Progesterone Receptor Levels in Estrogen-induced Renal Carcinomas after Serial Passage beneath the Renal Capsule of Syrian Hamsters

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ABSTRACT

Elevated concentrations of progesterone receptor, a sensitive indicator of estrogen responsiveness, have been measured in this study during early serial transplantation of estrogen-induced renal adenocarcinoma tissue in hamsters. A new approach of tumor transplantation beneath the renal capsule has been used. Fragments of primary tumor tissue transplanted subpannicularly in estrogen-treated hamsters increased less than 10% in mass during a 2-month period; however, transplantation of primary tumor tissue beneath the renal capsule resulted in a 20- to 30-fold increase in tumor mass after 2 months. There was no statistically significant difference in tumor concentration of progesterone receptor after the first or second serial passage relative to the concentration found in primary tumor tissue. Although the concentration of progesterone receptor was not significantly decreased after the second serial passage, there was a slower growth rate of tumors during this period. In addition to the reduced growth rate of the tumors, a lower concentration of progesterone receptor was found in untransformed kidney areas of hosts bearing second-serial-passage tumors relative to those with first-passage or primary tumors. The serum concentration of diethylstilbestrol in tumor-bearing hosts was 2 to 4 x 10^-4 M and apparently was not a factor in the change in growth rate of the tumors. The results are discussed in terms of a possible alteration in invasiveness or surface properties of estrogen-induced renal adenocarcinoma cells during early transplantation.

INTRODUCTION

Prolonged estrogen treatment of the Syrian hamster induces transformation of the proximal convoluted tubules of the kidney into adenocarcinoma, the growth of which is also estrogen dependent (3, 4, 6-9). The tumor remains estrogen dependent through serial transplant passages (2, 3, 4, 6, 7, 9) but acquires varying degrees of autonomy after the 12th to 20th serial passages (5). High-affinity receptors for both estrogen and progesterone are present in the renal primary tumor (13, 14). In other target tissues for estrogen, the concentrations of both progesterone receptors and estrogen receptors are under estrogenic control (10, 21). During estrogen-induced tumorigenesis in the hamster kidney, the concentration of progesterone-binding sites in kidney cytosol increases within 2 months after the initiation of hormone treatment (14). This change appears much earlier than does the incidence of tumor formation at 6 months or more and is the earliest biochemical change that has been detected in the kidneys of estrogen-treated hamsters (11, 17). The measurement of progesterone-binding sites is a useful probe to examine estrogen responsiveness (10, 18). Therefore, a study was made to determine whether progesterone receptor concentrations changed as a function of serial passage of tumor tissue, a process associated with gradual estrogen autonomy. We have examined the concentration of progesterone-binding sites in tumor tissue after serial transplantation of the estrogen-induced primary renal adenocarcinoma. A novel approach of tumor transplantation beneath the renal capsule has been used successfully and has resulted in growth rates of the first hormone-dependent passages that considerably exceed those obtained with the subpannicular transplants used by other investigators (5).

MATERIALS AND METHODS

Chemicals and Reagents. [1,2,6,7-3H]Progesterone (104 Ci/mmol), [3,4,6,7-3H]-17β-estradiol (85 to 105 Ci/mmol), and Biofluor were obtained from New England Nuclear, Boston, Mass. Unlabeled progesterone, estradiol, cortisol, Trizma base, Norit A, dextran 80, dithiothreitol, and bovine serum albumin were obtained from Sigma Chemical Co., St. Louis, Mo. Anhydrous glycerol was purchased from J. T. Baker Chemical Co., Phillipsburg, N. J. Zephiran was obtained from Winthrop Laboratories, New York, N. Y.

Animals and Tumor Induction. Young, mature castrated male Syrian hamsters (LVG:LAK, outbred strain, Charles River; Lakeview Hamster Colony, Newfield, N. J.) weighing 80 to 100 g were used. Pellets of diethylstilbestrol were administered as previously described (13). All animals were exsanguinated under ether anesthesia, and tissues were immediately placed on ice.

Tumor Transplantation. Tumor tissue was rinsed with sterile 0.9% sodium chloride solution, minced into 1- to 2-mm fragments, and stored on ice in sterile Petri dishes during the transplantation procedure. Nembutal anesthesia (50 mg/ml) was administered i.p. at a dosage of 0.15 ml/kg. All animals were exsanguinated under ether anesthesia, and tissues were immediately placed on ice.

