Endothelin Receptors in the Aetiology and Pathophysiology of Varicose Veins


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Introduction: Varicose veins are tortuous and poorly contractile. Their aetiology remains unclear. Neovascularisation has been suggested as a possible explanation. Endothelins are mitogenic, promoting proliferation and migration of endothelial cells via endothelin-B receptors. We hypothesise that endothelial cells and endothelin receptor density and distribution may play a role in the development of varicosis.

Methods: Saphenous vein segments from nine patients with varicose veins were compared to six controls. Slide-mounted sections were incubated in radioactive labelled endothelin-1 and receptor subtype-selective ligands and binding sites assessed using autoradiography. Endothelin-1 and endothelial cells were identified by immunohistochemistry and CD31-positive staining cells counted.

Results: Radioactive labelled endothelin-1 and endothelin-B receptor binding was reduced in varicose compared to control veins \( (p = 0.04) \). Endothelin-A receptor binding was diffuse, with no difference in density in both groups \( (p = 0.58) \). Endothelin-B receptor binding was diffuse with superimposed clusters. Although the density of medial endothelin-B receptor binding was reduced in the varicose group, more clusters were identified in this group compared to controls \( (p = 0.005) \). CD-31 staining identified these clusters as endothelial cells.

Conclusion: The reduced endothelin-1 binding and endothelin-B receptor density may be partially responsible for the reduced vasocontractility in varicose veins. We speculate that the increase in endothelin-B receptor binding CD31-positive endothelial cells in varicose veins may potentially stimulate mitogenesis and migration, leading to new vessel formation.

Key Words: Endothelin; Varicose veins.

Introduction

Varicose veins are the commonest feature of chronic venous disease affecting 10–45% of the adult population in Western societies. The primary cause of venodilatation, poor contractility and recurrence following treatment of varicose veins remains unclear. The aetiology, diagnosis and treatment are often explained on the basis of the existence of valvular insufficiency.

The primary aetiology of lower limb varicosity may however lie in the inherent venous wall weakness as shown by smooth muscle malfunction and abnormalities of the connective tissue collagen and elastin structure and content. Smooth muscle maintenance of venous tone is implemented in part by the local effect of endogenous vasoconstrictor agents, of which endothelin (ET-1) is the most potent. This endothelium derived 21-amino acid peptide is also a potent smooth muscle mitogen and stimulus for endothelial cell migration, proliferation and angiogenesis. It is produced and released from the endothelium in response to several factors including increased mechanical stress and the presence of thrombin, both of which are present in venous hypertension and varicose veins. Systemic levels of ET-1 and ET-1 receptor affinity have been reported as unchanged in patients with varicose veins. We hypothesise that the density, distribution and function of these endothelin receptors may contribute to the pathogenesis of varicose veins.

In addition, studies on recurrent varicose veins, which occur in 20–30% of cases following surgery, have suggested a role for neovascularisation in the aetiology of varicose veins. Venous tissue blocks excised from 28 legs undergoing groin re-exploration for recurrence showed evidence of neovascularisation in 27 groins with the latter as the sole cause of recurrence in 19 (68%) groins. A trial of long saphenous stripping in 100 patients (133 legs) reported neovascularisation (serpentine tributaries arising from the ligated saphenofemoral junction) as the commonest
cause of recurrence occurring in 52% of limbs. The stimuli for these structural and functional defects are unclear. Although these studies are limited to recurrent veins we speculate that the same process may be occurring to some degree in primary varicose veins.

This present study compared the density of ET-1 receptor binding and the distribution of ET<sub>A</sub> and ET<sub>B</sub> receptor subtypes in varicose veins with those in non-varicose veins using a combination of in-vitro autoradiography and immunohistochemistry.

Material and Methods

Patients

Venous tissue from nine patients (five males and four females, aged 54 ± 5 years) undergoing primary varicose vein surgery was obtained. Clinically three patients had venous disease with skin damage (severe, C4), two had varicose veins with venous oedema (moderate, C3) and four had gross varicosity with neither oedema nor skin damage (mild, C2). Although duplex ultrasound confirmation of long saphenous incompetence was obtained in the patients, selection was based on the clinical evidence of overt varicose veins. Tissue from six patients (four males and two females, aged 58 ± 4 years) with no evidence of venous disease and undergoing coronary artery bypass grafting using autologous saphenous vein was obtained as controls. Patients were informed of the procedure and consent obtained.

