Immunocytochemical Localization of Glutathione Peroxidase (GSH-PO) and Bcl-2 in the Rat Ventral Prostate

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In order to confirm the relationship between glutathione peroxidase (GSH-PO) and Bcl-2, we have studied the immunocytochemical localization of GSH-PO, Bcl-2, androgen receptor (AR) and apoptosis in the rat ventral prostatic cells in the presence and absence of testosterone. Male Crj:CD(SD) IGS rats were divided into four experimental groups. Group 1 consisted of untreated 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 daily for 3 or 7 days after two days of castration, respectively. In group 1, GSH-PO was predominantly observed in the glandular epithelial cells. Bcl-2 was localized exclusively in the cytoplasm of the glandular epithelial cells. AR was localized in the nuclei of the glandular epithelial cells. In group 2, the intensity of the staining of GSH-PO, Bcl-2 and AR markedly decreased. However, the number of apoptotic cells (apoptotic index) markedly increased. In groups 3 and 4, the immunoreactivities of GSH-PO, Bcl-2 and AR clearly recovered. These findings strongly suggest that the expression of GSH-PO and Bcl-2 in the glandular epithelial cells of the rat ventral prostate is testosterone-dependent. Furthermore, co-expression of GSH-PO and Bcl-2 in the prostatic cells are considered to be normal or adaptive aspects of the cells.

Key words: apoptosis, Bcl-2, glutathione peroxidase (GSH-PO), prostate, testosterone

I. Introduction

Glutathione peroxidase (GSH-PO) which effectively reduces the lipid peroxides is a selenium-dependent enzyme that exists as a homotetramer with each 22-kDa subunit containing a selenium atom incorporated within a catalytically active selenocystein residue [3]. There are three other members of the selenium-dependent GSH-PO family, although cytosolic GSH-PO is the predominant form [5]. The gene encoding GSH-PO is mapped on chromosome 3q 11–13 [2]. Because GSH-PO decomposes hydrogen peroxide and organic hydroperoxides produced during normal metabolism and after oxidative insults, GSH-PO prevents peroxide-induced DNA damage, lipid peroxidation, and protein degradation [7, 31]. P53, after being activated by DNA-damaging reagents, has been shown either to induce G1 growth arrest or apoptosis [14]. The p53 target genes that mediate or associate with p53-induced apoptosis induce Bax [12], Fas/APO 1 [7], as well as those involving generation of reactive oxygen species [22]. Tan et al. [27] have identified and characterized GSH-PO, an antioxidant enzyme, as also induced by p53. It appears paradoxical that p53, on the one hand, induces the gene responsible for reactive oxygen species generation, which mediates apoptosis [22], and on the other hand, induces expression of the protective antioxidant enzyme GSH-PO, which protects cells from oxidative damage and apoptosis [15]. It is known that p53-induced reactive oxygen species generation is a rather latter event [22]. Thus, p53 may regulate cellular redox status in a time-dependent manner; it increases antioxidant synthesis at an early stage followed by an increase in reactive oxygen species generation.

In our previous reports [18], immunocytochemical localization of GSH-PO was demonstrated in the rat ventral prostate under castration and testosterone administration. As a result, the intensity of GSH-PO staining 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 [18].

Both Hockenbery et al. [9] and Kane et al. [10] have proposed that apoptosis may be mediated by oxidative pathways which could be inhibited by Bcl-2. Bcl-2 is localized to intracellular sites of oxygen free radical generation including mitochondria, endoplasmic reticulum and nuclear membranes and is proposed to function as an antioxidant and as a free radical scavenger [9, 24, 26]. Thus, Bcl-2 in the rat ventral prostate suggests a very close relationship to the secretary 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 the rat ventral prostate is testosterone-dependent.

In order to confirm the above hypothesis, this study examined the immunocytochemical localization of GSH-PO, Bcl-2, androgen receptor (AR) and apoptosis in the rat ventral prostatic cells in the presence and absence of testosterone.

II. Materials and Methods

Animals

Male Crj:CD(SD) IGS 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±2°C with a relative humidity of 55±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. Then one week was allowed for the adjustment of the animals to laboratory conditions.

Experiments

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. All animals were sacrificed by exsanguination under pentobarbital anesthesia at the end of the experimental period and the ventral prostates were removed.

The animals were cared for according to the principles outlined in the guide for the care and use of laboratory animals prepared by the Japanese Association for Laboratory Animal Science and our institution.

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

GSH-PO

The ventral prostates were fixed in periodate-lysine-4% paraformaldehyde solution [16] 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 10 to 20% sucrose overnight at 4°C. Subsequently, 6 μm-thick frozen sections were prepared from the washed tissues in a cryostat, and were placed on glass slides. The sections were washed in 0.01 M PBS and then stained by direct peroxidase-labeled antibody method using rabbit anti-rat GSH-PO polyclonal antibody IgG Fab fragment [19]. 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) instead of anti-GSH-PO IgG Fab fragment labeled with HRPO. In the light microscopic investigation instead of anti-GSH-PO IgG Fab fragment labeled with HRPO.

Bcl-2

Formalin fixed and paraffin sections were used. With autoclaving, deparaffinized sections were set in a stainless steel rack and placed in a glass beaker containing a soaking solution (distilled water) heated to 121°C under 2 atom 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.

