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Summary. This study was designed to evaluate the role of the smooth muscle cell and the apoptosis in the pathogenesis of the varicose vein.

Segments of saphenous vein were obtained from

distension and reflux. The deformation of the wall is brought about by the smooth muscle and connective tissue components of the medial layer which may be affected by disease.

# Evaluation of the smooth muscle cell component and apoptosis in the varicose vein wall

J. BuJán<sup>1</sup>, J.A. Jimenez-Cossi<sup>2</sup>, F. Jurado<sup>1</sup>, M.J. Gimeno<sup>1</sup>, G. Pascual<sup>1</sup>, N. Garcia-Honduvilla<sup>1</sup>, B. Dominguez<sup>1</sup> and J.M. Bellón<sup>1</sup>

<sup>1</sup> Department of Morphological Sciences and Surgery (Surgical Research Laboratory), Faculty of Medicine, University of Alcalá, Madrid, and <sup>2</sup> Service of Vascular Surgery, La Paz Hospital, Madrid, Spain

healthy subjects and from those with varicose veins. The vein specimens were subdivided according to subject age (younger or older than 50 years) and according to the varicose vein source (distal or proximal). Morphological, ultrastructural, cell proliferation (antiPCNA method) and cell death (TUNEL method) analysis were performed.

The walls of healthy, control vein specimens acquired a more collagenous and papillomatous appearance with age. A slight increase in the number of TUNEL-positive cells was also observed in specimens from older subjects. The proportion of apoptotic cells was much greater in the varicose veins than in control specimens. Most cellular alterations were seen in proximal varicose segments obtained from young subjects. These specimens showed hypertrophic areas with a high degree of cellularity (both in the media and in the thickened intima). The highest proportion of apoptotic cells and collagenisation were also observed in these areas. The enhanced number of apoptotic cells in varicose veins observed mainly in proximal/young vein specimens could be responsible, at least in part, for the acceleration of the final fibrosclerotic process characteristic of the varicose vein wall.

**Key words:** Varicose vein, Smooth muscle cell, Apoptosis, Vein wall, Proliferation

## Introduction

Understanding the pathogenesis of the varicose vein involves the search for a primary fault in the vein wall itself which could lead to weakness and provoke

There is much controversy over the role of the smooth muscle cell in the varicose condition. Several authors report an increase in these cells in the varicose vein (Svejar et al., 1963; Obitsu, 1990) while others refer to a reduction in the cellularity of the smooth muscle layer with replacement by collagen (Rose and Ahmed, 1986) or report no difference in the amount of smooth muscle (Lees et al., 1992a,b). Indeed, the defective regulation of the number of smooth muscle cells in the varicose vein is entirely feasible. Apoptosis, or programmed cell death, is essential for the normal regulation of the number of cells in a given tissue and is known to play a significant role in the arterial lesion formation (Mitchinson et al., 1996; Bennett, 1999; Mallat et al., 1999). However, its role in venous pathology is yet to be established.

The aim of the present investigation was to gain insight into the development of the varicose vein with special reference to changes in the smooth muscle component of the wall and the proportion of apoptotic cells. Since the ageing process is closely related to apoptosis, the study specimens were divided according to age. The area of the saphenous vein from which the specimens were obtained (distal or proximal) was also defined, since these areas have been reported to contain different amounts of muscle (Thulesius, 1993; Travers et al., 1996) and may even show different numbers of apoptotic cells due to the variation in blood flow.

## Materials and methods

Saphenous vein specimens obtained from patients to be subjected to aorto-coronary by-pass who presented no pathology of the venous system served as controls. Varicose veins were obtained at the time of vein extraction from voluntary patients with venous insufficiency.

The control (n=11) and varicose (n=22) specimen groups were divided according to subject age into young (<50 years, age range 23 to 48 years, mean 36±7.69 years: 12 varicose and 5 controls) and elderly (2 50

Offprint requests to: Dra. Julia Bujan, Departamento de Ciencias Morfológicas y Cirugía, Facultad de Medicina, Universidad de Alcalá.

