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Dysregulated Apoptosis in Primary Varicose Veins

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Objective. Programmed cell death plays a critical role in various physiological processes. To investigate its possible pathogenic role in primary varicose veins we studied histological changes in surgical specimens from human varicose veins. In varicose and healthy veins, we also determined the number of cells in apoptosis, and investigated mediators regulating the intrinsic apoptotic mitochondrial pathway (Bax and caspase 9).

Methods. A total 23 varicose veins were obtained from 18 patients undergoing lower-extremity varicose vein surgery for primary varicose disorders. We used nine healthy veins obtained from nine patients undergoing distal arterial bypass grafting surgery as controls. The venous segment analysed was the distal part of the greater saphenous vein. Specimens for histological examination were stained with hematoxylin and eosin, trichromic and Victoria blue. Cell apoptoses and mediators of the mitochondrial pathway were detected in the media by immunohistochemistry using antibodies to peroxidase in situ apoptosis, Bax and caspase 9. Results were expressed as indexes for the three antibodies tested. The Mann–Whitney test was used to compare the results obtained in the two groups.

Results. Varicose vein specimens exhibited a more disorganised architecture than healthy veins and showed an increased number of collagen fibres and a decrease in the density and size of elastic fibres. All anti-apoptotic antibodies tested detected significantly fewer immunoreactive cells in tissue sections from the media of varicose veins than of healthy veins (peroxidase in situ, varicose veins (VV) median 2.4% (inter-quartile range 1.6–3.9) versus control (C) 14% (IQR 8.8–19); Bax, VV 1.4% (IQR 0.36–2.4) versus C 11% (IQR 7.6–15); and caspase 9, VV 1.7% (IQR 0.06–3.4) versus C 10% (IQR 9.1–12), $P=0.0001$ (Mann–Whitney test).

Conclusion. Apoptosis is down regulated in the medial layer of varicose veins. This dysregulation of the cellular mechanism that maintains normal tissue integrity is mediated through the intrinsic apoptotic pathway and may be among the causes of primary varicose veins.

Keywords: Apoptosis; Varicose vein; Primary varicose veins; Vascular apoptosis; Mitochondrial pathway.

Introduction

Even today, we know surprisingly little about how and why primary varicose veins develop. Despite the numerous aetiological factors including pregnancy,¹ obesity,² heredity or ethnicity,^{3,4} no satisfactory underlying mechanism has been proposed to explain the onset and progression of primary superficial venous insufficiency. When Estienne and Canano first discovered the venous valves, valvular failure at the sapheno-femoral junction was regarded as the primary event with secondary failure of more distal

valves and complete superficial venous insufficiency.^{5,6} Others later refuted this hypothesis.⁷

Recent evidence implies that superficial venous insufficiency arises from a structural weakness in the vein wall. These weaknesses could reflect the collagen or elastin content,^{8–10} or the concentration of metalloproteinases or inhibitors in the extracellular matrix of the venous wall.¹¹ A recent ultrasonic analysis of the sapheno-femoral junction and the distribution of varicosities support a primary wall abnormality predisposing to venous dilatation.¹²

Some evidence suggests that wall weakness in the varicose veins and the progressive replacement of normal components of the venous wall leading to dilatation and superficial venous insufficiency which is associated with a decrease in the number of cells undergoing apoptosis.^{13–15} Apoptosis, programmed

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cell death, has attracted great attention in recent years. Apoptosis is a normal process in development, morphogenesis and tissue regulation. Improperly regulated apoptosis, with defective DNA repair mechanisms and cell cycle alteration can nevertheless contribute to several pathological conditions including cancer, Alzheimer's disease, spinal muscular atrophy, ischemic cardiac damage and auto-immune syndrome.¹⁶⁻¹⁸ Apoptosis is a multifactorial phenomenon caused by specific activation of a family of intracellular cysteine proteases known as caspases (cysteine aspartyl-specific proteases) inducible by various stimuli.¹⁹ Schematically, two diverse pathways are distinguished. The intrinsic pathway (mitochondrial pathway or type I) regulated by apoptosis promoter proteins such as Bax or Bcl-2 and specific caspases (with motif caspase-associated recruitment domains—CARD) involves the participation of mitochondria, which release caspase-activating proteins into the cytosol, thereby triggering apoptosis. The extrinsic pathway (trans-membrane pathway or type II), regulated by proteins such as fas, and the tumour-necrosis factor receptor (TNF-R) family of proteins, involves specific caspases (with motif death effector domains—DED) connecting ligand binding at the cell surface to apoptosis induction.²⁰⁻²³

