Localization of Estrogen Receptors in Interstitial Cells of Hamster Kidney and in Estradiol-induced Renal Tumors as Evidence of the Mesenchymal Origin of This Neoplasm

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ABSTRACT

The mechanism of estrogen-induced and -dependent kidney carcinogenesis in Syrian hamsters and the cell of origin of the tumor are not well understood; they have been investigated in this study by mapping the cellular locations of estrogen receptor (ER) in estrogen-dependent tumors, in kidney tissue of hamsters treated with estradiol for 0.5 and 5.5 months, and in kidneys of age-matched controls. To validate the methods used, receptors have also been localized in uteri of hamsters and rats and in female hamster kidneys. ERs have been identified in cryostat sections by immunocytochemical techniques using an affinity-purified ER antibody, ER-715. Nuclei of tumors were intensely stained for ERs. In estrogen-treated kidneys and in controls, ER protein was identified in interstitial cells and capillaries, in arteries, and in renal corpuscles, particularly in podocytes and in the parietal layers surrounding the renal corpuscles. There was no ER protein in tubular epithelia even when tubuli were surrounded by tumor cells. The ER distribution in female hamster kidneys closely matched that in male kidneys. However, the staining intensity was stronger in female than in male kidneys. In hamster uteri, there was an intense ER-positive reaction in the nuclei of stroma, in stromal vessels, and in the luminal epithelia as demonstrated previously by others in rat uteri. ER mRNA has also been demonstrated by Northern blot analysis in estrogen-treated kidneys which contained tumors but was undetectable in untreated kidneys. The localization of ERs in estrogen-dependent tumors and in interstitial cell types but not in tubular epithelia supports previous conclusions of an interstitial origin of estrogen-induced hamster kidney tumors.

INTRODUCTION

Estrogens are increasingly associated with human cancers and induce tumors in laboratory animals (1–4). In Syrian hamsters, the chronic administration of estradiol for 6–7 months induces kidney tumors which serve as a convenient animal model of hormonal cancer (4, 5). The origin of this tumor is controversial and has been examined in this study by mapping hormone receptors in tumors and in kidneys. Both epithelial and mesenchymal origins of these tumors have been proposed previously (6–9). Early histological examinations led to suggestions of an epithelial origin of kidney tumors arising from proximal or distal convoluted tubules (6, 7). Based on the morphology, enzyme histochemical patterns, and cytochemical and immunocytochemical demonstrations of intracellular intermediate filaments and extracellular matrix proteins of estradiol-induced hamster kidney tumors, a mesenchymal origin has been considered to be more likely, particularly because early proliferating foci and tumors expressed vimentin and desmin but not cytokeratin (8, 9). However, later stages of these neoplasms contained cysts lined with cells coexpressing cytokeratin and vimentin but no desmin (9). These cytokeratin-positive tumors were thought to arise by metaplastic transformation to an epithelial phenotype during tumor progression (9). However, this expression of cytokeratin in estrogen-induced hamster kidney tumors, in addition to vimentin and desmin, was also taken as evidence of an origin of the tumor from blastemal-like interstitial cells programmed to express an epithelial cell type (10, 11). In an attempt to resolve the controversies concerning the cell type from which tumors develop, we examined the localization of ERs in tumors and in kidneys of control and estrogen-treated hamsters. These experiments were expected to permit the identification of a population of ER-positive kidney cells from which these hormone-dependent neoplasms might arise.

