IMMUNOHISTOCHEMICAL STUDY OF ANDROGEN RECEPTOR IN RAT PROSTATIC HYPERPLASIA —EFFECT OF ANTI-ANDROGEN TZP-4238—

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The effect of a synthetic steroidal anti-androgen, TZP-4238, on steroid-induced rat dorsolateral prostatic hyperplasia was investigated. Male Sprague-Dawley rats were divided into five experimental groups. Group 1 consisted of intact controls. The other animals were castrated. The castrated animals were treated for seven weeks with 1) testosterone 1 mg/animal plus 17 β -estradiol (E₂) 10 μ g/animal (Group 2), 2) testosterone plus E₂ plus TZP-4238 2 mg/kg (Group 3), 3) testosterone plus E₂ plus TZP-4238 8 mg/kg (Group 4) and 4) testosterone plus E₂ plus Tween 80 (Group 5) instead of TZP-4238. TZP-4238 was administered orally for four weeks after three weeks of treatment with testosterone plus E₂. In groups 2 and 5, glandular hyperplasia of the dorsolateral prostate was clearly observed, and the fibro-muscular stromal proliferation was also noted. The glandular epithelial cells showed uniformly intense nuclear immunostaining for androgen receptors (AR). Furthermore, AR was also localized in the nuclei of the proliferated fibro-muscular cells. In contrast, combined treatment with TZP-4238 (Groups 3 and 4) produced inhibition of the glandular hyperplasia. Furthermore, nuclear immunostaining of AR of both epithelial and stromal cells was remarkably decreased. These results indicate that the uptake of testosterone and/or its androgenic effect on the dorsolateral prostate may be suppressed by TZP-4238. Furthermore, the decreased AR immunostaining may be explained by the decrease in number of AR and/or antibody binding site for AR.

Previous studies using castrated steroid-treated dogs and rats have indicated that 17 α -acetoxy-6-chloro-2-oxa 4,6-pregnadiene-3,20-dione (TZP-4238), a new type of steroidal anti-androgen, inhibits the experimental induction of BPH (12, 13).

The dorsolateral prostate of the rat has been shown to be particularly sensitive to estrogen (3, 7, 18). Specific receptor for estrogen has been demonstrated in the dorsolateral prostate (19). Therefore, it should be a good model for human prostate, since, besides androgens, estrogens are supposed to have an important role in its physiology and pathology (20).

The purpose of the present study was to further examine the effect of TZP-4238 on rat dorsolateral prostatic hyperplasia model after combined treatment

with testosterone and 17 β -estradiol. Using a polyclonal antibody specific for the fusion protein of human androgen receptor (AR) NH27 (11, 13–15), attempts were made to localize the AR immunohistochemically in the dorsolateral prostate of normal, castrated, testosterone plus 17 β -estradiol and anti-androgen TZP-4238-administered rats.

MATERIALS AND METHODS

Animals and tissue preparation

Male Sprague-Dawley rats were purchased from Charles River Japan Inc. (Atsugi, Japan) at the age of seven weeks. The animals were kept in a barrier-maintained room, which was maintained at a temperature of 22 $\pm$ 2°C with a humidity of 55 $\pm$ 15%. The room was ventilated twenty one times per hr and provided with 12 hr of light (from 8:00 to 20:00). The animals were housed individually in plastic cages

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(CLEA Japan Inc.). Solid food (CE-2, CLEA Japan Inc.) and tap water were available to all animals *ad libitum*. Then one week was allowed for the adjustment of the animals to laboratory conditions.

The details of treatment are given in Fig. 1. Five animals served as controls (Group 1). The other animals were castrated and allowed two days for involution of the prostate before any hormonal treatment was begun. After this period, castrated animals were treated for seven weeks with 1) testosterone propionate (testosterone, Sigma Chemical Co., St. Louis, MO) 1 mg/animal plus 17β -estradiol (E_2 , Teikoku Hormone Mfg. Co. Ltd., Kawasaki) 10 μ g/animal (Group 2, $n=5$), 2) testosterone plus E_2 and TZP-4238 2 mg/kg (Group 3, $n=5$), 3) testosterone plus E_2 and TZP-4238 8 mg/kg (Group 4, $n=5$), 4) testosterone plus E_2 and distilled water containing 2% Tween 80 (Group 5, $n=5$). The dosages of testosterone and E_2 have been previously shown to cause prostatic hyperplasia (ventral prostate) in the castrated rat (13, 15). Testosterone and E_2 were dissolved in dimethylsulfoxide and were administered subcutaneously six times per week. TZP-4238 was suspended in distilled water containing 2% Tween 80 and was administered orally (six times per week) for four weeks after three weeks treatment with testosterone plus E_2 .

