A Study of Cytoplasmic and Nuclear Estrogen and Progestin Receptors in Gynecologic Neoplasms

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Abstract

A rapid method for simultaneous preparation of cytosol and nuclear estrogen (E) and progestin (P) receptors and their in vitro determination is described. The method was applied to several uterine or ovarian surgical specimens to evaluate their steroid hormone “dependence”. The results suggest that low cytoplasmic E receptor levels (ERc) are associated with higher nuclear E receptor (ERn) levels but no apparent correlation was observed between PRc and ERn levels. The method appeared to be suitable for screening steroid hormone receptor content in tumor tissues and may provide better estimation of steroid dependence since both cytoplasmic and nuclear compartments can be studied simultaneously.

Estrogen is known to promote cell growth in its target tissues through interaction with specific, high-affinity protein receptor molecules. Growth stimulation by estrogen has been suggested to play an important role in the proliferation of tumors which contain estrogen receptors18 and the quantitation of estrogen receptors in surgical specimens of gynecologic malignancy has been practiced to develop a possible use of estrogen antagonists such as tamoxifen (TMX) as an antineoplastic agent.20, 21 Up to the present time, the majority of studies of gynecologic malignancies have employed measurements of cytoplasmic estrogen receptors. Since cellular response to estrogenic stimulation is expressed through effects at the nuclear level, nuclear estrogen receptor levels would seem to more directly indicate the tissue responsiveness to estrogen. In the present study, we intended to develop a rapid in vitro method to measure both cytoplasmic and nuclear steroid receptors simultaneously in samples of human gynecologic malignancy.
Materials and Methods

I. Tissue
Twelve ovarian, endometrial or myometrial tissues surgically removed from primary or metastatic sites were studied in multiracial patients (Caucasians, blacks and Asians, Table 1). Surgical specimens were placed on ice immediately after excision, frozen on dry ice and transferred and kept in a deep freezer (−70°C). All materials were assayed within 2 to 3 weeks.

II. Buffers and Chemicals

TEGEB buffer: 50 mM Tris/HCl buffer pH 7.4 containing 1.5 mM ethylene diamine tetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), 10% (v/v) glycerol and 1 mg/ml bacitracin
SI buffer: 0.32 M sucrose, 1 mM KH2PO4, 3 mM MgCl2, 1 mM DTT, 10% glycerol and 1 mg/ml bacitracin, pH 7.3
SII buffer: As for SI buffer, but with 2.4 M sucrose
TEGDBK buffer: As for TEGD buffer, but with 1 M KCl

[2, 4, 6, 7-3H] estradiol-17B (115 Ci/mmol), [17-a-methyl-3H] R5020 (17, 21-dimethyl, 19-norpregna-4, 9-diene-3, 20-dione, 87 Ci/mmol) and non-radioactive R5020 were obtained from New England Nuclear (Boston, MA). The radio ligands were periodically repurified by Sephadex LH-20 (Pharmacia, Piscataway, NJ) column chromatography in the system (methylene chloride: methanol, 95:5) and the purity was maintained over 95%. Diethylstilbestrol (DES) was obtained from Sigma Chemicals (St. Louis, MO). All other reagents were of the highest purity available.

III. Tissue Cytosol and Nuclei Preparation
Surgical specimens (300–1000 mg) stored at −70°C were lightly thawed on ice and minced finely with a razor blade. All subsequent procedures were performed at 0–4°C. The tissues were homogenized in 20 volumes (ml/gm) of TEGDB buffer using a Polytron PT10ST homogenizer at the half maximal setting. The homogenate was filtered through two layers of cheese cloth. An aliquot (1–2 ml) of filtrate was mixed with an equal volume of SI buffer in a glass centrifuge tube (1.5×10.2 cm). Then an equal volume of SII buffer was added along the tube wall while the tube was tilted 45°. This produced two separate sucrose density layers. Tubes were placed upright on ice for 2 minutes to allow the layers to settle and then centrifuged at 20,000×g for 45 minutes in a sorvall RC2-B centrifuge using an HB-4 swinging-bucket rotor. After centrifugation, 1 ml of the top layer was removed and further centrifuged in a Beckman Airfuge at an air pressure of 30 psi (105,000×g) for 30 minutes to purify the cytosol. The cytosol was then passed through a Sephadex LH-20 column (0.5×3.0 cm) equilibrated with TEGDB buffer to remove free steroids and sucrose. The elute was diluted with TEGDB buffer to bring the final cytoplasmic protein concentrations to 1–2 mg/ml. The remaining layers of the supernatant were vacuum drained and discarded. The nuclear pellets and the tube wall were quickly washed with 4–5 ml of TEGDB buffer twice and the tube wall cleaned with cotton swabs. The pellets were resuspended in 0.3–0.5 ml of TEGDB buffer and then mixed with an equal volume of TEGDBK buffer. The nuclear receptors were extracted for 30 minutes by rapid mixing every 10 minutes and separated from the nuclear residue by centrifugation for 10 minutes at 20,000×g. The extracts were decanted and assayed immediately. DNA content of the pellets was determined by the method of Burton with calf thymus DNA as a standard and cytosol protein was measured as described by Bradford with bovine serum albumin as a standard.

