Influence of Hormonal Alteration of Host on Estrogen-binding Capacity in 7,12-Dimethylbenz(a)anthracene-induced Mammary Tumors

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SUMMARY

The estrogen-binding capacity of mammary tumors induced by 7,12-dimethylbenz(a)anthracene was measured in lesions from animals after either ovariectomy, deprival of insulin (diabetes), or treatment with lergotroline mesylate to inhibit prolactin secretion. The average estrogen-binding capacity was 30 fmoles/mg cytosol protein in growing or static carcinomas from intact (control) animals. A significant reduction in estrogen-binding capacity was observed in regressing but not static mammary tumors from ovariectomized animals. In regressing and static tumors from diabetic rats, estrogen-binding capacity was significantly lower than in lesions from intact animals; this effect was not seen in growing tumors from diabetic rats. Tumors that were growing or static in lergotroline-treated animals showed reduced capacity to bind labeled estradiol. The effects of duration of hormone treatment or time of tissue storage on estrogen-binding capacity were examined and did not appear to be correlated with the decreased binding in tumors from treated animals. The results suggest that hormones capable of producing altered neoplastic growth may influence the level of estrogen receptors.

INTRODUCTION

Hormone target tissues possess specific receptor molecules, and the interaction of the hormone with its receptor is thought to be an early and essential event for initiation of response. The presence of estrogen receptors in breast cancer tissue has been studied rather extensively; it is now well documented that absence of specific estrogen-binding capacity in human breast carcinomas usually predicts for failure to respond favorably to hormonal therapy (20). Considerably less information exists regarding those factors influencing the regulation of estrogen receptors in neoplastic tissue.

Administration of DMBA to rats will produce multiple mammary carcinomas, and these tumors can be classified as hormone dependent (11, 12). Growth of most of these tumors will cease after ovariectomy or hypophysectomy, demonstrating dependence of the tumor on ovarian steroids and/or pituitary hormones; a major role of estrogen and prolactin has been implicated repeatedly (11, 12, 21, 22). On this basis, some recent studies have explored the effects of prolactin on estrogen receptor levels in DMBA-induced tumors. Vignon and Rochefort (29) reported that administration of prolactin to ovariectomized rats bearing DMBA-induced tumors resulted in an elevation in estrogen-binding capacity in tumors. Similar results were reported by Leung and Sasaki (15), who extended these studies by also investigating the role of estrogen. They demonstrated that estrogen was needed for growth of these carcinogen-induced tumors; replenishment of estrogen-binding capacity was not achieved when an antiestrogen, nafoxidine, was administered concomitant with prolactin.

We recently confirmed the observation by Heuson et al. (5, 6) that insulin is required for growth of DMBA-induced tumors (3). Further, studies in our laboratory demonstrated that the levels of cyclic adenosine 3':5'-monophosphate were elevated in regressing carcinomas, regardless of the hormonal manipulation (ovariectomy, inhibition of prolactin secretion, or diabetes) that brought about the decrease in tumor mass (17). It seemed appropriate, therefore, to examine the estrogen-binding capacity of DMBA-induced tumors from hormone-modified hosts and to examine the data relative to tumor growth, i.e., regressing, static, or growing. The results presented here indicate that reduced estrogen-binding capacity was observed in growing and static tumors from animals that were treated with lergotroline mesylate (inhibits prolactin secretion), in static and regressing tumors from diabetic rats, and in regressing tumors from ovariectomized animals.

MATERIALS AND METHODS

Tumor Induction. Fifty-day-old Sprague-Dawley rats were intubated with DMBA as previously described (8). Tumors arose within 8 to 10 weeks after the last intubation; measurements with calipers in 2 perpendicular dimensions were performed 2 to 3 times/week. Tumor area was calculated by the product of the 2 perpendicular measurements. Hormonal manipulations of the hosts utilized were bilateral ovariectomy, induction of diabetes with streptozotocin, as described earlier (3), or inhibition of prolactin secretion by

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2 To whom requests for reprints should be addressed.
3 The abbreviation used is: DMBA, 7,12-dimethylbenz(a)anthracene.
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Classification of Tumor Growth. The 10-day period before sacrifice of each animal was the time chosen for classification of tumor growth. Tumors were classified as growing (>20% increase), regressing (>20% decrease), and static (<20% change). All tumors were characterized as adenocarcinomas after histopathological examination.