Ctra. Madrid-Barcelona, km 33.6, 28871 Madrid, Spain. Fax: 34-91-885

48 85. e-mail: mjulia.bujan@uah.es

years, age range 53 to 71 years, mean 63.6±5.35 years: 10 varicose and 6 controls).

The varicose specimen group was in turn divided according to the portion of the saphenous vein into proximal (at the level of the femoral condyle) and distal (at the level of the maleolus).

#### Light microscopy

The specimens for light microscopy were fixed by immersion in 10% formaldehyde solution and embedded in paraffin to obtain 5 µm-thick transverse and longitudinal sections. Specimens were deparaffinated, hydrated and stained. Haematoxylin-eosin and Masson's trichrome stains were used for morphological study.

#### Electron microscopy

The specimens for ultrastructural analysis were fragmented and fixed by immersion in 3% glutaraldehyde for 2 h. They were then placed in Millonig buffer (pH 7.3), postfixed in 1% osmium tetroxide, dehydrated in a graded series of acetone and embedded in Araldite to obtain thin cuts. The cuts were contrasted with lead citrate and observed under a Zeiss 109 transmission electron microscope.

Specimens for scanning electron microscopy were opened longitudinally and submerged in 3% glutaraldehyde for 2 h. They were then placed in Millonig buffer (pH 7.3) for several hours and dehydrated in a graded series of acetone reaching critical point in an E3000 Polaron with C02. Once metallised with gold-palladium, they were observed under a Zeiss 950 DSM scanning electron microscope.

#### Immunohistochemistry

In order to confirm the increase observed in the vasa vasorum network of varicose vein, the specimens embedded in paraffin were immunochemically labelled with rabbit anti-human von Willebrand Factor (vWF) (Sigma Product No. F-3520, St Louis, MO, USA) prepared at a dilution of 1:1000. The antigen-antibody reaction was detected using biotinylated goat anti-rabbit IgG and ExtraAvidin®-Peroxidase (Sigma Kit No. EXTRA-3, St Louis, MO, USA). The chromogenic substrate used was diaminobenzidine (DAB). Specimens were contrasted for 5 min using methyl green and examined under a light microscope (Zeiss, Jena, Germany).

#### Proliferation and cell death

Specimens were deparaffinated, hydrated and

samples were selected for the simultaneous evaluation of both cell processes:

##### a) In situ apoptotic cell labelling

Apoptotic nuclei were identified by modification of the TUNEL method (Negoescu et al., 1997). TUNEL is based on the in situ detection of nucleosomal DNA fragmentation characteristic of apoptosis. In this assay, terminal deoxynucleotidyl transferase (TdT) binds to exposed 3'-OH ends of DNA fragments generated in response to apoptotic signals and catalyses the addition of biotin-labelled and unlabeled deoxynucleotides. The detection of DNA fragmentation was performed using a TdT Fragment End Labelling kit (TdT FragEL™, Calbiochem, CN Biosciences INC., USA). Biotinylated nucleotides were detected using a streptavidin-horseradish peroxidase conjugate. The images were developed with a chromogenic substrate containing DAB. Specimens were contrasted for 5 min using methyl green.

##### b) Immunohistochemical labelling of proliferative cells

Proliferating cells were visualised by detection of the proliferative cell nuclear antigen (PCNA). Specimens were incubated overnight with a 1:2000 dilution of an anti-PCNA monoclonal antibody (Sigma, St Louis, MO, USA) in a humid atmosphere at 4 °C. The specimens were subsequently incubated (30 min at room temperature) with the secondary antibody (biotinylated anti-mouse, 1:20 dilution, Sigma). Sections were then treated with an avidin-peroxidase complex (Sigma, St Louis, MO, USA) at a 1:20 dilution for 30 min at room temperature. DAB was used as the chromogen and methyl green as the counterstain.

