Comparison of Macromolecular Binding of Estradiol in Hormone-dependent and Hormone-independent Rat Mammary Carcinoma

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SUMMARY

In rat mammary carcinoma, 2 types of cytoplasmic estradiol-binding sites, A and B, are present. The A sites are excluded on Sephadex G-100 chromatography, sediment at 8 S and 4 S in a low-ionic-strength sucrose gradient or at 4 S alone in a high-ionic-strength sucrose gradient, and form an insoluble complex with protamine sulfate. They have a very high affinity for estradiol (K_d ~ 1.2 x 10^{-10} M) and do not bind nonestrogenic steroids. The B sites are not seen on Sephadex G-100 chromatography, sediment at 4 S in a sucrose gradient, do not interact with protamine sulfate, and exhibit much weaker binding (K_d ~ 1 x 10^{-7} M) than the A sites. Only hormone-dependent tumors exhibit the highly specific A sites, while B sites are present in both hormone-dependent and autonomous tumors. Specific binding of estradiol within the nucleus is confined to hormone-dependent tumors.

INTRODUCTION

Huggins et al. (3) in 1961 described a carcinogen-induced mammary carcinoma in the rat which responded to endocrine gland ablation in a manner similar to human breast cancer. The hormone-dependent variety of these tumors has been shown to have an affinity for estrogen both in vivo and in vitro (4, 6, 10, 13, 16). More recent work demonstrates the rapid intranuclear localization of estrogen and suggests that specific estrogen-receptor molecules are present in the cytoplasm of these tumors (7).

This study was designed to compare the intracellular binding of estradiol in unequivocally proven hormone-dependent and hormone-independent experimental mammary tumors in order to understand better the mechanism by which hormones regulate tumor growth.

MATERIALS AND METHODS

Determination of Hormone Dependence. Female Sprague-Dawley rats, 50 days old, were given 20 mg of dimethylbenzanthracene (Eastman Organic Chemicals, Rochester, N. Y.) by gastric instillation. When mammary tumors appeared, size was monitored by frequent measurements with calipers. Rats with tumors regressing after oophorectomy were given 5 μg of 17β-estradiol (2) daily s.c. If the tumor began to grow again, it was considered hormone dependent. If the tumor failed to regress after oophorectomy or then failed to grow after estradiol administration, it was considered autonomous.

Preparation of Cytoplasmic Extracts. Rats were given i.p. injections of 0.1 μg of estradiol-17β-6,7-3 H (40 Ci/m mole), 0.5 hr prior to cervical dislocation. The tumors were rapidly excised, debrided of capsule and connective tissue, and cooled to 5° for all subsequent steps. They were homogenized in approximately 2 volumes of 0.01 M Tris-HCl, pH 7.6, containing 0.3 M KCl, over a 60-min period with intermittent homogenization of the pellet with a glass homogenizer. The supernatant remaining after centrifugation of this material at 105,000 X g for 60 min contained the soluble nuclear extract.

Kinetic Analysis of Estradiol Binding. Aliquots (0.2 ml) of nonradioactive cytosol diluted to approximately 1 mg/ml protein concentration with homogenization buffer were incubated with increasing quantities of estradiol-17β-6,7-3 H (0.05 to 2 pmole/0.25 ml final volume) for 16 hr at 5°. A 0.5-ml mixture of 0.25% Norit A and 0.0025% dextran in 0.01 M Tris-HCl, pH 7.6, was added to the incubation mixture for an additional 15 min. The suspension was centrifuged at 600 X g for 10 min, and the supernatant radioactivity, which represented the bound estradiol, was measured (8). The data were plotted according to the method of Scatchard (14). In certain experiments, the supernatant after the DCC treatment was incubated with protamine sulfate to form an insoluble protamine-estradiol-binding protein complex (15).