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sule with microtweezers. The total weight of tumor tissue transplanted per animal was 50 to 60 mg. The implanted fragments were pushed to separate sites on the exposed side of the kidney. The kidney was then returned to its normal position, the muscle layer incision was closed with suture, and the skin layer incisions were closed with metal clips. Three animals used for renal transplants and 1 animal without a renal transplant were used to passage a comparable amount of tumor tissue subpannicularly.

**Preparation of Cytosol.** The tumor tissue was freed of necrotic or hemorrhagic areas, weighed, and homogenized with glass-glass conical grinders in 5 to 10 volumes of TED-BSA-buffer containing 30% glycerol, at 0°C. Tissue homogenates were centrifuged for 60 min at 105,000 × g, and cytosol fractions were diluted to appropriate protein concentrations with TED-BSA buffer containing 30% glycerol. Cytosol was added at a final concentration of 1 µM to prevent the binding of labeled progesterone to cortisol-binding globulin (10).

**Assay of Progesterone Receptor.** Cytosols were incubated with 4 × 10⁻⁹ M tritiated progesterone and, to correct for nonspecific binding, other samples of cytosol preparation containing labeled progesterone and a 100-fold excess of unlabeled progesterone were incubated. After incubation to equilibrium (2 hr) at 0°C, unbound steroid was removed by treatment with dextran-coated charcoal (20) for 5 min. The amount of specific radioactive progesterone bound to the cytosol receptor was measured and expressed as the concentration of progesterone-binding sites for 1 mg of protein per ml of cytosol. Binding-site concentrations were assayed at 2 or more protein concentrations in the linear range and at a saturating concentration of progesterone (i.e., 4 × 10⁻⁹ M). The assay range under these conditions was less than 10% of the mean.

**Radioactivity Measurement.** Four ml of Biofluor were added to 0.5 ml of cytosol, and radioactivity was measured in a Packard Model 3002 scintillation spectrometer at 34% efficiency for tritium.

**Miscellaneous.** The protein content of the cytosol was measured by the procedure of Lowry et al. (15) with bovine serum albumin as a standard. Appropriate corrections were made for the serum albumin added to the homogenization buffer.

**Radioassay Assay.** Serum (0.1 ml) from estrogen-treated hamsters was diluted to 1 ml with water, extracted with 6 volumes of ethyl ether, dried under nitrogen, and redissolved in 0.2 ml of isoctane:benzene:methanol (90:5:5, v/v). Estrogen was fractionated on a column (1.0 × 4.5 cm) of Sephadex LH-20 prepared in benzene:methanol (90:10, v/v). Recovery of estrogen was routinely 70% as determined by including 2000 cpm of [³H]estradiol in the serum samples. Estrogen was eluted with 100% methanol, dried under nitrogen, and redissolved in ethanol, and 20 to 40 µl were used in a radioassay assay to quantitate diethylstilbestrol. All operations for the radioassay assay were carried out at 4°C. Estrogen receptor was prepared from rat uterine tissue 48 hr after ovariectomy. Uteri were homogenized in TED-BSA buffer and centrifuged for 1 hr at 105,000 × g. The cytosol fraction was diluted with buffer to 0.3 to 0.5 mg of cytosol protein per ml. One-ml incubations contained 3 × 10⁻¹⁰ M [³H]estradiol and diethylstilbestrol standards to give 0.3 to 30 × 10⁻¹⁰ M or unknown concentrations of diethylstilbestrol in 20 µl of ethanol. After incubation to equilibrium (2 hr), unbound steroid was removed by dextran-coated charcoal treatment (19) for 15 min. An aliquot of the supernatant (0.5 ml) was used to measure bound radioactivity. The concentration of diethylstilbestrol in unknown samples was calculated from the competition for [³H]estradiol binding to the uterine receptor protein by standard diethylstilbestrol concentrations. The index of precision (λ) for the assay was 0.15, and the interassay coefficient of variance was 3.2%.

**Statistical Analysis.** The significance of differences among groups was tested with Student’s t test.

**RESULTS**

**Serial Transplantation of Primary Renal Tumors.** After fragments of primary renal adenocarcinoma (i.e., 50 to 60 mg/animal) were transplanted beneath the kidney capsule of estrogen-treated hamsters, no increase in tumor mass was detectable by palpation for about 1 month. By the end of the second month, however, the tumor tissue had increased in mass to 1.30 ± 0.27 (S.E.; n = 5) g, and much of the normal kidney was transformed into tumor tissue (Fig. 1). The tumor growth led to severe kidney dysfunction: the peritoneal cavity of animals bearing transplants became filled with fluid. The appearance of the kidneys was indistinguishable from the kidneys of animals with the primary renal tumors (16). In contrast, the tumor mass in animals with subpannicular transplants had increased less than 10% after 2 months. At the end of 2 months, hamsters with first-passage tumors beneath the renal capsule were sacrificed, and 50 to 60 mg of the tumor fragments were transplanted beneath the kidney capsules of estrogen-treated hamsters. No palpable tumors could be detected even 5 months after transplantation. When animals were sacrificed at the end of 5 months, no kidney dysfunction was apparent, and the tumor fragments remained relatively small (Fig. 2). The fragments had dispersed and spread beneath the renal capsule, and the mass of the tumor tissue was 0.26 ± 0.08 (n = 4) g.