In this study of human surgical specimens, we evaluated histological changes in varicose veins. We also determined the number of cells in apoptosis detected by 3'/OH DNA termini on DNA fragments by a specific immunohistochemical assay in varicose and healthy veins. We also determined mediators regulating the intrinsic apoptotic mitochondrial pathway in varicose veins and healthy veins by detecting expression of Bax, considered a potent intrinsic apoptosis promoter,²⁴⁻²⁶ and caspase 9, a 46–48 kDa protein precursor of the mitochondrial apoptotic pathway.²⁷⁻²⁹ We did not detect expression of Bcl-2 because its role as a promoter or inhibitor of apoptosis remains ambiguous insofar as previous studies found no differences in Bcl-2 levels in varicose and healthy veins.¹³

Material and Methods

Cohort

A total of 23 varicose veins were obtained from 18 patients who were undergoing lower-extremity varicose vein excision. In five patients bilateral varicose veins were removed and included in the study. The characteristics of patients are given in Table 1. The

operative indications were distributed according to the clinical etiological anatomical and physiopathological (CEAP) classification as class 2 ($n=2$), class 3 ($n=10$) and class 4 ($n=6$). All patients had a primary varicose disorder with no specific venous risk factors and 16 of the 18 patients had a direct familial linkage. All patients were symptomatic for 1 year or longer. A total nine healthy veins (controls) were obtained from nine patients who were undergoing distal arterial bypass grafting surgery. For all the venous specimens, the segment analysed was the greater saphenous vein (GSV) 1 cm distal to its connection with the common femoral vein. The segment analysed in the varicose vein group was removed before vein stripping.

In all patients with varicose veins preoperative venous duplex ultrasound scanning showed greater saphenous vein reflux filling from the deep venous system and dilatation of GSV measuring 5 mm or more in diameter at the point of sampling. None of the patients had deep vein reflux or evidence of current or previous venous thrombosis. The control group consisted of patients in whom preoperative investigation and duplex ultrasound investigation prior to distal arterial bypass grafting surgery demonstrated no evidence of venous reflux or dilatation. None had diabetes or a history of venous disease. In one control patient the vein used for the bypass was partially dilated with no apparent global dilatation and was considered adequate for a distal bypass. In this patient, a distal segment of the GSV was removed for examination. In the control group, neither calcification nor parietal thrombus were histologically evident on the venous segment.^{30,31}

The study was approved by the hospital ethics committee and all the venous segments were removed with patients' informed written consent.

Tissues specimens

All venous segments were fixed in 10% neutral buffered formalin solution and paraffin embedded. Vein specimens were sectioned (3 μ m) (LEICA JUNG-RM-2035) perpendicular to the axis of the vessel; when the original venous segment was long enough the whole block was sectioned and sections were fixed each 5 mm (two to five sections per block/slide).

Histology

Specimens were stained with haematoxylin and eosin for routine histological evaluation. Changes in the localization, form and density of collagenous connective fibres in the parietal wall of varicose veins and

Table 1. Characteristics of the 27 patients with healthy and varicose veins

	Varicose veins (n=23)	Healthy veins (n=9)	P value
Number of patients	18*	9	
Gender			
Men:women	4:14	4:5	
Mean age (years)	54 (±16)	62 (±12)	<0.05
Height (cm)	165 (±6.1)	168 (±4.8)	
Weight (kg)	73 (±20)	70 (±11)	
Body mass index (BMI)	26.4 (±5.7)	24.8 (±2.9)	<0.05
<20	3	1	
20–26	6	5	
>26	9	3	
Clinical etiological anatomical and pathophysiological classification			
Type 2	2	–	
Type 3	10	–	
Type 4	6	–	

All data are expressed as means plus or minus SD.

* In five patients bilateral varicose veins were removed and included in the study.

healthy veins were detected by trichrome staining. Changes in the localization, form and density of elastic fibres in specimens from varicose and healthy veins were examined in Victoria blue-stained specimens.