ERs have been reported previously to be present in both cytosolic and nuclear preparations of estrogen-induced hamster kidney tumors (12–15). In addition, the location of estrogen-binding sites has been assayed by autoradiography in kidneys and in tumors of hamsters treated with injections of 3H-labeled estrogen (16, 17). Using this technique, Pantic et al. (16) reported an estrogen uptake by cytoplasm and some nuclei of proximal convoluted tubules and, to a lesser extent, by distal convoluted tubules of estrogen-treated hamster kidneys (16). In contrast, Tse et al. (17) reported the localization of 3H-labeled estrogen by autoradiography in nuclei of tumors but could not detect estrogen uptake in nonneoplastic cells (17). These discrepancies may have been caused by renal clearance processes of 3H-labeled estrogens, which occur in addition to any hormone binding to receptors and therefore distort the precise localization of estrogen binding. Therefore, the accurate mapping of estrogen-binding sites in kidneys of hamsters may be accomplished only by immunohistochemical demonstration of receptors in sections of hamster kidney. Such experiments have been carried out in our study in kidneys of male hamsters treated with estradiol for 0.5 and 5.5 months and in age-matched controls. The use of an estrogen receptor antibody has permitted the precise localization of ERs in tumors and in kidneys of hamsters. To validate this method, receptors were also localized in uteri of hamsters and rats and in kidneys of female hamsters.

MATERIALS AND METHODS

Treatment of Animals. Male Syrian hamsters (ages 4–6 weeks; Harlan Sprague-Dawley, Houston, TX) were housed in our animal facility with Purina rodent chow and water available ad libitum throughout the experiment. Eighteen animals received s.c. implants of estradiol (25 mg estradiol plus 10% cholesterol) as described previously (4, 5, 8, 9). Twelve of these hamsters received a second estradiol implant 3 months after initial treatment. A control group of 18 animals was left untreated. After 0.5, 5.5, and 7 months, six hamsters from each group were killed by decapitation, and their kidneys were excised, frozen, and stored in the same manner. The kidneys of each animal were quick-frozen in isopentane-liquid nitrogen at −140 to −150°C and stored in a −80°C freezer. Three untreated female Sprague-Dawley rats and three female hamsters were also killed, and their kidneys and uteri were excised, frozen, and stored in the same manner.

The abbreviations used are: ER, estrogen receptor; PPBS, potassium phosphate-buffered saline.

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Tissue Preparation for Immunocytochemistry. Sections 5–6 µm thick were cut on a cryostat (Jung Frigocut 2800E; Leica Instruments, Nussloch, Germany) at −20°C. The sections were mounted on previously silanized glass slides (18) and immediately fixed in 4% buffered formalin at room temperature for 10 min. The fixed sections were thoroughly washed with PPBS and kept for 5 min in methanol and then for 1 min in acetone at 4°C. The sections were further washed in PPBS for an additional 5 min and used directly for immunoocytochemical analysis or stored in a storage solution at −20°C (19). Kidney sections of estrogen-treated hamsters and of age-matched controls were mounted on the same slide to provide comparable cytochemical conditions.

Immunocytochemical Assay. Mounted sections were thoroughly washed in PPBS at room temperature for 5 min. Endogenous peroxidase activity was blocked after the application of the primary antibody by incubation in 100% methanol for 15 min followed by incubation in 0.03% hydrogen peroxide dissolved in PPBS for 30 min at room temperature (20). The slides were then washed with PPBS for 5 min. Nonspecific sites were blocked by covering sections with solutions of 1% bovine serum albumin (Sigma Chemical Co., St. Louis, MO) in PPBS for 30 min at room temperature. An affinity-purified ER antibody ER-715 (originally prepared by Dr. Jack Gorski and obtained from Dr. Koji Yoshinaga, NIH (21, 22)) was diluted (1:100) in 1% bovine serum albumin/PPBS. Sections were incubated in this antibody solution in a humidified chamber at 4°C for 18 h. Absorption control reactions were carried out by incubating the ER-715 antibody with a 100-fold molar excess of synthetic antigen (supplied together with ER-715 and originally used to raise the antisera) at 4°C for 18 h before applying the solution onto frozen sections (22).

