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Increased Vein Wall Apoptosis in Varicose Vein Disease is Related to Venous Hypertension

K. Filis [a,*], N. Kavantzas [b], T. Isopoulos [a], P. Antonakis [a], P. Sigalas [a], E. Vavouranakis [c], F. Sigala [a]

[a] *Division of Vascular Surgery, First Department of Propaedeutic Surgery, University of Athens Medical School, Athens, Greece*
[b] *Department of Pathology, University of Athens Medical School, Athens, Greece*
[c] *Department of Cardiology, University of Athens Medical School, Athens, Greece*

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Abstract *Objectives:* The study aimed to evaluate a wide range of apoptotic markers in the vein wall of patients with superficial chronic venous disease (SCVD) compared with normal veins.
Design: This was an observational study.
Methods: Vein specimens were obtained from 19 patients suffering from SCVD. From each patient, a specimen of the proximal part of the great saphenous vein (GSV), a specimen of the distal part of the vein and a specimen of a varicose tributary were obtained. Immunohistochemical analysis was used to localise the expression of BAX, p53, Caspase 3, BCL-2, BCL-6, BCL-xs, BCL-xl and Ki-67. Vein specimens from 10 healthy GSVs were used as controls.
Results: Saphenous vein specimens from patients with SCVD showed increased BAX, Caspase 3, BCL-xl and BCL-xs ($p < 0.01$ for all) and Ki-67 ($p = 0.02$) compared with healthy GSVs. In the venous disease group, GSV specimens from the distal ankle area showed increased BAX ($p < 0.01$) and BCL-xs ($p = 0.031$) compared with varicose tributaries specimens, which subsequently showed increased BAX ($p = 0.044$), Caspase 3 ($p = 0.028$) and BCL-xs ($p = 0.037$) compared with specimens from the proximal GSV. In addition, in the venous disease group, specimens from distal GSV showed increased BAX ($p < 0.01$), Caspase 3 ($p = 0.019$) and BCL-xs ($p = 0.014$) compared with the proximal GSV.
Conclusion: Varicose veins exhibit increased apoptotic activity, by means of increased BAX, Caspase 3, BCL-xl and BCL-xs, compared with normal veins. Patients with varicose vein disease show increased apoptosis in the distal saphenous trunk compared with the proximal saphenous trunk, suggesting an association between chronic venous hypertension and apoptosis.

* Corresponding author. 34B Faneromenis Str., 15562 Holargos, Greece. Tel.: +30 2106561429; fax: +30 2107707574.
E-mail address: kfilis@hotmail.com (K. Filis).

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The processes leading to varicose veins remain unclear. There is some evidence that altered apoptotic mechanisms may contribute to this. A number of studies have demonstrated programmed cell death in vessels that remodel postnatally.[1,2] Increased apoptotic activity within the media of varicose veins has been shown by Bujan et al., while Ascher et al. concluded that down-regulation of apoptosis occurred in primary varicose veins.[3,4] More recently, Urbanek et al. showed that during varicose vein development, activation of apoptosis is related to the early stages of venous incompetence, while down-regulation of the smooth muscle cell apoptosis leads to structural changes.[5] Down-regulation of apoptosis in the media layer of varicose veins was also shown by Ducasse et al., who concluded that deregulation of the intrinsic apoptotic pathway may be among the causes of the disease.[6] However, one of the most pressing questions to be answered is whether deregulated apoptosis is the cause or the effect of varicose vein formation.

It is known that apoptosis is the cause of 'normal' cell death. In this study, we evaluated a series of mediators regulating the apoptotic pathway and cell proliferation in human surgical specimens of varicose veins and healthy veins of the lower limbs. To determine if apoptotic mechanisms depend on the late effect of long-standing venous hypertension, we evaluated the apoptotic markers in three different anatomical locations: the proximal part of the GSV, a GSV tributary of the calf and the distal part of GSV.