Estrogen-binding Capacity Assay. Tumor-bearing rats were sacrificed by cervical dislocation. Excised tumor tissue was frozen immediately in liquid nitrogen and stored at –86°. Tissues for assay were thawed on ice, weighed, and minced, and a 25 to 33% homogenate was prepared using a Tris-EDTA-sucrose buffer, pH 8.0 (0.01 M Tris-HCl, 0.001 M EDTA, and 0.25 M sucrose), as diluent. Homogenization was performed on ice for 10 sec at a setting of 6, using a Polytron homogenizer (Brinkman Instruments, Lucerne, Switzerland). The homogenate was then centrifuged at 105,000 × g for 30 min to obtain a cytosol fraction. Incubation vials (0.5-dram glass; Kimble Glass Co., Toledo, Ohio) were prepared in triplicate with the addition of labeled 17β-estradiol (17β-2,4,6,7-[3H]estradiol; 90 Ci/m mole; New England Nuclear, Boston, Mass.). Usually, 5, but sometimes as many as 8, different concentrations of labeled ligand (1 to 4 nM) were used to obtain total binding for each cytosol. Nonspecific binding was determined by addition of 1000-fold excess of unlabeled 17β-estradiol along with the labeled ligand. Steroids were air-dried prior to addition to the cytosol preparation. The dextran-coated charcoal method of Korenman (13) was used with minor modifications; in these assays the dextran-coated charcoal was prepared using 0.5% Norit A (Pfanstiehl Chemical Co., Waukegan, Ill.) and 0.05% dextran (Schwarz/Mann, Orangeburg, N. Y.) and was suspended in the Tris-EDTA-sucrose buffer. To each incubation vial, 0.2 ml of the cytosol preparation was added and allowed to incubate at 0-3° for 16 to 18 hr (overnight). Reactions were stopped by addition of 1.0 ml of the dextran-coated charcoal mixture to each vial, followed by incubation for 10 min at 0°. The vials were centrifuged at 1500 rpm for 10 min at 4°, and 0.6 ml of the supernatant was carefully removed and transferred to scintillation vials containing 10 ml Bray’s fluor. Radioactivity in the vials was measured in a liquid scintillation counter (Isocap 300; Nuclear Chicago, Chicago, Ill.). The final value for specific estrogen-binding capacity of each cytosol was determined by subtracting the nonspecific binding from the total binding. These data were then analyzed according to the method of Scatchard (23), using linear regression analysis to obtain the best-fitting straight line. Data are presented as femoles (10⁻¹⁵ mole) of 17β-[3H]estradiol specifically bound per mg cytosol protein. Protein determinations were performed on a portion of the original cytosol preparation of each sample, using the method of Lowry et al. (16).

Some data appear in the tables as the mean ± S.E. Significance was determined by use of the Student t test, with a p value of 0.05 or less being considered as significant.

RESULTS

Characterization of Specific Estrogen-binding Capacity in Tumors. Using the dextran-coated charcoal method for determining estrogen binding, a typical result is illustrated in Chart 1. Specific binding, which is the difference between total bound and total bound in the presence of 1000-fold excess unlabeled 17β-estradiol demonstrated saturation at 3 to 4 nM. Representation of these data according to the method of Scatchard (23) yielded a straight line (from linear regression analysis). The affinity of the receptor for estradiol was ~1.2 × 10⁻⁹ m; the specific binding capacity for this tumor was calculated to be 35 femoles/mg cytosol protein. Specificity of binding of estradiol was measured by competition experiments, using a variety of unlabeled steroids at a 1000-fold excess in the presence of 17β-[3H]estradiol. These results, presented in Table 1, show that the other estrogens studied, estrone and estriol, were effective competitors and inhibited binding of the labeled ligand by 90% or greater. In contrast, none of the other steroids examined was capable of inhibiting binding of 17β-estradiol; 5α-dihydrotestosterone demonstrated marginal competi-

