Increased Expression of Hypoxia-Inducible Factor-1alpha and Bcl-2 in Varicocele and Varicose Veins

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Background: Primary vein wall abnormalities leading to secondary blood stasis and increased venous pressure that cause tissue hypoxia are observed in varicocele and varicose veins. Both types of diseased vessels are characterized by dilated thickened vein walls. Hypoxia upregulates Bcl-2 (antiapoptosis protein) expression in different human cell types. We studied the expression of hypoxia-inducible factor-1alpha (HIF-1α) and Bcl-2 in both venous diseases.

Methods: All vascular specimens, including the saphenous and internal spermatic veins, from patients with either varicocele or left inguinal herniorrhaphy (control group) were studied using immunoblotting, immunohistochemical staining, and double immunofluorescence staining. The data were analyzed using 1-way analysis of variance with Tukey comparison test.

Results: Protein analysis revealed that both venous diseases had a higher expression of HIF-1α and Bcl-2 compared with the control group (P < 0.05). Immunohistochemical staining and double immunofluorescence staining revealed that the greatest degree of HIF-1α and Bcl-2 colocalization occurred in the muscle layer of both diseased vessels. Moreover, under confocal microscopy, elevated Bcl-2 expression was found in the endothelium of both study groups compared with the control group.

Conclusions: Our findings revealed increased expression of HIF-1α and Bcl-2 in varicocele and varicose veins and increased Bcl-2 expression especially in the endothelium under hypoxia. Thus, Bcl-2 overexpression may protect cells against apoptosis and contribute to the dilated thickened walls seen in both types of diseased vessels.

INTRODUCTION

Varicocele is characterized by a dilated thickened wall in the internal spermatic veins. Although it is found in 15% to 20% of men1–3 and has a recurrence rate of approximately 2% to 14%,4 there are few studies of this vascular disease. The prevalence of varicose veins is underestimated, and it affects up to 40% of men and up to 51% of women.5 Both venous diseases are characterized by dilated thickened vein walls secondary to blood stasis and increased venous pressure causing tissue hypoxia and remodeling of the vessel wall as a compensatory result.3,6,7 It is not clear why hypoxia does not increase apoptosis and cause vessel atrophy in both venous diseases.

Hypoxia upregulates Bcl-2 expression in different types of human cells,8,9 Bcl-2 (antiapoptosis protein), which reduces apoptosis, may contribute to the thickened wall of varicose veins and varicocele under hypoxia. In the present study, we examined hypoxia-inducible factor-1alpha (HIF-1α) and Bcl-2 expression in both types of diseased vessels.

To our knowledge, this is the first report to study the relationship and distribution of HIF-1α and Bcl-2 simultaneously in both venous diseases.
involving the molecular changes in vascular diseases could improve future therapies.

MATERIALS AND METHODS

Patients and Venous Samples
This study included 42 young nonsmoking patients who were recruited between July 2008 and October 2010. One study group consisted of 15 patients aged from 20 to 25 years who had grade-3 left varicocele and received varicocele repair owing to scrotal pain. The varicocele was graded according to Dubin and Amelar. To prevent interobserver bias, all physical examinations were performed by one physician. The other study group consisted of 15 patients (seven men, eight women) with a mean age of 56 ± 2.2 years who were undergoing vascular stripping surgery for varicose saphenous veins of the lower leg after a duplex ultrasonographic survey. The operative indications were leg pain, edema, or venous stasis. All patients were characterized as having primary venous disease, and their symptoms had persisted for >6 months. According to the clinical, aetiological, anatomical and pathological (CEAP) classification for chronic lower-extremity venous disease, most of the patients were class 3 (n = 9) or class 4 (n = 6).

The control group consisted of 12 male volunteers (mean age: 25 ± 2.5 years) with an indirect left inguinal hernia, in whom a varicocele was ruled out by physical examination and color flow Doppler sonography (internal spermatic vein [ISV] diameter: <2 mm). A 1-cm venous segment was obtained during the operations, which included varicocelectomy, stripping of saphenous veins, or herniorrhaphy. Then, the vascular specimens were stored at −80°C for immunoblotting or fixed in 10% formalin and then embedded in paraffin blocks for immunohistochemical (IHC) and double immunofluorescence (IF) staining for confocal laser scanning microscopy. All specimens were collected only after written informed consent was obtained from the patients. This study was also approved by the Institutional Review Board of Taichung Armed Forces General Hospital.

