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Generation of Reactive Oxygen Metabolites by the Varicose Vein Wall

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Objectives: *to evaluate the content of lipid peroxidation products (expressed by the concentration of thiobarbituric acid-reactive substances; TBARS), the content of myeloperoxidase (MPO), and the localisation of xanthine oxidase (XO) in varicose veins (vv), varicose veins with superficial thrombophlebitis and unchanged saphenous veins.*
Methods: *varicose saphenous veins, varicose veins with superficial thrombophlebitis and normal saphenous veins obtained during varicose vein surgery on 36 patients as well as healthy saphenous veins from cadaver organ donors (control). Homogenates were prepared in which TBARS concentration and MPO content were determined. Immunohistochemical staining to detect XO was also performed.*
Results: *the highest concentration of TBARS occurred in vv with superficial thrombophlebitis, the lowest in donor vein. The highest content of MPO was observed in vv and slightly lower – in varicose veins with thrombophlebitis. A positive reaction for XO was seen in vv wall endothelium. Specimens of vv with thrombophlebitis revealed strong, intense staining in endothelium as well as in* vasa vasorum.
Conclusions: *varicose veins, especially those complicated with superficial thrombophlebitis revealed increased free radical generation. Its sources might be neutrophils, and in vv complicated with superficial thrombophlebitis–xanthine oxidase.*

Key Words: Varicose vein; Neutrophil activation; Xanthine oxidase; Free radicals.

Introduction

The aetiology of varicose veins is associated with incompetent valves and dilatation of the veins.[1] These are accompanied by blood stasis and hypoxia, which affects the luminal endothelium, as well as the other vessel layers and the surrounding tissues.[2] Endothelial cells are ubiquitous and located at the blood-tissue barrier. They have been proposed as the initial site of ischaemic injury. During hypoxia, the main metabolic disturbance is the breakdown of ATP and its degradation to hypoxanthine, with the conversion of xanthine dehydrogenase (XD) to xanthine oxidase (XO). This creates favourable conditions for excessive free radical generation.[3,4]

The role of leukocytes in varicose veins development has been well documented.[5–7] Not only neutrophils, but also monocytes, have been found on the endothelium and in the vein wall interstitium.[8] When stimulated, they release vast amounts of proteases, phospholipase products, and reactive oxygen metabolites, which are all highly toxic to the vessel wall and its surrounding tissues.[9] Reactive oxygen metabolites are generated through the action of membrane bound oxidases and myeloperoxidase. The activity of myeloperoxidase is commonly used as an indicator of neutrophil activation.[10] In addition, the interaction of activated neutrophils with endothelial cells resulting in conversion of XD to XO within endothelial cells has also been demonstrated.[11] One may expect that the effects of the chronic inflammatory state, such as lipodermatosclerosis, and leg ulcer might be caused by free radical action.

Previous studies indicate a benefit in the treatment of leg ulcers with the use of allopurinol – a xanthine oxidase inhibitor.[12] However, direct evidence for increased free radical generation in patients with venous insufficiency is still lacking.

The aim of the study was to evaluate the content of lipid peroxidation products (expressed by the concentration of thiobarbituric acid-reactive substances; TBARS), the content of myeloperoxidase (MPO), and the presence of xanthine oxidase (XO) in varicose

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veins, varicose veins with superficial thrombophlebitis and unchanged saphenous vein.

Material and Methods

Patients

The material studied consisted of segments of greater saphenous vein. We investigated varicose veins, varicose veins complicated with superficial thrombophlebitis and unchanged vein. The unchanged vein specimens were collected from competent segments of greater saphenous veins that were diseased (i.e. varicose) elsewhere. Those segments were used for analysis to determine whether alterations were present before venous dilatation. The material was obtained from 36 patients, 21 female and 15 male (median age 48 years, range 27–63 years) during varicose vein surgery. Patients with diabetes, hyperlipidaemia and smokers were excluded from the study, because oxidative–antioxidative balance is known to be disturbed in such patients. Normal veins, obtained during cadaver organ procurement, were used as a control–donor vein (DV). They were collected from 4 men and 2 women (median age 43 years, range 22–49 years), with no sign of venous insufficiency. The study protocol was approved by the Regional Bioethical Committee.

