ORIGINAL ARTICLE

Dysregulated Apoptosis Through the Intrinsic Pathway in the Internal Spermatic Vein of Patients With Varicocele

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Background/Purpose: Apoptosis plays a critical role in various physiological processes. Varicocele is the most common cause of male infertility in adults. The dilated and thickened wall of the internal spermatic vein (ISV) in varicocele is considered similar to that in varicose veins. We investigated apoptotic protein expression in the ISV of varicocele, including Bcl-2, Fas, caspase-8 and caspase-9, to determine the intrinsic or extrinsic pathway.

Methods: The study group consisted of 10 patients with grade 3 left varicocele. The control group consisted of 10 patients with left indirect inguinal hernia. A 1-cm section of ISV was resected, using left inguinal incision, from each patient in both groups. The ISV sections were used to detect the mediators that regulate the intrinsic (Bcl-2 and caspase-9) and extrinsic (Fas and caspase-8) apoptotic pathways, by immunoblotting and immunohistochemical staining. Results were analyzed using Student's *t* tests.

Results: Bcl-2, Fas, caspase-8 and caspase-9 immunoblots from both groups revealed a single band. The relative intensities of the Bcl-2 and caspase-9 protein bands differed significantly between the varicocele and control groups. Thickening of the smooth muscle layer of the ISV was found in patients with varicocele compared with the control group. Bcl-2 overexpression and downregulation of caspase-9 expression were noted in the varicocele group. There was no significant difference in Fas or caspase-8 expression in either group.

Conclusion: We showed overexpression of Bcl-2 and downregulation of caspase-9 expression in the ISV under hypoxic stress. This indicated dysregulated apoptosis through the intrinsic pathway in the ISV of patients with varicocele. To the best of our knowledge, this is the first study of the apoptotic pathway in the human ISV. Additional studies are needed to establish whether adjunctive hyperbaric oxygen therapy reduces the recurrence rate after varicocelectomy. [*J Formos Med Assoc* 2009;108(8):612–618]

Key Words: apoptosis, internal spermatic vein, varicocele

Apoptosis has an important role in normal development and in the pathogenesis of a variety of diseases.[1,2] Varicocele consists of an engorgement and dilatation of the pampiniform plexus above the testes. The pathogenesis of varicocele is similar to varicose vein development. Both the venous stasis and blood stagnation that are due to poor venous return contribute to diseased vessel formation.[3] It has been reported that dysregulated apoptosis involves the mitochondrial (intrinsic) pathway in the pathogenesis of human primary varicose veins.[2] We have previously reported overexpression of Bcl-2 in the internal spermatic vein (ISV) of patients with varicocele.[4] However, to our

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knowledge, no studies have examined the apoptotic pathway in ISV in humans with varicocele, although it is recognized as the most common cause of male infertility in adults and is responsible for 30–40% of cases.[5–7] Although varicocelectomy is a simple and safe technique, the recurrence rate of 2–14% is still an issue.[8]

Our understanding of how a cell undergoes apoptosis centers on the activation of caspases in vertebrates.[9] There are two pathways (intrinsic and extrinsic) that induce apoptotic cell death, which differ in how the death signal is transduced.[9]

In the present study, we investigated expression of Bcl-2, Fas, caspase-8 and caspase-9 in the ISV, to differentiate between the intrinsic and extrinsic apoptotic pathways. This study provides new insights on the mechanisms of varicocele formation and may help reduce recurrence in the future.

Materials and Methods

Patients and tissue samples

This study included 20 young patients between May and October, 2006, and all of them provided written informed consent. The study group consisted of 10 patients aged 20–25 years with grade 3 left varicocele, who were evaluated by physical examination and color flow Doppler sonography.[10,11] Varicocele was graded according to Dubin and Amelar in 1970 as follows: grade 1, varicocele palpable only during the Valsalva maneuver; grade 2, varicocele palpable in standing position; and grade 3, varicocele detectable by visual scrutiny alone.[10] To prevent interobserver bias, all physical examinations were performed by one physician. The control group consisted of 10 patients aged 20–25 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).[12]

All patients underwent left inguinal surgical incision,[13] and 1-cm segments of ISV were resected and stored at −80°C for immunoblotting for Bcl-2, Fas, caspase-8 and caspase-9, and immunohistochemical staining for Bcl-2 and caspase-9.

