Measurement of Androgen Receptor in Cytosols from Normal, Benign Hypertrophic and Cancerous Human Prostates

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Abstract

The binding of R 1881 in cytosols from the normal, benign hypertrophic and cancerous human prostates was examined in the presence of molybdate and triamcinolone acetonide. The addition of 10mM Na₂MoO₄ resulted in an increase in the stability of the binder without any change in Kd. Triamcinolone acetonide added to the incubation of cytosol with R 1881 modified the inhibition pattern by other steroids, and the binding in the presence of triamcinolone acetonide exhibited the characteristics of androgen receptor. The complex of cytosol and R 1881 formed in the incubation in the presence of triamcinolone acetonide was sedimented at 8.5S.

Kd of the binding to R 1881 of the normal and pathological prostates was almost identical, but maximum binding sites of the androgen receptor were larger in benign hypertrophic and cancerous prostates than in normal tissues. For the assay of binding capacity in needle biopsy specimens, one point determinations were performed using 2.5nM R 1881 as the ligand. The number of binding sites obtained by this method were well correlated with those obtained by the Scatchard plot in various prostatic tissues.

Many kinds of androphilic proteins bound to androgen in high affinity fashion in the human prostate have been reported (Menon et al., 1977b, Shain and Boesel, 1978, Ekman et al., 1979a, Bradlow and Gasparini, 1979). Among them, testosterone-binding globulin in blood plasma does not bind to R 1881 (methyltrienolone, 17β-hydroxy-17α-methyl-estr-4, 9, 11-trien-3-one), therefore, it has been claimed that androphilic proteins could be measured separately from the testosterone-binding globulin using R 1881 as the ligand (Bonne and Raynaud, 1976, Walsh et al., 1978, Shain et al., 1978, Ghanadian et al., 1978). However, it was reported that the human prostate contained a large amount of progestin-binding protein and this protein bound also to R 1881 in high affinity fashion (Menon et al., 1978, Gustafsson et al., 1979, Kodama et al., 1981). Therefore, the binding of cytosol from the human prostate to R 1881 seems to be the sum of the binding of the androgen receptor and of the progestin-binding protein.

It was reported that the addition of triamcinolone acetonide (9α-fluoro-11β, 16α, 17, 21-tetra-ol-pregna-1, 4-dien-3, 20-dione cyclic 16, 17 acetal with acetone) inhibited the binding of progestin-binding protein to R 1881 in cytosols from the human prostate (Zava et al., 1979), and that including molybdate ions in the incubation mixture stabilized steroid receptors (Leach et al., 1979). To assess the binding by the androgen receptor in cytosols from the human

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prostate accurately, the binding characteristics of the cytosols to R 1881 in the presence of triamcinolone acetonide and molybdate were examined in the present study.

Materials and Methods

Steroids
Labeled and unlabeled R 1881 (specific activity 87 Ci/mmol) were purchased from New England Nuclear, Boston, Mass, USA. Triamcinolone acetonide was obtained from Sigma Chemical Co., St. Louis, Mo, USA. Radioinert R 5020 (Promegeston, 17,21-dimethyl-19-nor-pregna-4,9-dien-3,20-dione) and ORG 2058 (16α-ethyl-21-hydroxy-19-nor-pregn-4-en-3,20-dione) were purchased from New England Nuclear and Radiochemical Centre, Amersham, UK, respectively. Dihydrotestosterone (17β-hydroxy-5α-androstan-3-one), progesterone and estradiol-17β were donated by the Teikoku Hormone Co., Kawasaki, Japan.

Materials
Tissues used in this study were obtained from patients admitted to Chiba University Hospital by surgical procedures (not by transurethral resection) or by perineal biopsy of prostatic cancer. Normal prostates were resected from cystectomized specimens of aged patients with bladder cancer. All tissues were examined histologically to confirm their pathological state.

Immediately after removal, tissues were cut into small pieces, placed on ice and carried to the laboratory. Some tissues were quickly frozen and kept at -80°C until processing.

