

Bcl-2 Overexpression in the Internal Spermatic Vein of Patients with Varicocele

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Background/Purpose: Varicocele is characterized by dilatation and tortuosity of the internal spermatic vein (ISV). Cross sections of ISV showed marked thickening of smooth muscle layer under microscopy. This study examined Bcl-2 (anti-apoptotic protein) expression in the ISV of patients with varicocele.

Methods: The study group consisted of eight patients with left varicocele, grade 3. The control group consisted of six volunteers with left indirect inguinal hernia. Using a left inguinal surgical incision, a 1-cm section of ISV was resected from each patient in both groups as specimens for immunoblotting and immunohistochemical staining of Bcl-2. Results were analyzed using Student's *t* test.

Results: Bcl-2 immunoblots from both groups revealed one single band. α -tubulin was used as loading control. The average relative intensity of the Bcl-2 was 25.82 ± 10.53 in the control group and 113.49 ± 27.49 in the varicocele group. Hematoxylin staining revealed a thickening ISV in the patients with varicocele, predominantly in the muscle layer, which was not found in the control group. Moreover, the intensity of Bcl-2 immunostaining was markedly higher in the study group than in the control group.

Conclusion: This study showed Bcl-2 overexpression in the ISV of patients with varicocele. Additional studies are necessary to clarify the molecular mechanisms of varicocele formation and recurrence. [*J Formos Med Assoc* 2007;106(4):308–312]

Key Words: Bcl-2, internal spermatic vein, varicocele

Varicocele consists of an engorgement and dilatation of the pampiniform plexus above the testis. It is found in 15–20% of men, with the left side being the most commonly affected.^{1–3} Pampiniform plexus drains into the internal spermatic vein (ISV) on the left side, which then drains into the renal vein. Varicocele has long been recognized as the most common cause of male infertility in adults. Approximately 30–40% of men evaluated for infertility were reported to have varicocele.^{3,4} Recently, increased expression of hypoxia-inducible factor-1 α (HIF-1 α) was reported in the ISV of

patients with varicocele.⁵ Hence, hypoxic stress was found to occur in these vessels but it did not cause vascular atrophy. Conversely, vascular dilatation and thickened ISV wall were present in patients with varicocele.⁵ What kind of molecular mechanism occurs in vascular cells under hypoxic stress?

Bcl-2 (anti-apoptotic protein) inhibits cell apoptosis by preventing the release of cytochrome c from outer mitochondrial membrane.^{6–8} Since the ISV is thicker and dilated in varicocele, it is hypothesized that hypoxic stress found in the ISV of patients with varicocele may upregulate

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Bcl-2 expression and lead to overexpression of Bcl-2. This study provides new insights into the mechanism of varicocele formation and recurrence. To our knowledge, this is the first report analyzing Bcl-2 expression in human ISV samples.

Methods

This study included 14 young patients between May and July, 2003. The study group consisted of eight patients aged between 20 and 25 years with left varicocele, grade 3, who underwent evaluation for varicocele by physical examination and color flow Doppler sonography.^{9,10} Varicocele was graded according to Dubin and Amelar in 1970⁹ as follows: grade 1, varicocele palpable only during the Valsava maneuver; grade 2, varicocele palpable in standing position; and grade 3, varicocele detectable by visual scrutiny alone. To prevent interobserver bias, all physical examinations were performed by one physician. The control group consisted of six volunteers (with written informed consent from each patient) aged from 20 to 25 years with indirect left inguinal hernia, for whom the possibility of varicocele was ruled out by physical examination and color flow Doppler sonography (ISV diameter < 2 mm).¹¹

The same left inguinal surgical incision was performed in all patients,¹² and 1 cm of ISV was resected and stored at -80°C for the following immunoblotting and immunohistochemical staining of Bcl-2.

Antibodies

All the antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Two primary antibodies were used in the present study: (1) Bcl-2 mouse monoclonal antibody (sc-7382, 200 $\mu\text{g}/\text{mL}$); (2) α -tubulin mouse monoclonal antibody (sc-5286, 200 $\mu\text{g}/\text{mL}$) used as loading control. The secondary antibodies for immunoblots were alkaline phosphatase (AP)-conjugated goat anti-mouse IgG (sc-2008). The secondary antibody for immunohistochemistry was horseradish

peroxidase (HRP)-conjugated goat anti-mouse IgG (sc-2005).