#### Cell counts and statistical analysis

The apoptotic and proliferative cells of the vein wall were counted in 20 transverse sections per vein specimen per group under a Zeiss Axiophot light microscope (Zeiss, Jena, Germany). Each tissue section was divided into four sectors and labelled cells were counted in two random optical fields per sector to determine the proportion of apoptotic, or proliferative, cells in each layer of the vein wall. This study was performed independently by two observers. The data obtained were compared using the Mann-Whitney U-test.

## Results

### Muscle and apoptosis in varicose veins

equilibrated in PBS pH 7.4. They were then subjected to microwave irradiation (SANYO EM-704T) for 5 min (350W) in 0.01M citrate buffer (pH 6). Following the inactivation of endogenous peroxidase activity (using 3% H<sub>2</sub>O<sub>2</sub> in methanol) and the blockage of unspecific sites (goat serum), consecutive sections of the vein

#### Morphology and ultrastructural analysis

##### a) Control vein specimens

The vein wall of specimens from the control young population showed well preserved layers of smooth muscle cells. Vein specimens obtained from elderly

subjects differed from those of younger subjects in the irregularity of the endoluminal portion which showed papilomatous formations and an enlarged extracellular matrix. The vein wall took on a more collagenous appearance with age showing alternating thick layers of this component throughout the wall. The smooth muscle cells of specimens from elderly subjects showed cytoplasm rich in secretory vacuoles and lysosomes. Some of these cells showed condensation and fragmentation of chromatin.

b) Varicose/young vein specimens

The most characteristic feature of the varicose vein wall from young subjects was the change in morphology observed in proximal segments. In some specimens, the

