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Bcl-2 and Bax are involved in experimental cryptorchidism-induced testicular germ cell apoptosis in rhesus monkey

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Abstract

Apoptosis occurs spontaneously during spermatogenesis. However, little is known about its regulation in primate. Using an experimental cryptorchidism model in rhesus monkey, we have investigated the relationship between apoptosis and the Bcl-2 family members, Bcl-2 and Bax. Apoptotic cells were identified by in situ end labeling of fragmented DNA. The expressions of Bcl-2 and Bax in the testes during the heat stress-induced testicular germ cell apoptosis were detected by immunohistochemistry and Western blot techniques. The results showed that the apoptotic signals increased after heat treatment and the most susceptible cell types were spermatocytes and spermatids. A redistribution of Bax from the cytoplasmic to nuclear localization in some germ cells was observed. However, its total expression levels in the cells remained unchanged in the cryptorchid testes as determined by Western blot analysis; on the other hand, Bcl-2 levels increased significantly in response to heat stress. The subcellular redistribution of Bax and the increase in Bcl-2 expression in the cryptorchid testis suggest an involvement of Bcl-2 family members in heat stress-induced germ-cell apoptosis.

Keywords: Rhesus monkey; Heat stress; Apoptosis; Bcl-2; Bax

1. Introduction

Cryptorchidism constitutes the most common genital abnormality in boys. The infertility associated with cryptorchidism is attributed to testicular suprascrotal temperature, in situ cooling of abdominal testes in dogs and pigs results in normal spermatogenesis [1,2]. Raising scrotal temperature such as varicocele and fever [3] even with high ambient temperature [4] can reduce sperm production. Previous work has shown that the germ-cell loss associated with cryptorchidism in primate occurs by apoptosis [5], but the resultant molecular mechanism has not been elucidated.

Of the genes that have been shown to be involved in apoptosis, the Bcl-2 family members have been most widely studied. The Bcl-2 family contains both antiapoptotic (e.g., Bcl-2 and Bcl-xl) and proapoptotic (e.g., Bax) members, the ratio of these molecules may be a critical determinant of cell fate such that elevation of Bcl-2 favors extension of cell survival, while increase in Bax expression accelerate cell death [6]. Studies using knockout mice and transgenic mice suggest that members of Bcl-2 family may play an important role in spermatogenesis. Bax-knockout mice are infertile as a result of the accumulation of premeiotic germ cells and absence of mature haploid sperm [7]. Transgenic mice expressing high level of Bcl-xl or Bcl-2 show an abnormal spermatogenesis accompanied by sterility [8].

Surgically induced cryptorchidism in the well-designed time points is a reproducible model to study spermatogenesis. Clinically, it is difficult to obtain human testes at the time points as expected, which greatly limits the study of spermatogenesis. We have established a unilateral surgical cryptorchid rhesus monkey model and examined the expression and possible role of Bcl-2 and Bax in the cryptorchism-induced germ-cell apoptosis.

2. Material and methods

2.1. Animal tissue preparation

Adult (5–7 years old) rhesus monkeys were obtained from Kunming Institute of Zoology (KIZ), Chinese Acad-

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emy of Sciences (CAS) and approved for the study by the academic committees of both the Institute of Zoology (IOZ) and the Kunming Institute Primate Research Center (KIPRC), CAS. To induce unilateral cryptorchidism, the monkeys were anesthetized and a midline incision was made in the abdomen. The gubernaculums was cut on the right side to displace the testis into the abdomen. Suturing the inguinal canal on the right side prevented the testis decent.

The testes were removed and weighed either 5, 10 or 15 days after surgery. The testes were cut into pieces. Some were snap frozen in liquid nitrogen, and stored at −70°C for later protein analysis and the others were fixed in Bouin's solution, embedded in paraffin and cut in 5-μm sections for immunohistochemistry.

2.2. In situ 3′ end labeling of testicular cell DNA fragmentation

Sections (5 μm) were deparaffinized in xylene and rehydrated in a graded ethanol series (100%, 95% and 70%). The slides were incubated with proteinase K (20 μg/mL) for 15 min at room temperature and washed in phosphate-buffered saline (PBS). After incubation with equilibrium buffer for 10 min, sections were then incubated with terminal deoxynucleotidyl transferase reaction mixture for 1 h at 37°C in a humidified chamber. After three washes in Tris buffer, the slides were incubated with a blocking buffer [100 mM Tris, 150 mM NaCl, pH 7.5, and 1% (wt/vol) blocking reagent] for 30 min at room temperature before addition of antidigoxigenin antibody conjugated to alkaline phosphatase. After incubation with the antibody at room temperature for 2 h in a humidified chamber, the slides were washed three times in Tris buffer and then stained with the substrates nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