Antibodies

Five primary antibodies used in the present study were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA): (1) Bcl-2 monoclonal antibody (sc-7382; 200 μg/mL); (2) caspase-9 polyclonal antibody (sc-8355; 200 μg/mL); (3) Fas polyclonal antibody (sc-7886; 200 μg/mL); (4) caspase-8 polyclonal antibody (sc-6134; 200 μg/mL); and (5) additional α-tubulin monoclonal antibody (sc-5286; 200 μg/mL), which was used as a loading control.

The secondary antibodies for immunoblotting were horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (31430; Pierce Biotechnology, Rockford, IL, USA; for detection of Bcl-2 and α-tubulin), goat anti-rabbit IgG (31460; Pierce Biotechnology; for detection of caspase-9 and Fas), or rabbit anti-goat IgG (305-035-003; Jackson ImmunoResearch Laboratories, West Grove, PA, USA; for detection of caspase-8). The secondary antibody for immunohistochemistry was HRP-conjugated goat anti-rabbit IgG (sc-2004; Santa Cruz Biotechnology).

Immunoblotting

Each 1-cm segment of ISV 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 homogenized on ice. Aliquots of 100 μg of the homogenate and pre-stained molecular weight standards (Bio-Rad, Hercules, CA, USA) were heated at 100°C for 5 minutes and fractionated by electrophoresis on 10% SDS–polyacrylamide gels performed at 140 V for 3.5 hours. Gels were then equilibrated for 15 minutes in 25 mM Tris–HCl, pH 8.3, which contained 192 mM glycine and 20% (v/v) methanol.

Electrophoresed proteins were transferred onto nitrocellulose membranes (Hybond-C Extra Supported, 0.45 Micro; 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 for Bcl-2, caspase-9, Fas,

caspase-8, and α-tubulin detected molecular weights of about 26, 46, 48, 55 and 55 kDa, respectively. Therefore, 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 different primary antibodies in the 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 secondary antibody buffer 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 the SuperSignal West Pico Kit (34082; Pierce Biotechnology) and Kodak Biomax light film (Cat #178 8207; Eastman Kodak Co., Rochester, NY, USA).

Immunoblots were photographed and imported as TIF files into the ID image analysis software package (Kodak Digital Science, 1998). The results were converted to numerical values in order to compare the relative intensities of the immunoreactive bands.

Immunohistochemistry of Bcl-2 and caspase-9

Formalin-fixed and paraffin-embedded sections (4 μm) of vessels from control and study groups were deparaffinized and stained with hematoxylin and eosin. For immunohistochemistry of Bcl-2 and caspase-9, deparaffinized sections were dehydrated and then immersed in 10^{-3} M sodium citrate buffer (pH 6.0). Sections were heated in a microwave oven at 60°C for 10 minutes. An avidin–biotin–peroxidase-complex method was used to detect Bcl-2 and caspase-9. Endogenous peroxidase was inactivated by incubating sections with 3% hydrogen peroxide, and nonspecific reactions were blocked by incubating sections in a solution that contained 5% normal horse serum and 1% normal goat serum. Sections were incubated with primary antibody overnight at 4°C. Bcl-2 and caspase-9 expression was assessed using peroxidase-conjugated rabbit polyclonal antibody IgG (Santa Cruz Biotechnology; dilution 1:100). After three rinses with phosphate-buffered saline, the sections were incubated with diaminobenzidine substrate for 5 minutes. Finally, they were rinsed with distilled water and counterstained with hematoxylin. Negative control experiments, in which goat serum was used instead of the primary antibody, were conducted (data not shown) to confirm the positive results. All sections were observed using an Olympus BX50 light microscope (Olympus Corp., Tokyo, Japan) and photographed with a Nikon CP5000 digital camera (Nikon Corp., Tokyo, Japan).

Statistical analysis

Data were analyzed using Student's *t* tests, with $p < 0.05$ considered statistically significant.