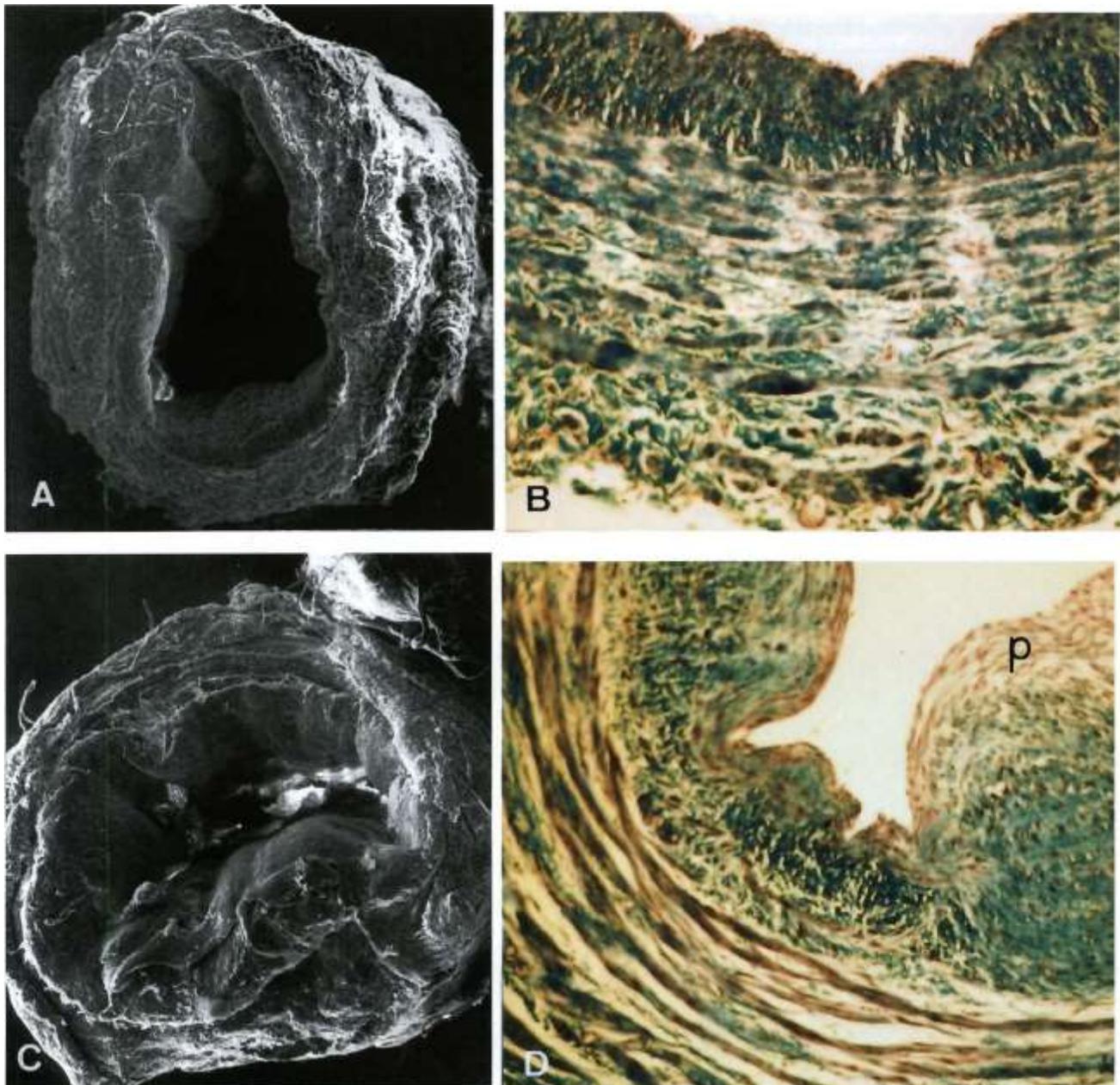


Fig. 1. A. Panoramic SEM view of the varicose vein wall in transverse section. The specimen was obtained from the proximal portion of the saphenous vein of a young subject. x 50. B. Light microphotograph showing the different layers of the same vein wall. x 160. C. Panoramic SEM view shows papilomatous appearance of the varicose vein in specimens from elderly subjects. x 40. D. Hypertrophic areas showing extracellular matrix accumulation in the varicose vein wall of an elderly subject (P).O.M. x 160

wall was well-preserved (Fig. IA,B) while other regions showed hypertrophic areas with a high degree of cellularity (both in the media and in the thickened intima) and collagenisation. The smooth muscle cells also contained small vacuoles, lysosomes and lamellar bodies. Frequently, the endothelium showed micropinocytosis vesicles. Many of the intimal and muscle cells also showed nuclear alterations. Particularly noticeable, was the increase in vascularisation approaching the luminal

surface of the wall itself (Fig. 2A,B).

Distal specimens obtained from young subjects showed hypertrophic characteristics but were also well structured.

c) Varicose/elderly vein specimens

In these specimens, there was a tendency towards fibrosclerosis of the wall with considerable changes

Table 1. Percentage of TUNEL-positive cells according to vein specimen group.

	CONTROL VEINS		VARICOSE VEINS			
			Proximal		Distal	
	years	>50 years	years	years	years	years
Endothelium	9.15±0.81	10.10±2.74	97.82±0.8*	52.22±1.81	42.98±10.72	51.96±9.09
Media	7.03±1.32	8.11±1.23	88.38±4.42*	44.45±20.19	48.43±16.37	41.45±7.6
Adventitia	7.10±1.65	8.31±2.3	88.95±12.49*	64.89±10.06	63.05±4.04	58.79±10.06

\*: statistical significance ( $p < 0.05$ ).