IV. Steroid Receptor Assay
Steroid receptors were assayed as reported previously. Briefly, aliquots (200 μl) of LH-20 purified cytosol or nuclear extract were incubated with either 3H-estradiol or 3H-R5020 for estrogen or progestine receptor (ER, PR) assay, respectively. The final concentration of the radioligand in each case was 5 nM. Non-specific binding was determined by parallel incubation of the samples in the presence of either a 50 molar excess of DES (for ER) or a 100 molar excess of R5020 (for PR). The final incubate volume was adjusted with TEGDB buffer to 300 μl. For a characterization of binding affinity and capacity, cytosol or nuclear extracts were incubated with varying doses of radio-ligand (range 0.1–5 nM) for Scatchard analysis. Incubation was carried out for either 4 hours at 0–4°C (for PR) or overnight at 0–4°C followed by 4.5 hours incubation at 25°C for exchange assay
of ER). After incubation, aliquots (100 µl) were withdrawn from the incubate and subjected to Sephadex LH-20 minicolumn chromatography as reported previously. Labeled receptors in the void volume were counted in 5 ml of Betafluor (National Diagnostic) in a Beckman LS 8000 liquid scintillation counter at an average counting efficiency of 50%.

V. Estimation of “Salt resistant” Fraction and Degradation of the Nuclear Estrogen Receptors

Since a fraction of the nuclear estrogen receptors has been shown to be resistant to the high salt extraction and tightly associated with the nuclear matrix, we measured the residual saturable estrogen specific binding of the salt extracted nuclei.

Purified nuclei were resuspended in TEGDB buffer and incubated at 37°C for 45 minutes with 5 nM of ³H-estradiol either in the presence or absence of 100 fold excess DES. After incubation, nuclei were separated by centrifugation (2,000 x g for 10 min.) and washed once with 0.05% TEGDB buffer containing 0.1% Triton X-100 and then with TEGDB buffer twice. These nuclei were extracted with 0.5 M KCl (500 µl) as described above. An aliquot (2000 µl) of the extract was applied to the LH-20 column and the number of salt extractable receptors was estimated. The residual nuclear matrix was solubilized in 5 M urea and 2 M NaCl (500 µl) for 15 min at 0°C as described by Aten et al. The mixture was centrifuged at 2,000 x g for 20 min at the end of solubilization and the radioactivity in the supernatant was counted. The final residue was further extracted with 500 µl of ethanol overnight at room temperature and the radioactivity of the extract was determined.

To estimate receptor degradation during the exchange incubation, ³H-estradiol labeled nuclear receptor (tissue from patient C. D.) was mixed with 5 nM of ³H-estradiol and incubated overnight at 0°C then 4.5 hours at 25°C. At the beginning of the 0°C and 25°C incubations as well as at the end of the 25°C incubation, an aliquot was withdrawn and the receptors were separated by LH-20 minicolumn chromatography.

VI. Competition of the unoccupied ERc with TMX in vitro

In order to examine the possibility of an in vitro screening assay for the tumor sensitivity to TMX, cytosol estrogen receptors were incubated with 5 nM of ³H-estradiol and varying concentrations (7 fM to 10 nM) of TMX for 18 hours at 4°C. The labeled receptors were collected and counted as described above.

