IMMUNOHISTOCHEMICAL EVALUATION OF THE EFFECT OF TZP-4238—POSSIBLE ANDROGEN RECEPTOR ANTAGONIST IN RAT PROSTATE—

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Rat prostate was stained immunohistochemically using polyclonal androgen receptor antibody. A rabbit polyclonal antibody referred as NH27 was raised against human androgen receptor. Male Sprague-Dawley rats were divided into four experimental groups. Group 1 consisted of intact controls. In group 2, rats were sacrificed two days after castration. In group 3, rats were administered subcutaneously 1 mg/kg of testosterone daily for three days after two days of castration. In group 4, rats were given 10 mg/kg of TZP-4238, an anti-androgen agent, orally by gavage once a day for 1 week. Frozen sections of the prostates were fixed in Zamboni's fixative. The sections were incubated overnight at 4°C with NH27. In the control rat prostate, positive staining for the antibody appeared in nuclei of the glandular epithelium. In addition, immunodetectable androgen receptor remarkably decreased 2 days after castration and returned to intact levels after testosterone-administration in the castrated rats. These findings are in agreement with results of previous work on the prostate. Furthermore, nuclear immunostaining of the androgen receptor remarkably decreased after the treatment with TZP-4238. The precise mechanism which produces atrophy of the prostatic glandular epithelium is not yet clear from the present study. We further speculated that TZP-4238 was considered to be a steroidal androgen receptor antagonist. It is concluded that immunohistochemical analysis using NH27 may be a very useful method for predication of the effects of anti-androgen treatment for rats.

Recently androgen and estrogen receptors have been demonstrated biochemically as well as immunohistochemically (8, 13, 14, 19). A rabbit polyclonal antibody referred as NH27 was raised against human androgen receptor (9). Previous immunohistochemical study using this antibody showed that androgen receptor is localized in nuclei of the human secretory cells and basal cells of the prostate, spermatogonia, spermatocytes, Sertoli cells and Leydig cells of the testis, epithelial cells of the rete testis, fibroblasts in the whole organ, squamous cells, sweat gland and hair follicle cells of the skin, and hepatocytes of the liver (6). On the other hand, androgen receptor is primarily under androgenic control

in prostatic tissue (2, 3, 16). Androgens have been shown to increase both androgen receptor synthesis (1, 17) and half-life of the receptor (17), which result in elevated receptor protein levels within the tissue. It is a well documented fact that immunodetectable androgen receptor rapidly decreased 3 days after castration and returned to the intact level after testosterone-administration (5). In contrast, prostatic androgen receptor mRNA levels are elevated after castration-induced androgen withdrawal and decline in the presence of testosterone under *in vivo* and *in vitro* conditions (7, 15, 20).

Recently, a new type of steroidal anti-androgen, 17 α -acetoxy-6-chloro-2-oxa-4,6-pregnadiene-3,20-dione (TZP-4238), was synthesized in our laboratory by introducing an oxygen atom at the C₂ position of chlormadinone acetate (CMA). TZP-4238 was found to be a more potent anti-androgen agent than CMA in

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the prostate (10–12). However, studies on the effect of TZP-4238 on androgen receptor levels in the prostatic tissue are scarce.

Using a polyclonal antibody specific for the fusion protein of human androgen receptor NH27, attempts were made to localize the androgen receptor immunohistochemically in the prostate of normal, castrated, testosterone- 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 five weeks. The animals were kept in a barrier-maintained room, which were maintained at a temperature of $22 \pm 2^\circ\text{C}$ with a humidity of $55 \pm 15\%$. The room was ventilated twenty one times per hour and provided with 12 hr of light (from 8:00 to 20:00). The animals were housed individually in plastic cages (CLEA Japan Inc.). Solid food (CE-2, CLEA Japan Inc.) and tap water were available to all animals *ad libitum*. Two weeks were allowed for the adjustment of the animals to the laboratory conditions. These animals were divided into four experimental groups. Group 1 consisted of five untreated controls. In group 2, five rats were sacrificed two days after castration. In group 3, five rats were administered subcutaneously 1 mg/animal of testosterone (Sigma Chemical Co., St. Louis, MO) daily for three days after two days of castration. In group 4, five rats were given 10 mg/kg of TZP-4238, an anti-androgen agent, orally by gavage once a day for 1 week. Each rat was killed by decapitation and the ventral prostate was removed immediately.