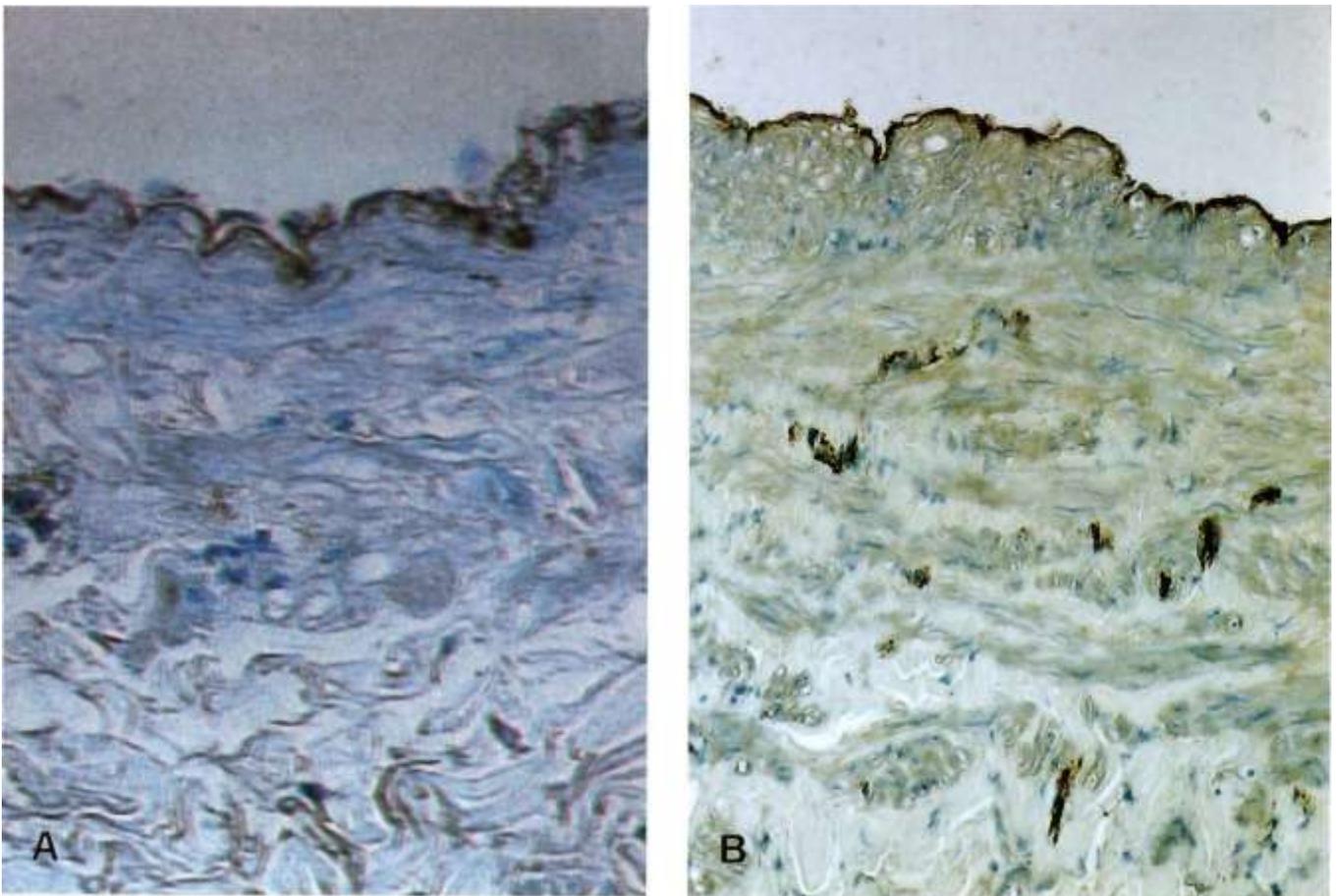


Fig. 2. Endothelial cells labelled with anti-von Willebrand Factor antibody (brown) in a control/young vein specimens (A) and a varicose/young vein specimens (proximal segment) (B). The photograph shows an increase in the vasa vasorum network approaching the luminal surface of the wall itself. A, x 400; B, x 250

observed in superficial layers including a papillomatous and highly fibrillar appearance (Fig. IC,D). The proximal vein wall was of disorganised appearance with cell rarefaction and loss of collagen and elastic laminae. These effects were less prominent in distal samples with the exception of a

network of vasa vasorum detected in the muscle layer in which highly dilated lumens were occasionally observed.

### Apoptosis

Table 1 shows the proportion of apoptotic cells detected in the different specimens.

