Androgen Receptor in Serially Subcultured Human Endometrial Fibroblasts

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Abstract

Specific dihydrotestosterone (DHT) binding was demonstrated in the whole cell sonicates obtained from serially subcultured human endometrial fibroblasts. This binding showed a high affinity and specificity for DHT (Kd=3.3×10^{-10} M). Various kinds of excess nonradioactive steroids were arranged, as a result of competition among them, in the following decreasing order of binding affinity: DHT, testosterone, cyproterone acetate, 17β-estradiol, progesterone, and 5α-androstane-3α, 17β-diol. Androsterone, 5α-androstanedione, cortisol, androstenedione and estrone hardly affected the binding even at 200 fold excess. Saturation analysis indicated that cultured endometrial and genital skin fibroblasts have a similar number of binding sites for DHT (14.3±8.5 vs 11.5±8.6 fmoles/mg cell protein). Sucrose density gradient centrifugation brought about a sharp peak of 3H-DHT in the 4S region in high ionic strength buffer. This binding was abolished by heating sonicates at 37°C for 1 hr, treating them with pronase or adding to them excess nonradioactive DHT. Endometrial fibroblasts also contained testosterone binding components which formed a peak in the 4S region and showed a similar binding affinity to that for DHT.

Uterine growth can be stimulated by androgens as well as estrogens and progesterone (Lerner, 1964). This effect of androgens, if administered in a physiological amount, appears to be mediated by the androgen-receptor system predominantly, and can be inhibited by antiandrogens (Lerner, 1964); (Heyns et al., 1976); Schmit and Katzenellenbogen, 1979).

However, few studies have been performed to substantiate the presence of androgen receptors as far as the human uterus is concerned. This paper describes some characteristics of androgen-binding components in cultured human endometrial fibroblasts.

Materials and Methods

Culture

Endometrial fibroblast strains were established by a modification of the method of Chen et al. (1973). Endometrial tissues were obtained from eight patients undergoing hysterectomy for myoma uteri. One of them was a postmenopausal woman (51 y.o.) and the others were premenopausal women (43-49 y.o.) who had menstruated normally. None of them had a steroid therapy before operation. Minced tissues were treated with 0.05% trypsin-0.02% EDTA at room temperature for 1 hr with continuous stirring. Precipitated tissue fragments were resuspended in Eagle’s minimal essential medium (MEM) supplemented with 10% fetal calf serum, and placed in Petri dishes. The culture medium was changed twice a week and the cells were serially subcultured every 7 days in a 75 cm² glass culture bottle. After 4 or 5 passages, cells were seeded at a concentration of approximately 1×10⁶ cell/ml into Petri dishes. Cells grown to a confluent in one or two weeks were used.

Received January 10, 1980.
for the experiment. Similar procedures were used for the culture of the skin fibroblasts, except that fine dissected specimens were placed in Petri dishes for the primary culture.

