Immunocytochemical Assay for Estrogen Receptors Applied to Human Prostatic Tumors

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Abstract

An immunocytochemical assay using a monoclonal antibody to estrogen receptor was applied to frozen sections of 43 prostatic tumors. In addition, in 16 tumors the results of the immunocytochemical assay were compared with those obtained using an enzyme immunoassay for estrogen receptor, a tritiated ligand binding assay, and a histochemical procedure using fluorescein-labeled estradiol conjugates. Specimens from 14 patients with benign prostatic hyperplasia and 29 patients with prostatic carcinoma were analyzed in assays in which human breast carcinoma specimens of high, medium, low, or negative estrogen receptor content acted as controls. The intensity of nuclear staining and the percentage of stained cells in the breast sample controls correlated well with estrogen receptor content as determined by both the enzyme immunoassay and the tritiated ligand binding assay. None of the fixed, frozen sections from the 43 prostatic tumors exhibited nuclear staining of either the benign or the malignant epithelial cells or of the stromal components. Negative results were also obtained with the tritiated ligand binding assay. The majority of prostatic samples were negative when using the enzyme immunoassay but four specimens had a mean value of 6.2 fmol/mg protein which is just above the sensitivity of the assay. Using fluorescein-labeled estradiol conjugates both cytoplasmic and nuclear binding was observed in the prostatic samples which did not correlate with the results obtained by the other three procedures.

Introduction

The physiological role of estradiol in the human prostate remains unresolved (1). This steroid has, however, been implicated in the development of BPH both from epidemiological evidence of an increase in the estradiol:androgen ratio with age (2, 3) and from experimental observations of the induction of canine prostatic hyperplasia after long term administration of 17β-estradiol and 5α-androstane-3α,17β-diol (4). Pharmacological concentrations of estrogen as administered in the treatment of prostatic carcinoma in contrast cause regression of certain prostatic components (5). This effect is probably not a direct action of estrogens on prostatic tissues but is mediated indirectly by reducing blood androgen supplies via the hypothalamic-pituitary-testicular axis (2).

Controversy exists as to the presence of a high affinity specific estrogen receptor in human prostatic tissues. Reports of estrogen binding in normal, benign, and carcinomatous prostate specimens vary considerably as to the concentration of estrogen receptor and the percentage of positivity even within the same pathologies (6–11). These discrepancies can be accounted for in part by methodological differences, but in addition the heterogeneous nature of prostate tissue, together with heat damage to many prostatic specimens, must affect substantially the estrogen receptor determinations.

The advent of estrogen receptor monoclonal antibodies has made it possible to examine both the presence and the location of this moiety in human breast tissue (12). In the prostate, assays for estrogen receptor using monoclonal antibodies offer distinct benefits over conventional ligand binding methods. These advantages are that the antigenic determinants are more stable than the steroid binding function, they are unaffected by endogenous steroid, and the presence of large amounts of other steroid binding proteins in this tissue will not interfere with these measurements. Predominantly stromal localization of estrogen receptor has been reported in the prostate (8, 13) and an immunocytochemical procedure should visually detect if this situation exists in unseparated specimens. In an alternative visual approach, fluorescein-labeled estrogen conjugates have been used to detect putative estrogen binding sites in breast and prostate tissues (14, 15). We have therefore undertaken to examine the presence and distribution of estrogen receptors in prostatic tumors using the ER-ICA (Abbott Laboratories) and the ER-EIA (Abbott Laboratories), a tritiated ligand binding assay, and a histochemical procedure utilizing fluorescein-labeled estradiol conjugates.