Tissue collection

The proximal 2 cm of the long saphenous vein was taken after ligation and division at the saphenofemoral junction. Utmost care was taken in the handling of the vein and crushed ends at ligation excised with a sharp blade. Samples were snap frozen in isopentane/liquid nitrogen and stored at −80 °C.

In-vitro autoradiography

Endothelin receptors were identified as previously described. Briefly, slide-mounted vein sections were pre-incubated in 50 mmol/L tris HCl buffer, pH 7.4, for 15 min at 22 °C in order to reduce endogenous peptide levels. Binding was determined by incubating slide-mounted sections in buffer containing radioligand alone (100–150 pmol/L [<sup>125</sup>I]-ET-1, specific activity 2000 Ci/mmol/L, Amersham Pharmacia Biotech, Buckinghamshire, U.K.). ET<sub>A</sub> binding sites were identified using [<sup>125</sup>I]-PD 151242 and ET<sub>B</sub> sites using [<sup>125</sup>I]-BQ 3020 (100–150 pmol/L, specific activity of both compounds 2000 Ci/mmol/L, Amersham Pharmacia Biotech, U.K.).

Non-specific binding for each radioligand was established by incubating alternate slides in the presence of 1 μmol/L unlabelled ET-1 (Bachem Fine Chemicals, Basel, Switzerland).

Film autoradiographs were produced by co-exposing radioligand incubated tissue sections with <sup>125</sup>I microscales to Hyperfilm-<sup>3</sup>H (Amersham Pharmacia Biotech) in X-ray cassettes for up to 4 days at 4 °C. Films were then processed according to the manufacturer’s instructions. Binding sites were localised at the cellular level by dipping incubated sections in molten nuclear emulsion (K2 emulsion, Ilford, Moberley, Cheshire, U.K.) and exposing for up to 8 days in lightproof boxes at 4 °C. Emulsion was then processed and tissue stained with Mayer’s haematoxylin and eosin for histology.

Quantification of binding

Receptor binding was assessed and quantified by densitometry using a GS 700 Imager and Densitometer (BioRAD Laboratories, Hercules, California, U.S.A.). Binding was calculated based on standard curves generated by <sup>125</sup>I microscales. Specific receptor binding was calculated by subtracting non-specific from total binding.

Endothelial cells were identified by immunohistochemistry using Vector’s avidin-biotin complex method (ABC Vector Laboratories, Petersborough, U.K.). Details of the protocol are as previously described. Platelet endothelial adhesion molecule-1 (PECAM, CD31) antibody for endothelial cells (1:1000 dilution, DAKO Ltd, High Wycombe, Buckinghamshire, U.K.) was used to identify the endothelial cells within the vein sections. ET-1 immunoreactivity was also identified in a limited number of control and varicose vein sections using an anti-ET-1 monoclonal antibody (Peninsula Labs, CA, U.S.A., diluted 1:500).

Cell counts

Microscopic images of vein sections were transferred onto a monitor via a CCD camera attachment on an
Olympus BX50 microscope. ET_α clusters and CD31 positive endothelial cells/sites were counted using a 500 × 500 μm grid at ×100-magnification and standardised to counts per section.

Results were analysed using Microsoft Excel 97 (Microsoft Inc., Washington DC, U.S.A.) and Graphpad Prism for Windows statistical packages. Densitometry was performed on four sections per subject expressed as mean (confidence intervals) in the text. The final reading for each patient was the mean total ET-1 binding minus non-specific ET-1 binding for four adjacent vein sections. The results obtained were then compared using Student’s unpaired t-test. A p-value <0.05 was considered significant. The CD31 and CD31 ET_α counts were per section and compared using Mann–Whitney U-test.

**Results**

There was demonstrable ET-1 binding which was associated with both ET_α and ET_β receptors in all the sections studied. Macroscopically, all three radioligands bound to the smooth muscle of the venous tunica media. Non-specific binding, in the presence of a saturating concentration of unlabelled ET-1 (1 μmol/L) accounted for 0–15% of total binding (specific binding therefore 85–100% total binding).