Organ weight

The weight of the dorsolateral prostates was recorded (absolute weight). Weight relative to body weight (relative weight) was calculated.

Histopathological examination

The dorsolateral prostates were fixed in 0.1 M phosphate-buffered 10% formalin, embedded in paraffin, mounted and stained with hematoxylin and eosin (HE).

Immunohistochemical staining AR

The tissues taken from the animals were frozen in dry-ice cooled ethanol. Frozen sections of 6 μ m thickness were prepared in a cryostat and mounted on albumin-coated glass slides. The sections were fixed for 10 min at 4°C in Zamboni's fixative (21). After washing in 0.01 M PBS containing 20% sucrose, the sections were soaked in absolute methanol containing 0.3% hydrogen peroxide for 30 min at room temperature to inactivate endogenous peroxidase. After washing in 0.01 M PBS, the sections were incubated overnight at 4°C with NH 27, a rabbit polyclonal anti-androgen receptor antibody (1:1000) (11, 13-15). After washing in 0.01 M PBS, the sections were covered with biotin-conjugated goat anti-rabbit IgG for 1 hr, washed and then treated with streptavidin-biotin-peroxidase complex (Histofine SAB-PO (R) kit, Nichirei, Tokyo) for 1 hr. After washing in 0.01 M PBS, the sections were treated in Graham-Karnovsky's reaction medium (6), which contained 20 mg% DAB and 0.005% hydrogen peroxide in 0.05 M Tris-HCl buffer, pH 7.6, for 5 to 10 min at room temperature. Then the sections were counterstained for nuclei with 1% methyl green dissolved in veronal acetate buffer, pH 4.2. As an immunologic negative control, normal rabbit IgG was substituted for the primary antibody.

Statistics

The data was expressed as means $\pm$ S.D. Homogeneity of variance was tested by Bartlett's method, and when the assumption of homogeneity of variance was met, one-way layout analysis of variance was performed. When a significant difference was observed, Scheff's or Dunnett's multiple comparative

FIG. 1. Experimental schedule.

TABLE 1. Influence of testosterone (T) plus 17 β -estradiol (E₂) or T plus E₂ and TZP-4238 on the weight of rat dorsolateral prostate

Group	Preparation	n	Treatment	Prostatic weight (mg)	
				Absolute	Relative
1	Intact control	5	—	298 $\pm$ 4.83	68 $\pm$ 12.1
2	Castrated	5	T + E ₂	593 $\pm$ 81.5*	108 $\pm$ 22.3**
3	Castrated	5	T + E ₂ + TZP-4238 2 mg/kg	380 $\pm$ 68.3	83 $\pm$ 41.3
4	Castrated	5	T + E ₂ + TZP-4238 8 mg/kg	235 $\pm$ 21.3	59 $\pm$ 8.3
5	Castrated	5	T + E ₂ + Tween	543 $\pm$ 40.3*	98 $\pm$ 21.3**

Values are mean $\pm$ S.D.

*, P < 0.05 vs. intact control (Dunnett's multiple comparison test).

**, P < 0.01 vs. intact control (Dunnett's multiple comparison test).

FIGS. 2a-d. a. Dorsolateral (DL) prostate of intact rat. b. DL prostate of castrated rat following treatment with combined testosterone plus E₂. Glandular hyperplasia is clearly observed. Interacinar stroma is also proliferated. c, d. DL prostate of castrated rat after treatment with testosterone plus E₂ and TZP-4238 2 mg/kg (c) or 8 mg/kg (d). Inhibition of the glandular hyperplasia is observed. H-E stain, $\times$ 90

test was performed between the intact control group and the other experimental groups.

RESULTS

1. Prostatic weight

The dorsolateral prostatic weight is shown in Table 1. Statistically significant increase was noted in the absolute ($p < 0.05$) and relative ($p < 0.01$) weight of the dorsolateral prostate of the castrated rats treated with testosterone plus E_2 (Group 2). Groups 2 and 5 showed a similar change of prostatic weight. The administration of testosterone plus E_2 and TZP-4238 (Groups 3 and 4) to the castrated rats resulted in a

reduction of the absolute and relative weight of the dorsolateral prostates. In particular, the value of group 4 was not different from that of the intact control (Group 1).

2. Histopathological and immunohistochemical findings

Group 1 (Intact control)

Epithelial cells lining the dorsolateral prostate were columnar (Fig. 2a). AR was localized exclusively in the nuclei of the epithelial cells (Fig. 3a). No immunostaining was observed in the nuclei when the sections were incubated with preimmune serum (data not shown).