Materials and Methods

Tissues. Prostatic specimens were obtained from Tru-cut needle biopsy, open prostatectomy, or transurethral resection operations. Immediately on receipt, the sample was embedded in Tissue-Tek II O.C.T. Mountant (Miles Laboratories, Inc., Naperville, IL) prior to freezing in liquid nitrogen, and if sufficient material was provided another portion was snap frozen in liquid nitrogen and stored at −70°C for biochemical estrogen receptor analysis. Breast tumor tissue was rapidly frozen after excision and transported to the Tenovus Institute on dry ice for biochemical estrogen receptor assays, and an adjacent portion was embedded as for the prostate samples. The embedded tissue was either immediately sectioned at −25°C or stored at −70°C for no longer than 1 week prior to sectioning.

Reagents. The ER-ICA and ER-EIA kits were generously supplied by Abbott Laboratories, North Chicago, IL. 17β-[2,4,6,7-3H]Estradiol (93 Ci/mmol) was obtained from the Radiochemical Centre, Atherstone, Buckinghamshire, United Kingdom.

Immunocytochemical Assay. The ER-ICA kit uses a peroxidase-antiperoxidase technique for the visualization of estrogen receptors in frozen tissue specimens, using monoclonal antibody H222Sp7 directed against human estrogen receptor (12). In essence, frozen sections (5 μm) were thaw-mounted on adhesive-coated slides and immediately fixed with formaldehyde-PBS (3.7%) for 15 min at 25°C, PBS for 5 min at 25°C, methanol for 4 min at −20°C, acetone for 2 min at −20°C, and PBS for 5 min at 25°C. The immunocytochemical procedure was carried out at 25°C on duplicate sections and involved incubation with normal goat serum for 15 min followed, without prior washing, by the monoclonal antibody H222Sp7 or control antibody for 30 min. Incubations with goat anti-rat antibody for 30 min, peroxidase-antiperoxidase (rat origin) for 30 min, and diaminobenzidine-4 HCl-hydrogen peroxide substrate solution for 6 min were each followed by washing in PBS for 10 min. Light counterstaining of sections with hematoxylin was required to aid morphological identification and to estimate the number of unstained cells. Where appropriate, sections were analyzed in a semi-quantitative manner by determining the predominant staining intensity from 0 to 3+ and the percentage of stained tumor cells. In each assay, breast carcinoma specimens with high, medium, low, or
negative estrogen receptor content were used as controls.

**Enzyme Immunoassay.** The ER-EIA kit is an enzyme immunoassay using monoclonal rat antibodies to MCF-7 human breast cancer estrogen receptors (16). Tumor tissue was pulverized after immersion in liquid nitrogen and homogenized at 4°C in Tris-HCl buffer (10 mM, pH 7.4) containing EDTA (1 mM), monothioglycerol (1 mM), and glyceraldehyde 3-phosphate dehydrogenase. A cytosol fraction was prepared by ultracentrifugation of the homogenate (1–2 mg protein/ml) at 105,000 g for 1 h. Cytosol (100 µl) was incubated at 4°C for 18 h with an antibody (DS54)-coated polystyrene bead followed by incubation at 37°C for 1 h with a second antibody (H222). After incubation with o-phenylenediamine 2HCl-hydrogen peroxide substrate solution for 30 min, the absorbance was measured at 492 and 600 nm. Each of these steps was followed by washing in distilled water and the enzyme reaction was stopped by the addition of 1 M sulfuric acid. Estrogen receptor concentration was determined from a standard curve of absorbance versus estrogen receptor standards (0.0 and dilutions of 2.5 pmol/ml stock).

**Tritiated Ligand Binding Assay.** Estrogen receptor concentration was measured by incubation of cytosol overnight at 4°C with a range of 17β-[2,4,6,7-3H]estradiol concentrations (0.1–10 nM; specific activity, 93 Ci/mmol). A 1000-fold excess of diethylstilbestrol was used to correct for nonspecific binding. Free steroid was removed by adsorption on to charcoal (Tris-HCl, pH 7.4, containing Norit A (0.5% w/v), dextran T-70 (0.05% w/v), and gelatin (0.1% w/v)). Estimates of binding parameters were made using Scatchard analysis (17).