Results

Characterization of the Receptor Assay

Cytosol and nuclear extract prepared separately as well as prepared by the above described method were assayed for total, ERc, ERn and PRn by Scatchard analysis. The receptor binding affinity and capacity in cytosol and nuclei from one patient are shown in Fig. 1. The dissociation constants (Kd) for estradiol and R5020 in cytosol were 0.1 nM and 3.5 nM and the binding capacity in the cytosol for estradiol and R5020 was 7.1 and 120.6 fmol/mg protein and that in the nuclear extract was 362.1 and 213.6 fmol/mg DNA. These values for Kd are essentially equal to the values obtained from cytosol and nuclear extracts prepared separately by the previous method. The cell nuclear pellets appeared to be free of contamination by cell debris when observed with the electron microscope.

Nuclear Matrix Associated Estrogen Binding and the Receptor Degradation

After incubating nuclear pellets with ³H-estradiol, 131.0 pmoles/g tissue of saturable binding was recovered by KCl extraction and urea-salt solubilization further released 30.0 pmoles/g tissue of the binding. The ethanol extraction of the final residue was found to contain 42.5 pmoles/g tissue of ³H-estradiol but this fraction of the binding was non-specific and non-saturable (Fig. 2).
Fig. 1. Cytosol and nuclear extracts prepared by the present method from surgical tissue of patient C. S. were incubated with varying doses (0.1 to 5 nM) of $^3$H-estradiol or $^3$H-5020 under the exchange conditions for the Scatchard analysis of estrogen and progestin binding characteristics. Each point represents the mean value for duplicate incubations. CP; cytoplasmic protein.

$^3$H-Estradiol labelled estrogen receptors extracted from nuclei by 0.5 M KCl were further incubated with 5 nM of $^3$H-estradiol overnight at 4°C and then at 25°C for 4.5 hours. At the end of each incubation, more than 98% of the initial specific binding was found to remain (Fig. 3).

Steroid Receptors in Human Tissue

The results of simultaneous determination of total PRc, ERc, PRn and ERn in a limited number of human materials are shown in Table 1. Eleven out of thirteen specimens contained estrogen and progestin receptors in the cytoplasmic as well as in the nuclear compartment. In one sample, an attempt was made to demonstrate in vitro competition for unoccupied cytoplasmic estrogen receptors between $^3$H-estradiol and TMX. TMX competed for
Fig. 2. Purified nuclei were incubated with 5 nM of $^3$H-estradiol and extracted serially by 0.5 M KCl, urea-NaCl and ethanol. The highest specific bound was recovered by 0.5 M KCl extraction.

Fig. 3. Extracted nuclear estrogen receptors were incubated with $^3$Hestradiol and the loss of specific binding was studied. There was no appreciable loss of specific binding at the lower temperature. At the exchange temperature, the binding began to decrease if incubation time exceeded 4.5 hours.

