HISTOPATHOLOGICAL AND IMMUNOHISTOCHEMICAL STUDIES OF THE RAT DORSOLATERAL PROSTATE — EFFECTS OF TESTOSTERONE AND TESTOSTERONE PLUS ESTROGEN —

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Abstract: The effects of testosterone and 17β -estradiol (E_2) on the dorsolateral prostate of castrated rats were investigated by histopathological and immunohistochemical procedures. Male Sprague-Dawley rats were divided into four experimental groups. Group 1 consisted of intact controls. The other animals were castrated. In group 2, rats were sacrificed 2 days after castration. The castrated animals were treated for 6 weeks with 1) testosterone 1 mg/head (Group 3) and 2) testosterone 1 mg/head plus E_2 10 μ g/head (Group 4). A significant increase in the dorsolateral prostatic weight occurred after 6 weeks treatment with testosterone plus E_2 (Group 4). Histopathologically, glandular hyperplasia with fibro-muscular stromal proliferation was clearly observed, and the number of bromo-deoxyuridine (BrdU)-positive cells showed a significant increase over that induced by testosterone alone. Immunohistochemical localization of glutathione peroxidase (GSH-PO) which effectively reduces the lipid peroxides, was clearly observed in the glandular epithelial cells of the dorsolateral prostate following testosterone alone or testosterone plus E_2 -treatment. Therefore, it appeared that GSH-PO protein synthesis in the glandular epithelium of the dorsolateral prostate can be enhanced (induced?) by sex hormones such as testosterone and E_2 . In the dorsolateral prostate of intact rats, positive staining for androgen receptor (AR) was observed in nuclei of the glandular epithelial cells. In addition, immunodetectable AR decreased within 2 days after castration, but returned to intact levels after administration of testosterone. These findings agree with previous work on the ventral prostate. Furthermore, in group 4, AR was also detected in the nuclei of the proliferated stromal fibro-muscular cells. It is concluded that immunohistochemical analysis, using GSH-PO and AR, may be a useful method for predication of the effects of androgen action on the rat prostate. (*J Toxicol Pathol* 7: 387~395, 1994)

Key words: Testosterone, 17β -estradiol, Prostatic hyperplasia model, Glutathione-peroxidase (GSH-PO), Androgen receptor

Introduction

In a previous report¹, it was shown that glandular hyperplasia of the ventral prostate developed in castrated rats after combined treatment with testosterone and 17β -estradiol. It is generally accepted that estrogens play a critical role in the etiology of canine and human benign prostatic

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hyperplasia²⁻⁵.

The dorsolateral prostate of the rat has been shown to be particularly to estrogen⁶⁻⁸. Specific receptor for estrogen has demonstrated in the dorsolateral prostate⁹. 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 pathology⁴.

The purpose of the present study was to examine the effects of testosterone, and testosterone plus 17 β -estradiol, on the castrated rat dorsolateral prostate. In addition, an analytical examination for cell proliferation activity, using 5-bromo-deoxyuridine (BrdU)-positive cell ratio and an attempt to observe immunocytochemical localization of androgen receptor (AR) and glutathione-peroxidase (GSH-PO), which effectively reduces the lipid peroxides, were also investigated.

Materials and Methods

Animals

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 was maintained at a temperature of 22 $\pm$ 2°C and humidity of 55 $\pm$ 15%. The room was ventilated twenty-one times per hour and provided with 12 hr of light (from 800 hr to 2,000 hr). 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*. Then one week was allowed for the adjustment of animals to laboratory conditions.