Antibodies

Four types of primary antibody were used in this study: 1) HIF-1α, two rabbit polyclonal antibodies for immunoblotting (#3716, Cell Signaling, Beverly, MA; dilution 1:200) and immunostaining (sc-10790, Santa Cruz, Santa Cruz, CA; dilution 1:50); 2) Bcl-2, a mouse monoclonal antibody (sc-7382, Santa Cruz; dilution 1:50) for immunoblotting and immunostaining (dilution 1:400 and 1:50, respectively); and 3) actin, a rabbit polyclonal antibody for immunoblotting (sc-1616-R, Santa Cruz; dilution 1:5000).

The secondary antibodies for immunoblotting were horseradish peroxidase-conjugated goat antimouse IgG (#0031430, Pierce, Hercules, CA) or goat anti-rabbit IgG (#0031460, Pierce). The secondary antibodies for immunostaining were provided in the commercial kit (PicTure; Zymed, South San Francisco, CA) used for IHC staining; the Alexa-Fluor 488 conjugated goat anti-rabbit antibody (Molecular Probes, Eugene, OR; dilution 1:50) and the Alexa-Fluor 546 conjugated goat antimouse antibody (Molecular Probes; dilution 1:200) were used for IF staining. Preliminary experiments of negative control specimens (sections stained with only primary or secondary antibodies) demonstrated the lack of nonspecific staining and overstaining of the background (data not shown) in IF staining.

Immunoblotting

The method used was modified from that of our previous study. The samples, which had been stored at −80°C, were homogenized on ice and then centrifuged at 13,000 × g at 4°C for 20 minutes. The supernatants were used to determine protein concentration and for immunoblotting. The protein concentration was measured with reagents from the BCA Protein Assay Kit (#23229, Pierce), using bovine serum albumin as the standard (#23209, Pierce). For immunoblotting, the antibodies for HIF-1α, Bcl-2, and actin detected bands with molecular weights of approximately 120, 26, and 42 kDa, respectively. The blots were cut into upper and lower portions, incubated at 4°C overnight with the diluted primary antibodies, and then incubated with diluted secondary antibody for 1 hour. Finally, the protein bands were detected using the SuperSignal West Pico Detection Kit (#34082, Pierce) and imaged using a cooling-CCD image sensor (Chemidoc XRS+, Bio-Rad, Hercules, CA) with associated software (Quantity One version 4.6.8, Bio-Rad). The immunoreactive bands were analyzed using Image Lab software version 3.0 (Bio-Rad). The results were converted to numerical values to compare the relative protein abundance of the immunoreactive bands.

IHC Staining

The method used was modified from our previous studies. The deparaffinized ISV sections (4 μm) were rinsed with phosphate-buffered saline. Endogenous peroxidase was inactivated by incubating the sections with 3% hydrogen peroxide. The sections
were stained with primary antibody (HIF-1α or Bcl-2) before being analyzed with the commercial kit. Negative control experiments, in which phosphate-buffered saline was used instead of the primary antibody, were conducted to confirm the positive results seen with HIF-1α or Bcl-2. Finally, the sections were counterstained with hematoxylin (catalog no. 1.05175.0500; Merck, Darmstadt, Germany) and rinsed with tap water. The sections were observed with a light microscope (BX50; Olympus, Tokyo, Japan).

**Double IF Staining and Confocal Laser Scanning Microscopy**

The method used was modified from our previous study.16 The sections were incubated at 4°C overnight with diluted primary antibody (HIF-1α or Bcl-2) and then exposed to the respective secondary antibodies for 1 hour. Finally, the sections were covered by a slip with mounting solution (Zymed) before confocal laser scanning microscopy.

**Statistical Analysis**

The data were analyzed using 1-way analysis of variance with Tukey test. *P* < 0.05 was considered to be statistically significant.