Group 2 (Castration plus Testosterone plus E_2)

The glandular epithelial cells were high, column-

FIGS. 3a-e. a. Dorsolateral (DL) prostate of intact rat. AR is localized exclusively in the nuclei of the epithelial cells. b. DL prostate of castrated rat following treatment with combined testosterone plus E_2 . The glandular epithelial cells showed uniformly intense nuclear immunostaining for AR. Furthermore, AR is also localized in nuclei of the fibro-muscular cells (c). d, e. DL prostate of castrated rat after treatment with testosterone plus E_2 and TZP-4238 2 mg/kg (d) or 8 mg/kg (e). The immunoreaction for the AR is negative or very weak in both glandular epithelial cells and fibro-muscular cells. ABC method, $\times 110$, $\times 300$ (c)

nar, and showed an increased number of papillary projections extending into acini (Fig. 2b). In addition, the epithelium showed a highly secretory appearance. Interacinar stroma was also proliferated. The glandular epithelial cells showed uniformly intense nuclear immunostaining for AR (Fig. 3b). Furthermore, AR was also localized in the nuclei of the fibro-muscular cells (Fig. 3c).

Group 3 (Castration plus Testosterone plus E₂ plus TZP-4238 2 mg/kg)

Severity of the glandular hyperplasia was less marked than that in group 2 (Fig. 2c). The immunoreaction for the AR was weak in both glandular epithelial cells and fibro-muscular cells (Fig. 3d).

Group 4 (Castration plus Testosterone plus E₂ plus TZP-4238 8 mg/kg)

Inhibition of the glandular hyperplasia was observed (Fig. 2d). The immunoreaction for the AR was negative or very weak in both glandular epithelial cells and fibro-muscular cells (Fig. 3e).

Group 5 (Castration plus Testosterone plus E₂ plus Tween)

This appearance was nearly the same as that of Group 2.

DISCUSSION

In the present study, glandular hyperplasia of the dorsolateral prostate was seen in castrated rats after treatment with testosterone in combination with E₂. It is known that E₂ induces glandular atrophy of the prostate in normal castrated animals (1, 2). The suppressive effects of E₂ on the male genital organs have been attributed not only to gonadotropin suppression but also to the direct effect of E₂ on the gonads and their accessories (4, 17). On the contrary, it is a well documented fact that E₂ exerts a synergistic effect with androgen in promoting prostatic growth such as glandular hyperplasia or dysplasia (10). Therefore, glandular-type prostatic hyperplasia was thought to be the main feature of rat prostatic hyperplasia occurring experimentally as a result of treatment with steroid hormones. These findings are in good agreement with previous reports (13, 15). Fibro-muscular stromal proliferation was also seen in the group of rats receiving the combination treatment. This feature might be explained by the action of E₂, since growth of stromal tissue following estrogen administration has been demonstrated in the prostate of non-human primates and dogs (9).

Combined treatment with TZP-4238 to the castrated rats resulted in a reduction of the absolute and relative weight of the dorsolateral prostates. In fact, the average prostatic weight following treatment

with TZP-4238 (8 mg/kg) was not different from that of the intact control. Histologically the combined treatment with TZP-4238 produced inhibition of the glandular hyperplasia. These results indicate that the uptake of testosterone and/or its androgenic effect on the dorsolateral prostate may be suppressed by TZP-4238. In fact, atrophy of the glandular epithelium induced by TZP-4238 was compensated by fibro-muscular stromal proliferation (12). This feature was considered to be the action of E₂.

A rabbit polyclonal antibody referred as NH 27 was raised against human AR (11). The specificity of the antibody in immunohistochemical reactions has been described elsewhere (13-15). In the present work, nuclear immunostaining of AR of both epithelial and stromal cells was remarkably decreased after combined treatment with TZP-4238.

Takezawa *et al.* (16) reported that TZP-4238 inhibited prostatic growth through 1) inhibition of the AR, 2) a decrease in nuclear AR and 3) a decrease in 3 α -hydroxysteroid oxyreductase activity to prevent a metabolic shift to dihydrotestosterone accumulation. It is a well documented fact that the prostatic nuclear AR contents after treatment with gonadotropin-releasing hormone (GnRH) agonist (5) as well as cyproterone acetate (8), an anti-androgen agent, were decreased. Based on our data and these facts, decreased immunostaining for AR after combined treatment with TZP-4238 may be explained by the decrease in number of AR and/or antibody binding site for AR.

The precise mechanism by which TZP-4238 produces atrophy of the prostatic glandular epithelium is not yet clear from the present immunohistochemical investigation. In this context, measurements of nuclear and/or cytosolic ARs, and of nuclear concentrations of TZP-4238 in the prostatic hyperplasia, would seem to be important for clarifying this problem. Further work along this line is now in progress in our laboratory.

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