Table 1

<table>
<thead>
<tr>
<th>Steroid added</th>
<th>17β-[3H]Estradiol bound (fmoles/mg protein)</th>
<th>Competition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>23</td>
<td>0</td>
</tr>
<tr>
<td>Estradiol-17β</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Estrone</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Estriol</td>
<td>2</td>
<td>91</td>
</tr>
<tr>
<td>Progesterone</td>
<td>23</td>
<td>0</td>
</tr>
<tr>
<td>Triamcinolone acetonide</td>
<td>23</td>
<td>0</td>
</tr>
<tr>
<td>Hydrocortisone</td>
<td>23</td>
<td>0</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>23</td>
<td>0</td>
</tr>
<tr>
<td>Testosterone</td>
<td>23</td>
<td>0</td>
</tr>
<tr>
<td>5α-Dihydrotestosterone</td>
<td>20</td>
<td>12</td>
</tr>
</tbody>
</table>

* Steroids (unlabeled) were added at 1000-fold excess of 17β-[3H]estradiol (2.5 nM).
tion. In another experiment, ovine prolactin was used as a potential competitor for estradiol binding; prolactin did not compete for binding. Thus, these data agree well with several previous reports on estrogen binding in DMBA-induced tumors (2, 12, 15, 18, 19).

**Estrogen-binding Capacity in Tumors from Hormone-deprived Hosts.** Tumors classified as growing, static, or regressing were analyzed for specific estrogen-binding capacity. These results are illustrated in Chart 2 and are presented for each tumor in each category. Growing or static tumors in intact (control) animals showed similar levels of estrogen binding, with a combined mean (± S.E.) of 30.1 ± 3.1 femoles/mg cytosol protein (1917 ± 245 femoles/gm tumor). However, the mean estrogen-binding capacity for all tumors from ovariectomized (16.8 ± 2.1 femoles/mg cytosol protein; 1158 ± 131 femoles/g tumor), diabetic (13.9 ± 2.4 femoles/mg cytosol protein; 866 ± 131 femoles/g tumor), and lergotrine-treated (14.3 ± 1.9 femoles/mg cytosol protein; 913 ± 134 femoles/g tumor) animals was significantly reduced compared to that obtained in tumors from intact animals. These data were further analyzed according to tumor growth characteristics. As illustrated in Chart 2, neoplasms in diabetic rats that were classified as static (8.3 ± 2.4 femoles/mg protein) or regressing (8.5 ± 0.7 femoles/mg protein) demonstrated significantly lower specific estrogen-binding capacity as compared to growing or static tumors from intact animals, as well as compared to the unresponsive (insulin-independent or growing) lesions (25.0 ± 3.4 femoles/mg cytosol protein) in the insulin-deprived hosts; these differences were significant (p < 0.05) when binding capacity was expressed on either a protein basis or per g tumor. Although we are aware that the number of responsive tumors in each category in diabetic rats was small, the values for specific binding were quite similar and did not overlap with any of those values from intact animals or with those in insulin-independent lesions.

The average estrogen-binding capacity of neoplasms from lergotrine-treated animals was significantly lower than that found in lesions from intact animals. There were several tumors from the drug-treated animals that had levels of specific estrogen binding equal to growing or static lesions in untreated rats. However, levels of estrogen binding below those obtained in tumors from untreated animals were observed in 6 of 11 growing and 4 of 7 static tumors from lergotrine-treated animals. Unfortunately, we did not have sufficient tissue from regressing tumors for analysis. A comparable distribution of estrogen-binding capacity was obtained in tumors from ovariectomized animals, although it should be noted that the number of tumors assayed was smaller than in the other groups. Thus, removal of endogenous insulin or inhibition of the secretion of prolactin appeared to influence the capacity for DMBA-induced tumors to bind labeled 17β-estradiol.

**Distribution of Tumors by Growth Classification.** The criterion used here for classifying tumors was based on changes in area, calculated by measurement of each lesion with calipers. Table 2 summarizes the average change for carcinomas categorized according to growth characteristics. These data show that the changes in size, as estimated by caliper measurements, were quite similar in each of the treatment groups.