Tissue preparation
Tissues were homogenized in 5 volumes of 0.01 M Tris-HCl buffer (pH 7.4) containing 1 mM EDTA, 1 mM β-mercaptoethanol and 10% (w/v) glycerol (TEMG buffer) using a glass homogenizer and filtered through nylon cloth. Cytosols were obtained after centrifuging the homogenates at 105,000×g for 60 min.

Sucrose density gradient centrifugation
The cytosol was incubated with 4.6 nM of 3H-R 1881 and 10 mM Na2MoO4 in the presence or absence of 1000-fold molar excess of radioinert R 1881 and/or triamcinolone acetonide at 4°C for 20 h. After incubation, the same amount of TEMG buffer containing 0.05% dextran (T 70, Pharmacia, Uppsala, Sweden)-coated 0.5% charcoal (Norit A, Wako Pure Chemicals, Osaka, Japan) was added, centrifuged, and 0.5 ml of the resultant supernatant was applied on the top of a 5-20% sucrose gradient in TEMG buffer (13 ml) containing 10 mM Na2MoO4. Centrifugation was performed in a Hitachi Ultracentrifuge (55P-72, Hitachi Co., Tokyo, Japan) using a RPS40T rotor at 165,500×g for 23 h. After centrifugation, the gradient was fractionated into 0.4 ml fractions with a density gradient fractionator (Isco, Model 640, Lincoln, Nebraska, USA).

Saturation analysis of the binding
Unless otherwise indicated, cytosols were incubated in 0.5 ml of TEMG buffer containing 10 mM Na2MoO4 and various concentrations (0.25-5 nM) of 3H-R 1881 at 4°C for 20 h. 3H-hormone was dissolved in 10 μl of ethanol and added to the incubation medidm. When the effect of the addition of various steroids was examined, another 5-10 μl of the ethanol solution of the additives was added to the incubation medium.

After incubation, 0.5 ml of TEMG buffer containing dextran-coated charcoal was added to the incubation mixture, centrifuged, and the radioactivity in an aliquot of the supernatant was measured. In parallel incubations, 1000-fold molar excess of unlabeled ligand was added to the incubation tube. Nonspecific binding which was not displaced under these conditions was subtracted in all instances. The dissociation constant (Kd) and binding capacity (maximum binding sites) were determined by the method of Scatchard (1949).

One point determination of the binding capacity
Samples obtained by needle biopsy was homogenized in TEMG buffer, filtered through nylon cloth, and centrifuged to make cytosols. The cytosols were incubated in 0.3 ml of TEMG buffer containing 10 mM Na2MoO4, 2.5 μM triamcinolone acetonide and 2.5 nM 3H-R 1881 at 4°C for 20 h. After incubation, unbound steroid was removed by adding the dextran-coated charcoal and the radioactivity was measured. Nonspecific binding was also subtracted.

Analytical methods
Protein was determined by the biuret method (Gornall et al., 1949, Zishka and Nishimura, 1970) or the method of Lowry et al. (1951). Radioactivity was determined in toluene containing 0.4% PPO and 0.01% POPOP in a liquid scintillation counter (LKB 1215 Rackbeta, Wallac OY, Finland).
Results

Effect of supplement of molybdate and triamcinolone acetonide on the $^3$H-R 1881 binding in cytosols from the human prostate

The time course of the binding of cytosol to $^3$H-R 1881 in the presence of 10 mM Na$_2$MoO$_4$ and 1000-fold molar excess of triamcinolone acetonide was examined (Fig. 1). The binding increased gradually with time, and the specific binding reached almost the maximum level after 16 h of incubation. Therefore, subsequent incubations for determination of $^3$H-R 1881 bin-

![Image of Fig. 1](image1)

![Image of Fig. 2](image2)

Fig. 1. Time course of binding of the cytosol to $^3$H-R 1881 in the presence of 10 mM Na$_2$MoO$_4$ and 1000-fold molar excess of triamcinolone acetonide. The cytosol from the benign hypertrophic prostate (4.1 mg as protein/tube) was incubated with 1 nM $^3$H-R 1881, 10 mM Na$_2$MoO$_4$, and 1 mM triamcinolone acetonide at 4°C for various time periods. Nonspecific binding was obtained from parallel incubations which contained 1 mM radioinert R 1881. Specific binding was calculated by subtracting nonspecific binding from the total binding. Closed circle: total binding, open circle: specific binding, closed triangle: nonspecific binding.

