EXPERIMENTAL STUDY

The expression and regulation of Bcl-2-related ovarian killer (Bok) mRNA in the developing and adult rat testis

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Abstract

Objective: To study the role of *Bcl-2-related ovarian killer (Bok)* in the regulation of apoptosis in the testis of developing and adult rat.

Methods: Bok mRNA expression was analyzed by Northern hybridization before and after culturing rat seminiferous tubules *in vitro*. Seminiferous tubules were cultured with different hormones and growth factors. Changes in the expression level of Bok mRNA during testicular development was analyzed by Northern hybridization. Localization of Bok mRNA was verified by *in situ* hybridization.

Results: Bok mRNA was highly expressed in the rat testis, varying during development. Highest expression levels were found in immature rats. Highest hybridization intensity appeared to be in spermatogonia, pachytene spermatocytes and Sertoli cells. Treatment with FSH was able to inhibit spontaneous increase of Bok mRNA expression that occurred in the defined stages of the rat seminiferous epithelium.

Conclusions: FSH protects germ cells from apoptosis and this protective effect may at least partly be due to the inhibition of *Bok* gene expression. The amount of apoptosis varies during testicular development and highest expression of Bok mRNA occurs at the time of apoptosis, suggesting a possible role for Bok in its regulation.

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Introduction

A massive cell death occurs during spermatogenesis. It has been estimated that up to 75% of the hypothetical sperm number is lost due to cell death, which has been shown to be due to apoptosis (1–5). In mice, there are two peaks of apoptotic cell death during testicular germ cell development: the first around the 13th day of gestation and the second peak around the 10th day after birth (6). Our understanding of the molecular mechanisms of apoptosis has expanded recently, but the specific apoptotic machinery in germ cells is poorly understood (7).

The Bcl-2 family of proteins plays an important role in the control of apoptosis. It consists of both pro-survival (e.g. Bcl-2, Bcl-xL, Bcl-w, Mcl-1) and pro-apoptotic (e.g. Bok, Bcl-xS, Bax, Bak, Bad, Bik 1) proteins. They are related by homology and they operate via hetero- and homodimeric interactions (7–10). It appears that the balance between death promoting and antagonizing proteins determines how a cell will respond to an apoptotic signal (11, 12).

The exact role of apoptosis-regulating Bcl-2 gene products during spermatogenesis is unknown, but several

lines of evidence suggest that it has to be important. Gene knock-out experiments of anti-apoptotic *Bcl-w* and pro-apoptotic *Bax* in mice lead to impaired spermatogenesis, suggesting that the balance of pro- and anti-apoptotic mediators is crucial for the maintenance of spermatogenesis (8, 10, 13).

Bok (Bcl-2-related ovarian killer) is a pro-apoptotic member of the *Bcl-2* gene family. It was isolated from a rat ovarian fusion cDNA library by using the yeast two-hybrid system (14) and the mouse homolog (termed mtd) was found from the gene bank (15). In immature rats, the expression of *Bok* was found to be predominant in reproductive tissues, suggesting an important role for it in these tissues.

Bok expression has been studied in the ovaries (14) but its role in the testis is unknown. In the present study, our objective was to study the role of *Bok* in the regulation of apoptosis in the testis. We analyzed steady state levels of Bok mRNA in the developing and adult rat testis. Localization of Bok mRNA was performed by *in situ* hybridization. In addition, we studied the regulation of the Bok mRNA expression in the seminiferous tubules by reproductive hormones and growth factors.

Materials and methods

Experimental animals

Sprague–Dawley rats of 0, 5, 10, 21, 41 days and 2–3 months of age were housed in a constant temperature (20 °C) and light–darkness cycle (lights on, 0600–2000 h) with free access to food and water. Ethylene dimethane sulfonate (EDS) was used to kill specifically Leydig cells in studies aiming to analyze hormonal regulation. The animals were injected i.p. with a single dose of EDS (75 mg/kg body weight (BW)). EDS was synthesized as previously described (16) and dissolved in DMSO–water (1:3, vol./vol.). Control animals received injection of vehicle. Rats were killed on days 1, 2, 3, 4, 7, 10, 20 and 40 after EDS administration. Serum testosterone decreases to an undetectable level within 48 h and remains as such until day 10 after EDS treatment. Testosterone levels start to increase from day 20 onward and nearly reach control levels on day 40 (17). Most of the Leydig cells are depleted by apoptosis due to EDS treatment within 48 h (17).