Experiments

Group 1 consisted of 5 untreated controls. In group 2, 5 rats were sacrificed 2 days after castration. In group 3, 5 rats were treated subcutaneously 1 mg/head of testosterone (Sigma Chemical Co., St. Louis, MO, U.S.A.) daily for 6 weeks after 2 days of castration. In group 4, 5 rats were treated subcutaneously 1 mg/head of testosterone plus 10 μ g/head of 17 β -estradiol (E₂, Teikoku Hormone, Mfg. Co. Ltd, Kawasaki) daily for 6 weeks after 2 days of castration. Testos-

treone was dissolved in dimethylsulfoxide (Wako Pure Chemical Industries, Osaka) and E₂ was dissolved in triolein (Sigma Chemical Co., St. Louis, MO, U.S.A.). One hour prior to sacrifice, animals were given an intraperitoneal injection of 5-bromo-2-deoxyuridine (BrdU, Sigma Chemical Co., St. Louis, MO, U.S.A.), at a dose of 100 mg/kg body weight. Each rat was killed by decapitation, and the dorsolateral prostate was removed.

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

1) BrdU

Formalin fixed and paraffin sections were used. Sections (4 μ m) were cut, deparaffinized with xylene and rinsed thoroughly with ethanol. The sections were then placed in absolute methanol containing 0.3% hydrogen peroxide for 30 min at room temperature to inactivate endogenous peroxidase activity. Sections were washed twice in 0.01 M phosphate-buffered saline (PBS), pH 7.4, 5 min per wash. Thereafter, sections were immersed in 2 N HCl at 25°C for 20 min to denature the DNA. To neutralize the HCl, the sections were rinsed in 0.1 M borate buffer, pH 7.6. After washing in 0.01 M PBS, the sections were blocked with 5% normal goat serum for 30 min, then incubated for 2 hr with 1:50 mouse monoclonal anti-BrdU antibody (Becton Dickinson immunocytometry Systems, San Jose, CA). After washing in 0.01 M PBS, the sections were covered with biotin-conjugated goat anti-mouse IgG for 1 hr, washed and then incubated with streptavidin-biotin complex (Histofine SAB-PO (M) Kit, Nichirei, Tokyo) for 1 hr. After washing in 0.01 M PBS, the sections were incubated in Graham-Karnovsky's reaction medium¹⁰ which contained 3, 3'-diaminobenzidine-tetra-hydrochloride (DAB,

Wako Pure Chemical Industries, Osaka) and 0.005% hydrogen peroxide in 0.05 M Tris-HCl buffer, pH 7.6, for 10 min at room temperature. Then the sections were counter-stained for nuclei with hematoxylin. As an immunologically negative control, 1:50 diluted nonimmune mouse serum was used. In addition, the BrdU-positive cell ratio (%) was calculated as the positive cell number in one acinus/total cell number in the acinus $\times$ 100.

2) GSH-PO

The dorsolateral prostates were fixed in periodate-lysine-4% paraformaldehyde solution (4% PLP)¹¹ for 6 hr at 4°C under constant agitation. The fixed tissues were then washed in 0.01 M PBS, containing sucrose from 10 to eventually 20% overnight at 4°C. Subsequently, 6 μ m frozen sections were prepared from the washed tissues in a cryostat, and were placed on albumin coated glass slides. The sections were washed in 0.01 M PBS and then were stained by Nakane's direct peroxidase-labeled antibody method with anti-GSH-PO IgG Fab fragment¹². For light microscopic observations of GSH-PO, 6 μ m frozen sections were incubated with the antibody labeled with horseradish peroxidase (Sigma Chemical Co., St. Louis, MO, U.S.A.) for 1 hr. After the incubation was completed, the immuno-peroxidase staining was performed as described above.

3) AR

Fresh dorsolateral prostates were frozen in dry-ice cooled ethanol. 6 μ m thickness of frozen sections were prepared in a cryostat and attached to albumin-coated glass slides. The sections were fixed for 10 min at 4°C in Zamboni's fixative¹³. After washing with 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 in inactivate endogenous peroxidase activity. After washing in 0.01 M PBS, the sections were incubated overnight at 4°C with NH27, a rabbit polyclonal anti-AR antibody (1:1000)¹⁴. After washing in 0.01 M PBS, the sections were covered with biotin-conjugated goat anti-rabbit IgG for 1 hr, washed and then incubated with streptavidin-biotin complex (Histofine SAB-PO (R) Kit, Nichirei, Tokyo) for 1 hr. After washing in 0.01 M PBS, the immuno-peroxidase staining was performed as described above.