Fig. 2. Binding of the cytosol to $^3$H-R 1881 in the presence of 1000-fold molar excess of triamcinolone acetonide and various concentrations of Na$_2$MoO$_4$. The cytosol from the benign hypertrophic prostate (4.1 mg as protein/tube) was incubated with various concentrations (0.25-5 nM) of $^3$H-R 1881 and 1000-fold molar excess of triamcinolone acetonide in the presence or absence of Na$_2$MoO$_4$ (closed circle: no addition, open circle: 0.2 mM, closed triangle: 10 mM, open triangle: 20 mM). Kd and maximum binding sites calculated by Scatchard plot are as follows: no addition; 0.6 x 10$^{-9}$M and 13 fmol/mg protein, 0.2 mM molybdate; 0.6 and 13, 10 mM; 0.5 and 14, 20 mM; 0.6 and 16, respectively.
ding were performed for 20 h.

To examine the effect of molybdate on the binding, the cytosol was incubated with various concentrations of \(^3\)H-R 1881 and 1000-fold molar excess of triamcinolone acetonide supplemented with or without 0.2–20 mM \(\text{Na}_2\text{MoO}_4\). The addition of molybdate did not change the \(K_m\) significantly, but increased the number of maximum binding sites significantly (Fig. 2). Therefore, subsequent incubations were carried out in the presence of 10 mM \(\text{Na}_2\text{MoO}_4\).

The cytosol prelabeled with \(^3\)H-R 1881 in the presence of a 1000-fold molar excess of triamcinolone acetonide was fractionated in the sucrose density gradient centrifugation (Fig. 3). A peak of approximately 8.5 S was evident after incubation whether there was a triamcinolone acetonide supplement or not, and this peak disappeared after introducing a 1000-fold molar excess of the radioinert R 1881 to the incubation mixture.

**Inhibition of \(^3\)H-R 1881 binding in cytosol from the benign hypertrophic prostate by various steroids**

The cytosol was incubated with 1 nM \(^3\)H-R 1881 and 10 mM \(\text{Na}_2\text{MoO}_4\) supplemented with various concentrations (10–1000 nM) of additives (Fig. 4A). Synthetic progestins such as R 5020 and ORG 2058 were potent inhibitors of the binding, while the inhibition by dihydrotestosterone and estradiol-17β was rather weak.

When 1 μM triamcinolone acetonide was added to the incubation mixture, dihydrotestosterone inhibited the binding markedly, while the inhibition by the synthetic progestins was relatively weak (Fig. 4B). Therefore, the binding of cytosol to R 1881 in the presence of an excess amount of triamcinolone acetonide seemed to be androgen-specific.
Fig. 4. Effect of triamcinolone acetonide on the inhibition of the binding of cytosol to $^3$H-R 1881 by various steroids in the incubation mixture containing 10 mM Na$_2$MoO$_4$. The cytosol from the benign hypertrophic prostate (4.1 mg [Fig. 4A] or 4.4 mg [Fig. 4B] as protein/tube) was incubated with 1 nM $^3$H-R 1881 and 10 mM Na$_2$MoO$_4$ in the presence (Fig. 4B) or absence (Fig. 4A) of 1 µM triamcinolone acetonide at 4°C for 20 h. Various concentrations (10, 100 and 1000 nM) of additives were also included (closed circle; R 1881, open circle; dihydrotestosterone, open triangle; R 5020, closed triangle; ORG 2058, closed square; progesterone, open square; estradiol-17β). Results are expressed as % of the control (0.145 pmol in Fig. 4A and 0.06 pmol in Fig. 4B).