After incubation of the sections with ER-715, slides were rinsed in PPBS three times (10 min for each rinse) and incubated with peroxidase-conjugated, affinity-purified F(ab’)2 fragment donkey anti-rabbit IgG (Jackson Immuno Research Laboratories, West Grove, PA) (1:50 dilution in PPBS) for 30 min at room temperature. Slides were then rinsed in PPBS three times (5 min for each rinse), and the staining was developed by incubating with 3,3’-diaminobenzidine tetrachloride/H2O2 (Sigma Chemical Co., St. Louis, MO) for 2–5 min. The 3,3’-diaminobenzidine tetrachloride/H2O2 was prepared according to the manufacturer’s recommendations. The slides were then rinsed in distilled water, dehydrated in ethanol/water baths with decreasing water content, and finally rinsed in xylene before being mounted using a permanent mounting medium. Rat and hamster uteri were used to validate the procedure (22). Control incubations were carried out by either preabsorbing the antibody against its antigen or by omitting the primary antibody.

RNA Preparation and Northern Blot Analysis. Total kidney RNA was prepared by the method of Chomczynski and Sacchi (23) using RNAzol B (Tel-Test, Inc., Friendswood, TX). Polyadenylated RNA was prepared from total RNA using an oligodeoxynucleotide-cellulose column (Pharmacia, Alameda, CA) according to the manufacturer’s recommendations. Samples of total RNA (10 µg) or polyadenylated RNA (4 µg) from hamsters treated with estradiol for 7 months and from controls were denatured for 30 min in 15 mM methymercuric hydroxide (Alfa Chemical Co., Salt Lake City, UT) and separated by electrophoresis on a 1% agarose gel containing 20% formaldehyde. Gels were stained with ethidium bromide, and ribosomal bands were viewed under UV light to ensure that a constant amount of RNA was applied to each lane. After destaining, RNA was transferred to Duralon (Stratagene, La Jolla, CA) by electroblotting (18 h; 500 mA in 25 mM sodium phosphate buffer, pH 6.5). Membranes were UV-cross-linked, dried, and prehybridized in a solution containing 50% formamide, 1.25% saline-sodium phosphate-EDTA, 0.05% Denhardt’s solution, 0.75% dextran sulfate, 0.01% sodium dodecyl sulfate, and 100 µg/ml heat-denatured salmon sperm DNA. The prehybridization was carried out for 6–8 h at 42°C. A 32P-labeled estrogen receptor complementary DNA probe (provided by Dr. G. M. Stancel, University of Texas Health Science Center, Houston, TX) was prepared using a 1.4-kilobase EcoRI-purified insert (24) and a multiprime DNA labeling system (Amersham, Arlington Heights, IL) according to the manufacturer’s recommendations. [α-32P]dCTP (3000 Ci/mmol) was obtained from ICN Radiochemicals, Irvine, CA. Hybridization was performed overnight at 42°C. The hybridization mixture was similar to the prehybridization mixture except that it contained 50 µg/ml of tRNA. After hybridization, blots were washed with 2X standard saline citrate, 0.1% sodium dodecyl sulfate (twice, 15 min at room temperature; one time, 15 min at 55°C), 0.2X standard saline citrate-0.1% sodium dodecyl sulfate (once, 15 min at 55°C) and exposed to X-ray film.

RESULTS

Tumors and Proliferating Foci. Kidneys of male hamsters treated with estradiol for 5.5 months contained tumors and proliferating foci as reported previously (4, 8, 9). Nuclei of tumor cells were intensely stained for ER protein in all areas of the tumor mass (Fig. 1). In addition to the nuclear staining, a positive reaction occurred also in filamentous structures surrounding tumor cell groups. ER-positive tumor cell nests often were located in close contact with renal arteries as had been observed previously (8, 9).

Early proliferating foci were also strongly ER positive (Fig. 2). Small foci often consisting of only a few tumor cells were detected in the renal interstitium and often were located close to renal arteries as has been recognized for larger tumors (Fig. 3). In the vicinity of one of these small tumor foci, three single, strongly ER-positive cells were detected in the renal interstitium and adjacent to a tubule (Fig. 3). These three cells may have been strongly ER-positive interstitial cells, proneoplastic cells, or early neoplastic cells.