Table 1

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Clinico-Pathological Diagnosis</th>
<th>Tissue Studied</th>
<th>ERc</th>
<th>PRC</th>
<th>ERn</th>
<th>PRn</th>
<th>Preoperative Steroid Administration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) G. K.</td>
<td>35</td>
<td>Pulmonary lumphotoangio leimato-sis and cellular leiomyoma</td>
<td>Endometrium</td>
<td>9.4</td>
<td>5.8</td>
<td>500.4</td>
<td>114.4</td>
<td>TMX and DepoProvera for 15 months</td>
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<tr>
<td></td>
<td></td>
<td>Same</td>
<td>Myometrium</td>
<td>0</td>
<td>29.9</td>
<td>735.0</td>
<td>331.6</td>
<td>Same</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Same</td>
<td>Fibroid</td>
<td>6.1</td>
<td>6.4</td>
<td>809.2</td>
<td>203.1</td>
<td>Same</td>
</tr>
<tr>
<td>2) R. A.</td>
<td>22</td>
<td>Serous cystoadenocarcinoma of ovary. Grade 3</td>
<td>Tumor</td>
<td>31.1</td>
<td>10.9</td>
<td>134.4</td>
<td>8.6</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Same</td>
<td>Omentum with Metastasis</td>
<td>81.0</td>
<td>46.8</td>
<td>137.7</td>
<td>15.3</td>
<td>None</td>
</tr>
<tr>
<td>3) W. S.</td>
<td>19</td>
<td>Endodermal sinus tumor</td>
<td>Tumor</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>None</td>
</tr>
<tr>
<td>4) C. S.</td>
<td>25</td>
<td>Myxoid neurofibroma</td>
<td>Tumor</td>
<td>7.1</td>
<td>20.0</td>
<td>362.1</td>
<td>213.6</td>
<td>Orthonovum for 8 1/2 yr</td>
</tr>
<tr>
<td>5) S. B.</td>
<td>54</td>
<td>Endolympathic Stromal Myosis</td>
<td>Tumor</td>
<td>46.9</td>
<td>11.8</td>
<td>nd</td>
<td>nd</td>
<td>Premarin preoperatively</td>
</tr>
<tr>
<td>6) C. D.</td>
<td>34</td>
<td>Serous cystoadenocarcinoma of ovary. Borderline</td>
<td>Tumor</td>
<td>5.0</td>
<td>21.0</td>
<td>500.0</td>
<td>26.0</td>
<td>None</td>
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<td>7) M. D.</td>
<td>34</td>
<td>Mucinous cystoadenocarcinoma of ovary. Grade 1</td>
<td>Tumor</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>Secondary amenorrhea, recent Methadone</td>
</tr>
<tr>
<td>8) R. D.</td>
<td>22</td>
<td>Endometriid adenocarcinoma Grade 2</td>
<td>Tumor</td>
<td>146.0</td>
<td>180.3</td>
<td>324.4</td>
<td>222.5</td>
<td>None</td>
</tr>
<tr>
<td>9) L. D.</td>
<td>66</td>
<td>Serous cystoadenocarcinoma of ovary. Grade 3</td>
<td>Tumor</td>
<td>32.8</td>
<td>300.0</td>
<td>480.6</td>
<td>327.4</td>
<td>None</td>
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</tbody>
</table>

Cytoplasmic receptor levels: fmoles/mg cytoplasmic protein
Nuclear receptor levels: fmoles/mg DNA
the unoccupied estrogen receptors with $^3$H-estradiol in a dose dependent manner and the apparent ED50 for the cytoplasmic receptors was 4.5 nM (Fig. 4).

**Relationship between Estrogen and Progestin Receptor concentrations**

There was no apparent correlation observed between any two combinations of cytoplasmic or nuclear estrogen and progestin receptor levels. Interestingly, however, when all receptor positive samples were divided into two arbitrary groups with low (less than 10 fmoles/mg protein) or high (greater than 10 fmoles/mg protein cytoplasmic estrogen receptor concentrations, the tissue nuclear receptor levels appeared to be greater in the former group (0.01 < p < 0.05, Fig. 5).

**Discussion**

Malignant neoplasms arising from female reproductive tissues have been studied for their possible "dependence" on estrogen by *in vitro* measurement of putative cytoplasmic estrogen receptors. Recently, however, it has been suggested that cell estrogen receptors reside predominantly in the cell nucleus and not in the cytoplasm *in vivo*. This agrees with the observation that uterine growth or RNA polymerase activities induced after estrogen administration correlate positively to the cell nuclear estrogen receptor concentrations. These data seem to question the use of cell cytoplasmic estrogen receptor "positiveness" as a sole indicator of the estrogenic status of the tumor or possible involvement of estrogen in tumor growth, and it has become necessary to assess the steroid receptor positiveness in both nuclear and cytoplasmic compartments. The procedure described in this paper has been adopted and modified from a recently developed method for measuring estrogen receptors in rat hypothalamus and has several advantages;

(1) The ability to determine cytoplasmic and nuclear estrogen and progestin receptors...
simultaneously. This can also be achieved by the conventional method but it is laborious and requires several steps involving centrifugation and washing that make receptor loss inevitable, especially when starting tissue weight is less than a gram.

(2) Rapidity. From tissue homogenization to the beginning of incubation requires no more than 120 minutes and thus reduces the chances of receptor loss during preparation and increases the number of samples that can be handled in a day.