1 This work was supported in part by USPHS Grant CA 11378 and American Cancer Society Grant T-510.

2 The trivial name used is: 17β-estradiol, estra-1,3,5,(10)-triene-3,17β-diol. The abbreviation used is: DCC, dextran-coated charcoal.
Sephadex G-100 Chromatography. Samples (3 ml) of cytosols were applied to Sephadex G-100 columns (2.5 x 25 cm), equilibrated, and eluted at 10 ml/hr with 0.01 M Tris HCl, pH 7.6. Fractions (5 ml) were collected, and 0.5-ml aliquots were taken for radioactivity and absorbance measurements. The fractions corresponding to the macromolecular effluent were determined with blue dextran.

Sucrose Density Gradient Analysis. A 0.2-ml cytosol fraction was applied to a 5 to 20% sucrose gradient and centrifuged in a Spinco SW 50.1 rotor for 8 to 12 hr at 40,000 to 50,000 rpm. The bottoms of the tubes were pierced, and 7-drop fractions were collected for radioactivity measurements. Sedimentation values were assigned relative to the migration of a bovine serum albumin standard.

Miscellaneous. Estradiol-17β-6,7-3H was obtained from New England Nuclear (Boston, Mass.) and used without further purification. All reagents were analytical grade. Protein was quantitated by the method of Lowry et al. (9). Radioactivity was measured in a Beckman Biosolve mixture in an ambient-temperature liquid scintillation counter.

RESULTS

Our original intention was to examine estradiol binding in all mammary tumors in the rat colony. This proved to be impossible because when tumor growth charts were studied, 3 patterns appeared: (a) unequivocally hormone-dependent tumors, (b) unequivocally hormone-independent tumors, and (c) tumors which behaved in an equivocal fashion. This latter group displayed various growth patterns such as growing to a certain size, then plateauing or spontaneously regressing. Occasionally, they would regress after ovariectomy but fail to grow after estradiol. In addition, certain tumors appeared suitable for use from their growth curves but were found to contain significant amounts of hemorrhage or necrosis which prevented their use. Therefore, the data in this report are confined to those tumors which were unequivocally hormone-dependent or independent and were free from significant hemorrhage or necrosis. Representative growth curves can be seen in Chart 1.

Macromolecular Cytoplasmic Binding of Estradiol. The results of gel filtration chromatography of a cytosol from a hormone-dependent tumor are shown in Chart 2. The major portion of estradiol was located in the macromolecular protein fraction excluded from the column (Fractions 9 to 13) as determined by blue dextran. A smaller amount of unbound estradiol was retarded on the column. Cytosols from 13 hormone-dependent tumors have been examined by this technique with identical results. By contrast, no significant binding of estradiol to the macromolecular fraction of the autonomous tumors was found (Chart 3). As an additional control, rat muscle cytosol was prepared and chromatographed under identical conditions; binding of estradiol to macromolecules was not demonstrated. These experiments show that, in hormone-dependent mammary carcinoma, macromolecular binding is responsible for the majority of estradiol found in the cytoplasm.

Identification of 2 Species of Cytoplasmic Estradiol-binding Macromolecules. To further characterize the macromolecular binding of estradiol, a cytosol from a hormone-dependent tumor was applied to a 5 to 20% sucrose gradient (Chart 4). The radioactive peak near the bottom of the gradient (Fraction 8) corresponds to an S value of 8 to 9, and the peak at Fraction 15 corresponds to an S value of 4 to 5. In the presence of 0.3 M KCl, the estradiol migrates only in the 4 to 5 S region suggesting that the 8 S binding site has a salt-dissociable 4 S binding component. In contrast to the hormone-dependent cytosols, the cytosols from autonomous tumors never contain the 8 S binding site, yet appreciable 4 S binding may be observed if the cytosol is incubated with excess estradiol-3H in vitro (Chart 5B). Thus, whereas the 8 S component seems specific for hormone-dependent tumors, 4 S binding can be found in both hormone-dependent and independent tumors.