Immunohistochemical detection

Cell apoptoses and mediators of the intrinsic mitochondrial pathway were detected with three primary antibodies: Apoptag® peroxidase *in situ* Apoptosis Detection Kit-S7101 (BIOTECH, Andover, MA, USA) (diluted: 33:110); rabbit polyclonal bax P-19-SC526 (SANTA CRUZ Biotechnology, INC) (diluted 1:40); and rabbit polyclonal antibody caspase 9 Ab-4 (Cat RB-1205-P1ABX) (NEOMARKERS, Fremont, CA, USA) (diluted 1:40).³² Serial formalin-fixed paraffin-embedded tissue sections were dewaxed and rehydrated by sequential immersions in three changes of xylene for 5 min each wash followed by immersion in absolute, 95 and 70% ethanol for 3 min each bath and in phosphate-buffered saline (PBS) for 5 min. The tissue was pretreated by applying proteinase K (20 µg/ml) for 15 min at room temperature directly onto the slide (60 µl/5 cm²). The specimen was washed in two changes of dH₂O for 2 min each wash. Endogenous peroxidase was quenched in PBS containing 3% hydrogen peroxide for 5 min at room temperature. Equilibration buffer was applied directly to the specimen (75 µl). For the peroxidase *in situ* detection, working strength TdT enzyme was immediately applied onto the section. The specimen was then incubated in a humidified chamber at 37 °C for 1 h.

Working strength stop buffer was applied for 10 min at room temperature. Anti-digoxigenin conjugate was applied to each specimen (65 µl/5 cm²) and specimens were incubated in a humidified chamber for 30 min at room temperature. Specimens were washed in four changes of PBS for 2 min per wash. For Bax and caspase 9 detection, biotinylated link was applied and incubated 10 min at room temperature. Specimens were rinsed with wash solution and placed in a wash solution bath for 10 min. Streptavidin-HRP was applied and specimens were incubated for 10 min at room temperature. Specimens were rinsed with wash solution and placed in a wash solution bath for 10 min. For all specimens, peroxidase substrate was applied (75 µl/5 cm²) to stain the specimens for 3–6 min. Specimens were washed in three changes of dH₂O for 1 min each wash. Slides were incubated in dH₂O for 5 min at room temperature. Specimens were stained in 0.5% methyl green for 10 min at room temperature and washed in three changes of dH₂O. Slides were then immersed 10 times each in the first and second washes, and in the third wash 30 s without agitation. The specimens were washed in three changes of 100% *N*-butanol, mounted on silanised slides and viewed under the optical microscope. On samples from carcinoma the anti-apoptosis antibody normally detects a large number of immunoreactive cells.

Quantification

For the histological analysis two fields per section were examined for staining at 400× magnification. The immunohistochemical expression of apoptosis was determined according to the intensity of nuclear staining and the density and morphology of chromatin. Results were evaluated by two independent investigators who were blinded to the patients' clinical findings; only inter-observer errors <2% were allowed. Peroxidase *in situ*, Bax and caspase 9-immunoreactive cells considered as positive for apoptosis in the media layer were counted manually at 400× magnification and seven random fields per section were counted. Two to five sections per specimen were evaluated. Immunohistochemical positive cell staining was not investigated in the intima and vasa-vasorum because previous studies found no differences in intimal apoptosis in varicose and healthy veins.¹⁴ Nor was positive cell staining investigated in the adventitia because preliminary experiments showed that this layer was severely and diffusely disorganized and contained few positive cells.^{13,14} The apoptotic index was calculated as the

number of positive cells in each field divided by number total of cells in the field multiplied by 100.

Statistical analysis

All data are expressed as medians and interquartile ranges. Immunohistochemical results are expressed as the apoptotic index for the three antibodies tested. Data obtained were analyzed by the Mann–Whitney test comparing the results obtained in the two groups. A *P* value less than 0.05 was considered to be statistically significant. Statistical analyses were performed with StatView (SAS Institute, Cart, NC) software.

Results

Histology

Varicose vein specimens exhibited a more disorganised architecture than healthy veins. Trichrome staining demonstrated an increased number of collagen fibres in the media with a specific decrease in smooth muscle cells in the sub-intimal layer associated with inhomogeneous thickness and organisation in the media layer (Fig. 1(a) and (b)). The adventitial layer was thinner in varicose veins than in healthy veins and contained more collagen fibres. In varicose veins, Victoria blue staining demonstrated a decrease in the density and size of elastic fibres and increased fibre degradation in the adventitial layer. In the media, elastic fibres were fragmented with constant parcelled interruption of the elastic lamina.