**Monolayer binding assay**

Fibroblasts in thirty Petri dishes (60×15 mm) were employed in each experiment. Confluent monolayers were washed twice with serum-free MEM, and incubated with various concentrations of 3H-1, 2, 4, 5, 6, 7-α-dihydrotestosterone (SA 110 Ci/mM, New England Nuclear Corp.) or 3H-1, 2-testosterone (SA 45 Ci/mM, New England Nuclear Corp.) in MEM containing 15 mM Hepes at 37°C under an atmosphere of 5% CO2, 95% air at a high humidity. To assess nonspecific binding, the fibroblasts were incubated with a 250 fold excess of nonradioactive DHT. After incubation, media were discarded and monolayers were washed 5 times with 1.5 ml of phosphate buffered saline at 0°C. All subsequent procedures were carried out at 0°C. The cells in each Petri dish were harvested by scraping with a rubber policeman in 1 ml of 0.32 M sucrose buffer (0.32 M sucrose, 20 mM Tris-HCl, pH 7.4, 1 mM MgSO4). An aliquot was removed for protein determination. Cells suspended in 0.32 M sucrose buffer were precipitated by centrifugation at 800×g for 10 min. The cell pellets were suspended in 2 ml of 0.5 M KCl in 20 mM Tris-HCL, pH 7.4 and 1.5 mM EDTA, sonicated with a Sonifier Cell Distributor Model W185 (Heat Systems-Ultrasonics, Inc., N.Y.) at a 40 W power with a 15 seconds burst, and allowed to stand for 30 min before centrifugation at 800×g for 10 min. In order to remove free steroids, the supernatants were mixed, for 30 min, with dextran-coated charcoal pellets which were prepared by centrifuging 1.5 ml of suspension of 0.5% charcoal and 0.05% dextran. After removing charcoal by centrifugation, the radioactivity of the supernatants was counted with an ISOCAP liquid scintillation counter in toluene Nonion HS210, Nihon Yushi Co., (2:1, v/v) containing 1% PPO and 0.03% POPOP. Counting efficiency was about 40%. Bmax and Kd were calculated from Scatchard plots (Scatchard, 1949) of the data. In a competition study, monolayers were incubated with 0.4 nM 3H-DHT and 10 or 200 fold excess of various kinds of nonradioactive steroids. Bound radioactivity was determined by the dextran-coated charcoal method.

**Sucrose density gradient centrifugation**

Four-tenth milliliter of 0.5 M KCl supernatant, which had been prepared out of ten Petri dishes and treated with the dextran-coated charcoal method as described above, was layered on the top of 4.6 ml of 5-20% sucrose density gradient containing 0.5 M KCl, 20 mM Tris-HCL, pH 7.4 and 1.5 mM EDTA. Ultracentrifugation was carried out at 48,000 rpm (190,000×g) for 18 hr at 4°C. Determination of sedimentation constants for the androgen binding macromolecules was made by the method of Martin and Ames (1961). Bovine serum albumin (S20, w=4.6) and yeast alcohol dehydrogenase (S20, w=7.4) were used as standards.

**Thin layer chromatography**

Steroids were extracted from a mixture of cells and medium by the method of Folch et al. (1947), and were analyzed by thin layer chromatography in the 98.25% chloroform: 1.75% methanol system described by Gomez and Hsia (1968). After chromatography, the radioactivities found in steroid fractions were counted in a liquid scintillation counter. In another experiment, steroids were extracted from the peak fractions of a sucrose density gradient centrifugation profile (Fraction No. 10-No. 20) and analyzed by the thin layer chromatography mentioned above.

**Enzymes**

Deoxyribonuclease I and ribonuclease A were obtained from Sigma, and pronase E from Kaken-Kagaku Kogyo Co., Tokyo, Japan.

**Results**

Figure 1 displays the time course of specific binding in human endometrial fibroblasts incubated with 0.1 or 0.7 nM 3H-DHT at 37°C. The specific binding increased lineally until 30 min, and reached a plateau after 45 min at either concentration. When steroids were extracted from a mixture of cells and medium 30 min after incubation and analyzed and identified by thin layer chromatography and recrystallization to a constant specific activity, about 80% of the radioactivity was found to be unmetabolized DHT.

The values for nonspecific binding were less than 20% of the total binding in the range of 0.1-0.6 nM DHT (Fig. 2, a). Scatchard analysis of the data revealed a high affinity (Kd=0.27 nM) and a low capacity (6.6 fmole/mg protein) binding for DHT (Fig. 2, b). The specific DHT Bmax and Kd for the individual endometrial fibroblast strains are shown in Table 1. Some of these strains were also found to
Fig. 1. Time course of specific binding in endometrial fibroblasts. Monolayers were incubated with $^3$H-DHT (0.1 or 0.7 nM) with or without 250 fold excess of nonradioactive DHT at 37°C. Specific binding was calculated by subtracting nonspecific binding from total binding. For the separation of free and bound steroids, the dextran-coated charcoal method was used. Three Petri dishes were used for the assay of total or nonspecific binding. Protein content per three Petri dishes averaged 750-900 µg.