Considering the apoptotic cell fraction as an indicator of damage to the wall, most injury was seen in the proximal region of specimens from young subjects (Fig. 3). A 5-fold increase in the number of apoptotic cells was recorded in the varicose veins with respect to control specimens while proximal specimens from young subjects showed a 2-fold increase with respect to distal or elderly specimens. Relative to the other layers, the intima showed marked damage. This phenomenon was attenuated with age and eventually became general to each of the wall layers. The proportions of apoptotic cells were similar in specimens from both age groups, whether distal varicose veins or control specimens, although much greater in the varicose veins, compared to the control veins. These observations corresponded to the condensation and fragmentation of chromatin observed

Fig. 3. A. TUNELpositive cells (arrows) seen in the intimal (i) and medial (m) layers of varicose/young vein specimens. x 400. B. Microphotograph of the tunica media of the varicose vein of a young subject showing

smooth muscle cells and collagen fibres of irregular size. x 4,400

by transmission electron microscopy.

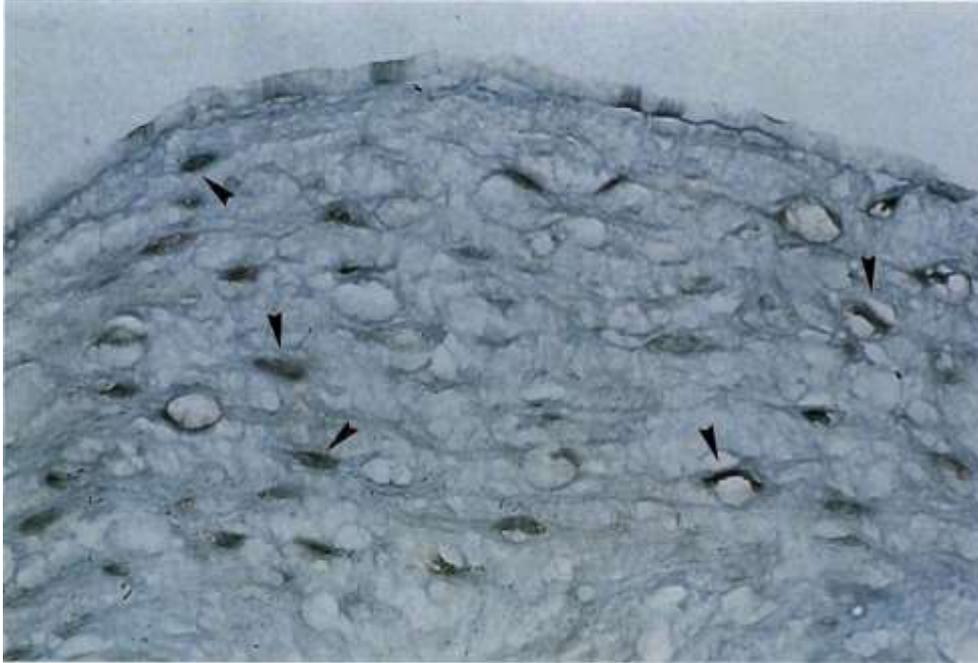
### Cell proliferation

Anti-PCNA antibody labelling indicated an increase in smooth muscle cell proliferation ( $54 \pm 3.8\%$ ) in varicose vein specimens from very young subjects (23 years) (Fig. 4). The proportion of proliferative cells

fell in subjects of 38-40 years showing a clear increase in cellularity (probably due to early proliferation), cell hypertrophy and apoptosis. In varicose/elderly specimens and in the control specimens, the proportion of proliferative cells was much lower and stable (data not shown).

## Discussion

Although the pathology of the varicose vein does not appear to be attributable to a deficiency in muscle content (Travers et al., 1996), the inability of the



muscle to maintain the required tone in the vein wall suggests that the smooth muscle cell is ultimately responsible for the entire varicose process occurring in the wall (Fischer et al., 1996). There is much controversy over the number of cells involved in the varicose condition (Svejcar et al., 1963; Rose and Ahmed, 1986; Obitsu, 1990; Travers et al., 1992; Krasinski et al., 1997) and some authors even attribute smooth muscle cells a compensatory role in the varicose vein (Orlov and Erofeev, 1977).