Methods

All segments were washed in 0.15 M NaCl solution. Loose, perivascular fascia, fat, blood and thrombi were carefully removed. Next, all segments were dried, immediately frozen and stored at $-70\,^{\circ}C$. Dishes with the dissected and weighed fragments of veins were placed in ice, then homogenised in 5 volumes of glacial 0.15 M saline solution for 2 min, using a knife-shaped homogeniser. The homogenates were then centrifuged at 3000 r.p.m. for 30 min at $+4\,^{\circ}C$. Supernatant was used for determination of protein by the Lowry method. TBARS were measured using the TBA technique described by Buege and Aust.[13] When lipid peroxidation by oxygen derived free radicals takes place aldehyde end-products occur. Malondialdehyde (MDA) is the most abundant individual aldehyde resulting from lipid peroxidation and its determination by thiobarbituric acid (TBA) is one of the most common assays in free radical studies. Aldehydes reacting with TBA are named as TBA–reactive substances.[14] The basis of the method used is the reaction of MDA with thiobarbituric acid to form a colour complex that can be quantified spectrophotometrically from its visible absorbance (E_{max} 532 nm). Myeloperoxidase was measured using Bioxytech MPO Enzyme Immunoassay System (Oxis International, Inc. U.S.A.). The method is based on "sandwich" ELISA. Antigen captured by solid phase monoclonal antibody is detected with biotin-labelled goat polyclonal anti-MPO. An avidin alkaline phosphatase conjugate then binds to the biotinylated antibody. The alkaline phosphatase substrate *p*-nitrophenyl phosphate (pNPP) is then added and the yellow product (*p*-nitrophenol) is monitored at 405 nm. Small vein sections (10×12 mm) were fixed in glacial ($-4\,^{\circ}C$) acetone (HPLC-grade, Sigma, U.S.A.), according to AMeX method and then embedded in paraffin.[15] Immunohistochemical staining was performed using avidin-biotin complex based on Vectastain Kits (Vector Laboratories, Burlinghame, U.S.A.) with the use of diaminobenzidine (DABA, Sigma, U.S.A.) as a substrate. Anti-xanthine oxidase serum, collected by immunising New Zealand white rabbit with purified human xanthine oxidase protein was used to detect the localisation of the studied enzyme.

Statistical analysis

All values are reported as median and 25–75% interquartil range (IQR), in text and figures. Results were analysed using Mann–Whitney *U*-test. Statistical significance was set at $p < 0.05$.

Results

The content of TBARS in control veins amounted to 25.9 (22.3–30.2) nmol/mg protein, in varicose veins – 34.9 (28.7–43.1) nmol/mg protein, and in unchanged ones – 35.7 (28–40), whereas it was significantly higher in varicose veins with thrombophlebitis – 45.1 (32.4–62.1) nmol/mg protein, $p = 0.009$. The values in varicose and unchanged veins did not significantly differ, but they were greater than in control ($p = 0.042$ and $p = 0.034$ respectively) (Fig. 1).

The highest content of myeloperoxidase was observed in varicose vein – 48.2 (34.8–58.2) ng/mg protein, $p = 0.011$, and was lower in segments with superficial thrombophlebitis – 34.5 (25.1–47.8) ng/mg protein. The content diminished in unchanged saphenous vein wall without varicosities – 30.9 (25.8–37.3) ng/mg protein, approaching the values of healthy vein in control group – 29.1 (23.9–33.2) ng/mg protein (Fig. 2).

Expression of xanthine oxidase is presented on Figure 3. The enzyme was seen in the endothelium

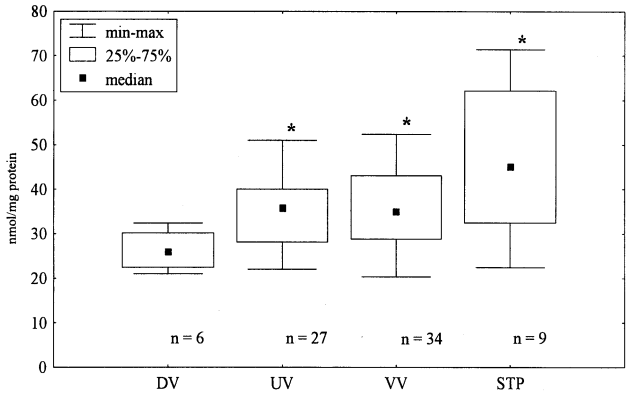

Fig. 1. Content of TBARS. Data are presented as median and 25–75% IQR. * $p < 0.05$ comparing to control. n: number of specimens; DV: donor vein (control); UV: unchanged vein; VV: varicose vein; STP: varicose vein complicated with superficial thrombophlebitis.