Results

The immunoblots revealed a single band for Bcl-2 (26 kDa), caspase-9 (46 kDa), Fas (48 kDa) and caspase-8 (55 kDa) in all patients (Figures 1–4). The relative intensity of the Bcl-2 band was approximately fourfold higher in patients with varicocele than the control group (110.42 ± 8.72

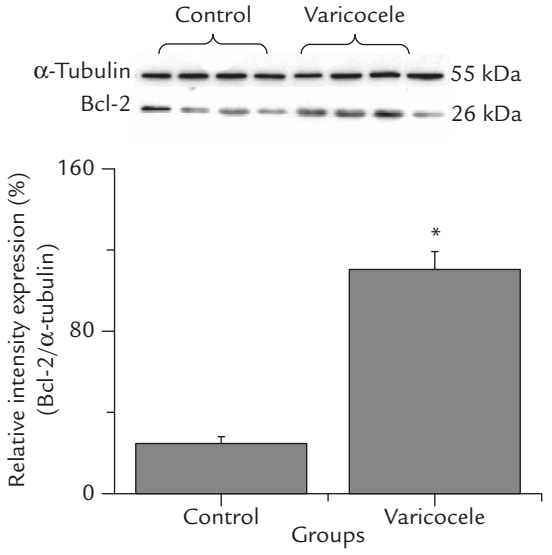

Figure 1. Representative immunoblot and relative intensity of Bcl-2 in the control (inguinal hernia) and varicocele groups. α-Tubulin was used as the loading control. Values of Bcl-2 expression are normalized relative to α-tubulin expression.

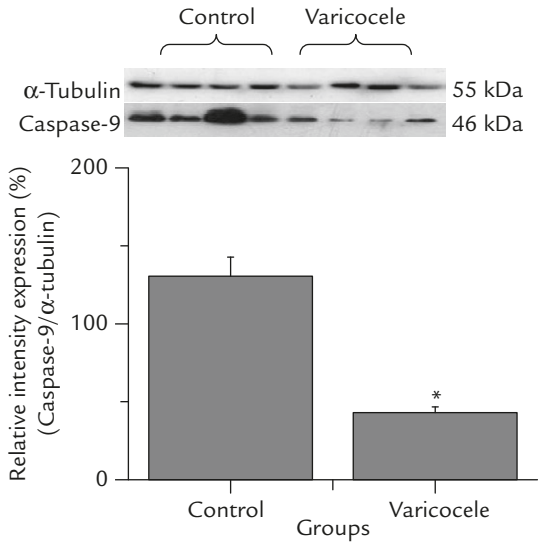

Figure 2. Representative immunoblot and relative intensity of caspase-9 in the control and varicocele groups. α-Tubulin was used as the loading control. Values of caspase-9 expression are normalized relative to α-tubulin expression.

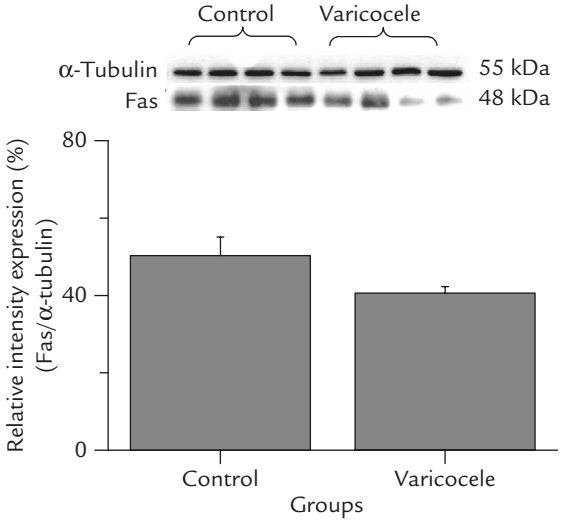

Figure 3. Representative immunoblot and relative intensity of Fas in the control and varicocele groups. α-Tubulin was used as the loading control. Values of Fas expression are normalized relative to α-tubulin expression.

vs. 24.72 ± 3.30; Table, Figure 1). In contrast, the relative intensity of the caspase-9 band was threefold higher in the control group than in the varicocele group (130.61 ± 12.22 *vs.* 43.11 ± 3.72; Table, Figure 2). There was no significant difference in Fas and caspase-8 expression between the two groups (50.27 ± 4.82 *vs.* 40.60 ± 1.68, and 463.52 ± 27.22 *vs.* 411.80 ± 56.10, respectively;