Immunocytochemical staining

The tissues taken from the animals were frozen in dry-ice cooled ethanol. Frozen sections of $6 \mu\text{m}$ 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 with 0.01 M phosphate-buffered saline (PBS), pH 7.4, 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 NH27, rabbit polyclonal anti-androgen receptor antibody (1:1000) (9). 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 SABPO (R) Kit, Nichirei, Tokyo) for 1 hr. After washing in 0.01 M PBS, the sections were treated in Graham-Karnovsky's reaction medium (4), which contained 20 mg% 3,3'-diaminobenzidine (DAB, Wako Pure Chemical Industries, Osaka) 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.

RESULTS

Intact control

The glandular epithelium of prostate consisted of single-layered, cylindrical cells which showed pronounced eosinophilic staining of the cytoplasm, and the basal location of nuclei. Not frequently, papillary projections of the glandular epithelial cells into the acinar lumen were also noted. Androgen receptor was localized exclusively in the nuclei of the epithelial cells (Fig. 1). No immunostaining was observed in the nuclei when the sections were incubated with preimmune serum (Fig. 2).

Castration

Two days after castration, the cells of the glandular epithelial lining were lower in height than those of the intact controls. Under this condition, the immunoreaction for the androgen receptor was negative

FIG. 1. Immunohistochemical localization of androgen receptor in control rat prostate. Androgen receptor is localized exclusively in the nuclei of the epithelial cells. ABC method. $\times 120$

FIG. 2. Using control serum (normal rabbit serum), no immunohistochemical reaction is seen in prostate. ABC method. $\times 100$

FIG. 3. Immunohistochemical localization of androgen receptor in rat prostate after two days of castration. The immunoreaction for the androgen receptor is negative or very weak in the glandular epithelial cells. ABC method. $\times 200$

FIG. 4. Immunohistochemical localization of androgen receptor in prostate after testosterone- administration for three days to the castrated rat. Nuclear immunostaining for androgen receptor in the glandular epithelial cells is clearly seen. ABC method. $\times 200$

FIG. 5. Immunohistochemical localization of androgen receptor in rat prostate after TZP-4238 (anti-androgen)- administration for 1 week. The immunoreaction for androgen receptor is negative or very weak in the glandular epithelial cells. ABC method. $\times 180$

or very weak in the glandular epithelial cells (Fig. 3).

Castration + testosterone-administration

The height of the glandular epithelial lining was similar to that of the intact controls. Nuclear immunostaining for the androgen receptor in the glandular epithelial cells was clearly seen (Fig. 4).

TZP-4238-administration

The cells of the glandular epithelial lining were low in height. The immunoreaction for the androgen receptor was negative or very weak in the glandular epithelial cells (Fig. 5).

DISCUSSION

In the present study, we found that immunodetectable androgen receptor remarkably decreased 2 days after castration, and that it was clearly recovered by testosterone-administration in the castrated rats. These findings are in agreement with the results of previous works on the prostate (8, 13, 19). Androgen is known to down-regulate its own receptor (20). A possible explanation for the loss of androgen receptor immunostaining in the absence of androgen is that androgen receptor may be easily lost during tissue preparation and fixation since it is loosely associated with cellular components. Recovery of the nuclear immunostaining in the castrated animals by the testosterone treatment suggests that androgen stimulates the synthesis of new androgen receptor.

In the present work, the nuclear immunostaining for androgen receptor remarkably decreased after treatment with TZP-4238. As the mechanism by which TZP-4238 produces atrophy of the prostatic glandular epithelium, Takezawa *et al.* (18) reported that TZP-4238 inhibited prostatic growth through 1) inhibition of the androgen receptor, 2) a decrease in nuclear androgen receptor and 3) a decrease in 3α -hydroxysteroid oxyreductase activity to prevent a metabolic shift to dihydrotestosterone accumulation. The precise mechanism which produces atrophy of the prostatic glandular epithelium is not yet clear from the present study. We further speculated that TZP-4238 was considered to be a steroidal androgen receptor antagonist.

It is concluded that immunohistochemical analysis using NH27, may be a very useful method for predication of the effects of anti-androgen treatment for rats.

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