When the ER-715 antibody was preabsorbed to its antigen and then applied to the sections, no immunoreactivity was observed for ERs in the tumor or in kidney tissue (Fig. 4), demonstrating specificity for the antigen. Moreover, nonspecific binding of the secondary antibody was not observed when the primary antibody was omitted from the immunocytochemical incubations (data not shown).

Kidneys of Estrogen-treated Hamsters. In kidney tissue of hamsters treated with estradiol for 5.5 months, no ER protein could be detected in proximal or distal tubuli (Figs. 1–3). Moreover, tubuli surrounded and enclosed by tumor were also ER negative (Fig. 1). Positive staining for ER protein was observed in the muscle layer as well as in the endothelium of renal arteries (Fig. 2), in vasa recta, and in arterioles, which were positive for ERs in pericytes and in endothelia (data not shown). In addition, interstitial cells (Fig. 3), interstitial capillaries, and renal corpuscles expressed ER protein (Fig. 5). Particularly intense staining was observed in parietal layers, podocytes, and capillary endothelia within renal corpuscles. No ER protein could be detected in any cell types of the renal papilla (data not shown). In kidneys of hamsters treated with estradiol for 15 days, intense ER-positive staining occurred in podocytes, cells of the parietal layers of renal corpuscles, endothelia of arteries, and interstitial cells and capillaries, as was observed in hamster kidneys after more prolonged estrogen exposure.

Untreated Kidneys and Uteri. ER localization in kidneys of untreated hamsters was comparable to that in kidneys of estrogen-treated

Fig. 1. Immunocytochemistry of a kidney tumor induced by treatment of male Syrian hamsters with 17β-estradiol for 5.5 months. Cryostat sections were incubated with ER-715 for 18 h at 4°C. Note the highly positive nuclear staining for tumor (T) and filamentous structures surrounding tumor cell nests, which partially or totally enclose ER-negative tubules (TB). × 148.
**ER LOCALIZATION IN HAMSTER KIDNEY AND TUMOR**

**Fig. 2.** Immunocytochemistry of the kidney of a male hamster treated with 17β-estradiol for 5.5 months. Cryostat sections were incubated with ER-715 for 18 h at 4°C. Proliferating foci (large arrowhead) were strongly ER positive. Also, arteries (A), cells of the vasa recta (small arrowheads), and interstitial cells (IC) stained for ER. X 367.

**Fig. 3.** Immunocytochemistry of the kidney of a male hamster treated with 17β-estradiol for 5.5 months. Cryostat sections were incubated with ER-715 for 18 h at 4°C. Note the positive staining for ER of three interstitial cells (arrowheads) and of tumor foci (arrow). X 367.

**Fig. 4.** Preabsorption control incubation of a kidney tumor induced by treatment of a male hamster with 17β-estradiol for 5.5 months. ER-715 was incubated for 18 h at 4°C with a 100-fold excess of the oligopeptide used to raise the antibody. This preabsorbed antibody was applied to the sections and then incubated for another 18 h. Note the negative reaction of tumor cells (TC) and of tubuli (TB). The aperture had to be narrowed to make structures visible. X 367.

**Fig. 5.** Immunocytochemistry of the kidney of a male hamster treated with 17β-estradiol for 5.5 months. Note the intense nuclear staining of the parietal layer (PL) and of podocytes (PC) of the renal corpuscle. Note also the absence of staining of tubules (TB). X 230.

**Estrogen Receptor mRNA in Kidney and Tumors.** Estrogen receptor mRNA expression was analyzed in RNA extracts of tumor-containing kidneys of hamsters treated with estradiol for 7 and 9 months, which contained increasing tumor masses with increasing treatment periods. ER mRNA was detected in tumors plus kidneys of hamsters treated with estradiol for 9 months using total RNA and in tumors plus kidneys of hamsters treated with estradiol for 7 months using polyadenylated RNA but not in untreated controls (Fig. 8). These data demonstrate that the content of ER message increases as the tumor RNA:total RNA ratio or tumor mass:kidney mass ratio increases. These data are in agreement with the immunocytochemical results of intense ER protein expression in the tumor.

