and in its metabolic role. Preliminary data has already been gathered in our laboratory and is now in preparation.

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