2.3. Immunohistochemistry

The testicular tissues were dehydrated and embedded in paraffin. Sections (5 μm) were cut and processed with the adivin-biotin-peroxidase complex (ABC) method. Following deparaffinization, the sections were hydrated and incubated for 20 min in 0.3% H_2O_2 in distilled water to quench endogenous peroxidase activity. The sections were then digested with 0.4% pepsin in 0.1 M HCl for 10 min at 37°C and incubated overnight at 4°C with the polyclonal antibody against Bax (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted in PBS containing 10% normal goat serum (NGS). After washing twice in PBS the sections were incubated with horse antimouse biotinylated immunoglobulin (Vector) for 30 min at 1:200 dilution in 2% NGS, and with peroxidase-conjugated strepavidin for 30 min at room temperature and developed with diaminobenzidine, and then dehydrated in ethanol and mounted.

2.4. Western blot analysis

The testes were homogenized in Dounce homogenizer (Fisher Scientific, Pittsburgh, PA, USA). The supernatants were obtained by centrifugation at 10,000 × *g* for 30 min. After boiling for 3 min at 100°C the aliquots equivalent to 50 μg of protein were separated in sodium dodecyl sulfate-polyacrylamide (12%) slab mini gels. Separated proteins were transferred for 3 h at 75 V to nitrocellulose membrane, and the membranes were then soaked in blocking solution (5% nonfat milk in PBS, pH 7.6) for 1 h at room temperature and then incubated with the primary polyclonal antibodies, at the dilutions of 1:300 for Bcl-2 (Santa Cruz Biotechnology) and 1:400 for Bax overnight in blocking solution at 4°C. After extensive washing with PBS the membranes were incubated with a peroxidase-conjugated second antibody (goat antimouse immunoglobulin; Gackson), at 1:2000 in blocking solution. The filters were developed with an enhanced chemiluminescence (ECL) Western blotting analysis, following the procedure described by the manufacturers (Pierce Co, Rockford, IL, USA).

2.5. Statistical analysis

Testes from three individual monkeys for each group were analyzed. Experiments were repeated at least three times, and one representative from at least three similar results is presented. The quantitative data represent mean ± SD of three results from at least three animals. Statistical analysis was conducted using the paired or unpaired Student's t test. P-values <0.05 were considered statistically significant.

3. Results

3.1. Testicular weight loss in cryptorchid testes

To evaluate effects of heat stress on testes, unilateral cryptorchidism was experimentally induced in rhesus monkeys. The testicular weights were recorded over a 15-day period (Fig. 1). The weights of the scrotal testes were unchanged over the study period and the cryptorchid testicular weights decreased in a time-dependent manner; a 17% weight loss on day 5 and a further 31% weight reduction was noted from day 5 to 15, for a total weight loss of 48%.

3.2. Identification of apoptotic cells using in situ end labeling

A comparison of apoptotic cell labeling in the scrotal and the cryptorchid testes is shown in Fig. 2. Condensed nuclei of apoptotic germ cells from the scrotal testes are occasionally observed. The most predominantly labeled cell types were spermatocytes and round spermatids (Fig. 2a). Strong positive staining was found from the cryptorchid testis 5

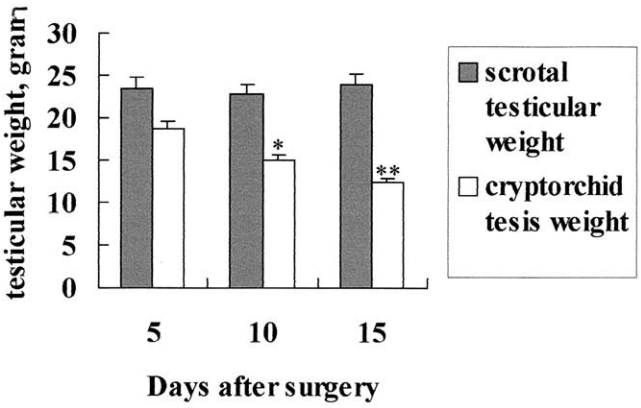

Fig. 1. Testicular weights of the scrotal and the cryptorchid testes. The data are expressed as the mean ± SEM from three rhesus monkeys. Statistically significant differences: *$p < 0.05$, **$p < 0.01$.

days after operation (Fig. 2b). Ten days after operation, in the seminiferous epithelium, a marked decrease in the signs of apoptosis was noted. Very few spermatocytes and round spermatids survived beyond 10 days (Fig. 2c).