AR

The ventral prostates were frozen in dry ice-cooled ethanol. 6 μm-thick frozen sections were prepared in a cryostat and mounted on glass slides. The sections were fixed for 10 min at 4°C in Zamboni’s fixative [33]. 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 NH27
Fig. 1
Fig. 2
Fig. 3
Fig. 4
(55 KD recombinant human AR), a rabbit polyclonal antiandrogen receptor antibody (1:1000). After washing 0.01 M PBS, the sections were covered with biotin-conjugated goat anti-rabbit IgG for 1 hr, washed in 0.01 M PBS, 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 immuno-peroxidase staining was performed as described above.

**Apoptosis staining**

Apoptosis detection by labeling of 3’OH ends of DNA breaks using terminal deoxynucleotidyl transferase was done using the ApopTag detection Kit (Intergen Co., NY). Formalin fixed and paraffin sections were used. After deparaffinization and hydration, the sections were incubated with 20 μg/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. Multiple fragmented DNA 3’OH ends on the sections were labeled with digoxigenin-dUTP in the presence of terminal deoxynucleotidyl transferase (TdT), and then peroxidase-conjugated anti-digoxigenin antibody was reacted with the sections. Apoptotic nuclei were visualized in a diaminobenzidine (DAB, Wako Pure Chemical industries, Osaka) solution [8]. Then the sections were counterstained for nuclei with 1% methyl green dissolved in veronal acetate buffer, pH 4.2. Apoptotic index was 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, significant difference from control.

**III. 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 cells. Immunocytochemical localization of GSH-PO was predominantly observed in the glandular epithelial cells (Fig. 2A). No reaction products were seen in the 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). AR was extensively localized in nuclei of the glandular epithelial cells (Fig. 4A). To evaluate the frequency of apoptosis, we counted the positive cells by ApopTag kit. The apoptotic index was about 0.21±0.23 (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 immunoreaction for the Bcl-2 protein was negative or very weak in the glandular epithelial cells (Fig. 3B). The AR immunoactivity was also decreased (Fig. 4B). The apoptotic index was about 9.5±3.01 (Table 1).

![Fig. 1. A: Prostate of intact rat. B: Prostate of castrated rat. C and D: Prostate of castrated rat following treatment with testosterone. HE ×100 (A–D).](image1)

![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 is clearly detected. Peroxidase-labeled antibody method, ×90 (A–D).](image2)

![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 protein staining is markedly decreased. C and D: Prostate of castrated rat following treatment with testosterone. Bcl-2 protein in the glandular epithelial cells is clearly detected. Peroxidase-labeled antibody method, ×100 (A–D).](image3)

![Fig. 4. A: Prostate of intact rat. AR is observed in the nuclei of the glandular epithelial cells. B: Prostate of castrated rat. The intensity of AR staining is markedly decreased. C and D: Nuclear localization of AR in the glandular epithelial cells is clearly detected. Peroxidase-labeled antibody method, ×100 (A–D).](image4)
IV. Discussion

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 AR was clearly detected in the nuclei of the glandular epithelial cells (Fig. 4C). The apoptotic index was about 0.67±0.38 (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 cytoplasm of the glandular epithelial cells (Fig. 3D). AR was clearly observed in the nuclei of the glandular epithelial cells (Fig. 4D). The apoptotic index was about 0.31±0.05 (Table 1).

IV. 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. It is well recognized that the metabolic and secretory activities of the prostate are regulated by testosterone. The secretory acid phosphatase in the glandular epithelial cells of the prostate appears to be testosterone-dependent, since it disappears after castration and returns after treatment with testosterone [28]. Therefore, an enzyme histochemical or immunohistochemical staining of acid phosphatase has been thought to be a good marker of androgen action in the rat prostate. Based on our data and theses facts, GSH-PO in the prostate appears to be testosterone-dependent, since it disappears after castration and returns after treatment with testosterone [28]. Therefore, an enzyme histochemical or immunohistochemical staining of acid phosphatase has been thought to be a good marker of androgen action in the rat prostate. Based on our data and these facts, we considered the increased expression of GSH-PO and Bcl-2 on the prostatic glandular epithelial cells by testosterone-administration to be a preventive mechanism for increased amount of free radical or lipid peroxides.

The significant increase of GSH-PO protein triggered by lipid peroxides has been demonstrated in an experimental system of inactivation and reactivation of the arachidonic acid cascade in the rat peritoneal macrophages [31]. These findings indicate that increased levels of lipid peroxides (or peroxidation) enhance the expression of GSH-PO, or, in other words, decreased levels of lipid peroxides likely reduces the expression of the enzyme. Lowered lipid peroxidation in cancer cells has been detailed by several investigators [1, 29] and has been related to changes in fatty acid composition [4]; antioxidants such as vitamin E [4] and a reduced form of glutathione [6]; enzyme activities of lipid peroxide-scavenging enzymes, including GSH-PO [6, 11]; catalase [6]; superoxide dismutase [6, 21] and glutathione-S-transferase [17, 20]. Therefore, the suppressed expression of GSH-PO in cancer cells may be related to the low amount of lipid peroxides within cells. It is well known that overexpression of Bcl-2 is noted in the cancer cells. Therefore, co-expression of GSH-PO and Bcl-2 in the prostatic cells are considered to be normal or adaptive aspects of the cells.

V. References

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