Immunohistochemical staining

Peroxidase in situ apoptosis detection

The apoptotic index of positive cell staining differed significantly in the two groups: varicose veins contained fewer peroxidase *in situ*-positive cells than healthy veins (Fig. 2(a) and (b)) (Table 2). Microscopy without quantification showed that the vasa-vasorum in the medial layer of varicose veins contained fewer positive stained cells than healthy veins.

Bax detection

The apoptosis promoter index of positive cell staining differed significantly in the two groups: varicose veins contained fewer Bax positive cells than healthy veins (Fig. 3(a) and (b)) (Table 2).

Caspase 9 detection

Again, the apoptosis promoter index of positive cell staining differed significantly in the two groups: varicose veins contained fewer caspase 9 positive cells than healthy veins (Fig. 4(a) and (b)) (Table 2). At microscopic examination of varicose veins, caspase 9 stained cells were localized in the sub-adventitial area of the medial layer; no stained cells were detected in the sub-endothelial media. In healthy veins, caspase 9 stained cells were homogeneously distributed in the medial layer.

Discussion

Our study has confirmed previous histological and immunohistochemical reports,^{13–15} but in addition, by analysing data from a large number of vein specimens in a standardised and reproducible manner, we now provide evidence implicating the mitochondrial (intrinsic) pathway in the pathogenesis of human primary varicose veins. By evaluating histological changes in the architectural organisation and determining the quantity, morphology and distribution of collagen and elastic fibres in varicose veins, we found that immunoreactivity to apoptotic detection in the medial layer was less prominent in varicose veins than in healthy veins. Immunohistochemical staining detected fewer specific mediators regulating the intrinsic apoptosis pathway (Bax and caspase 9) in the medial layer of varicose veins than of healthy veins. These cellular findings suggest that deregulated programmed cell death through the intrinsic mitochondrial apoptosis pathway promoter is among the possible causes of primary varicose veins in humans. Hence, we may need to add programmed cell death to the list of previously suggested causes of primary varicose veins including valvular changes or parietal recasting by tissue metalloproteinases.^{5,6,11}

The architecture and distribution of collagen and elastic fibres in the varicose veins appeared diffusely disorganised. This cellular disorganisation corresponded to an increased number of collagen fibres in the media and adventitia layers and a specific increase in elastin fibre degradation. These pathologic histological features correspond exactly to fibrotic degradation of the parietal wall and a loss of mechanical properties in varicose veins.^{14,33}

Deregulated apoptosis is a fundamental mechanism in a variety of diseases including cancer, autoimmune disorders, immunodeficiency, inflammation, ischemic heart disease, stroke and neurodegenerative diseases. For example, inappropriate expression of apoptosis promoters on lymphocytes and other immune cells

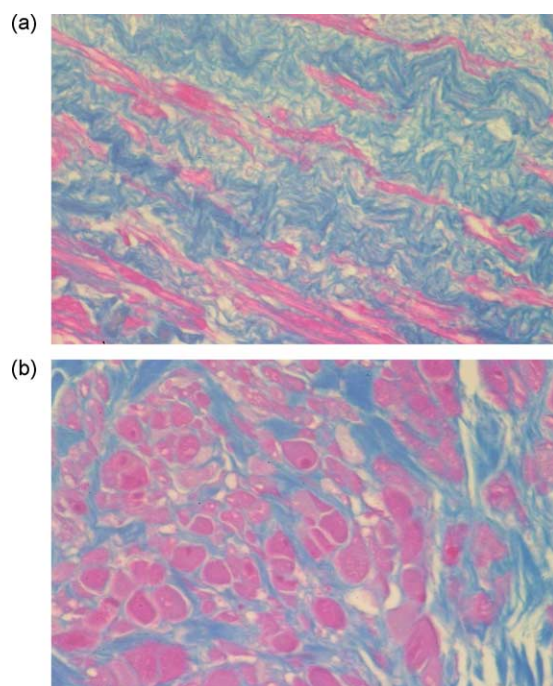


Fig. 1. (a, b) Trichrome staining showing an increased number of collagen fibres in the media (stained in blue) associated with a non homogeneous organisation and fewer smooth muscle cells in varicose veins (a) than in healthy veins (b) (original magnification, $\times 400$).