Results

1. Prostatic weights

The dorsolateral prostatic weight is shown in Table 1. Those of the intact and castrated groups were comparable. 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 .

2. Histopathological and immunocytochemical findings

Group 1 (Intact control)

Epithelial cells lining the dorsolateral prostate were columnar and the nuclei were located mainly in basal portion of the cells (Fig. 1A). A few BrdU-positive nuclei were observed in the glandular epithelial cells (Table 1). In the glandular epithelial cells, immunohistochemical localization of GSH-PO was weak or negative (Fig 2A). AR

Table 1. Influence of Testosterone (T) or T Plus 17 β -estradiol (E_2) on the Weight of Rat Dorsolateral Prostate and BrdU-positive Cell Ratio

Group	Preparation	n	Treatment	Prostatic weight (mg)		BrdU-positive cell ratio (%)
				Absolute	Relative	
1	Intact control	5	—	298 $\pm$ 48.3	68 $\pm$ 12.1	0.31 $\pm$ 0.12
2	Castrated	5	—	285 $\pm$ 31.5	61 $\pm$ 9.8	0.08 $\pm$ 0.01
3	Castrated	5	T	399 $\pm$ 98.6	88 $\pm$ 42.3	1.35 $\pm$ 0.81
4	Castrated	5	T + E_2	593 $\pm$ 81.5*	108 $\pm$ 22.3**	3.94 $\pm$ 0.23**

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).

was localized exclusively in the nuclei of the epithelial cells (Fig. 3A). No immunostaining was observed in the nuclei when the section were incubated with preimmune serum.

Group 2 (Castration)

Two days after castration, the height of glandular epithelial cells was slightly reduced (Fig. 1B). BrdU-positive nuclei in the glandular epithelial cells were rarely seen (Table 1). Both GSH-PO (Fig. 2B) and AR (Fig. 3B) were negative or very weak in the glandular epithelial cells.

Group 3 (Castration + Testosterone)

The glandular epithelial cells were of a high columnar shape (Fig. 1C). The number of BrdU-positive nuclei was increased compared with intact control (Table 1). GSH-PO was stained in those glandular epithelial cells (Fig. 2C). In addition, nuclear immunostaining of AR in the glandular epithelial cells was also observed (Fig. 3C).

Group 4 (Castration + Testosterone + E₂)

The glandular epithelial cells were high, columnar, and showed an increased number of papillary projections extending into acini (Fig. 1D). In addition, the epithelium showed a highly secretory appearance. Interacinar stroma was also proliferated. The number of BrdU-positive nuclei was greatly increased compared to the other experimental groups (Table 1). Immunohistochemical localization of GSH-PO staining was stronger than that of Group 3. In addition, the glandular epithelial cells showed uniformly intense nuclear immunostaining for AR (Fig. 3D). Furthermore, AR was also detected in the nuclei of the stromal fibro-muscular cells.

Discussion

In the present study, the weight of the dorsolateral prostate was markedly increased by testosterone plus E₂ to the castrated rats. In addition, it was seen that the castration-induced decrease in the number of BrdU-positive cells was reversed by testosterone. The stimulatory effects of testosterone were strongly potentiated by the addition of E₂, resulting in the highest BrdU-positive cell ratio. Histologically, glandular hyperplasia was clearly seen in this group. It is known that E₂ alone induces glandular atrophy of

the prostate in normal and castrated animals^{5,15}. 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 accessories¹⁵⁻¹⁷. On the contrary, it is a well documented fact that E₂ exerts a synergistic effect with androgen in promoting prostatic growth in the castrated dog or rat, a fact of considerable importance in understanding the pathogenesis of prostatic hyperplasia. 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 dogs¹⁸. Furthermore, Rohr *et al.*¹⁹ demonstrated that treatment of castrated dog with E₂ resulted in activation of the stromal tissue in association with smooth muscle cells.