Rats in all experiments were killed by CO₂ asphyxiation, and different tissues were removed for subsequent analysis.

Microdissection of the seminiferous tubules, tissue and cell culture and stimulation

Testes were decapsulated and 5 mm seminiferous tubule segments were isolated under a transilluminating stereomicroscope. Stages of the seminiferous epithelial cycle were identified as described previously (18, 19). Microdissection and tissue culture experiments were performed in DMEM–Ham's F-12 medium (1:1; DMEM/F12; Life Technologies, Paisley, UK) supplemented with 15 mmol/l HEPES, 1.25 g/l sodium bicarbonate, 10 mg/l gentamicin sulfate, 60 mg/l G-penicillin, 1 g/l BSA and 0.1 mmol/l 3-isobutyl-1-methylxanthine (Aldrich Chemie, Steinheim, Germany). In tissue culture

experiments, twenty 5 mm seminiferous tubule segments were incubated in the presence and absence of follicle-stimulating hormone (FSH; 10 ng/ml) (Organon, Oss, The Netherlands), testosterone (10^{-7} mol/l) (Sigma Chemical Co., St. Louis, MO, USA) and recombinant mouse stem cell factor (SCF; 100 ng/ml) (Genzyme Transgenics Corp., Cambridge, MA, USA) for 8 or 30 h. In cell culture experiments, we used MSC-1 cells, which are an immortalized Sertoli cell line derived from a testicular tumor of a transgenic mouse carrying a fusion gene composed of human anti-Müllerian hormone transcriptional regulatory sequences linked to the coding sequence of the SV40 virus T-antigen. Our MSC-1 cells were stably transfected with a cDNA plasmid encoding the rat FSH receptor (20). MSC-1 cells were incubated in DMEM, supplemented with gentamicin and 10% fetal calf serum (Bioclear UK Ltd., Devizes, Wilts, UK).

Preparation of cRNA probes

The rat complementary DNA of *Bok* was cloned into pGEM4Z (Promega Corp., Madison, WI, USA). The cloned plasmid containing a 589 bp insert, spanning nucleotides 46–635 of the published rat *Bok* cDNA, was linearized with HindIII or EcoRI restriction enzymes for the preparation of sense and antisense probes respectively. Control hybridization was made by using a 1.3 kb BamHI fragment of *pI-19* cDNA clone of the mouse 28S rRNA (21). *In vitro* transcription reactions were performed as recommended by the manufacturer (Promega). The radionuclides used were [³⁵S]UTP (for *in situ* hybridization) and [³²P]UTP (for Northern hybridization, *Bok*) and [³²P]CTP (for Northern hybridization, 28S) (Amersham, Aylesbury, Bucks, UK). For *in situ* hybridization, sense and antisense RNA probes were adjusted to the same radioactivity (1×10^5 c.p.m./μl).

Figure 1 Expression of *Bok* mRNA transcripts in the rat tissues. The expression level in adrenal gland is designated as 100% and other values are expressed as percentages of it. *Bok* mRNA expression was found to be high in the adult rat testis, ovary, uterus and adrenal gland ($n = 3$). A.D.U., arbitrary densitometric units.

Figure 2 Localization of Bok mRNA in adult rat testis by *in situ* hybridization. The bright-field (A–D) and corresponding darkfield (A'–D') photomicrographs of sections from an adult rat testis are shown. The specific signals (A, A') are mainly confined to the layer where spermatogonia and meiotic cells are located. In high magnification (C, C') the signals are mainly found to be confined to Sertoli cells (Sc), spermatogonia (Sg) and pachytene spermatocytes (Sp). The background signals were evaluated according to the sense probe (B, B' and D, D'). Note that bundles of elongated spermatids give auto-fluorescent signals that do not represent any labeling. Bars, 50 μ m.