Materials and Methods

Patients

Lower limb vein specimens were obtained from two groups of patients. Group I (venous disease group) consisted of 19 patients (10 women) suffering primary superficial venous disease, at stages C_2, E_P, A_S and P_R without history of superficial vein thrombosis. Group II (control group) consisted of 10 patients (three women, mean age 63 years), who underwent distal GSV excision for using it as a graft in cardiac bypass. Group I (mean age 48 years) had a mean disease duration of 12 years (from 4 to 25 years, median 8.5 years), and a preoperative lower-limb duplex ultrasound assessment confirming the extent of venous reflux in the GSV. No evidence of current or previous deep or superficial venous thrombosis was found. Control patients underwent preoperative (before cardiac bypass) venous duplex ultrasonography scan as quality control for the vein segment to be used as a graft during aortocoronary bypass grafting and to confirm the absence of deep or superficial venous thrombosis.

Patients in group I underwent stripping of the GSV from the ankle to the saphenofemoral junction and with excision of tributaries. Before saphenous vein stripping, a 2-cm vein segment was excised from the saphenofemoral junction and the GSV at the ankle. Another 2-cm vein segment was excised from a calf varicosity. In patients of group II, the distal 2 cm of the GSV was used as control vein.

Ethical approval for the study was obtained from the University of Athens Medical School.

Tissue preparation

All vein specimens were divided into two portions along the longitudinal axis. The specimens collected were fixed in 10% neutral-buffered solution containing approximately 4% formaldehyde for 24 h, and embedded in paraffin for conventional histology and immunohistochemistry.

Histology

Routine haematoxylin and eosin staining was performed for histological evaluation of the specimens. Gomori's one-step trichrome staining was used to indentify increased collagenous connective fibres and to differentiate between collagen and smooth muscle fibres in both normal and varicose vein specimens. Verhoeff's elastic tissue stain (with van Gieson's stain to counterstain) was performed to assess the pathological changes in the elastin network in all the veins included in the study. Histology was performed for confirmation that the sections used for immunohistochemical analysis contained the classical findings of varicose veins and as well as those of healthy veins in group II.

Immunohistochemistry

Antibodies

For immunohistochemical analysis, the following antibodies were used: BCL-6 (clone: PG-B6p) mouse monoclonal antibody (Dako, Kalifronas, Athens, Greece) in a dilution 1:10; p53 (clone: DO-7) mouse monoclonal antibody (Dako, Kalifronas, Athens, Greece) in a dilution of 1:50; Ki-67 (clone:MIB-1) mouse monoclonal antibody (Dako, Kalifronas, Athens, Greece) in a 1: 50 dilution; BCL-2 (clone:BCL-2/100/D5) mouse monoclonal antibody (NCL-L-BCL2-Novocastra,Meranini Diagnostics, Athens, Greece) in a 1:50 dilution; Caspase 3 (clone:3CSPO3) mouse monoclonal antibody (Neomarkers, Bioanalytica, Athens, Greece) in dilution 1:50; BAX (clone:930Rb) rabbit polyclonal antibody (Santa-Cruz Biotechnology, Bioanalytica, Athens, Greece) in 1:200 dilution; BCL-xs (clone: PC89) mouse monoclonal antibody (Calbiochem, Chemilab, Athens, Greece) in 1:100 dilution; and BCL-xl (clone:H-62) rabbit polyclonal antibody (Santa-Cruz Biotechnology, Bioanalytica, Athens, Greece) in 1:100 dilution.

Method

Immunohistochemistry was performed according to an indirect streptavidin—biotin—peroxidase method. In brief, 5-μm paraffin sections were placed on poly-L-lysine-coated slides, dewaxed, rehydrated and incubated for 30 min with 0.3% hydrogen peroxide to quench the endogenous peroxidase activity. Unmasking of the related proteins was carried out. The sections were incubated with the primary antibody at a 1:50 dilution at 4 °C overnight. Biotin-conjugated secondary antibody was added at a 1:200 dilution for 1 h at room temperature. The next stage comprised 30-min incubation in Strept—AB complex (1:100 stock biotin solution, 1:100 stock streptavidin—hyperoxidase solution) (Dako, Kalifronas, Athens, Greece). For colour development, we used 3,3′-diaminobenzidine tetrahydrochloride (DAB, Sigma-Hellas, Athens, Greece) and haematoxylin as a counterstain.