Table 1. Binding of cytosols from the normal, benign hypertrophic and cancerous prostates to $^3$H-R 1881.

<table>
<thead>
<tr>
<th>Cytosol Type</th>
<th>No. of cases</th>
<th>$K_d$ (10$^{-9}$M)</th>
<th>fmol/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal prostate</td>
<td>6</td>
<td>0.4±0.1</td>
<td>8.8±0.8</td>
</tr>
<tr>
<td>Benign hypertrophic prostate</td>
<td>14</td>
<td>0.4±0.1</td>
<td>13.2±1.1*</td>
</tr>
<tr>
<td>Cancerous prostate</td>
<td>3</td>
<td>0.6±0.2</td>
<td>22.1±2.8**,#</td>
</tr>
</tbody>
</table>

Data are shown as M±S.E. Statistical differences from values for the normal prostate (*; p<0.02, **; p<0.01) and from values for the benign hypertrophic prostate (#; p<0.01).

Cytosols were incubated with various concentrations of $^3$H-R 1881 supplemented with 10 mM Na$_2$MoO$_4$ and 1000-fold molar excess of triamcinolone acetonide.

Binding to $^3$H-R 1881 of cytosols from normal, benign hypertrophic and cancerous prostates measured by incubations in the presence of 10 mM Na$_2$MoO$_4$ and 1000-fold molar excess of triamcinolone acetonide

All cytosols examined in the present study which were obtained from normal and pathological human prostates showed a single high affinity binding to $^3$H-R 1881. $K_d$ and maximum binding sites in these cytosols are summarized in Table 1. $K_d$ in the normal and the pathological prostates were similar to each other, while significant differences were noticed in the maximum binding sites of the normal, benign hypertrophic and cancerous prostates.
One point determination of the binding capacity to $^3$H-R 1881

Specimens obtained by needle biopsy are too small to perform the saturation analysis. To establish the one point determination of the binding which can be applicable to small specimens, small tissue specimens were taken by a biopsy needle from the tissues which were removed surgically and used for the saturation analysis described in the former section. Using these small specimens one point determination was performed. A ligand concentration of 2.5 nM was chosen, because the specific binding at this concentration was close to the saturation level and the nonspecific binding was relatively low. The nonspecific binding was increased when more than 2.5 nM of the ligand was used.

The maximum binding sites obtained by the saturation analysis and the binding sites obtained by the one point determination performed in the same specimens are depicted in Fig. 5. The values estimated by these two methods were well correlated, and the binding sites obtained by the one point determination were almost 80% of the maximum binding sites by the saturation analysis.

Fig. 5. Relationship between the maximum binding sites calculated by the saturation analysis and the binding sites measured by the one point determination. Open triangle; normal prostate, closed circle; benign hypertrophic prostate, open circle; cancerous prostate.

The binding sites in twenty-one needle biopsy samples of prostatic cancer were measured by the one point determination (Fig. 6). Weight of the samples used for the determination were approximately 100 mg wet weight. In Fig. 6, values for the normal and the benign hypertrophic prostates estimated in the same way and described in the former section are also included. The binding sites in the normal prostate seemed to be smaller than the pathological prostates. Although Table 1 showed a significant difference between the maximum binding sites in the benign hypertrophic prostate and the cancerous tissues, the one point determination did not show any difference between these two pathological tissues. This might be due to variations
in the samples used for the experiments. The samples of prostatic cancer described in Table 1 were taken from the localized stage and the patients had received no endocrine treatment previously, while the cancerous tissues used for the assays depicted in Fig. 6 consisted of various tissues from different stages and different hormone sensitivity. However, in some cases of prostatic cancer the number of binding sites was obviously higher. There was no correlation between the number of binding sites and the histological grading in prostatic cancer (data not shown).