RNA extraction and Northern blot hybridization

RNA extractions were made by using the single-step method (22). RNA was size-fractionated in denaturing 1% agarose gels. The gel was stained with ethidium bromide to verify an even loading of RNA. The RNA was transferred onto Hybond-N+ nylon membrane (Amersham). RNA was fixed to the membrane by u.v. cross-linking.

Filters were prehybridized in 50% formamide, 3 $\times$ SSC, 5 $\times$ Denhart's solution (1 mg/ml Ficoll, 1 mg/ml polyvinylpyrrolidone and 1 mg/ml BSA), 1.2% SDS, 10%

dextran sulfate, 1 mmol/l EDTA (Sigma) containing 10 μ g yeast transfer RNA at 65 $^{\circ}$ C for 4–16 h. Hybridization was performed at the same temperature for 14–24 h by adding the 32 P-labeled probe. Filters were washed for 20 min with 2 $\times$ SSC–0.1% SDS at room temperature, followed by two washes in 0.2 $\times$ SSC–0.1% SDS in 65 $^{\circ}$ C for 20 min and two washes of 20 min in 0.1 SSC–0.1% SDS at 65 $^{\circ}$ C. The filters were stripped after hybridization with Bok probe by pouring the boiling 0.1% SDS onto the filters. Control hybridization was done subsequently with mouse 28S rRNA at 42 $^{\circ}$ C. Filters were exposed to Fuji Rx 100 film at –70 $^{\circ}$ C between intensifying screens.

Figure 3 *In situ* hybridization of Bok mRNA in the immature rat testis using anti-sense probe is shown in the left panels (A–C). The control hybridization with sense probe is on the right (A'–C'). At 10 (A) and 20 (B) days of age, signals can be seen in Sertoli cells (Sc), spermatogonia (Sg) and pachytene spermatocytes (Sp). Spermatids (Sd) do not show signals as shown in 20- and 40-day-old rat testis (C). Bar, 50 μ m. Bok mRNA expression in the Sertoli cells was also verified by Northern hybridization analysis (D). The locations of the 28S and 18S ribosomal RNAs are marked on the left.

Histology and *in situ* hybridization

Testes were fixed in PBS-buffered 4% paraformaldehyde at 4 °C for 24 h, dehydrated in ethanol, cleared in xylene, and embedded in paraffin. Five μ m sections were cut, and *in situ* hybridization and autoradiography were performed as described previously (23).

Densitometric analysis of Northern hybridization results

The X-ray films of Northern hybridization were first scanned by a UMAX scanner (Super Vista S-20, Binuscan, Inc., Mamaroneck, NY, USA) and a Binuscan Photoperfect software package (Binuscan, Inc.). The images were saved as TIFFs (*.tif, Microsoft Corp. and

Aldus Co., New York, NY, USA) and then quantified by Tina 2.0 densitometric analytical system (Raytest Isotopenmesgerate GmbH, Straubenhardt, Germany) according to the manufacturer's instructions.

Statistical analysis

In the Northern hybridization analyses, the densitometric values of the signals of Bok mRNA were first normalized to values obtained from the pI-19 control probe, to correct loading differences, then the highest densitometric value was designated 100%. Other values were expressed as percentages of the highest one. The values from all experiments were pooled for calculation of the means and their S.E.M.s and for one-way ANOVA

Figure 4 (A) *Bok* gene expression during testicular development. *Bok* mRNA expression was found to be high during the first 21 days of life. Highest expression was found at 10 days after birth and it was designated as 100%. The level of *Bok* mRNA expression during adult life was approximately one-third of the highest level. Each bar represents the mean $\pm$ S.E.M. of three independent experiments. A.D.U., arbitrary densitometric units. (B) A representative Northern hybridization. EtBr, ethidium bromide.

and Tukey's *post hoc* test using SPSS 9.0 (SPSS Inc., Chicago, IL, USA). *P* values less than 0.05 were considered statistically significant.