**Histochemical Procedure.** Conjugates of estradiol linked via position 6 to either BSA-FITC or fluoresceinamine were synthesized as reported previously (18). Frozen sections (5 µm) were incubated with the conjugates for 24 h at 4°C. Sections were then washed in phosphate-buffered saline and incubated for 30 min with the antibody, indicating that this was nonspecific. After thorough rinsing in buffer, sections were mounted and assessed microscopically for fluorescence (λ = 490 nm) on a semiquantitative scale.

**Results**

**Immunocytochemical Assay.** Frozen sections of a human breast tumor containing estrogen receptor (380 fmol/mg protein) were included in every assay, together with sections from breast tumors with either a medium or a low estrogen receptor content. Nuclear staining only was observed in these breast tumors, except in two cases in which ductal contents also stained; this was also seen with the control antibody on the duplicate sections. Good correlations were seen between the intensity of nuclear staining together with the percentage of stained cells and the results obtained using the biochemical assays for estrogen receptor. This is in agreement with the studies on a large number of breast samples by King et al. (12).

Prostate samples analyzed by ER-ICA consisted of 14 BPH specimens, 6 of which were from open prostatectomy and 8 of which were from transurethral resection operations. No stained nuclei were found in either the epithelium or the stromal cells of any of the specimens. A few foci of stained cells arising in the epithelium were seen in 3 specimens which were also observed on the duplicate sections incubated with the control antibody, indicating that this was nonspecific.

Prostatic carcinoma specimens examined by ER-ICA consisted of 4 Tru-cut needle biopsies and 25 specimens from transurethral resection operations. Specimens were obtained from 2 patients who had previously received endocrine treatment. One patient had received diethylstilbestrol (1 mg 3 times a day) for approximately 3 years and the other received the luteinizing hormone-releasing hormone (Zoladex; ICI, United Kingdom) for 2 months. The primary Gleason grading of the tumors included 1 grade 2, 6 grade 3, 6 grade 4, and 12 grade 5. None of these tumors displayed nuclear staining on frozen sections when using ER-ICA. No brown staining was seen in either the neoplastic cells or the stroma on the sections, which indicates a lack of estrogen receptor. A few small foci of stained cells were observed on 2 samples, both with the primary monoclonal antibody and the control antibody.

**Enzyme Immunoassay.** Six specimens of BPH and 10 prostatic carcinoma specimens supplied sufficient material to measure their cytosolic estrogen receptor content by enzyme immunoassay. Using this assay, 3 BPH and 1 prostatic carcinoma specimen had measurable estrogen receptor just above the cutoff point with a mean value of 6.2 fmol/mg protein. All of the other tumor specimens were negative.

**Trititated Ligand Binding Assay.** Cytosols from the same 6 BPH specimens and 10 prostatic carcinoma specimens were measured by Scatchard analysis using the tritiated ligand binding assay. Negative results were obtained in all 16 specimens.

**Histochemical Assay.** Consecutive frozen sections to those used in the ER-ICA assay were incubated separately with the estradiol-6-BSA-FITC and estradiol-6-fluoresceinamine conjugates. Assessment of fluorescence was carried out by scoring and determining fluorescence seen in cellular components. Nuclear staining of + to 2+ was detected in the epithelium of the BPH specimens after estradiol-6-BSA-FITC incubation. Cytoplasmic staining of epithelial cells and the stromal matrix was seen after incubation with the estradiol-6-fluoresceinamine. Essentially similar locations and intensities of staining were observed with all of the carcinoma specimens. No prostate samples gave negative results with this procedure.