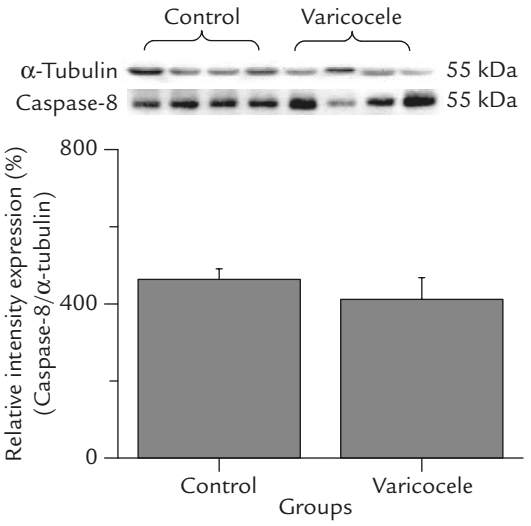

Figure 4. Representative immunoblot and relative intensity of caspase-8 in the control and varicocele groups. α-Tubulin was used as the loading control. Values of caspase-8 expression are normalized relative to α-tubulin expression.

Table, Figures 3 and 4). Thickening of the smooth muscle layer of the ISV and more Bcl-2 deposition were found in patients with varicocele (Figure 5B) compared with the control group (Figure 5A). Conversely, caspase-9 expression was higher in the control group (Figure 5C) than in the varicocele group (Figure 5D).

Discussion

Apoptosis plays a major role in tissue homeostasis and maintenance.[1,2] In the human apoptotic pathway cascade, 14 caspases (cysteinyl aspartate-specific proteinases) have been found to date. Two pathways can induce apoptotic cell death: the intrinsic (or mitochondrial) pathway is induced by cellular stress, which involves Bcl-2 (antiapoptotic protein), mitochondrial outer-membrane permeability, and caspase-9;[1,14] and the extrinsic (or death receptor) pathway is induced by specific ligands that engage death receptors, which involves Fas and binding and activation of caspase-8.[14]

Varicocele is abnormal tortuosity and dilatation of gonadal veins within the spermatic cord,[6,7] and its pathogenesis is similar to that of varicose

Table. Relative apoptotic protein abundance in both groups*

	Protein			
	Bcl-2	Caspase-9	Fas	Caspase-8
Control group	24.72 ± 3.30	130.61 ± 12.22	50.27 ± 4.82	463.52 ± 27.22
Varicocele group	110.42 ± 8.72†	43.11 ± 3.72†	40.60 ± 1.68	411.80 ± 56.10

**Values for protein expression are normalized relative to α-tubulin expression and presented as mean ± standard error of the mean; †$p < 0.05$.*