In contrast, the separation of smooth muscle cells by collagen fibres in the varicose condition is a fact which appears to be well established (Rose and Ahmed, 1986; Porto et al., 1995) although it is nevertheless unclear whether this occurs as the result of an intrinsic anomaly of the muscle (followed by the infiltration of collagen) or by a defect in collagen production. The vein wall from the present control population took on a more collagenous appearance with age showing thick layers of this component interspersed with smooth muscle and a tendency to become papilomatous due to the slow and irregular growth of the wall.

Here, the TUNEL method was used to estimate cell death, although it has been recently shown that this technique also identifies necrotic cells (Grasl-Kraupp et al., 1995), cells in a state of transcription (Kockx et al., 1998), calcified matrix vesicles (Kockx et al., 1996) and DNA repair (Kanoh et al., 1999). However, the most common use of this method is the detection of the apoptotic cell fraction (Kressel and Groscurth, 1994; Mesner and Kaufmann, 1997; Negoescu et al., 1997; Peng and Liu, 1997).

It is of note that in this study, control specimens also showed a high degree of apoptosis. This may partly be due

to the lengthy, aggressive (but unavoidable) extraction process that these specimens are subjected to.

The number of apoptotic cells in the varicose vein specimens showed a marked increase with respect to control specimens. Most damage was seen in proximal segments obtained from young subjects, where segments showing muscle hypertrophy and collagenisation and those in which the thickness of the muscle layer was preserved could be identified. Leu (1980) and Porto et al. (1995) reported similar observations.

Anti-PCNA antibody labelling indicated an increase in smooth muscle cell proliferation in varicose vein specimens from very young subjects. Although PCNA immunoreactivity requires a careful tissue preparation

Fig. 4. Cells labelled with an antiPCNA antibody (arrows) in a proximal varicose vein specimen from a 23year-old subject. x 630

## Muscle and apoptosis in varicose veins

(Yu et al., 1992) and this antigen is also expressed during DNA repair (Shivji et al., 1992), The PCNA immunohistochemical method is preferred to determine the cellular proliferation.

Most of the apoptotic cells were identified in areas of muscular hypertrophy. Apoptosis is perhaps the final expression of cell hypertrophy. It is well known that cells in culture may follow one of two routes: they may either divide normally or may undergo a substantial increase in size and finally enter the apoptotic phase. The increase in apoptosis observed here may reflect an attempt on behalf of the vein wall to regulate the number of cells which, paradoxically, eventually favours the collagenisation of the muscle layer. The apoptotic cells retract and separate from neighbouring cells thus permitting the infiltration of collagen.

At the same time, the non-apoptotic cells may be stimulated to secrete matrix components. Thus, smooth muscle cells are thought to adopt secretory characteristics and produce collagen leading to the thickening and disorganisation observed in varicose veins (Rose and Ahmed, 1986). It has been suggested that these changes in smooth muscle could be the result of changes in the endothelium provoked by hypoxia during venous stasis (Michiels et al., 1996). Further, the intimal layer of the varicose vein often shows focal endothelial denudation or necrotic cells. In the present study, proximal segments of varicose veins of young subjects also showed a higher proportion of apoptotic cells in this layer. Endothelial damage may stimulate the proliferation of smooth muscle cells and the synthesis of extracellular matrix components (Dormandy, 1996; Michiels et al., 1997) accounting for the increase in cellularity and collagenisation observed in certain areas of elderly vein specimens and in proximal portions of veins from young subjects.

The changes observed when the varicose disease is added to the age factor have previously been described as morphological changes (Bouissou et al., 1988; Pieraggi et al., 1992; Bigel and Taccoen, 1996) rather than as stages in the development of the same process. As the varicose process becomes chronic, cellularity decreases and is related more to age than to the pathological process.

The present results suggest that the varicose process involves a series of steps (normal vein - failure compensatory hypertrophy - new failure - apoptosis atrophy - sclerosis) among which apoptosis may be the regulatory mechanism underlying the compensatory

hypertrophy of the vein wall. Further, the increase in the number of apoptotic cells, particularly in young subjects, may eventually lead to the final fibrosclerosis of the vein wall.

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## Muscle and apoptosis in varicose veins

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