**Discussion**

The good correlation observed among the immunocytochemical assay, the enzyme immunoassay, and the tritiated ligand binding assay in human breast samples used in this study was consistent with previously reported data (12). This methodological agreement was also seen with the prostate specimens except that in 4 of 16 tumors, estrogen receptor could be detected just above the cutoff point of the ER-EIA, whereas both the trititated ligand binding assay and ER-ICA were negative. Estrogen receptor positivity in breast cancer tumors is quoted above 10 fmol/mg protein by the tritiated ligand binding assay, and a detection limit of 5 fmol/mg protein for ER-EIA is used in this laboratory. In our experience, some breast tumor samples determined as being receptor negative using the biochemical assay procedures contained a few cells which displayed staining, indicative of estrogen receptor. In consequence these tumors can contain populations of cells with or without receptor which, depending on the proportion of each type, will affect the concentration measured by biochemical assay procedures. Since no visual detection of estrogen receptor was observed in the prostate sections with ER-ICA, there appears to be no population of cells in the prostate exhibiting estrogen receptor concentrations comparable to those detected in breast tumors. The possibility exists that the low concentrations of estrogen receptor measured by ER-EIA are the result of the wide distribution of this moiety over a large number of cells in amounts well below the detectability of ER-ICA. In addition, any deleterious effects of fixation may be more pronounced at lower receptor concentrations. Immunocytochemical procedures have been used previously to visualize estrogen receptors in prostatic tissue, in which the results obtained were in agreement with this report although both different fixation procedures and monoclonal antibodies were used (15, 19).
ESTROGEN RECEPTORS IN HUMAN PROSTATIC TUMORS

The monoclonal antibodies used in this study for both ER-ICA and ER-EIA were raised against estrogen receptor purified from breast MCF-7 cells. It is not known whether antigenic sites on estrogen receptors present in different tissues are identical, although it is most likely that the domain containing the estrogen binding site will be conserved in these functional macromolecules. The antibody H222Spγ used in ER-ICA binds to a site in this domain (20) and should be able to demonstrate estrogen receptor in a variety of human tissues. Ideally, a panel of monoclonal antibodies to the various domains of the receptor molecule should be tested in an immunocytochemical procedure to confirm whether estrogen receptors display antigenic differences in non-breast tissues.

The wide range of estrogen receptor concentrations reported in the literature (6–11) in prostate tissue must relate to the various factors which can interfere with conventional procedures. Low values can be the result of poor handling of surgical specimens leading to receptor degradation or due to endogenous estrogen occupancy of receptor sites. High values quoted by some investigators could be attributable to an overestimation of the binding sites due to high concentrations of other binding proteins, such as sex hormone binding globulin and albumin present in prostatic tissue. Indeed, Ekman et al. (19) have described two binding classes of estrogen receptor, a type I, classical high affinity receptor and a type II, lower affinity site in the prostate, and his studies indicated that these type II sites can influence the determination of the classical receptor. A similar range of values for type I sites was reported to those observed in a few tumors in this study. Stromal localization of estrogen receptor was not seen with ER-ICA, which was surprising in view of the high values found in this compartment by others in the human prostate (8) and in the canine prostate (13). Such values in human breast tumors are easily visualized with ER-ICA.

The lack of correlation of both breast and prostate samples with fluorescent-labeled estradiol conjugates with ER-ICA, ER-EIA, and the tritiated ligand binding assay confirmed that this method does not visualize specific receptor sites in tissue sections. These findings are contrary to a recent report by Perschuk et al. (15) who observed a correlation with their estradiol BSA-FITC conjugates and a dextran-coated charcoal receptor assay in prostatic tumors. Whether binding of these fluorescent conjugates is to a type II (21) lower affinity site requires further experimentation and a possible correlation with clinical response needs to be assessed.

The functional importance of such low amounts of estrogen receptor which have been previously reported by others (19) and found in this study in human prostatic tumors is uncertain. Estrogen action at the physiological level might result from mechanisms not involving the receptor system and this could be an interesting area for further research. The pharmacological action of estrogens on prostatic tumors is more likely, from the available evidence, to be mediated indirectly by reducing androgen supplies although direct effects cannot be discounted.

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