**Influence of Duration of Treatment on Estrogen-binding Capacity.** The foregoing classification of tumors was based on the growth characteristics for the 10-day period prior to sacrifice of the animal. These data were also analyzed to determine whether the overall duration of treatment of the host would influence the level of estrogen binding. This was done because the rapidity of tumor response was somewhat variable; i.e., tumor regression was apparent at earlier times after ovariectomy than after induction of diabetes. The data
presented in Table 3 do not demonstrate that estrogen-binding capacity was related to the duration of treatment. However, most carcinomas were obtained within 30 days after hormonal modification of the host. This study does not preclude that estrogen-binding capacity might be altered at longer time intervals after removal of endogenous hormones.

**Effect of Storage Time on Estrogen-binding Capacity.** Some of the tumors used for the present study were obtained during an earlier experiment and had been stored at −86°C. Since it is well accepted that estrogen receptors are subject to degradation, we compared results on estrogen-binding capacity of tumors according to the date of assay and the date that the animal was sacrificed. The majority of assays were performed within 60 days from the date that the carcinoma was excised. As shown in Table 4, no clear-cut trend in the relationship between estimated estrogen-binding capacity and storage time was seen. Low estrogen-binding capacity was measured in regressing tumors from diabetic rats at each of the time intervals; reduced receptor levels were observed in growing or static carcinomas from lergotrine-treated rats. In addition, portions of 1 large tumor, classified as growing in an intact host, were assayed at 1, 12, 21, and 97 days after freezing; estrogen-binding capacity was estimated as 18, 26, 28, and 20 fmoles/mg cytosol protein, respectively. Thus, although it may be advisable to perform the assay as soon as possible (31), the data presented here indicate that the lower receptor level in some of the carcinomas was not due simply to lability.

**DISCUSSION**

The data presented here demonstrate that estrogen-binding capacity of DMBA-induced carcinomas was altered by subjecting the host to a variety of hormonal treatments. This conclusion is based on the presumption that the estrogen-binding capacity at the time of hormonal alteration was in the same range as that measured in tumors from intact animals. This assumption is supported by the data showing an overall decrease in estrogen-binding capacity obtained in regressing tumors from ovariectomized and diabetic rats, as well as the lower values seen in carcinomas from animals treated with lergotrine mesylate. Furthermore, the apparent lack of relationship between decreased binding capacity and duration of therapy, or time of tissue storage, supports the conclusion that the hormonal manipulations were responsible for the change in estrogen receptors.

The hormonal modifications used here were chosen on the basis of response of these tumors; regression of neoplasms was anticipated (3, 5, 11, 21, 22). However, reports from several laboratories and our own have indicated that response to a single hormonal manipulation may vary. In any group of tumor-bearing animals, not all lesions will regress; some will continue to grow, while others will become static. This also has been observed for intact, untreated animals (1, 3, 28). Because of this, it was advisable to analyze the data according to the growth characteristics of each lesion prior to sacrifice of the animal. When using this approach, as reported by us earlier (3), it was found that
estrogen-binding capacity was similar in tumors classified as growing or static from intact rats. The values obtained, 15 to 60 fmoles/mg cytosol protein, agree with other reports. The procedure used here for measurement of specific estrogen-binding capacity is influenced by the relative amount of nonspecific binding, which is defined as that amount of binding that occurs in the presence of excess unlabeled competitor. To determine whether nonspecific binding was constant in tumors from each of the treatment groups, we calculated the amount of nonspecific binding (fmoles/mg cytosol protein) in each sample that occurred at the saturating level of ligand. Although this varied from tumor to tumor, the overall range was similar in each treatment group. Thus, the reported decrease in specific estrogen-binding capacity in the static and regressing tumors from diabetic rats, and in the tumors from lergotriole-treated animals, could not be attributed to an increase in nonspecific binding, which would in turn lead to a decrease in specific binding. Also, the values reported here for specific estrogen binding are similar to those reported for DMBA-induced tumors in intact rats when assayed by sucrose gradient analyses (2, 4, 18) or by dextran-coated charcoal technique (15, 29). No attempts were made to define the molecular species of estrogen receptors, but earlier reports have clearly demonstrated the presence of 8 to 9 S estrogen receptors in the cytosol (2, 18, 19). The data obtained here indicate ligand specificity for estrogen binding similar to that reported by others (31). However, it should be noted that the presence of cytoplasmic estrogen receptors does not assure ovarian dependence of mammary neoplasms (2, 26).