3.3. Bax protein expression in the scrotal and cryptorchid testes

Changes in the in vivo pattern of Bax expression during the heat stress-induced germ cell apoptosis were examined by immunohistochemistry (Fig. 3). Immunoreactive staining for Bax in the scrotal testis showed its localization in the cytoplasm of germinal cells, in a granular or punctated pattern suggestive of association with subcellular structure (Fig. 3a). In addition, the Sertoli and Leydig cells also showed a cytoplasmic Bax staining. A change of Bax expression from cytoplasmic to nuclear localization was observed in the cryptorchid testis on day 5 (Fig. 3b) and on day 10 (Fig. 3c).

3.4. Western blot analysis of Bcl-2 and Bax in the cryptorchid testis

The expression of Bcl-2 and Bax in the heat stress-treated testes was examined (Fig. 4). Although we observed the change in Bax localization by immunohistochemical analysis, the Bax protein levels in the total testicular lysates were unchanged after the heat stress. However, Bcl-2 level began to increase by 5 days after surgery and attained statistical significance ($p < 0.05$) by 5 days after the surgery.

4. Discussion

Our previous work demonstrated that the expression of TR2, which modulates many signal pathways, was completely repressed in the testes by the cryptorchid operation in rat and rhesus monkey [9,10]. Further studies have shown that a feedback control mechanism between TR2 and p53/Rb tumor suppressor might play an important role in germ cell apoptosis in cryptorchid testis [11].

In the present study, we further examined the possible involvement of Bcl-2 and Bax in the heat-induced germ cell apoptosis. Immunohistochemistry revealed that Bax localized in the cytoplasm of spermatogonia, spermatocytes and spermatids in the seminiferous epithelium of the scrotal testis. However, the molecule redistributed from cytoplasmic to perinuclear or nuclear localization in the cryptorchid testis. Consistent with our result, Yamamoto et al. have previously demonstrated that redistribution of proapoptotic Bax protein is the early step in an apoptotic pathway leading to germ cell death induced by mild testicular hyperthermia in rats [12]. Our present data also suggest that the proapoptotic Bax protein may be one of the molecules responsible for cryptorchidism-induced germ cell apoptosis in rhesus monkeys. It has been reported that Bax translocation from cytoplasm to either close proximity of the nucleus or into the nucleus has been observed in various cell lines upon

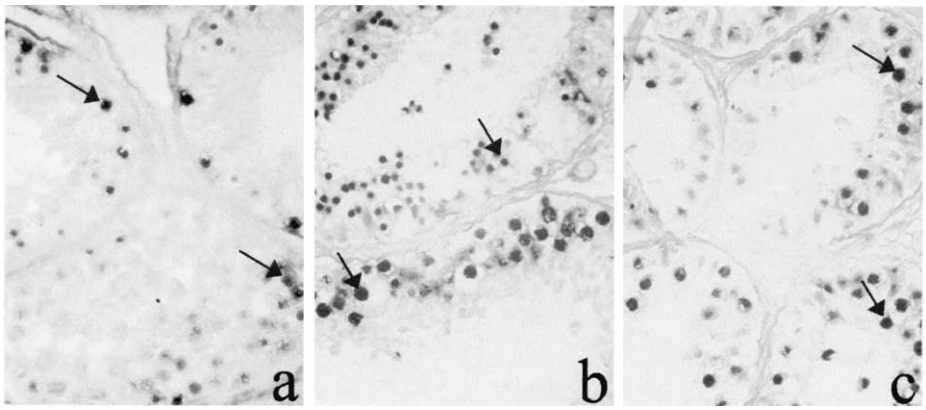

Fig. 2. Apoptotic cells in the scrotal (a) and the cryptochid (b,c) testes identified by in situ end labeling. The arrows indicate the positively labeled cells which appear as darkly stained. The scrotal testis (a) shows few apoptotic germ cells in the seminiferous tubules; the cryptorchid testis on day 5 (b) shows the most strong positive staining, on day 10 after surgery the apoptotic cells are considerably reduced. Original magnification ×400.