**RESULTS**

Higher HIF-1α expression was observed in the study groups (varicocele and varicose veins) compared with the control group (138.56 ± 6.23, 142.45 ± 6.45, and 35.85 ± 3.61, respectively, *P* < 0.05; Table I and Fig. 1). Additionally, higher Bcl-2 expression was found in the study groups compared with the control group (134.77 ± 7.32, 130.32 ± 7.73).
7.73, and 47.18 ± 5.99, respectively, P < 0.05; Table I and Fig. 2). Similar profiles were obtained between HIF-1α and Bcl-2 protein expression of these three groups. By IHC staining, the higher levels of HIF-1α and Bcl-2 protein were found in the muscle layer of both diseased veins compared with the control group (Fig. 3). Similar results were found in double IF staining (Fig. 4D–I), and majority of the HIF-1α and Bcl-2 protein of the study groups were colocalized (Fig. 4K, L). Moreover, in the endothelium of the study groups, HIF-1α protein was rarely found (Fig. 4E, F), and the level of Bcl-2 protein was elevated under confocal laser scanning microscopy (Fig. 4H, I).

**DISCUSSION**

Both varicocele and varicose veins were characterized by dilated thickened vein walls, which have a similar etiology of hypoxia. These two diseased vessels develop because of poor venous return and blood stasis, causing tissue hypoxia that is associated with remodeling of the vessel walls. Hence, hypoxic stress has been proven to occur in these vessels, but it does not increase vascular cell death or vascular wall atrophy. On the contrary, the vein walls become engorged and thickened under hypoxia.

We reported that increased HIF-1α expression in the internal spermatic veins of patients with...
varicocele indicates hypoxia-related pathophysiological changes that have occurred in the varicocele. Moreover, higher levels of Bcl-2 were found in the varicocele and varicose veins compared with the control group. This study showed that in both diseased vessels, most of the HIF-1α and Bcl-2 in the smooth muscle layer were colocalized. The endothelium is the first target, and it responds to hypoxia and then expresses antiapoptotic genes, including Bcl-2, Bcl-XL, and survivin, which also contribute to angiogenesis and vascular remodeling. In this study, the Bcl-2 immunoreaction in the endothelial layer was higher in both types of diseased vessels than the control group under confocal microscopy. In addition, hypoxia-activated endothelial cells secrete growth factors that trigger smooth muscle cell proliferation and the synthesis of extracellular matrix components. Hence, hypoxia may be one of the factors responsible for Bcl-2 regulation, as Bcl-2 protein expression is increased in different cells under hypoxic conditions to protect cells against apoptosis.

Michiels et al. studied varicose veins and observed that hypoxia resulting from venous stasis induces the activation of endothelial cells, which then release inflammatory mediators to activate neutrophils and induce their infiltration and stimulate growth factors for smooth muscle cells. Bcl-2 inhibits cell apoptosis by preventing the release of cytochrome c from the outer mitochondrial membrane, which may be involved in the mechanism of thickened walls in both types of diseased vessels. Bcl-2 overexpression could decrease vascular cell apoptosis under hypoxic conditions and lead to vascular cell

Fig. 4. Representative sections of control (A, D, G, J), varicocele (B, E, H, K), and varicose saphenous vein (C, F, I, L) after double immunofluorescence staining and confocal laser scanning microscopy for the visible-light image (A–C), HIF-1α (green color; D–F), Bcl-2 (red color; G–I), and merged image (J–L). L, lumen; SM, smooth muscle layer; arrows, endothelium. Magnification: 200×.
proliferation, causing dilated thickened walls in varicoceles. A limitation of our study is the small number of cases and lack of normal saphenous veins as a control group. An additional study is needed to investigate whether hyperbaric oxygenation therapy is effective in reducing disease recurrence after surgical treatment.

CONCLUSIONS

Taken together, the results show increased HIF-1α and Bcl-2 expression in varicocele and varicose veins, and that Bcl-2 is expressed in the endothelium under hypoxia. Thus, Bcl-2 overexpression may protect cells against apoptosis and contribute to the dilated thickened walls of the diseased vessels.

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