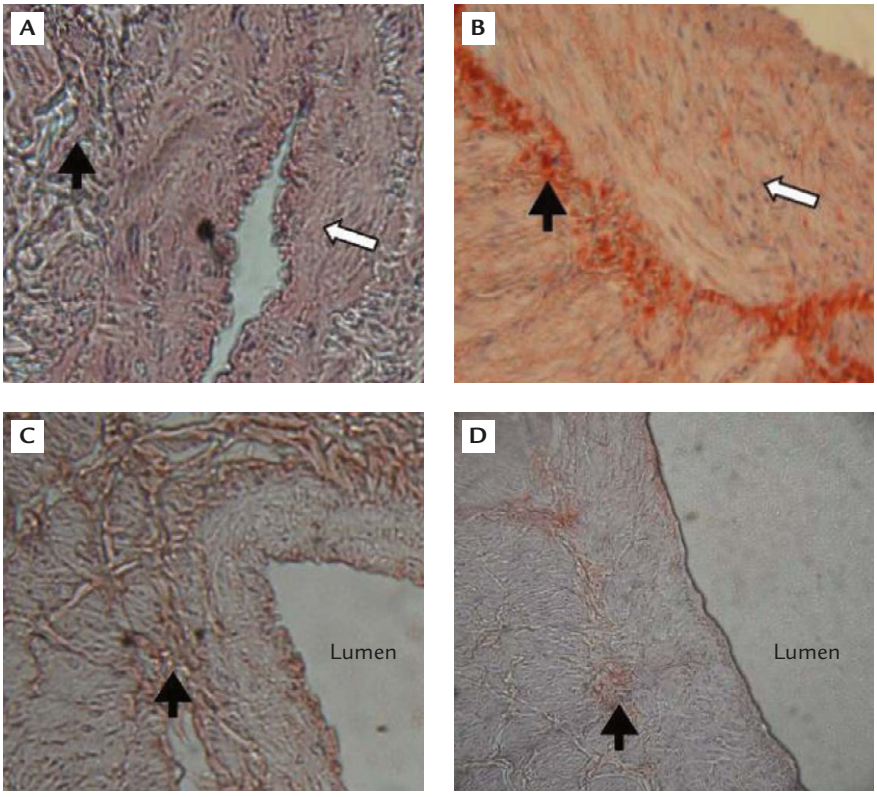

Figure 5. Micrographs of representative ISV sections after Bcl-2 (A and B) or caspase-9 (C and D) immunostaining. (B, D) Varicocele group; (A, C) control group (inguinal hernia patients). The smooth muscle layer of the ISV (white arrows) was thicker in the varicocele group (B) compared with the control group (A). The intensity of Bcl-2 immunostaining (red deposition, black arrows) was markedly higher in the varicocele group (B) than in the control group (A). The intensity of caspase-9 immunostaining (brown deposition, black arrows) was markedly higher in the control group (C) than in the varicocele group (D). Magnification: 200×. ISV = internal spermatic vein.

veins. Hypoxic stress that is caused by venous blood stagnation and poor venous return contributes to formation of diseased vessels in both conditions.[3] There are several treatment modalities for varicocele, including incisional ligation of the ISV, percutaneous embolization, and laparoscopy. Although all procedures are safe and effective, recurrence still troubles most urologists. We have previously reported a sevenfold increase in hypoxia-inducible factor-1α in the ISV of patients with varicocele,[15] which indicates that hypoxia-related pathophysiological changes occur in the diseased vessels. Under conditions of hypoxic stress in the ISV, our study revealed overexpression of Bcl-2 and downregulation of caspase-9 expression in the varicocele group. 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 (apoptotic protease activating factor-1;

apoptosome) pathway, through stabilization of the mitochondrial outer membrane.[1] The presence of these enzymes stimulates caspase-9 activation, which leads to cell apoptosis.[1,14]

Since the ISV is thicker and dilated in varicocele, it is hypothesized that hypoxic stress in the ISV upregulates Bcl-2 expression and leads to less apoptosis than in the control group.[4] Moreover, hypoxia-activated endothelial cells secrete growth factors that trigger smooth muscle cell proliferation.[3,16,17] Hence, hypoxia may be one of the factors responsible for Bcl-2 regulation, as Bcl-2 expression is increased in different cells under hypoxic conditions, such as primary varicose veins and pulmonary hypertension.[18,19] Whether adjunctive hyperbaric oxygen therapy can reduce the recurrence rate after varicocelectomy is an interesting issue. Previous studies have reported that hyperbaric oxygen therapy can reduce testicular injury under hypoxic stress in animal models and infertile patients with varicocele.[20–22]

In this study, we demonstrated downregulation of caspase-9 expression in the varicocele group. This resulted in a decrease in the rate of cellular apoptosis in the ISV through the intrinsic pathway, and may have caused the dilation and thickening of the ISV under hypoxic conditions.

In conclusion, the present study showed overexpression of Bcl-2 and downregulation of caspase-9 expression in the ISV of the varicocele group. There was no significant difference in Fas or caspase-8 expression in either group. This indicated dysregulated apoptosis through the intrinsic pathway in the ISV of patients with varicocele. To the best of our knowledge, this is the first study of the apoptotic pathway in the human ISV. Additional studies are necessary to investigate the specific promoter proteins in the apoptotic pathway and the effect of hyperbaric oxygenation, for better treatment of varicocele and prevention of recurrence.

Acknowledgments

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