IMMUNOLocalIZATION OF GLUTATHIONE-PEROXIDASE (GSH-PO), APOPTOSIS, AND *BCL-2* PROTEIN IN THE RAT VENTRAL PROSTATE — EFFECTS OF CASTRATION AND ADMINISTRATION OF TESTOSTERONE —

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Abstract: Immunolocalization of glutathione-peroxidase (GSH-PO), apoptosis, and *bcl-2* protein in the rat ventral prostate was investigated under the presence or absence of androgen. 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 groups 3 and 4, rats were administered subcutaneously 1 mg/animal of testosterone-propionate daily for three or seven days after two days of castration. The intensity of GSH-PO staining in the glandular epithelial cells of the ventral prostate was remarkably decreased after castration (Group 2), and it was clearly recovered by testosterone-administration (Groups 3 and 4) in the castrated rats. Furthermore, castration (Group 2) induced apoptosis in the prostatic glandular epithelial cells and apoptosis was reduced by testosterone-administration (Groups 3 and 4) in the castrated rats. In groups 3 and 4, expression of *bcl-2* protein was clearly detected in the glandular epithelial cells of the ventral prostate. These findings strongly suggest that expression of GSH-PO and *bcl-2* protein in the glandular epithelial cells of the rat ventral prostate is considered to be testosterone-dependent. (J Toxicol Pathol 9: 57~64, 1996)

Key words: Glutathione-peroxidase (GSH-PO), *Bcl-2*, Apoptosis, Ventral prostate, Testosterone

Introduction

Androgen ablation is thought to induce apoptosis of prostatic cancer cell. This is based on observations that castration induces rapid involution of rat prostatic epithelium that appears to be mediated by apoptosis of epithelial cells¹.

The *bcl-2* gene was first discovered because of its involvement in the *t*(14; 18) chromosomal translocations commonly found in non-Hodgkin's lymphomas². The specific mechanism by which the *bcl-2* protein extends cell survival remains enigmatic, inasmuch as no biochemical activity has thus far been ascribed to this oncoprotein, but high levels of *bcl-2* protein have been shown to delay or block apoptosis

in variety of circumstances³⁻⁶. Both Kane *et al.*⁷ and Hockenbery *et al.*³ have proposed that apoptosis may be mediated by oxidative pathways which could be inhibited by *bcl-2*.

In our previous reports^{8,9}, immunocytochemical localization of glutathione-peroxidase (GSH-PO), which effectively reduces the lipid peroxides, was demonstrated in the rat ventral prostate under castration and testosterone-administration. We postulated that GSH-PO in the glandular epithelial cells of the rat ventral prostate was testosterone-dependent, and that its staining pattern was a useful marker for testosterone action^{8,9}.

The aim of this study is to examine the immunocytochemical localization of GSH-PO, *bcl-2*, and apoptosis in the rat ventral prostate of normal, castrated and testosterone-administered rats.

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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 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 hr 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*. Then one week was allowed for the adjustment of the animals to laboratory conditions.

Five animals served as controls (Group 1). In group 2, five rats were sacrificed two days after castration. In groups 3 and 4, five rats were administered, subcutaneously, 1 mg/animal of testosterone-propionate (testosterone, Sigma Chemical Co., St. Louis, MO) daily for 3 or 7 days after two days of castration. Testosterone was dissolved in dimethyl sulfoxide. Each rat was killed by decapitation and their ventral prostates were removed immediately.

Histopathological examination

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

Immunocytochemical staining

(1) *GSH-PO*

The ventral prostates were fixed in periodate-lysine-4% paraformaldehyde solution¹⁰ for 4 to 6 hr at 4°C under constant agitation. The fixed tissues were then washed in 0.01 M phosphate-buffered saline (PBS) containing sucrose from 10 to eventually 20% overnight at 4°C . Subsequently, 6 μm -thick 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 using rabbit anti-rat GSH-PO polyclonal antibody IgG Fab fragment¹¹.

For light microscopic observations of GSH-PO,

6 μm -thick frozen sections were incubated with the antibody labeled with horseradish peroxidase (HRPO, Sigma Chemical Co., St. Louis, MO) for 1 hr. After the incubation was completed, the sections were treated in Graham-Karonsky's reaction medium¹², 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, pH7.6, for 5 to 10 min at room temperature. Then the sections were counterstained for nuclei with 1% methyl green dissolved in veronal acetate buffer, pH4.2.