Steggles and King (15) observed that protamine sulfate could form an insoluble complex with specific estradiol receptors but not with estradiol bound to nonspecific proteins. We evaluated the 4 S binding site in both hormone-dependent and hormone-independent tumors with this technique. In Chart 5A, protamine has completely precipitated the 8 S region suggesting that the 8 S binding site has a salt-dissociable 4 S binding component. In contrast to the hormone-dependent cytosols, the cytosols from autonomous tumors never contain the 8 S binding site, yet appreciable 4 S binding may be observed if the cytosol is incubated with excess estradiol-3H in vitro (Chart 5B). Thus, whereas the 8 S component seems specific for hormone-dependent tumors, 4 S binding can be found in both hormone-dependent and independent tumors.
autonomous tumor cytosol lacks the 8 S site, and the 4 S site is minimally affected by protamine. It appears, then, that in hormone-dependent tumors a large part of the 4 S binding is also specific for estradiol but that in the autonomous tumors the 4 S binding is nonspecific.

**Kinetic Analysis of Estradiol Binding.** Another method of distinguishing specific from nonspecific estradiol binding is to compare the affinity of the steroid for the various binding molecules. We added increasing amounts of estradiol-$^3$H to a constant amount of tumor cytoplasm, using the DCC method to separate bound from free estradiol. The values were plotted according to the method of Scatchard (14), yielding an estimate of the dissociation constant ($K_d$) of the binding reaction. The $K_d$ derived from Chart 6 was $1.2 \times 10^{-10}$ M. This relatively high degree of binding affinity (low $K_d$) suggests specific estradiol-macromolecular interaction and distinguishes this binding from nonspecific binding of steroid molecules to proteins such as albumin (12). The $K_d$'s of our autonomous tumors have all been greater than $1 \times 10^{-7}$ M, whereas our hormone-dependent tumors range from $3.3 \times 10^{-9}$ to $1.0 \times 10^{-10}$ M.

**Steroid Specificity.** In order to show that the binding sites are specific for estrogen molecules, we have tried to inhibit the estradiol-binding reaction (DCC method) with a variety of other steroids (Table 1). In concentrations 1000 to 10,000-fold greater than that of the added estradiol-$^3$H, the nonestrogenic steroids, 11ß, 17a, 21-trihydroxyprogestagen-4-ene-3,20-dione, 17ß-hydroxyandrost-4-en-3-one; pregen-4-en-3,20-dione; 5ß-androstane-17ß-ol-3-one failed to inhibit significantly estradiol binding. Very low concentrations of...
Chart 4. Sucrose gradient 5 to 20% centrifugation of an in vitro-labeled hormone-dependent mammary tumor cytosol in the presence (○) or absence (♦) of 0.3 M KCl. Conditions are described in the text.

Chart 5. Sucrose gradient 5 to 20% centrifugation of an in vitro-labeled mammary tumor cytosol before (♦) and after (○) protamine sulfate precipitation. A, hormone-dependent tumor cytosol; B, autonomous tumor cytosol.

Chart 6. In vitro kinetic analysis of macromolecular binding of estradiol by a hormone-dependent mammary tumor cytosol. Conditions are described in the text. The dissociation constant is obtained by dividing the abscissa by the ordinate.

unlabeled 17β-estradiol successfully completed with 17β-estradiol-³H for the binding sites.

Nuclear Binding of Estradiol. The data thus far demonstrate a lack of specific cytoplasmic estradiol binding in the autonomous tumors. Since it is possible that nuclei might be able to concentrate estradiol by a mechanism independent of cytoplasmic receptors, we compared the nuclear uptake of estradiol administered in vivo in hormone-dependent and autonomous tumors.

Chart 7 shows estradiol radioactivity of a 0.3 M KCl nuclear extract from a hormone-dependent tumor migrating in the 5 S region of a 5 to 20% sucrose gradient. When autonomous tumors are treated identically, there is no intranuclear 5 S estradiol binding.