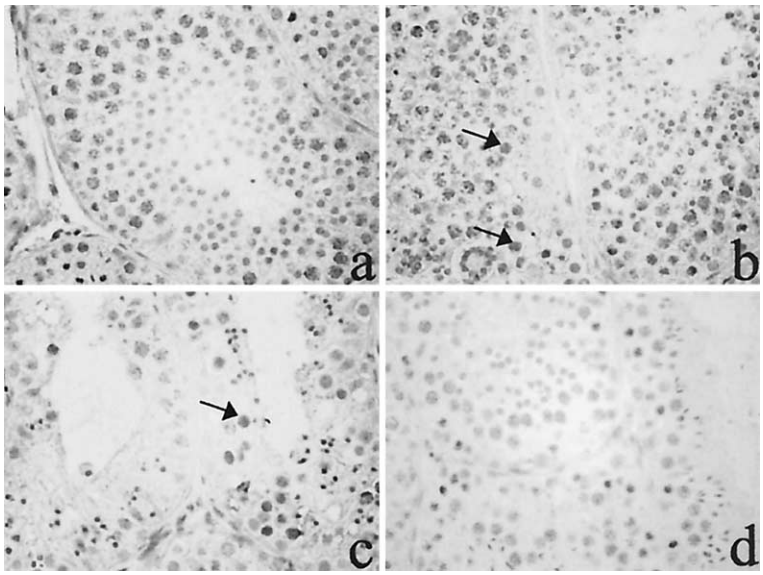

Fig. 3. Immunohistochemical analysis of Bax expression in the monkey testes. Light micrographs from the scrotal testis (a) and the cryptorchid testes on day 5 (b) and 10 (c) after surgery exhibiting localization of Bax in the germ cells. A change of Bax localization from cytoplasm to the nucleus was observed in the cryptorchid testes (b,c) as compared with the scrotal testes (a). The arrows indicate the nuclear localization of Bax. (d) A negative control with normal rabbit immunoglobulin G. Original magnification ×400.

induction of apoptosis [13]. The expression of Bcl-2 protein was up-regulated in the cryptorchid testes. It has also been found that increased myocyte apoptosis occurred in the decompensated human heart in spite of enhanced expression of Bcl-2 [14]. A rise in Bcl-2 protein levels in these cells may be very important to maintain mature germ cells in the testis, physiologically, acting as a survival mechanism to guarantee the atrophied tissue responding to normal situations. Other Bcl-2 family members, such as Bak [15], Bok [16], Mcl-1 [17] and Bcl-w [18,19] have been identified in testis. It is possible that these Bcl-2 family members also play an important role in germ cell apoptosis induced by heat stress.

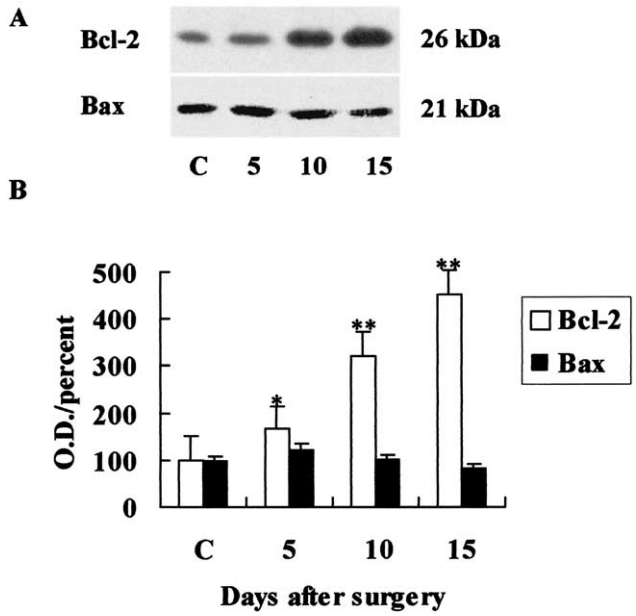

Fig. 4. Western blot analysis of Bcl-2 and Bax expression in the testes. (A) Proteins in the testicular extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotted with anti-Bcl-2 and anti-Bax polyclonal antibody. (B) Quantitative analysis of Bcl-2 and Bax protein expression determined from Western blot analysis. The data are presented as percentage of scrotal values. O.D. = optical density. Statistically difference was determined between the control (C) and cryptorchid groups at various times after surgery. *$p < 0.05$, **$p < 0.01$.

In conclusion, cryptorchidism in rhesus monkey induces increase in apoptotic germ cells and redistribution of Bax protein from the cytoplasm to the nucleus of the testicular cells. The concomitant elevation of Bcl-2 expression in the testes may represent a physiological survival mechanism to ensure that the germ-cell–depleted testis could respond to normal temperature.

Acknowledgments

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