As an immunologic negative control, normal rabbit serum (NRS) IgG Fab fragment labeled with HRPO was applied on light microscopic investigation instead of anti-GSH-PO IgG Fab fragment labeled with HRPO.

(2) *bcl-2*

Formalin fixed and paraffin sections were used. With autoclaving, deparaffinizing sections which were set in a stainless steel rack and placed in a glass beaker containing a soaking solution (distilled water) received 121°C heat under 2 atm for 5 min at the peak. After washing in 0.01 M PBS, 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 for 1 hr at room temperature with mouse monoclonal antibody against *bcl-2* oncoprotein (*bcl-2*, 124; DAKO, Copenhagen, Denmark) diluted at 1:50. After washing in 0.01 M PBS, the sections were covered with biotin-conjugated goat anti-mouse IgG for 1 hr, washed and then treated with streptavidin-biotin-peroxidase complex (Histofine SAB-PO (M) kit, Nichirei, Tokyo) for 1 hr. After washing in 0.01 M PBS, the immunoperoxidase staining was performed as described above.

(3) *Apoptosis*

Apoptosis detection by labeling of 3' OH ends of DNA breaks using terminal deoxynucleotidyl transferase^{13,14} was done using the ApopTag detection Kit (Oncor, Gaithersburg, MD). Formalin fixed and paraffin sections were used. After deparaffinization and hydration, the sections were incubated with 20 $\mu\text{g}/\text{ml}$ proteinase K (Sigma Chemical Co., St. Louis, MO) at room temperature for 15 min, and then inactivated by covering the sections with absolute

methanol containing 0.3% hydrogen peroxide. The sections were washed with 0.01 M PBS, equilibrated, and then incubated with terminal deoxynucleotidyl transferase in a reaction buffer containing digoxigenin dUTP at 37°C for 10 min. The reaction was stopped, and the sections were washed for 30 min, after which the sections were incubated with anti-digoxigenin antibody coupled to peroxidase for 30 min at room temperature. After washing in 0.01 M PBS, the immuno-peroxidase staining was performed as described above.

Results

Group 1 (Intact control)

The glandular epithelial cells of intact animals appeared as single-layered, cylindrical epithelial cells (Fig. 1A). The cytoplasm of the glandular epithelial cells showed remarkably eosinophilic staining, and the nuclei were located mainly in the basal portion of the cell. The acini of the ventral prostate were relatively large and the shallow papillary projections were present into the acinar lumen. Immunocytochemical localization of GSH-PO was predominantly

Fig. 1. A: Prostate of intact rat. B: Prostate of castrated rat. C and D: Prostate of castrated rat following treatment with testosterone. HE, $\times 100$ (A, B, C, D).

Table 1. Effects of Testosterone and Castration on Apoptosis of Rat Ventral Prostate

Control	Castration	Castration + testosterone 3-day	Castration + testosterone 7-day
0.24±0.33	9.7±3.18**	0.87±0.35	0.36±0.09

Apoptotic index is calculated as the positive cell number in one acinus/total cell number in the acinus×100.

Values are the mean±S.D.

**, $p < 0.01$ vs control (Dunnett's multiple comparison test).

Fig. 2. A: Prostate of intact rat. GSH-PO is predominantly observed in the glandular epithelial cells. B: Prostate of castrated rat. The intensity of GSH-PO staining is markedly decreased. C and D: Prostate of castrated rat following treatment with testosterone. GSH-PO in the glandular epithelial cells are clearly detected.

Peroxidase-labeled antibody method, ×90 (A, B, C, D).

Fig. 3. A : Prostate of intact rat. *Bcl-2* protein is observed in the glandular epithelial cells. B : Prostate of castrated rat. The intensity of *bcl-2* staining is markedly decreased. C and D : Prostate of castrated rat following treatment with testosterone. *Bcl-2* protein in the glandular epithelial cells are clearly detected. Peroxidase-labeled antibody method, $\times 100$ (A, B, C, D).

observed in the glandular epithelial cells (Fig. 2A). No reaction products were seen in interstitial tissues. The control serum (NRS) was negative for immunocytochemical localization of GSH-PO in the rat ventral prostate (data not shown). *Bcl-2* protein was localized exclusively in the cytoplasmic matrix of the glandular epithelial cells (Fig. 3A). No immunostaining was observed in the cytoplasmic matrix when the sections were incubated with preimmune serum (data not shown). To evaluate the frequency of apoptosis, we counted the positive cells stained by

ApopTag Kit. The apoptotic index was about 0.24 ± 0.33 (Table 1).