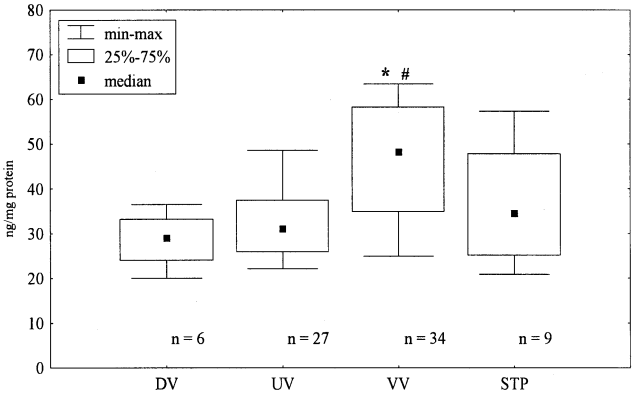

Fig. 2. Content of myeloperoxidase. Data are presented as median and 25–75% IQR. * $p < 0.05$ comparing to control, # $p < 0.05$ comparing to unchanged vein (UV). n: number of specimens.

of unchanged saphenous vein as single granules (a). Similar, weak staining was observed in healthy control vein. A positive reaction was seen in the endothelium of varicose veins (b). Specimens of varices with thrombophlebitis revealed strong, intense staining in luminal endothelium as well as in the *vasa vasorum* (c).

Discussion

The study set out to check whether an increased free radical generation took place in varicose veins, and to identify its sources. Varicose saphenous veins were compared to undilated segments of those veins and to healthy saphenous veins obtained during cadaver organ procurement. Healthy tissues of organ donors are used as a control material in an increasing number of studies. Isolated brain damage, leading to death in a short time, does not affect the function of other organs significantly. This allows to use them for transplantation, and for scientific purposes, including analysis of blood vessels.[16] Saphenous veins used in the present study were assessed microscopically by a pathologist before inclusion to the control group.

Our results show that oxidative–antioxidative balance is altered in varicose vein wall, especially after superficial thrombophlebitis. Superficial thrombophlebitis occurs as a complication of varicose veins.[17] Its incidence is rather bound with individual susceptibility than progression of disease.[18] Accompanying inflammatory state with leukocyte infiltration and release of aggressive proteolytic enzymes leads to degradation of the connective tissue.

Vascular remodelling may contribute to the development of varicose veins. Some authors have described changes in collagen content and in muscle mass.[19] It has been suggested that the transformation of the extracellular matrix, disruption of elastin fibers and hypertrophy of smooth muscle cells leads to weakness of the venous wall.[20] Stereometric analysis of varicose vein has shown the thickening of adventitia which is rich in *vasa vasorum*, fibroblast and inflammatory cells.[21] Certain cells (leukocytes, platelets, fibroblasts) possess a specific proteolytic activity. The role of proteolytic enzymes in connective tissue remodelling in the pathogenesis of varicose vein has been widely studied. Destruction can also be caused by reactive oxygen metabolites – ROMs, also known as free radicals.[22]

ROMs cause lipid peroxidation, oxidative modification of proteins and carbohydrates, leading to damage to lipid membranes and organelles, denaturation of enzymatic and structural proteins and of polysaccharide components of the interstitium. All of these processes are involved in vascular pathology.[23]

This study indicates that varicose veins and especially varices with superficial thrombophlebitis produce significantly more TBARS (lipid peroxidation products) than segments of normal veins. This implies that intensification of lipid peroxidation occurs under the influence of ROMs. Free radicals activate blood platelets and influence components of haemostasis (e.g. they decrease antithrombin III activity).[9] This may be of significance in the thrombotic complications of varicose veins, such as superficial thrombophlebitis. An increased content of lipid peroxidation products, occurring in unchanged segments of patients with varices (unchanged vein (UV) group, $p = 0.034$), suggests its low effectiveness as a graft for arterial reconstruction.