(3) Low nonspecific binding. Direct incubation of purified nuclei from tumor tissue with radio-labeled ligand has been reported to have high (ca. 30%) nonspecific binding. The extraction of nuclear receptors with salt reduced the nonspecific binding to less than 10%, thereby increasing the assay sensitivity.

Several animal studies have indicated that a considerable portion of the nuclear receptors are not extractable with high concentrations of salt and are closed associated with the nuclear matrix. In the present study, this portion was estimated at about 22%. However, this residual binding which can be solubulized with urea-NaCl had higher nonspecific/specific bound ratio (ca. 4/1) than the initial KCl extracted binding (ca. 1/9) which could obliterate a reliable estimation of the specific receptor binding fraction. Therefore it would be appropriate to limit the vigorous solubilization in those cases with high nuclear receptor levels.

It has not yet been established whether the estrogen receptor positiveness of malignant tumour is a reliable parameter of its sensitivity to anti-estrogen therapy. Clinically, however, Schwartz et al. have reported a possible stabilizing effect of TMX on the advance of ovarian carcinoma. Therefore an evaluation of the positive competition of estrogen receptors by TMX in vitro may be of clinical value in estimating the therapeutic potential of the antiestrogen more directly on an individual basis. Also TMX competition assay should be performed if tissues from metastatic sites have estrogen receptors, as was seen in patient R. A. (Table 1) to seek the advantages of the adjuvant therapy.

When cytoplasmic estrogen receptor concentrations are low, it may reflect two entirely opposite conditions: either poor tissue response or refractoriness to estrogen or a nuclear receptor dominance as a consequence of increased translocation. The latter is a false negative interpretation of tissue receptor “positiveness” and in fact this seems to occur not infrequently because the present data showed that some of the cancerous tissues with lower cytoplasmic estrogen receptors had higher nuclear receptor concentrations. This does not seem due to a greater nuclear/cytoplasm volume ratio in the cancerous specimen because some nonmalignant tissues included in the present study also showed low cytoplasmic estrogen receptors with high nuclear receptor accumulation (patient K. G.).

This reversed relationship between cytoplasmic and nuclear estrogen receptor levels has also been suggested in a recent study of Schwartz, et al. in which 74 cases of epithelial ovarian malignancies were studied for the cytoplasmic estrogen and progestin receptors. Among 55 materials with positive cytoplasmic estrogen or progestin bindings, 23 (42%) had low or barely detectable cytoplasmic estrogen receptors but high progestin receptors (Schwartz et al., unpublished data). Since high progestin binding indicates an active estrogenic effects, these tissues were likely to have had significant nuclear estrogen accumulation. Another reports from Vihko’s laboratory also demonstrated that in endometrial carcinoma, the ratio of cytoplasmic and nuclear receptor concentrations significantly lower than that in intact endometrium due to a rise in nuclear receptor levels.

Our data confirmed the presence of
nuclear progestin receptors in cancerous endometrium. Progesterone has been shown to antagonize estrogen action including an inhibitory effect on the replenishment of cytoplasmic estrogen receptors and has established a position in a hormonal therapy of certain gynecologic malignancies such as endometrial carcinoma or endolymphatic stromal myosis. Although positive cytoplasmic as well as nuclear progestin receptors in tumor tissues may support the introduction of progestin therapy such as medroxyprogesterone acetate administration, if estrogen and progestin receptors are both high, concomitant use of anti-estrogens with occasional discontinuation of progestin may artificially create progesterone withdrawal to enhance the formation of anti-estrogen and estrogen receptor complexes (see ref. 5 for a recent discussion on the intracellular progesterone withdrawal) and decrease complications due to the prolonged use of progestins.

Since it has been shown that in normal cycling woman, TMX can raise the serum FSH, estrogen, and progesterone levels, it may be necessary to consider possible stimulation of cancerous portion of ovaries by TMX. It would be tempting, therefore, to investigate theoretical therapeutic value of a combination regimen of synthetic gonadotrophin-releasing hormone antagonists to turn off the endogenous gonadal steroid synthesis and medroxyprogesterone acetate +TMX to inhibit receptor mediated tissue response in receptor positive gynecologic malignancies. Also it seems that the rapid screening of tumor receptors has additional value in follow-up patients if tissue is available at the second-look procedure.

References