Group 2 (Castration)

Two days after castration, the height of the glandular epithelial cells was slightly reduced (Fig. 1B). The intensity of GSH-PO staining was markedly decreased (Fig. 2B). The immuno-reaction for the *bcl-2* protein was negative or very weak in the glandular epithelial cells (Fig. 3B). The apoptotic index was about 9.7 ± 3.18 (Table 1) (Fig. 4).

Fig. 4. Apoptosis measured by terminal deoxynucleotidyl transferase assay. They are labeled with the ApopTag Kit (Oncor). Peroxidase-labeled antibody method, $\times 100$.

Group 3 (Castration plus testosterone 3 days)

By testosterone administration to the castrated animals, the height of the glandular epithelial cells was slightly increased (Fig. 1C). GSH-PO was intensely stained in the glandular epithelial cells (Fig. 2C). Furthermore, *bcl-2* protein was localized exclusively in the cytoplasmic matrix of the glandular epithelial cells (Fig. 3C). The intensity of *bcl-2* staining was stronger than that of Group 1. The apoptotic index was about 0.87 ± 0.35 (Table 1).

Group 4 (Castration plus testosterone 7 days)

The glandular epithelial cells were hypertrophic and showed an increased number of papillary projections extending into the acini (Fig. 1D). GSH-PO was intensely stained in the glandular epithelial cells. The intensity of GSH-PO staining was stronger than that of Group 3 (Fig. 2D). *Bcl-2* protein was observed in the cytoplasmic matrix of the glandular epithelial cells (Fig. 3D). The apoptotic index was about 0.36 ± 0.09 (Table 1).

Discussion

In the present study, we found that the intensity of GSH-PO staining in the glandular epithelial cells of the rat ventral prostate was remarkably decreased after castration, and that it was clearly recovered by testosterone-administration to the castrated rats. It was reported that the active center of GSH-PO consisted of selenocystein¹⁵. In addition, disappearance of GSH-PO in selenium deficient rat liver was im-

munochemically and immunocytochemically proved by us¹⁶ and others¹⁷. We found that only negligible amount of GSH-PO mRNA was exhibited in the case of the selenium deficient rat liver, while the mRNA coding for β -actin¹⁸ was outstandingly increased with the selenium deficiency. Therefore, we postulated that the transcription of GSH-PO mRNA was regulated by selenium and the *de novo* synthesis of GSH-PO would be thus regulated at transcription level by selenium¹⁸. Based on our data and these facts, it is suggested that the transcription of prostatic GSH-PO mRNA was regulated by testosterone and *de novo* synthesis of GSH-PO would be thus regulated at transcription level by testosterone.

It has been shown that rat prostatic glandular epithelium undergoes apoptosis within hours of castration and results in involution of the gland within 7 days¹. The morphological assessment of apoptosis was confirmed using the ApopTag assay in which the production of DNA breaks is monitored by labeling the 3' OH ends of the DNA *in situ* with dUTP coupled to digoxigenin using terminal deoxynucleotidyl transferase. This is followed by detection of the digoxigenin using peroxidase-conjugated antibodies¹³. The morphological appearance of apoptosis coincided exactly with peroxidase staining using this assay. In the present study, castration induced apoptosis in the prostatic glandular epithelial cells and that apoptosis reduced by testosterone-administration to the castrated rats. We found that expression of *bcl-2* protein greatly reduces the induction of apoptosis. *Bcl-2* protein has the oncogenic function of blocking apoptosis¹⁹⁻²⁰. Therefore, it

was strongly suggested that expression of *bcl-2* protein in the glandular epithelial cells of the rat ventral prostate was considered to be testosterone-dependent.

Low lipid peroxidation in cancer cells has been emphasized by several investigators²²⁻²⁶ in relation to changes in the fatty acid composition^{24,27}, levels of anti-oxidants such as vitamin E²⁴ and reduced form of glutathione^{28,29}, and enzyme activities of lipid peroxide-scavenging enzymes, including GSH-PO³⁰⁻³², catalase³⁰, superoxide dismutase^{32,33} and glutathione-S-transferases³³⁻³⁵. In addition, the suppressed expression of GSH-PO in cancer may thus be related to the low amount of lipid peroxides within the cell. We speculated that *bcl-2* expression might enable prostatic cancer cells to survive in an androgen-deprived environment and confer resistance to androgen withdrawal therapies. The relationship between *bcl-2*, GSH-PO-expression, apoptosis, and testosterone-stimulation of the prostatic glandular epithelial cells require further study.

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