Fig. 2. Saturation curves (a, left) and Scatchard analysis (b, right) of DHT binding in endometrial fibroblasts. Monolayers of endometrial fibroblasts were incubated with various concentrations (0.1-0.6 nM) of $^3$H-DHT with or without 250 fold excess of nonradioactive DHT at 37°C for 30 min. Specific binding ($\circ$--$\circ$) was obtained by subtracting nonspecific binding ($\times$--$\times$) from total binding ($\bullet$--$\bullet$).

Table 1. Androgen binding in cultured human endometrial fibroblasts

<table>
<thead>
<tr>
<th>Strain</th>
<th>Aeg (yr)</th>
<th>Kd (nM)</th>
<th>Bmax (fmoles/mg protein)</th>
<th>Kd (nM)</th>
<th>Bmax (fmoles/mg protein)</th>
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<tbody>
<tr>
<td>E-1</td>
<td>47</td>
<td>0.27</td>
<td>6.6</td>
<td>n.d.</td>
<td>n.d.</td>
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<tr>
<td>E-3</td>
<td>43</td>
<td>0.68</td>
<td>31.9</td>
<td>n.d.</td>
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<tr>
<td>E-4</td>
<td>45</td>
<td>0.37</td>
<td>20.0</td>
<td>n.d.</td>
<td>n.d.</td>
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<tr>
<td>E-5</td>
<td>51</td>
<td>0.25</td>
<td>11.7</td>
<td>0.25</td>
<td>3.85</td>
</tr>
<tr>
<td>E-6</td>
<td>46</td>
<td>0.21</td>
<td>9.4</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>E-7</td>
<td>45</td>
<td>0.36</td>
<td>11.8</td>
<td>0.20</td>
<td>3.30</td>
</tr>
<tr>
<td>E-8</td>
<td>44</td>
<td>0.15</td>
<td>6.7</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>E-9</td>
<td>49</td>
<td>0.37</td>
<td>16.6</td>
<td>0.17</td>
<td>3.46</td>
</tr>
<tr>
<td>Average</td>
<td>0.33 ± 0.16 (S.D.)</td>
<td>14.3 ± 8.5</td>
<td>0.21 ± 0.04</td>
<td>3.54 ± 0.28</td>
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</table>
have testosterone binding components. The amount of DHT binding was nearly 4 times greater than that of testosterone binding, though Kd's for DHT and testosterone were similar. We also investigated DHT binding characteristics in cultured skin fibroblasts by the same method for comparison. As previously reported (Griffin et al., 1976), fibroblasts derived from genital skin gave larger Bmax values than nongenital skin. These two groups did not appear to differ significantly from each other with respect to Kd values (Table 2). Thus, endometrial fibroblasts were found to possess binding sites similar in number to those in genital skin fibroblasts.

Steroid specificity of the DHT binding components was examined (Fig. 3). Monolayers of endometrial fibroblasts were incubated with 0.4 nM 3H-DHT in the presence or absence of 10 or 200 fold excess of various competing steroids at 37°C for 30 min. DHT and testosterone decreased the binding by more than 60% at 10 fold excess, while 17β-estradiol and cyproterone acetate remarkably inhibited the binding at 200 fold excess. Other steroids thus far tested did not decrease the binding significantly. These data indicate that the androgen binding component of endometrial fibroblasts is specific for DHT and testosterone.

Sucrose density gradient centrifugation of 0.5 M KCL extract from endometrial fibroblasts incubated with 3H-DHT or 3H-testosterone consistently produced a peak radioactivity at 4S which was eliminated by the addition of excess non-radioactive DHT or testosterone (Fig. 4 and 5). When the radioactivity in the 4S peak was extracted and analyzed and identified by thin layer chromatography and recrystallization, 75% of the radioactivity was found to be DHT in experiments with 3H-DHT, while 50% was testosterone in those with 3H-testosterone.