has been documented in patients with HIV infection and has been implicated in the loss of lymphocytes that characterises this immunodeficiency syndrome.³⁴ The down regulation of apoptosis has also been recognised in patients with severe forms of spinal muscular atrophy, a motor-neuron degenerative disease, and in most non-Hodgkin's lymphomas.³⁵⁻³⁷ Conversely, upregulation of apoptosis has been documented in autoimmune lymphoproliferative syndromes, and in many common types of cancer.³⁸⁻⁴⁰ Although less is known about cell turnover in the vascular wall, recent evidence shows that apoptosis is inversely correlated with restenosis related to arterial intimal hyperplasia after angioplasty and venous

Table 2. Immunohistochemical quantification of the specimens from healthy and varicose veins

Number of positive stained cells (in %)	Varicose veins	Healthy veins	*P value
Peroxidase <i>in situ</i> apoptosis detection	2.4 (1.6-3.9)	14 (8.8-19)	0.0001
Bax detection	1.4 (0.36-2.4)	11 (7.7-15)	0.0001
Caspase 9 detection	1.7 (0.06-3.4)	10 (9.1-12)	0.0001

All data are expressed as median (interquartile range).

* By Mann-Whitney test.

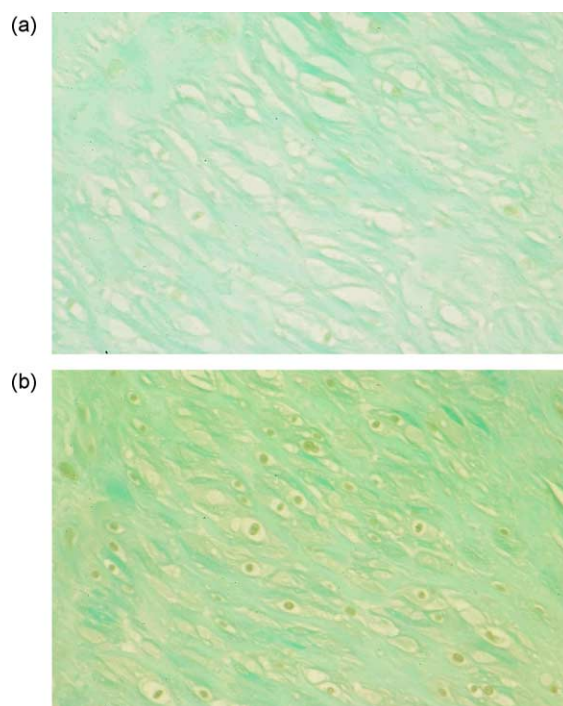


Fig. 2. (a, b) Peroxidase *in situ* apoptosis detection showing fewer peroxidase *in situ*-positive cells in varicose veins (a) than in healthy veins (b) (original magnification, $\times 400$).

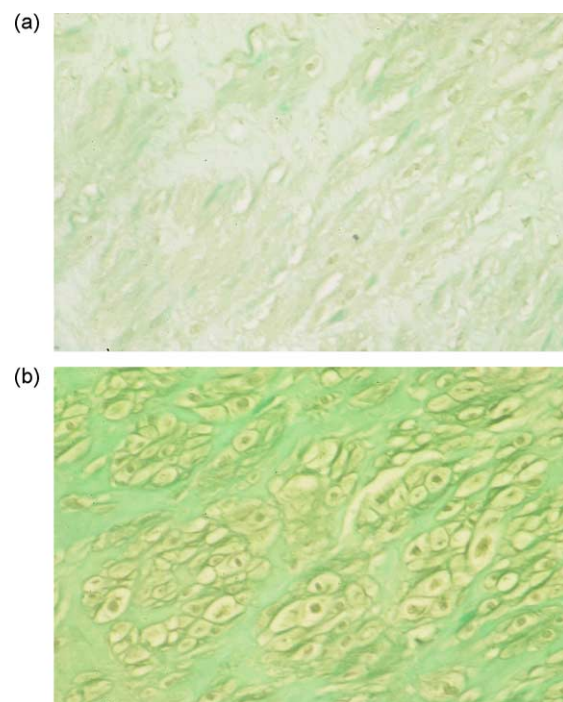


Fig. 3. (a, b) Bax detection showing fewer positive cells in varicose veins (a) than in healthy veins (b) (original magnification, $\times 400$).