Free radicals can be generated in many conditions. Leukocytes and xanthine oxidase are sources

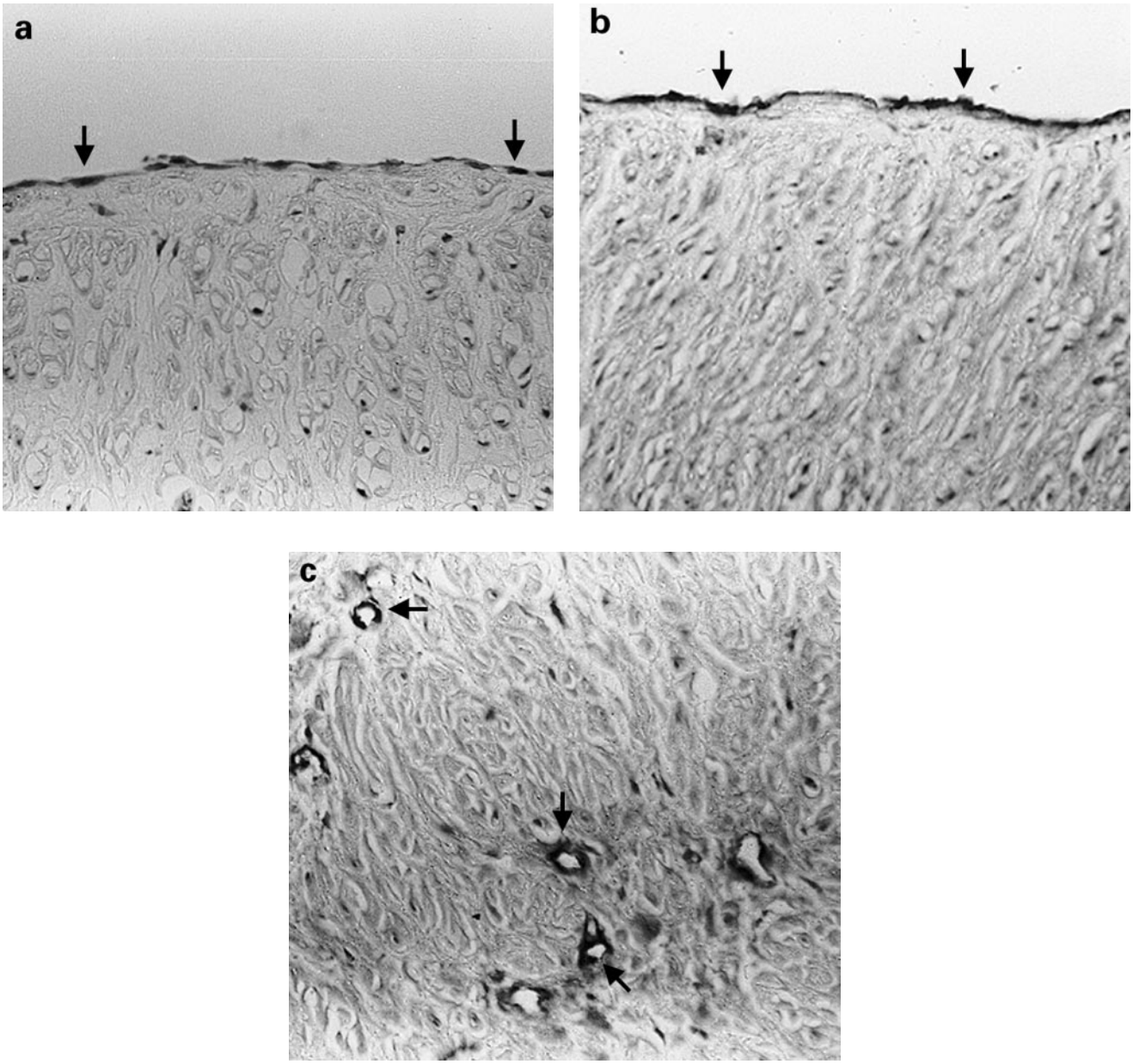

Fig. 3. Expression of xanthine oxidase in the normal vein (a), varicose veins (b) and varicose veins complicated with superficial thrombophlebitis (c). Positive reaction is shown in black colour, and indicated with an arrow (↑). Single black-stained endothelial cells can be seen in the normal vein, they form an evident black layer in the luminal surface of the varicose veins as well as black rings of *vasa vasorum* in adventitia of varicose veins with thrombophlebitis.