Results of ET-1 binding and ET_α and ET_β receptor distribution are depicted in Figure 1. Macroscopic evaluation of the low-resolution autoradiographs was suggestive of a lower density of radioligand binding in the varicose vein group compared to controls. Results of densitometric evaluation of receptor binding confirming a reduced binding in varicose veins are shown in Figure 2. ET_α receptor binding was diffusely distributed across the media while ET_β receptors were distributed diffusely but with obvious clusters of binding also located within the media. The mean ET_α binding was significantly higher than ET_β receptor binding in both varicose and control groups (p<0.01 respectively). There was however, no difference in ET_α receptor density between the varicose and control groups (p = 0.58; Fig. 2). Mean ET_β receptor density was lower in the varicose vein group compared to the control group (p = 0.04; Figs 1 and 2).

There was a significant increase (p = 0.005; Mann–Whitney) in ET_β clusters in varicose veins (30 (5–56)) compared to controls (3 (0–6)). CD31-positive staining identified the clusters as endothelial cells. There was also a significant increase (p <0.001; Mann–Whitney) in the mean number of CD31 positive cells/sites in the varicose veins per section (537 (441–634)) compared to controls (167 (117–217)). The ratio of CD31 staining endothelial cells to ET_β clusters in the varicose vein sections was 0.06 whereas this ratio in the control

![Figure 1](image_url)
Receptor binding and density
ET binding (dpm × 10³/mm² activity)

Fig. 2. Low-resolution densitometric analysis comparing ET-1 binding and ETA/ETB receptor densities in varicose (VV) versus control (C) saphenous vein sections.

veins was 0.02. These results indicate that the varicose veins had a higher proportion of ET₄ binding endothelial cells in the media than control veins.

Immunohistochemistry further demonstrated that ET-1 was associated with the endothelial cells lining the lumen of control (not shown) and varicose veins (Fig. 5), whereas the endothelial cells in the media of the varicose veins did not exhibit ET-1 immunoreactivity. Conversely, medial endothelial cells showed ET₄ binding while luminal endothelial cells did not (Figs 3 and 4).

**Discussion**

Varicose veins are distended tortuous and poorly contractile veins of uncertain aetiology. They have a familial tendency and are prone to recurrence even after meticulous surgery. Varicose veins exhibit diminished contractile responses to potent vasoactive agents – norepinephrine, angiotensin and endothelin. Of these vasoactive agents, endothelin is the most potent. This discussion is based on the two main findings in our study. Firstly, the diminished medial endothelin binding and receptor density in varicose veins, which may in part account for the poor contractile response in these vessels. Secondly, the marked increase in ET₄ receptor binding to endothelial cells in the varicose vein wall, which we speculate, may mediate neovascularisation as a potential cause of varicose veins.

The maintenance of venous tone is dependent on the action of the sympathetic nervous system and vasoactive agents such as noradrenaline, angiotensin and ET-1. ET-1 is a 21 amino acid peptide with a short plasma half-life. Since ET-1 was first isolated from porcine endothelial cells in 1988, it has been implicated in a host of cardiovascular diseases and pathologies involving altered smooth muscle contractility in tubular organs. Its action is likely to be at local tissue level rather than systemic. ET-1 induced vasocontractility is mediated via both ETA and ETB receptor subtypes. The presence of both receptor subtypes has been demonstrated in human long saphenous vein.

Further studies have shown similar plasma levels of ET-1 and
**Endothelin Receptors**

Fig. 4. ET\_A receptor distribution on sections of varicose vein. (a): Section used for the identification of endothelial cells (positive CD31 staining shown by brown reaction product). (b): High resolution autoradiograph of ET\_A receptors ([\(^{125}\)I]-BQ3020 binding) on an adjacent section of varicose vein (haematoxylin and eosin stained, binding shown as dark grain accumulations). (c): Dark-field illumination of (b), where binding sites shown as white grains on a dark background. These clusters of ET\_A binding sites are associated with a proportion of endothelial cells within the media of the varicose vein but not with the lumenal endothelium. Scale bar = 50 \(\mu\)m.

Fig. 5. Endothelin-1 immunoreactivity on varicose vein. (a): Positive ET-1 immunoreactivity (brown stain) associated with lumenal endothelium, with some staining also of medial smooth muscle. (b): CD31 immunostaining of an adjacent section identifying the lumenal endothelium and "migrating" cells within the media. ET-1 immunostaining is restricted to the lumenal endothelium. Scale bar = 50 \(\mu\)m.