Results

Expression of *Bok* mRNA transcripts in rat tissues

Bok mRNA is widely expressed in the adult rat tissues. The expression levels were found to be high in adrenal gland, testis, ovary and uterus and *Bok* mRNA was expressed also in prostate, spleen, liver (Fig. 1) and heart, intestine, kidney, brain and lung (data not shown) analyzed by Northern hybridization.

Localization of *Bok* mRNA

The *Bok* gene was expressed in the compartment where spermatogonia and meiotic cells are located in the

seminiferous tubules (Fig. 2). Highest hybridization intensity appeared to be in Sertoli cells, spermatogonia and pachytene spermatocytes (Fig. 2). Strong hybridization signals were seen during the early stages of testicular development (Fig. 3A). *Bok* mRNA expression in the Sertoli cells was verified also by Northern hybridization of mRNA isolated from MSC-1 Sertoli cell line (Fig. 3D).

Bok gene expression during testicular development

The highest level of *Bok* mRNA was found at 10 days after birth (Fig. 4). The expression was high from newborn to 21 days of age. Thereafter, it decreased and remained at a constant level.

Hormonal regulation of *Bok* mRNA expression

The steady state mRNA levels of *Bok* increased spontaneously about 2.5-fold in stages IX–XII of the rat seminiferous epithelium during a 30-h incubation *in vitro* (Fig. 5). FSH stimulation inhibited the increase of *Bok* mRNA accumulation. In stages VII–VIII of the rat seminiferous epithelium FSH also showed some inhibition of *Bok* mRNA expression, but the effect was not statistically significant. Unlike FSH, SCF and testosterone failed to show any effect in the regulation of *Bok* mRNA levels.

Effect of EDS treatment on *Bok* gene expression

EDS causes a reversible decrease in the level of testosterone by killing the Leydig cells. The first apoptotic germ cells are shown to occur at day 3 (24). Steady state *Bok* mRNA levels were unaltered during the acute decrease of serum testosterone levels for the first 48 h after EDS administration (Fig. 6). On the third day after EDS administration, *Bok* mRNA expression dropped to approximately half of the control level. Seven days after EDS treatment the *Bok* mRNA levels were again comparable to those seen in the control.

Discussion

Bok is a novel member of the *Bcl-2* gene family and it was originally isolated from a rat ovarian fusion cDNA library (14). The mouse version of *Bok* cDNA, termed mtd, has also been isolated (15). Because the expression of *Bok* mRNA was shown to be abundant in the immature female reproductive tissues, we anticipated that it might also have a role in the male reproductive physiology. The *Bok* mRNA expression was high in the adult rat adrenal gland, uterus and ovary. As expected, *Bok* mRNA was abundant also in the rat testis, suggesting that it may act as an apoptotic regulator during spermatogenesis.

Figure 5 (A) Effect of FSH, testosterone and SCF on Bok gene expression. A spontaneous increase in the steady state level of Bok mRNA took place in stages IX–XII of the rat seminiferous epithelium during a 30-h culture *in vitro*. FSH stimulation inhibited the increase of Bok mRNA accumulation. SCF and testosterone did not show any effect on Bok mRNA levels. The expression level of Bok mRNA in stages XIII–I after 30-h stimulation *in vitro* was designated as 100%. Statistical comparisons were made using ANOVA and Tukey's *post hoc* test. * $P < 0.002$. Each bar represents the mean $\pm$ S.E.M. of three independent experiments. A.D.U., arbitrary densitometric units. (B) A representative Northern hybridization. EtBr, ethidium bromide.