![Diagram showing binding sites of cytosols from normal, benign hypertrophic, and cancerous prostates](image)

**Discussion**

It has been reported that the addition of molybdate in incubation mixtures does not modify the Kd, sedimentation profile, steroid specificity and rate of dissociation of androgen receptor from rat prostate, but only stabilizes the receptor probably protecting protein from denaturation (Gaubert et al., 1980). A similar effect of molybdate has been also observed in other steroid-receptor systems (Noma et al., 1980, Bevins and Bashirelahi, 1980). In a previous study it was observed by Kodama et al. (1980) that the binding of prostatic cytosol to R 1881 decreased after 6 h in the incubation without molybdate but in the present experiment the binding progressively increases and is maintained as long as 20 h by adding molybdate. It is also noticed that the 8.5S peak of the binding is clearly observed in the presence of molybdate but without molybdate the 8.5S peak is not shown clearly (Menon et al., 1977a, Kodama et al., 1980).
R 1881 binds to both androphilic and progestin-binding proteins of cytosols from the human prostate, but the addition of triamcinolone acetonide selectively inhibits the binding to progestin-binding protein (Asselin et al., 1979). Consequently, R 1881 binding in the presence of triamcinolone acetonide represents the binding of the androphilic protein and excludes that of the progestin-binding protein. Hicks and Walsh (1979) have reported that the rate of inhibition by various steroids of R 1881 binding in cytosols of the human prostate is changed when the incubation mixture includes triamcinolone acetonide; in the absence of triamcinolone acetonide the binding of R 1881 is markedly inhibited by many progestins, while in the presence of triamcinolone acetonide the inhibition of progestins diminishes and the binding of R 1881 is almost identical to that of dihydrotestosterone. From this evidence and the results of the present inhibition study, most of the R 1881 binding to androphilic protein demonstrated under triamcinolone acetonide seems to be attributable to the cytosolic androgen receptor.

In a previous report from this laboratory (Nozumi et al., 1981), it was shown that approximately 20% of the maximum binding sites for R 1881 in cytosols from the human prostate were not inhibited by the addition of triamcinolone acetonide. Therefore, it was assumed that 20% of the R 1881 binding was attributable to the androgen receptor. However, in the present study, the rate of inhibition by triamcinolone acetonide is approximately 55% of the total binding to R 1881 (data not shown). Therefore, 45% of the R 1881 binding in cytosols from the human prostate is attributable to the androgen receptor. This is probably due to the effect of the molybdate supplement which stabilizes the androgen receptor, and prolonged incubation time may also increase the rate of exchange of the binding sites to R 1881 which have been already occupied by endogeneous androgens.

There have been many reports concerning measurement of androgen binding in various human prostates. A higher content of the androgen binding components in the cancerous prostate has been reported by several workers (Snochowski et al., 1977, Krieg et al., 1978, Voogt and Dingjan, 1978, Ekman et al., 1979b). In the other hand, the whole binding of cytosols to R 1881 is similar in both normal and the pathological prostates (Shain et al., 1980, Nozumi et al., 1981). In the present study, cytosolic androgen receptor is compared in the normal and the pathological prostates, and a higher amount of androgen receptor in benign hypertrophic and cancerous prostates is observed than that in normal tissues. It has also been reported that cancerous tissues which are sensitive to endocrine therapy contain more binding components for R 1881 in cytosols than those being refractory to estrogen (Gustafsson et al., 1978, Mobbs et al., 1978, Ekman et al., 1979c, Sidh et al., 1979, Martelli et al., 1980).

Kd in the binding of cytosols from the human prostate to R 1881 is fairly constant. Therefore, a one point determination assayed at a saturable concentration of the ligand should be applicable for comparison of the binding capacity in cancerous prostates. Hicks and Walsh (1979) and Trachtenberg et al. (1981) used 5 nM R 1881 as the concentration of the assay and claimed that 76% of the maxmum binding sites are estimated by their one point determinations. In the present study, similar results are obtained. Using the one point determination for biopsy specimens, the examination of the androgen receptor will be able to be performed easily at various stages of prostatic cancer.
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References