Bcl-2 immunoblotting

Each ISV sample (1 cm, about 100 mg) stored at -80°C was mixed with 0.5 mL lysis reagent (Cat. No. E1531; Promega, Madison, WI, USA) and 5 μL proteinase inhibitor (10 mg antipain, 5 mg leupeptin, and 50 mg benzamidine dissolved in 5 mL aprotinin), and then homogenized on ice. The aliquots of 100 μg of the homogenate and prestained molecular weight standards (Bio-Rad, Hercules, CA, USA) were heated at 100°C for 5 minutes and fractionated by electrophoresis on 10% SDS-polyacrylamide gel performed at 140 V for 3.5 hours. Gels were then equilibrated for 15 minutes in 25 mM Tris-HCl, pH 8.3, containing 192 mM glycine and 20% (V/V) methanol. Electrophoresed proteins were transferred onto nitrocellulose (Hybond-C Extra Supported, 0.45 μm ; Amersham, Piscataway, NJ, USA) using a Transphor Unit (Hoefer Scientific Instruments, San Francisco, CA, USA) at 100 mA for 14 hours. According to the manufacturer's manual, the antibodies of Bcl-2 and α -tubulin revealed molecular weights of about 28 and 50 kDa, respectively. So the transferred nitrocellulose membrane (blot) was cut into upper and lower parts at 40 kDa. The upper and lower blots were incubated separately at room temperature for 2 hours in blocking buffer and then for 3.5 hours with Bcl-2 antibody (lower blot) and α -tubulin antibody (upper blot) diluted at 1:200 for each antibody in antibody binding buffer (100 mM Tris-HCl pH 7.5, 0.9% [W/V] NaCl, 0.1% [V/V] Tween-20, and 1% [V/V] fetal bovine serum). Blots were washed three times in blotting buffer and incubated in AP-conjugated goat anti-mouse IgG (lower blot) and AP-conjugated goat anti-mouse IgG (upper blot) for 1 hour (diluted 1:1000 for each antibody in binding buffer). Blots were washed three times in blotting buffer for 10 minutes and signals were developed with nitro-blue tetrazolium, 5-bromo-4-chloro-3-indolyl-phosphate (Chemicon, Temecula, CA, USA). Immunoblots were scanned and the

relative intensity of the immunoreactive bands was measured.

Immunohistochemistry for Bcl-2

Formalin-fixed and paraffin-embedded sections (4 μm) of patients' ISV were stained with hematoxylin and eosin. For Bcl-2 immunohistochemistry, sections were dehydrated and immersed in 10⁻³ M sodium citrate buffer (pH 6.0). Sections were then heated at 60°C for 10 minutes. An avidin-biotin-peroxidase complex was used to detect Bcl-2. Endogenous peroxidase was inactivated by incubating sections with 3% hydrogen peroxide, and nonspecific reactions were blocked by incubating sections in a solution containing 5% normal horse serum and 1% normal goat serum. Sections were incubated with the primary antibody overnight at 4°C. Bcl-2 expression was assessed using HRP-conjugated goat-anti-mouse IgG (Santa Cruz Biotechnology) (dilution 1:100). After three rinses with phosphate-buffered saline, sections were incubated with diaminobenzidine substrate for 5 minutes. Finally, sections were rinsed with distilled water and counterstained with hematoxylin. Sections were observed using an Olympus BX50 light microscope and photographed with a Nikon CP5000 digital camera.

Statistical analysis

Statistical analysis was conducted using Student's *t* test (SPSS Inc., Chicago, IL, USA), and significance was established when *p* < 0.05.

Results

The immunoblots revealed a single band of Bcl-2 protein (at 28 kDa) in all patients. The relative

intensity of the Bcl-2 band was approximately 4-fold higher in patients with varicocele than in the control group (Table and Figure 1). Hematoxylin staining showed a thickened ISV in the patients with varicocele, predominantly in the muscle layer (Figure 2C), which was not found in the control group (Figure 2A). Moreover, expression of Bcl-2 in the muscle layer of the ISV was higher in patients with varicocele (Figure 2D) than in the control group (Figure 2B).