Similar ET receptor affinities in varicose and non-varicose veins. Our study demonstrated a reduced density of ET-1 binding and ET\_B receptor subtypes in varicose veins and suggests a fundamental local reduction in receptor availability (Fig. 2). These findings are in keeping with similar studies on pulverised veins. Whether the reduced contractility is due to desensitisation, internalisation, degenerative or genetically determined lowering in absolute receptor numbers is unclear. Although our study was limited to leg veins, Blochl-Daum *et al.* have reported defective contractile responses to vasoactive agents in the hand veins of patients with varicose veins compared to controls without varicose veins. Decreased binding and receptor availability may partly account for the impaired tone and contractility in varicose veins. Whether this is the primary insult or the result of disease progression is unclear at this stage.

Varicose veins have been shown to have a higher blood flow rate and increased blood oxygenation than non-varicose veins. These observations have been associated with the often quoted but rather debatable arteriovenous fistulae theory in the aetiology of varicose veins. Experimental arteriovenous fistulae in canine veins have been shown to be associated with a modulation of contractility. While the increased shear associated with increased flow upregulated ET receptors in arteries, the reverse was the case in A-V fistulated canine veins with receptor down regulation and a reduced maximal contractility response to ET-1. Although these studies are yet to be confirmed in humans, we speculate that the chronic exposure of varicose veins to high flow, stress or oxygenation may be contributory to the desensitisation or reduction in ET receptors as shown by our study.

In addition to its effect on vascular tone, ET-1 has been shown to have mitogenic and angiogenic properties. ET-1 is produced by endothelial cells. Growth and migration of endothelial cells control the vascular remodelling that is necessary for healing and is associated with various vascular diseases. Morbidelli *et al.* have shown that ET-1 stimulates endothelial cell

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growth and mobilisation, thus contributing to neo-
vascularisation. 36 This autocrine effect is mediated via 
ETB receptors. Our study showed a reduction in ETB 
receptor binding to the tunica media in the varicose 
vein group, whereas there was discrete clustering of 
ETB receptors in this region (Figs 3 and 4). Initially, it 
was thought that these clusters might represent bind-
ing to vasa vasorum as previously reported in coronary 
arteries 36 leading to the possibility of altered blood 
flow to the vascular smooth muscle, impairing its 
function and overall vessel contractility. Immuno-
histochemistry, however, revealed that these clusters 
were collections of endothelial cells within the media. 
Interestingly, the immunohistochemical studies 
showed that those endothelial cells that exhibited ETB 
binding failed to exhibit ET-1 immunoreactivity, unlike 
the luminal endothelial cells, which exhibited positive 
ET-1 immunostaining. Both groups of endothelial cells 
may well represent different phenotypes. We found 
higher numbers of ETB receptor bearing endothelial 
cells in the media of the varicose vein sections com-
pared to control vessels. The proliferation and mi-
gration of these cells promoted by ET-1 via ETB 
receptors may play an important role in vessel wall 
remodelling and neovascularisation associated with 
varicose veins.

In putting forward these findings, the authors re-
recognise that there are limitations to our study. We 
have concentrated on a relatively small sample size 
totalling 15 patients. Our methodology however, in-
volved taking several tissue sections from each of the 
patients and using the mean readings for the final 
analysis. The borderline statistical significance in ET-
1 binding and ETB receptor densities (p = 0.04) may 
be due to the relatively small sample size in our study. 
The “mismatch” of CD31 staining/ETB binding is 
mainly due to the fact that not all endothelial cells 
appear to possess ETB receptors. However, it is clear 
that exact “matching” of CD31 staining on serial 10 μm 
sections would not occur due to alterations in the 
pattern of “migrating” endothelial cells along the seg-
ments of vein used.

In summary, we have explored a potential role for 
ET receptors in the pathophysiology of varicose veins 
using autoradiographic and immunohistochemical 
techniques. The reduction in medial ET-1 binding and 
density of the ETB receptor subtype may contribute 
to the decreased contractile response recognised in 
varicose veins. At this stage, we cannot be certain 
whether the altered ETB binding identified in varicose 
veins is associated with the primary cause or the effect 
of previously described venous wall remodelling in 
varicose veins. The mitogenic and angiogenic potential 
of the clusters of ETB receptors on endothelial cells in 
varicose veins may enhance neovascularisation as a 
potential cause of primary and recurrent varicose 
veins.

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