**DISCUSSION**

Our results precisely localized ER protein in the nuclei of tumors and of several kidney interstitial cell populations but not in renal tubular epithelia of control or estrogen-treated hamsters. Our immunocytochemical experiments confirm the suspected radiolabeling of nuclei of tumor cells after injection of ³H-labeled estrogen into tumor-bearing hamsters (17). The binding of estrogen to cytoplasmic recep-

hamsters except that the latter were more intensely stained and thus had a higher receptor content than the controls. ER was expressed as described above in podocytes, mesangial cells, and cells of the parietal layers of the renal corpuscles and in interstitial cells and capillaries, whereas tubuli were devoid of any estrogen-binding protein (Fig. 6). ER protein was also present in blood vessels, such as intralobular arteries and vasa recta (data not shown).

For validation purposes, ER protein localization was also carried out in uteri of rats and hamsters and in kidneys of female hamsters. There was an intense ER-positive reaction in nuclei of stroma, stromal vessels, and luminal epithelia of hamster (Fig. 7) and rat uteri (data not shown). In addition, nuclei of longitudinal smooth muscle layers, intramural capillary endothelia, and cells of connective tissue septa contained ER protein (data not shown). The endothelia as well as the muscle layers of blood vessels were also strongly ER positive. These data in hamster uteri are in agreement with receptor protein localization in rat uteri (data not shown), which confirms results published previously (22).

The receptor distribution in kidneys of untreated female hamsters matched that in males, but the intensity of staining was higher than in male kidneys. Intense staining for ER protein occurred in renal corpuscles, in arteries and capillaries of kidneys, and in interstitial cells (data not shown).
which served as a positive control. 3 and 5) was prepared and analyzed for the ER transcript by blot analysis as described in (17).

estrogen by tubuli after tubular excretion of the hormone had peaked not surprising. The tumors depend on estrogen for growth and de-

Fig. 6. Immunocytochemistry of the kidney of a male hamster (untreated). Cryostat sections were incubated with ER-715 for 18 h at 4°C. Note the positive staining for ERs in the renal corpuscle (arrow), interstitial capillaries (ICP), and interstitial cells (IC) but absence of staining in the tubules (TB). × 230.

Fig. 7. Immunocytochemistry of a uterus of an adult untreated hamster. Cryostat sections were incubated with ER-715 for 18 h at 4°C. Note the intense staining of the nuclei of stroma, stromal vessels (SV), and luminal epithelium (LE). × 148.

Fig. 8. Northern blot analysis of ER-mRNA levels in animals treated with estrogen implants for 7 and 9 months. Total (Lanes 2, 4, and 6) and polyadenylated RNA (Lanes 3 and 5) was prepared and analyzed for the ER transcript by blot analysis as described in “Materials and Methods.” Lanes 2 and 3, RNA prepared from control kidney; and Lanes 4 and 5, RNA prepared from tumor-bearing kidney which had been exposed to estrogen implants for 7 months; Lane 6, RNA prepared from tumor-bearing kidney of a hamster treated with estrogen implants for 9 months; Lane 1, total RNA prepared from rat uterus which served as a positive control.

tors and tubular epithelial cells, as reported on the basis of a previous autoradiography study and of binding studies with cell fractions, may thus reflect diffusion artifacts (12–16). The absence of receptors in tubular cells, even after 5.5 months of chronic treatment with estradiol, also is in agreement with the low levels of uptake of 3H-labeled estrogen by tubuli after tubular excretion of the hormone had peaked (17).

The presence of ERs in estrogen-induced hamster kidney tumors is not surprising. The tumors depend on estrogen for growth and de-

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