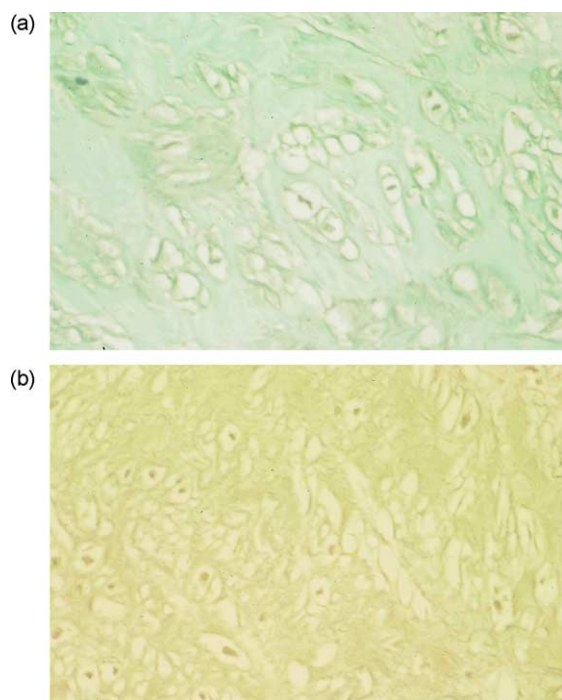


Fig. 4. (a, b) Caspase 9 detection showing fewer positive cells staining in varicose veins (a) than in healthy veins (b) (original magnification, $\times 400$).

graft.^{41,42} In human specimens, recent investigations of apoptotic activity in advanced atherosclerotic lesions in segments of renal, coronary and carotid arteries have shown increased apoptosis and intrinsic pathway promoters including Bax.⁴³ Because apoptosis plays a major role in tissue homeostasis and maintenance, the decreased cell turnover we observed in this study could be a major determinant of the parietal weakness in primary varicose veins.

Our cellular study of primary varicose veins showed that fewer cells stained for the peroxidase *in situ* apoptosis detection in varicose veins than in healthy veins in the media layer. This cellular finding confirms the only previous investigations indicating that the process of programmed cell death is inhibited in varicose veins.^{13,14} Nevertheless, one of these studies reported a statistically significant decrease in the apoptotic index in the adventitia of varicose veins compared with control veins and no difference in the medial layer.¹³ The discrepancy presumably reflects the high sensitivity of the anti-apoptosis antibodies we used for our immunohistochemical assays. Yet cell cultures from varicose veins show that the main component of the venous wall, smooth-muscle cells, principally localised in the media, transform from normally contractile to secretory cell types thus leading to fibrotic degeneration in the varicose vein

wall.⁴⁴ Cell cycle changes in varicose veins mediate smooth-muscle cell dedifferentiation from a contractile to a secretory phenotype.⁴⁵ In accordance with our study and previous findings, the fibrotic degradation typical of the varicose venous wall is associated with dysregulation of apoptosis in the media layer.

The distinctive finding in this study was the significantly weaker concomitant Bax and caspase 9 immune expression in varicose veins than in healthy veins. In previous studies, decreased Bax expression in varicose veins was associated with Bcl-x (without specifying whether the immunoreactivity detected was anti-apoptotic Bcl-x_l or pro-apoptotic Bcl-x_s) or with Bcl-2 (an anti-apoptotic protein).^{13,14} Because, we found no significant difference for either Bcl-x or Bcl-2 proteins in patients and controls we cannot specify the precise role of the intrinsic pathway in apoptotic dysregulation. The intrinsic pathway can be induced by mitochondrial release of cytochrome c, induced by various stimuli, including elevated levels of the pore-forming pro-apoptotic Bcl-2 family, including Bax. Within the cytosol, cytochrome c binds and activates Apaf-1, thus binding and activating pro-caspase 9.^{46–48} Active caspase 9 serves as the apical caspase of the intrinsic (mitochondrial) pathway, and directly cleaves and activates the apoptotic effector protease, caspase 3.^{49,50} By associating Bax and caspase 9 in our study we clearly showed that the decreased cell turnover is mediated by the intrinsic pathway.

A limitation of our study is the possible sex-linked effect related to the preponderance of women (14 out of 18) within the varicose vein group. This matter calls for further investigation in studies determining possible associations between demographic characteristics and immunohistochemical findings.

In conclusion, our results provide evidence of decreased programmed cell death in the medial layer of human primary varicose veins. Assay of specific proteins indicates that the physiological decrease in normal apoptosis is mediated through the intrinsic mitochondrial-dependent pathway. Our findings on the intrinsic pathway call for further investigations to identify the specific promoter proteins involved. Another pressing question is whether decreased apoptosis is the cause or effect of varicose vein formation.

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