Discussion

Varicocele is the most common etiologic factor associated with male infertility.¹⁻⁴ The dilated and thickened ISV wall in varicocele was defined similar

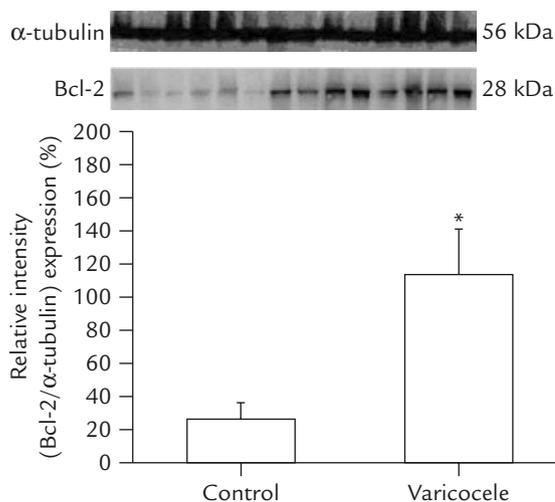


Figure 1. Representative immunoblot and relative intensities of Bcl-2 protein in the control (inguinal hernia) and study (varicocele) groups. α-tubulin was used as the loading control. Values of Bcl-2 expression were normalized relative to α-tubulin expression. The mean amount of Bcl-2 protein in patients from the study group was four times higher than that in the control group. *Significant difference between the control and study groups.

Table. Relative protein abundance of Bcl-2 in the control and varicocele groups*†

	Case no.								Mean	SD
	1	2	3	4	5	6	7	8		
Control group	16.74	19.67	22.97	17.69	41.77	36.09	–	–	25.82	10.53
Varicocele group	79.35	119.67	130.1	150.85	94.67	147.79	89.30	96.22	113.49	27.49

*Values of Bcl-2 expression are normalized relative to α-tubulin expression; †*p* < 0.01. SD = standard deviation.