commonly studied in the circulatory system. Activated neutrophils increase their oxygen metabolism several fold, this process is called "oxygen burst".[24] Vast amounts of reactive oxygen metabolites are released. This is accompanied by an excessive production of proteolytic enzymes, as well as leukotrienes, prostaglandins, and other inflammatory mediators. Increased myeloperoxidase content confirms leukocyte activation in varicose veins and suggests their potential role in free radical generation at the stage of varicose vein formation.

We postulate that endothelial and the whole venous wall damage is a consequence of activated neutrophils releasing both free radicals and proteolytic enzymes. Free radicals cause degradation of structural proteins (collagen and elastin). Lipid peroxidation results in cell membrane damage, which releases additional proteases.[25] Both these factors

(proteolytic enzymes and free radicals) act synergetically to damage venous wall.

Xanthine oxidase (XO) is another important source of reactive oxygen metabolites. Endothelial cells containing this enzyme are capable of free radical generation.[4] Additionally, interaction of activated neutrophils with endothelial cells causes conversion of xanthine dehydrogenase to oxidase.[11] Our immunohistochemical study showed an increased content of the XO in the varicose vein wall, particularly in segments with superficial thrombophlebitis. XO expression is distinct in both luminal endothelium and the *vasa vasorum*. This implies that hypoxia related mechanisms activate the endothelium in varices with superficial thrombophlebitis. Hypoxia induced xanthine oxidase becomes a principal source of free radicals.

We have shown previously that antioxidative potential is decreased in varicose vein. The activity of a superoxide dismutase – the key enzyme scavenging superoxide radical arising during the action of xanthine oxidase or activated neutrophils is low in varicose veins.[26] High content of lipid peroxidation products in varicose veins is a results of both increased free radical generation and insufficient antioxidant protection.

The study has shown a disturbed prooxidative–antioxidative balance not only in large varices, but also in the undilated saphenous vein, coming from patient with venous insufficiency. The present paper suggests, that disturbed vein metabolism (leading to the extracellular matrix remodelling of the vessel wall) is a first step in the pathogenesis of varicose veins, and the vein dilatation with haemodynamic consequences – the next. Oxygen derived free radicals are important mediator of the vein wall damage during varicose veins formation. They also augment degradation of collagen and glycosaminoglycans thus contributing to the progress of disease.[27] Alterations in the connective tissue even before valvular incompetence have been shown in the study on proteolytic activity of saphenous vein.[28]

Since free radicals are involved in the pathogenesis of the varicose veins, antioxidants and free radical scavengers may have a beneficial effect. The levels of superoxide anion and lipid peroxides can be decreased by administration of the small molecule antioxidant vitamin C and E.[29] Vitamin C is of special importance because apart from antioxidant properties it is essential for collagen synthesis.[30]

Some of the flavonoids can effectively protect cells and tissues against the deleterious effects of reactive oxygen species.[31] Their antioxidant activity results from scavenging of free radicals and other oxidising intermediates.[32] They can also prevent the activation cascade induced by hypoxia, thus inhibiting the infiltration of neutrophils and subsequent free radical generation.[33]

Antioxidant vitamins and flavonoids inhibit destroying effects of free radicals, so they may reduce degradation of the vein wall and progress of varices. Such pharmacotherapy should be recommended particularly to patient with small varices, exposed to prolonged standing position and with positive family history.

Conclusions

Varicose veins, and especially those complicated with superficial thrombophlebitis revealed increased free radical generation. Its sources are neutrophils, and in complicated varicose veins – additionally xanthine oxidase. The results of the study suggest the primary role of the venous wall damage in the varices development, whereas vein dilatation, valves insufficiency and hypertension are its consequences. Proved contribution of free radicals to the pathogenesis of varicose veins implies that administration of safe antioxidants (vitamin C and E, flavonoids) may have a benefit outcome.

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