**Measurement of Serum Diethylstilbestrol Concentrations in Hosts Bearing Primary and Serial-Passage Tumors.** For determination of whether a difference in circulating estrogen could account for the reduced growth of tumors after the second serial passage, the serum concentration of diethylstilbestrol was measured with a radioassay assay. As shown in Table 1, the mean serum concentration of diethylstilbestrol among groups ranged from 2 to 4 × 10⁻⁹ M and could not account for the difference observed.

**Concentrations of Progesterone-binding Sites In Cytosols of Primary and Serial-Passage Transplant Renal Adenocarcinoma.** Progesterone binding as a function of cytosol protein concentration was linear to approximately 0.5 mg of protein per ml of cytosol. The concentration of progesterone-binding sites in the primary renal tumors ranged from 0.3 to 4.0 × 10⁻⁹ M when expressed for 1 mg of protein per ml of cytosol; the mean concentration was

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3 The abbreviation used is: TED-BSA buffer, 0.01 M Tris-Cl, pH 7.5:1.5 mM disodium EDTA:1 mM dithiothreitol:0.1% bovine serum albumin.
Table 1
Concentration of serum diethylstilbestrol in hormone-treated hamsters

<table>
<thead>
<tr>
<th>Time after initiation of hormone treatment (mos.)</th>
<th>Serum concentration of diethylstilbestrol (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>23.2 ± 1.5 (3)†</td>
</tr>
<tr>
<td>2</td>
<td>35.1 ± 6.8 (3)</td>
</tr>
<tr>
<td>3–4</td>
<td>41.8 ± 4.9 (4)</td>
</tr>
<tr>
<td>10–11 (animals bearing primary renal tumors)</td>
<td>40.8 ± 5.6 (11)</td>
</tr>
<tr>
<td>3–4 (animals bearing first-serial-passage tumors)</td>
<td>18.4 ± 2.1 (5)</td>
</tr>
<tr>
<td>6–7 (animals bearing second-serial-passage tumors)</td>
<td>20.1 ± 3.5 (3)</td>
</tr>
</tbody>
</table>

† Numbers in parentheses, number of determinations.

1.80 ± 0.24 (n = 17) \( \times 10^{-9} \) M (Chart 1). When 3 separate nodules of a primary tumor from 1 animal were assayed separately for concentration of progesterone-binding sites, the values were 1.14, 1.44, and 1.80 \( \times 10^{-9} \) M. The mean concentration of progesterone-binding sites in tumors after the first serial passage was 1.83 ± 0.42 (n = 6) \( \times 10^{-9} \) M, a value nearly identical with that of the primary tumors. The mean concentration of binding sites in tumors from the second serial passage was 1.14 ± 0.17 (n = 4) \( \times 10^{-9} \) M when expressed for 1 mg of protein per ml of cytosol. The difference in concentration of progesterone-binding sites was not statistically significant (p > 0.05).

Progestosterone-binding-Site Concentrations in Cytosols of Nontumorous Kidney Areas in Hosts with Primary Tumors or Serial Passage Transplants. After removal of renal tumor tissue, the concentration of progesterone-binding sites in the cytosol of the remaining untransformed kidney of hosts with primary tumors was measured. This value was compared with the concentration of binding sites in kidney cytosols of animals treated for 3 to 4 months with estrogen prior to tumor development. The mean progesterone-binding-site concentration in cytosol from untransformed kidney areas of hosts with primary tumors was 0.23 ± 0.03 (n = 10) \( \times 10^{-9} \) M (Chart 2), relative to 0.045 ± 0.009 (n = 4) \( \times 10^{-9} \) M in kidney cytosol from animals treated for 3 to 4 months with estrogen. Cytosol from untransformed kidney areas of hosts with first-serial-passage tumors contained a mean progesterone-binding-site concentration of 0.15 ± 0.02 (n = 5) \( \times 10^{-9} \) M, a value similar to that in cytosols from regions of untransformed kidneys of hosts with primary tumors. However, the average binding site concentration in cytosol from untransformed kidney areas after the second serial passage was 0.05 ± 0.008 (n = 4) \( \times 10^{-9} \) M, a value similar to the binding site concentration in kidney cytosol after 3 to 4 months of estrogen treatment and approximately one-fourth to one-third lower (p < 0.01) than the concentration in the kidneys of hosts with primary of first-serial-passage tumors.