We also tested whether DHT binding components were proteins or nucleic acids. After cell sonicates were digested with DNase, RNase or pronase (100 µg/ml each) at 25°C for 1 hr, samples were analyzed by sucrose density gradient centrifugation. Incubations with these enzymes reduced the amount of DNA, RNA or protein to nearly 50%. Neither DNase nor RNase digestion decreased binding significantly, while pronase treatment caused a 75% decrease in

<table>
<thead>
<tr>
<th>Source</th>
<th>Number of Materials</th>
<th>Kd (nM)</th>
<th>Bmax (fmoles/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genital</td>
<td>8</td>
<td>0.42±0.38 (S.D.)</td>
<td>11.5±8.6 (S.D.)</td>
</tr>
<tr>
<td>Nongenital</td>
<td>17</td>
<td>0.33±0.17 (S.D.)</td>
<td>4.1±2.2 (S.D.)</td>
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Table 2. DHT binding in cultured human skin fibroblasts

Fig. 3. Effect of various nonradioactive steroids on 3H-DHT binding in endometrial fibroblasts. Monolayers of endometrial fibroblasts were incubated with 0.4 nM 3H-DHT and 10 fold (open bar) or 200 fold excess (hatched bar) of various non-radioactive steroids at 37°C for 30 min. Free and bound steroids were separated by using dextran-coated charcoal.
Fig. 4. Sedimentation of DHT binding components in endometrial fibroblasts by sucrose density gradient centrifugation. Monolayers were incubated with 1.5 nM $^3$H-DHT with or without 250 fold excess of nonradioactive DHT at 37°C for 30 min. After cell sonicates in 0.5 M KCL were treated with dextran-coated charcoal, an aliquot was applied to 5-20% sucrose gradient containing 20 mM Tris-HCL, 1.5 mM EDTA, pH 7.4. Protein content layer on the top of the sucrose gradient averaged 6.2-7.5 mg/ml. □□□□, 1.5 nM $^3$H-DHT, ⧫เท้า(1,1), 1.5 nM $^3$H-DHT+250 fold excess nonradioactive DHT.

BSA: bovine serum albumin.

ADH: alcohol dehydrogenase.

binding. The result indicates that the binding components are protein in nature.

Heat stability of DHT binding components was tested. Sucrose density gradient analysis disclosed that the radioactivity in the 4S peak was reduced to 60% and 30% of the values at 0°C by standing at 25°C and 37°C, respectively.

Discussion

The present study demonstrated the specific androgen-binding components in cultured human endometrial fibroblasts. The binding characteristics such as dissociation constants, number of binding sites, steroid specificity and sedimentation constants on sucrose gradient analysis resembled those reported previously in other androgen target organs (Danzo et al., 1973); (Blondeau et al., 1975); (Griffin et al., 1976); (Takayasu, 1978). These results suggest that the human uterus as well as the rat uterus (Heyns et al., 1976) possesses androgen receptors.

One might wonder whether cultured endometrial fibroblasts were contaminated with serum binding protein like testosterone-estradiol binding globulin (TeBG). Such contamination can be neglected for the following reasons. First, using a similar procedure, we could not detect an appreciable amount of DHT binding in cultured skin fibroblasts obtained from patients with testicular feminization (unpublished data). Second, TeBG is stable at 45°C for 1 hr (Vermeulen and Verdonck, 1968), while DHT binding in endometrial fibroblasts was significantly reduced at 37°C.

Since androgens are reported to stimulate uterine growth (Lerner, 1964); (Heyns et al., 1976); (Schmidt and Katzenellenbogen, 1979), it seems that this culture
system is suitable for investigating the mechanisms of action of androgens in the human uterus.

References