Previously, we demonstrated that the intensity of GSH-PO staining in the glandular epithelial cells of the ventral prostate decreased following castration, and could be reversed by testosterone-administration to the castrated rats¹. We therefore postulated that prostatic GSH-PO may be testosterone-dependent. In the present study, we found that immunohistochemical staining of GSH-PO in the glandular epithelium of the dorsolateral prostate was clearly responsive to testosterone alone or in combination with E₂. Therefore, it seems likely that GSH-PO protein synthesis in the glandular epithelium of the dorsolateral prostate can be enhanced (induced ?) by sex hormones such as testosterone and E₂. In fact, the significant increase of GSH-PO protein triggered by lipid peroxides has also been demonstrated in an experimental system of inactivation and reactivation of the arachidonate cascade in rat peritoneal macrophages^{20,21}. These findings indicated that increased levels of lipid peroxides (or peroxidation) enhanced the expression of GSH-PO, or, in other words, the decrease of lipid peroxides might reduce the expression. We suggest that lipid peroxidation was increased in the dorsolateral prostate by the testosterone alone, or testosterone plus E₂-treatment. Further work in this area is

Fig. 1A

Fig. 1B

Fig. 1C

Fig. 1D

Fig. 1. A: Dorsolateral (DL) prostate of intact rat. B: DL prostate of castrated rat. The glandular epithelial cells are atrophic. C: DL prostate of castrated rat following treatment with testosterone. The glandular epithelial cells are of a highly columnar shape. D: DL prostate of castrated rat following treatment with combined testosterone and E_2 . Glandular hyperplasia with stromal proliferation is clearly observed.
HE $\times 100$ (A, B, C, D).

Fig. 2A

Fig. 2B

Fig. 2C

Fig. 2D

Fig. 2. A : Dorsolateral (DL) prostate of intact rat. B : DL prostate of castrated rat. C : DL prostate of castrated rat following treatment with testosterone. GSH-PO in the glandular epithelial cells clearly detected. D : DL prostate of castrated rat following treatment with combined testosterone and E₂. The intensity of GSH-PO staining is remarkably increased. Peroxidase-labeled antibody method, $\times 90$ (A, B, C, D).

Fig. 3A

Fig. 3B

Fig. 3C

Fig. 3D

Fig. 3. A: Dorsolateral (DL) prostate of intact rat. AR is localized exclusively in the nuclei of the glandular epithelial cells. B: DL prostate of castrated rat. AR is negative in the glandular epithelial cells. C: DL prostate of castrated rat following treatment with testosterone. AR is clearly noted in the nuclei of the glandular epithelial cells. D: DL prostate of castrated rat following treatment with combined testosterone and E_2 . AR is localized in the nuclei of the glandular epithelial cells. Furthermore, AR is also detected in the nuclei of the stromal cells. Avidin-biotin-peroxidase-complex method, $\times 100$ (A, B, C, D).

now in progress in our laboratory.

A rabbit polyclonal antibody referred as NH27 was raised against human AR¹⁴. The specificity of the antibody in immunohistochemical reactions has been described elsewhere^{1,22,23}. In the present work, we found that immunodetectable AR was decreased 2 days after castration, and that it was clearly recovered testosterone-administration to the castrated rats. These findings are in agreement with the results of previous work on the ventral prostate. AR was also detected in the nuclei of the proliferated stromal fibro-muscular cells. Human prostate epithelial cells in benign prostatic hyperplasia showed uniformly intense nuclear staining for AR²⁴. Furthermore, intense AR staining has been observed in stromal cells of fibro-muscular hyperplasia²⁴. The intense staining of AR in the epithelium is also in contrast with the abundance of 5 α -reductase in the stroma, which converts testosterone into dihydrotestosterone (DHT) in the prostate. It is possible that the epithelium utilizes DHT supplied by the stroma.

It is concluded that immunohistochemical analysis, using GSH-PO and AR, may be a very useful method for predication of the effects of androgen action on the rat prostate.

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