Northern hybridizations of testicular mRNA at different developmental stages showed that the level of Bok gene expression varies during testicular development. Highest expression was found at day 10 postnatally, when germ cell meiosis starts. Expression was high from newborn to day 21 when the first haploid cells appear (25). Also the amount of apoptosis varies during testicular development. The first apoptotic peak appears during prenatal development, when primordial germ cells immigrate into gonads. The second apoptotic peak occurs during the first round of spermatogenesis between days 10 and 20 of postnatal life (6). It has been suggested that the second apoptotic wave is

required to maintain a proper cell ratio between maturing germ cells and Sertoli cells (11, 26). Disruption of this apoptotic peak by overexpressing *Bcl-2* or *Bcl-xL* or by inactivating *Bax* or *Bcl-w* in the testis, results in sterility in mice (8–11, 13). The second apoptotic peak and the high expression of Bok mRNA occur at the same time, suggesting a possible role for Bok in this phenomenon. Similarly, in our previous studies, we have shown a similar expression pattern with pro-apoptotic proteins Bax and Bad, suggesting that they act in concert with Bok in order to maintain cellular homeostasis during this critical developmental stage (27).

Figure 6 (A) *Bok* mRNA levels were on a constant level for the first 48 h after EDS administration. Steady state level of *Bok* mRNA decreased to approximately half on the third day. After 1 week *Bok* mRNA levels rose to the level seen in control animals. The expression level of *Bok* mRNA at 40 days after EDS injection was designated as 100%. Statistical comparisons were made using one-way ANOVA and Tukey's *post hoc* test. * $P < 0.02$ compared with control. Each bar represents the mean $\pm$ S.E.M. of three independent experiments. A.D.U., arbitrary densitometric units. (B) A representative Northern hybridization. EtBr, ethidium bromide.

In situ hybridization showed *Bok* mRNA expression predominantly in spermatogonia and primary spermatocytes. Spermatocytes are the major germ cell type undergoing apoptosis upon hormone withdrawal caused by gonadotropin-releasing hormone (GnRH) antagonist treatment (28). The expression of pro-apoptotic *Bok* in these cells indicates that it may be a constituent of the cellular apoptotic machinery in these spermatogenic cells. The decrease in *Bok* mRNA expression after 21 days of postnatal life probably reflects a relative decrease of spermatogonial and spermatocyte numbers as spermatogenesis progresses, rather than absolute decline.

We have shown previously that FSH and SCF can protect germ cells from apoptosis (29) and that FSH stimulation elevates SCF mRNA steady state levels during 30 h stimulation (30). In this study, we wanted to find out if *Bok* gene expression can also be regulated by these factors. A spontaneous increase of *Bok* mRNA expression in seminiferous tubule segments from stages IX–XII of the rat seminiferous epithelium was observed. Treatment with FSH inhibited the increase of *Bok* gene expression significantly, but SCF and testosterone failed to show a significant effect. Our previous observations on the hormonal regulation of germ cell apoptosis demonstrated that both FSH and SCF have a pro-survival effect and that FSH action is partially mediated by SCF (29). However, in this study, only FSH was able to regulate *Bok* expression, indicating that this effect was independent of SCF regulation. The protective effect of FSH on germ cells (29, 31) may at least partly be due to the inhibition of *Bok* gene expression.

Testosterone alone can maintain spermatogenesis, but for quantitatively normal sperm production, FSH is also

needed (32–34). *In vitro* data showed no effect for testosterone in the regulation of the *Bok* gene. However, in an *in vivo* experiment, when testosterone-producing Leydig cells were depleted by EDS treatment, an acute fall in the intratesticular testosterone concentrations was reflected as decreased *Bok* mRNA expression. Therefore, it seems that FSH can regulate spermatogenesis by fine-tuning the expression of *Bok* and other pro-apoptotic *Bcl-2* family proteins, while testosterone is the main driving force for ongoing spermatogenic processes.

In summary, we have studied the expression and hormonal regulation of *Bok*, a pro-apoptotic *Bcl-2* family member in the adult and developing rat testis. The expression pattern of *Bok* suggests that it may act as a regulator of germ cell apoptosis during critical points of germ cell maturation in the immature and adult rat testis.

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