Immunolocalization of Glutathione-Peroxidase (GSH-PO) and Bcl-2 in the Rat Ventral Prostate —Effects of Castration and Administration of Testosterone—

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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 subcutaneously administered 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) to the castrated rats. The prostatic GSH-PO mRNA levels were diminished in the castrated rat ventral prostate but greatly increased by testosterone (Groups 3 and 4). Furthermore, castration (Group 2) induced apoptosis in the prostatic glandular epithelial cells and apoptosis was reduced by testosterone-administration (Groups 3 and 4) to 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.

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

I. Introduction

In our previous reports [11, 12], 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. As a result, the intensity of GSH-PO staining was decreased by castration and it was clearly recovered by testosterone-administration to the castrated rats. We postulated that GSH-PO in the glandular epithelial cells of the rat ventral prostate was testosterone-dependent [11, 12].

Androgen ablation is thought to induce apoptosis of prostatic cancer cells. This is based on observations that

castration induces rapid involution of rat prostatic epithelium that appears to be mediated by apoptosis of epithelial cells [9]. Both Kane *et al.* [8] and Hockenberger *et al.* [6] have proposed that apoptosis may be mediated by oxidative pathways which could be inhibited by bcl-2. The bcl-2 gene was first discovered because of its involvement in the *t* (14; 18) chromosomal translocations commonly found in non-Hodgkin's lymphomas [18].

Bcl-2 is localized to intracellular sites of oxygen free radical generation including mitochondria and endoplasmic reticulum and nuclear membranes and is proposed to function as an antioxidant and as free radical scavengers [6, 15–17]. Thus, bcl-2 in the rat ventral prostate suggests a very close relationship to the secretory or metabolic status of the glandular epithelial cells. It is well recognized that the metabolic and secretory activities of the prostate are regulated by testosterone. Therefore, it was suggested that bcl-2 in the glandular epithelial cells of

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the rat ventral prostate was considered to be testosterone-dependent.

The first part of this study examined the levels of GSH-PO mRNA in the ventral prostate of normal, castrated and testosterone-treated rats. The second part determined, by immunocytochemistry, the localization of GSH-PO, bcl-2 and apoptosis in the rat ventral prostatic cells in the presence and absence of androgen.

II. 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 $22 \pm 2^\circ\text{C}$ with a relative 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 subcutaneously administered 1 mg/animal of testosterone-propionate (testosterone, Sigma Chemical Co., St. Louis, MO) daily for 3 or 7 days after two days of castration, respectively. 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 solution [10] 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 fragment [13].

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-Karnovsky's reaction medium [5], 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 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 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 immuno-peroxidase staining was performed as described above.

3) Apoptosis

Apoptosis detection by labeling of 3'OH ends of DNA breaks using terminal deoxynucleotidyl transferase [4, 7] 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.

Biochemical study

Northern blotting

The GSH-PO mRNA was measured in extracts prepared by rapidly homogenizing tissues with guanidinium isothiocyanate solution [2]. Total mRNA was

prepared by a standard cesium chloride-ultracentrifugation method [14]. Fifteen micrograms of total RNA were electrophoresed and nick-transferred onto nitrocellulose filters. Hybridization was performed, as reported previously, using a [^{32}P]-labeled complementary deoxyribonucleic acid (cDNA) probe for GSH-PO [3], while a cDNA probe for chicken beta-actin [1] was used as an internal positive control (house-keeping gene mRNA). The density of the bands on X-ray films was analyzed by a computer-aided densitometric system (Carl Zeiss, Vidas, Germany).

III. Results and Discussion

Group 1 (Intact control)

Immunocytochemical localization of GSH-PO was predominantly 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 cytoplasm of the glandular epithelial cells (Fig. 3A). No immunostaining was observed in the cytoplasm when the sections were incubated with preimmune serum (data not shown). To evaluate the frequency

of apoptosis, we counted the positive cells stained by Apop-Tag 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). The prostatic GSH-PO mRNA levels recorded as relative hybridization are shown in Table 2. The prostatic GSH-

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	$9.7 \pm 3.18^{**}$	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 $\times 100$. Values are the mean $\pm$ S.D.

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

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–D).

Fig. 2.

Fig. 3.

Table 2. The levels of the prostatic glutathione-peroxidase (GSH-PO) mRNA

Group	Treatment	β -actin mRNA	GSH-PO mRNA	GSH-PO/ β -actin	Relative hybridization (%)
1	Intact	3076	226	0.073	100.0
2	Castration	5273	176	0.033	45.4
3	Castration + T 3 days	3564	526	0.148	200.9
4	Castration + T 7 days	3967	570	0.144	195.6

T: Testosterone

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

PO mRNA level was much lower, being only 45% of the control value.

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 cytoplasm 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). The prostatic GSH-PO mRNA level, as relative hybridization, was significantly higher than that of controls (Table 2).

Group 4 (Castration plus Testosterone 7 days)

The glandular epithelial cells were hypertrophic and showed an increased number of papillary projections exten-

ding 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 cytoplasm of the glandular epithelial cells (Fig. 3D). The apoptotic index was about 0.36 ± 0.09 (Table 1). The prostatic GSH-PO mRNA level, as relative hybridization, was comparable to that of Group 3 (Table 2).

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. In addition, the prostatic GSH-PO mRNA level was diminished in the castrated rat ventral prostate, but was increased by testosterone administration. Based on our data, it is suggested that the transcription of prostatic GSH-PO mRNA is regulated by testosterone and *de novo* synthesis of GSH-PO is thus regulated at the 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 days [9]. In the present study, castration induced apoptosis in the prostatic glandular epithelial cells and apoptosis was reduced by testosterone-administration to the castrated rats. We found that the intensity of bcl-2 staining in the prostatic glandular epithelial cells were greatly decreased after castration, and that it was clearly recovered by testosterone-administration to the castrated rats. This staining pattern was similar to that of GSH-PO, it suggesting a comparable relationship to testosterone action in the cells. Therefore, it is strongly suggested that expression of bcl-2 protein in the glandular epithelial cells of the rat ventral prostate is considered to be testosterone-dependent.

The relationship between bcl-2-, GSH-PO-expression,

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, $\times 90$ (A–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–D).

apoptosis, and testosterone-stimulation of the prostatic glandular epithelial cells require further study.

IV. References

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