DISCUSSION

The presence of a high-affinity progesterone receptor (\( K_d = 10^9 \) M\(^{-1} \)) in the estrogen-induced primary adenocarcinoma of the hamster kidney was demonstrated previously (14). The concentration of progesterone receptors in the untransformed hamster kidney and in the uterus is under estrogenic control (10, 14). In the hamster kidney the earliest detectable biochemical change during tumorigenesis is an increased concentration of progesterone receptor by the second month of estrogen treatment (14, 17). The eventual loss in estrogen dependency of this particular tumor led us to ask if the concentration of progesterone receptor changed with serial passage of the tumors. Earlier studies showing that the hamster kidney contained specific estrogen-binding sites (12) suggested that these sites might be important in the estrogen-induced transformation and growth of renal tumors in hamsters. Therefore, serial passage of tumors beneath the kidney capsule was used rather

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than the subpannicular method used by other laboratories (5). With this technique first-generation tumors could be passaged after 2 months, whereas subpannicular transplants were passaged by other investigators after 1 year. Our results confirm that the growth of tumors transplanted subpannicularly was much slower than that obtained when tumors were transplanted beneath the renal capsule for the first serial passage. The growth of tumors after the second serial passage beneath the renal capsule was considerably reduced; even after 5 months the increase in tumor weight was relatively small. The factor(s) that accounts for this difference is unknown. The serum concentration of diethylstilbestrol in tumor-bearing hosts was very similar and suggested that differences in hormone levels among groups of animals could not account for the change in growth rate of the tumors.

The concentration of progesterone-binding sites in the cytosol of primary and transplant tumors was similar to that reported for the hamster uterus at estrus (i.e., $1.5 \times 10^{-9} \text{M}$ when expressed for 1 mg of protein per ml of cytosol) (10). The uterus, a target tissue for estrogens and progestins, contains the highest tissue concentration of these hormone receptors. Some tumors contained a concentration of binding sites exceeding the uterine concentration at proestrus (i.e., $3.0 \times 10^{-9} \text{M}$) (10). There was no statistically significant difference in the concentration of progesterone receptor sites of renal tumors after the first or second serial passage relative to the primary tumor. It appears that early passage of estrogen-induced renal adenocarcinoma into hormone-treated animals is not associated with significant changes in the concentration of progesterone-binding sites. It may be that a gradual change in binding site concentration occurs and would not be detected unless measurements of tumor binding site concentrations were made after the fifth or tenth serial passages. These studies are under way.

The progesterone-binding-site concentration in the untransformed portions of the kidneys of hosts with primary tumors was approximately one-tenth of the concentration in the primary tumor but about 4-fold higher than the concentration in cytosols of kidneys from hamsters after 3 to 4 months of estrogen treatment. This elevated binding site concentration is possibly due to microscopic foci of tumor cells, which exist in the "untransformed" tissue. The presence of similar concentrations of binding sites in the untransformed kidneys of hosts with first-generation renal transplants as with primary tumors might be the consequence of migration of tumor cells into nontumorous areas after transplantation rather than the presence of the tumor foci in the nontumorous regions as early as 4 months after estrogen treatment. The reduced growth of the tumors after the second serial passage was associated with low concentrations of progesterone-binding sites in the untransformed kidney of tumor-bearing hosts, and these were comparable to the concentration in the kidneys of animals after 3 to 4 months of estrogen treatment. The slower growth of the tumors after the second serial passage may then in some way relate to an invasive property or surface change in the tumor cells rather than to significant changes in hormone receptor site concentrations within the cells. The pattern of tumor growth observed in these studies did not appear to be expansive, with compression and atrophy of the surrounding tissue, but rather to be infiltrative, as described for certain tumor types (1, 19).

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REFERENCES

Fig. 1. Hamster kidneys bearing tumors 2 months after first serial passage beneath the renal capsule of estrogen-treated animals. Much of both kidneys is composed of tumor tissue. × 15.

Fig. 2. Hamster kidneys bearing tumors 5 months after the second serial passage beneath the renal capsule of estrogen-treated animals. The implanted tumor nodules have spread beneath the renal capsule, and relatively little tumor growth has occurred. × 15.
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