Examination of tumors from animals treated with lergotriole mesylate, a synthetic ergeline compound that inhibits secretion of prolactin by the pituitary (27), showed that estrogen-binding capacity was significantly reduced. The reduction in estrogen binding was seen in tumors classified as growing or static (although some tumors regressed during treatment, insufficient amounts of tissue were available for assay in this series of experiments). These results suggest that prolactin may play a role in maintaining estrogen-binding capacity of DMBA-induced carcinomas and could be interpreted to agree with the earlier reports (15, 29), indicating that administration of prolactin stimulates estrogen binding capacity. However, whether prolactin actually stimulates synthesis of estrogen receptor or inhibits degradation, or both, is not specifically known.

No ovary-independent neoplasms were examined in these studies. Although few in number, ovary-dependent lesions, those that regressed after ovariectomy, showed significantly lower estrogen-binding capacity than tumors from intact animals. The data, expressed as fmoles/g tissue, also showed a lower binding capacity in ovary-dependent tumors compared to that obtained in growing tumors from intact rats; the values obtained, 1153 ± 157 fmoles/g tissue, compare favorably with a mean of 980 ± 245 fmoles/g tumor reported by DeSombre et al. (4) for hormone-dependent carcinomas. Only 2 carcinomas were classified as static; 1 tumor had 13 fmoles/mg cytosol protein and the other had 27 fmoles/mg cytosol protein. Since submission of this paper, 2 additional reports appeared, which confirm our findings. Vignon and Rochefort (30) demonstrated that estrogen receptor content decreased in DMBA-induced tumors after ovariectomy; regressing tumors contained about 10% of the level of cytosol receptors found in intact carcinomas. Holdaway and Friesen (10) reported that administration of bromoergocryptine (1.5 mg/kg/day for 3 weeks) resulted in a significant reduction of estrogen-binding capacity in DMBA-induced tumors; similar reductions were seen in hormone-responsive and hormone-independent tumors, although these groups were too small to permit adequate statistical evaluation. As reported here, ovariectomy or inhibition of prolactin secretion appeared to reduce the level of cytosol estrogen receptor, suggesting that alteration of the hormonal milieu of the host can lead to significant changes in estrogen-binding capacity.

Tumors from diabetic rats were obtained that satisfied each of the growth criteria used. The results in Chart 2 demonstrated that insulin-independent lesions, those that continued to grow in diabetic animals, had estrogen-binding capacity similar to that of tumors from intact animals. However, even with the small number of samples, regressing and static tumors from insulin-deprived animals demonstrated significantly lower estrogen-binding capacity. These results imply that estrogen-binding capacity may be influenced directly, by the presence of insulin, or, indirectly, as a reflection of the process of regression, i.e.,, dying or metabolically defective cells. In a preliminary report, Shafie and Brooks (24) indicated that the human breast cancer cell line MCF-7 showed a decline in estrogen receptor content when kept in hormone- and serum-free media for 18 days. Supplementation with insulin, however, restored the estrogen receptor level, and they concluded that insulin plays a role in the response of these cells to estrogen. In support of the latter suggestion, the activation of lysosomal enzymes occurring during early stages of regression (14, 25) may enhance breakdown of intracellular proteins, and the protein nature of estrogen receptors may make them susceptible to such degradation. The fact that estrogen receptor capacity was not decreased in growing tumors from diabetic rats lends support to the latter proposal that insulin may act indirectly rather than directly. Previous work on alterations in enzyme activities at various times after ovariectomy also showed that certain enzymes decreased earlier than others (7, 9). Thus, any hormonal modification of the host that leads to regression of tumor mass may result in the loss of specialized cellular components, 1 of which is estrogen-binding capacity.

Data were also obtained in these experiments relating to stability of estrogen binding in tissue samples stored for extended periods of time. We did not observe any definite trend indicative of decreased estrogen-binding capacity apparently due to tissue storage at —86°. Low levels of estrogen binding were obtained in regressing tumors from diabetic animals assayed soon after sacrifice of animals or at 270 days later. In addition, no apparent relationship between levels of estrogen binding and duration of hormonal treatment was seen. Unfortunately, the design of the experiments here could only provide us with a measurement of
estrogen-binding capacity at 1 point in time and, as such, does not provide any information as to the prognostic value of such a measurement as related to response.

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