DISCUSSION

Our findings indicate that the cytoplasm of mammary tumors contains 2 types of estradiol-binding sites (Table 2). In the terminology of Erdos et al. (2), these are A and B. The A sites (a) are excluded on Sephadex G-100 chromatography, (b) sediment at 8 S and 4 S in low-ionic-strength sucrose gradients and 4 S in high-ionic-strength sucrose gradients, (c) form an insoluble complex with protamine sulfate, and (d) are characterized by unusually high affinity binding (low Kₐ). These A sites are found only in unequivocally hormone-dependent mammary tumors.
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Table 1

Inhibition of estradiol binding in hormone-dependent mammary carcinoma

Hormone-dependent cytosol (0.1 ml) was incubated at 4° for 30 min with the indicated quantities of nonradioactive steroids. 17β-Estradiol-3H (0.2 pmole) was then added for an additional 30 min. Bound radioactive estradiol was determined by the DCC method.

<table>
<thead>
<tr>
<th>Competitor</th>
<th>pmoles</th>
<th>% of estradiol-3H bound after competitor preincubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>17β-Hydroxyandros-4-en-3-one</td>
<td>200</td>
<td>88</td>
</tr>
<tr>
<td>Pregn-4-ene-3,20-dione</td>
<td>2000</td>
<td>92</td>
</tr>
<tr>
<td>11β, 17α, 21-Trihydroxy pregn-4-ene-3,20-dione</td>
<td>1721</td>
<td>100</td>
</tr>
<tr>
<td>5β-Androstan-17β-ol-3-one</td>
<td>2000</td>
<td>95</td>
</tr>
<tr>
<td>17β-Estradiol</td>
<td>2</td>
<td>12</td>
</tr>
</tbody>
</table>

Table 2

Cytoplasmic estradiol-binding sites in mammary carcinoma

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Site A</th>
<th>Site B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Excluded by Sephadex G-100</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Protamine precipitable</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Sucrose gradient</td>
<td>8 S, 4 S</td>
<td>4 S</td>
</tr>
<tr>
<td>Kinetic analysis, Kα =</td>
<td>~1.2 x 10^-10 M</td>
<td>&gt;1 x 10^-7 M</td>
</tr>
<tr>
<td>Structural specificity for estrogens</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Nuclear binding dependence</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Hormone-dependent tumor</td>
<td>Yes</td>
<td>Yesb</td>
</tr>
<tr>
<td>Autonomous tumor</td>
<td>No</td>
<td>Yes</td>
</tr>
</tbody>
</table>

a B sites with respect to estradiol binding could show specific binding with other steroids.
b If A sites are saturated.

The B sites are not apparent on G-100 Sephadex chromatography, possibly because the affinity of the estradiol for the Sephadex is greater than for the B site. The B sites sediment at 4 S in sucrose gradient, are not precipitable by protamine sulfate, and exhibit a dissociation constant several orders of magnitude higher (lower affinity) than the A sites. The B sites can be demonstrated in hormone-dependent tumors if the estradiol concentration is high enough to saturate the A sites. In the autonomous tumors, only B sites are found. These data indicate that B site binding of estradiol is nonspecific. The presence and absence of a nuclear 5 S estradiol-binding protein in hormone-dependent and autonomous tumors, respectively, suggest that the presence of the A site is prerequisite to the specific intranuclear localization of estradiol in mammary tumors.

The presence of A sites does not necessarily mean that estradiol is responsible for the growth of hormone-dependent tumors. It does indicate that hormone-dependent tumors have retained certain biochemical characteristics of normal estradiol target tissue which permits normal hormonal regulation of growth to be superimposed on the basic malignant growth potential of the cancer cell. There is a growing body of evidence that demonstrates estrogen control of prolactin secretion by the rat pituitary (1). It has been suggested that this might be the primary influence of estrogen in mammary carcinoma growth and that these tumors are primarily prolactin dependent (5, 11). This hypothesis would not explain the apparent correlation between the response of the
tumor to oophorectomy and the presence of specific cytoplasmic estradiol receptors. Further work on the relationship between estradiol and prolactin at the cellular level must be done to clarify this problem.

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REFERENCES

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