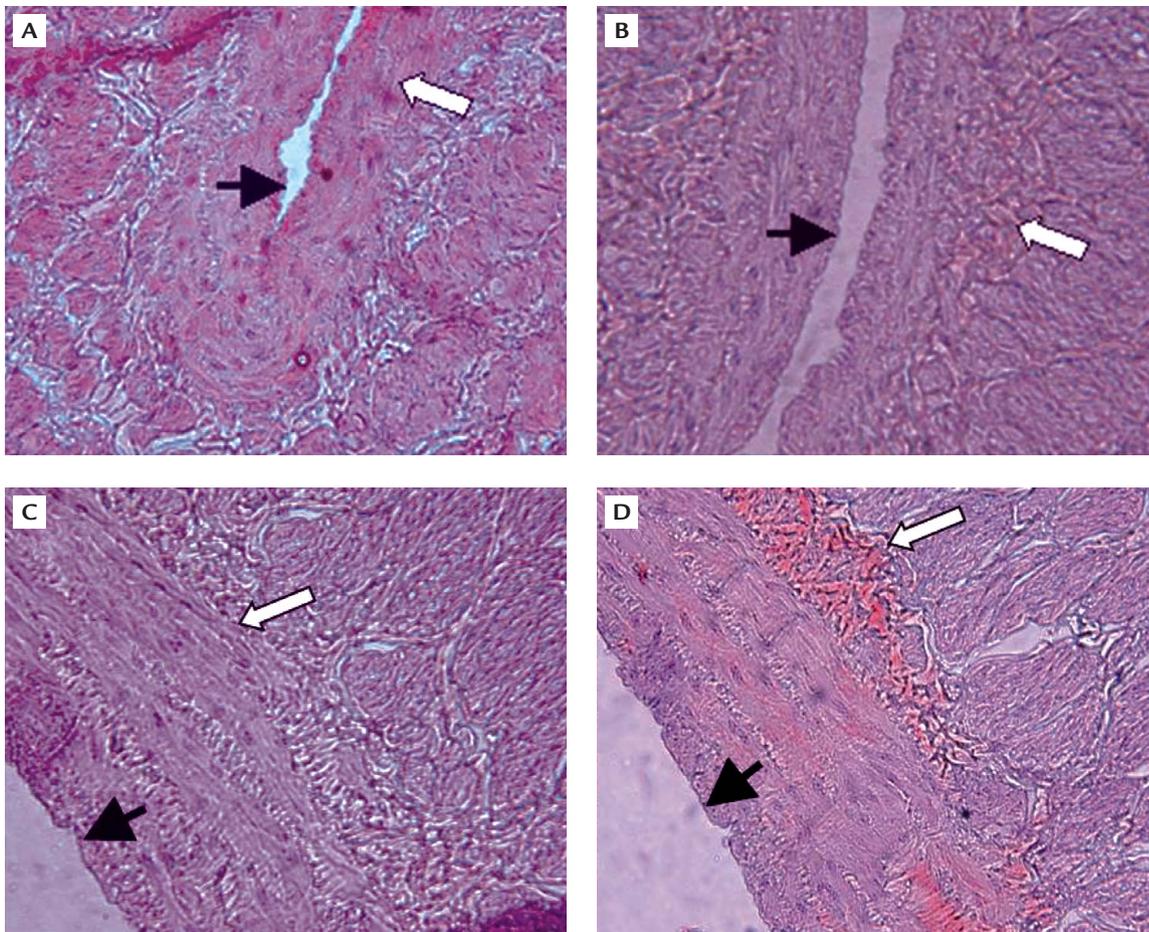


Figure 2. Micrographs of representative internal spermatic vein (ISV) sections after hematoxylin staining (A and C) or Bcl-2 immunostaining (B and D). Panels A and B: control group (inguinal hernia patients); panels C and D: study group (varicocele patients). The smooth muscle layer of the ISV (white arrows) was thickened in the study group (C) when compared to the control group (A). The intensity of Bcl-2 immunostaining (red deposition) was markedly higher in the study group (D) than in the control group (B). Black arrows: endothelium of the ISV. Magnification: 200 \times .

to that of varicose veins. The venous stasis due to blood stagnation has involved in the development of varicose veins¹³ as poor venous return of varicocele. Studies on varicose veins showed that many neutrophils adhered to the endothelium of vein incubated in hypoxic conditions rather than in normoxia.¹³ Hypoxia-activated endothelial cells secreted growth factors which triggered proliferation of smooth muscle cells.¹¹⁻¹⁵ Hypoxia was also a stimulus to vascular smooth muscle cell proliferation that occurred in rat aorta via increasing fms-like tyrosine kinase (Flt-1) activity.¹⁶ Higher HIF-1 α expression in the ISV of patients with varicocele was reported recently, which indicated that hypoxia-related pathophysiologic changes have occurred in the ISV of patients with

varicocele.⁵ In low or zero oxygen concentrations, mammalian cells undergo cell death through apoptosis, but not necrosis.⁸ Apoptotic signaling during oxygen deprivation occur through the release of cytochrome c and Apaf-1 mediated caspase-9 activation leading to cell apoptosis.^{7,8}

The Bcl-2 gene was first identified in association with the t(14;18) chromosomal translocation in follicular lymphoma.¹⁰ The oncogenic properties of Bcl-2 have been attributed mainly to its ability to inhibit apoptosis by interfering with the activation of the cytochrome c/Apaf-1 (apoptosome) pathway through stabilization of the mitochondrial outer membrane.⁷ The presence of these enzymes stimulate caspase-9, which in turn stimulates caspase-3 as part of the pathway

to apoptosis because caspase-3 is essential for apoptotic death in mammalian cells.⁶⁻⁸

Hence, hypoxia might be one of the factors responsible for Bcl-2 regulation, as Bcl-2 protein expression was increased in different cells under hypoxic conditions for survival and proliferation.^{6,8,17-20} The present study demonstrated that Bcl-2 expression in the varicocele group increased significantly compared to that in the control group. Bcl-2 overexpression may decrease vascular cell apoptosis in the hypoxic condition and lead to vascular cell proliferation (predominant in muscular layer) causing dilated and thickened ISV wall in patients with varicocele. This is the first report describing Bcl-2 overexpression in human ISV. Additional studies are necessary to clarify the molecular mechanisms of varicocele formation and recurrence.

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