Venous Morphology Predicts Class of Chronic Venous Insufficiency

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Objectives: this study aimed to determine specific morphological differences in long saphenous veins from patients with various grades of chronic venous insufficiency.

Design: comparable veins from a control group were compared with patients with either primary varicose veins or those with associated skin changes including venous ulcers.

Materials: below-knee segments of saphenous vein were examined from a total of 64 patients.

Methods: veins were examined for elastic-tissue disruption and endothelial-cell changes and comparisons made between clinical groups.

Results: elastic-tissue disruption, as measured by fragmentation of the elastic lamina and the percentage of the intimal-medial boundary containing elastin, increased with increasing severity of venous disease. Moreover, endothelial cells became more densely packed, as measured by endothelial cell and endothelial-cell nuclei density, with increasing severity of disease. Other measures such as the density of multinucleated “giant” endothelial cells and the number of nuclei per “giant” cell did not correlate with venous disease, however.

Conclusions: this study demonstrates that several morphological characteristics of superficial saphenous veins correlate with severity of venous disease. In particular, the alterations to the structure of elastic tissue within these veins appears indicative of the progressive nature of chronic venous insufficiency.

Key Words: Chronic venous insufficiency; Elastic tissue; Endothelium; Human; Venous ulcers; Varicose veins.

Introduction
The importance of valvular incompetence, venous reflux and high ambulatory venous pressure in the pathogenesis of venous ulceration has been well documented.1 Why only a proportion of patients with significant reflux go on to develop severe disease is not clear, and why similar findings on duplex examination are associated with varying levels of physiological dysfunction2 has not been well explained.

Flow-loading of veins, due to experimental arteriovenous fistulae, results in elastic-tissue failure, gross dilatation, tortuosity, valve failure, mural atrophy, intimal thickening3-6 and increased endothelial numbers (cell density) associated with morphological alterations.6 Veins associated with therapeutic arteriovenous fistulae in man have been shown to develop phlebsclerosis, increased endothelial-cell densities and degeneration of elastic elements compared to control veins.7

If venous reflux acts on the vein wall in a similar fashion to arteriovenous flow-loading, then similar elastic tissue and endothelial-cell alterations may be expected in patients with chronic venous insufficiency (CVI). It is therefore of interest as to whether the severity of these histological changes correlates with the relative severity of venous disease in this patient group.

This study aims to investigate the changes to superficial vein wall associated with venous reflux and the development of various stages of chronic venous insufficiency (due to superficial vein incompetence alone). Particular emphasis will be placed on alterations to the elastic tissues and endothelial cells.

Methods

Patient selection and tissue sampling

Samples of long saphenous varicose veins from below the knee (Table 1) were obtained from patients undergoing superficial vein surgery as part of their routine clinical management. Control material consisted of below-knee segments of long saphenous vein made superfluous after coronary-artery-bypass grafting.
Table 1. Below-knee saphenous vein specimens examined.

<table>
<thead>
<tr>
<th>CVI class</th>
<th>Patients (bilateral)</th>
<th>Limbs</th>
<th>Elastic tissue preparation</th>
<th>Endothelial-cell preparation</th>
<th>Both preparation</th>
<th>Mean age (±1 s.d.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>23 (1)</td>
<td>24</td>
<td>21</td>
<td>12</td>
<td>9</td>
<td>65 ± 10</td>
</tr>
<tr>
<td>2</td>
<td>18 (4)</td>
<td>22</td>
<td>16</td>
<td>13</td>
<td>7</td>
<td>54 ± 21</td>
</tr>
<tr>
<td>4-6</td>
<td>23 (7)</td>
<td>30</td>
<td>24</td>
<td>12</td>
<td>6</td>
<td>61 ± 10</td>
</tr>
<tr>
<td>Totals</td>
<td>64 (12)</td>
<td>76</td>
<td>61</td>
<td>37</td>
<td>22</td>
<td>59 ± 15</td>
</tr>
</tbody>
</table>

Patients have been grouped by class of chronic venous insufficiency (CVI) using the CEAP reporting standards (*). No visible venous disease (0), primary varicose veins (2) and skin changes including venous ulcers (4-6). The number of specimens examined for elastic-tissue morphology (elastic-tissue preparation), endothelial-cell morphology (endothelial-cell preparation) or both techniques are indicated.

Two of the controls were long saphenous veins recovered from limbs following below-knee amputation (secondary to peripheral vascular disease). All limbs from which control veins were collected were examined visually so as to exclude those with varicose veins. In total, 76 limbs from 64 patients were sampled (Table 1). The dissection, removal and handling of all vein specimens was performed gently to minimise stretching and damage to the venous wall. No stripped products were examined, as this technique both removed the endothelium and damaged the underlying vein wall.

All specimens were assigned a clinical classification of chronic lower-extremity venous disease using the CEAP reporting standards. In brief, these were: no visible signs of venous disease (class 0), primary varicose veins (class 2), skin changes ascribed to venous disease (class 4), skin changes ascribed to venous disease with healed ulceration (class 5) or skin changes ascribed to venous disease with active ulceration (class 6). For the purposes of this study, specimens from classes 4 to 6 were grouped together for analysis. No patients with class 1 (reticular veins) or class 3 (oedema without skin changes) disease were examined in this study. All CVI patients examined in this study were confirmed as having venous reflux disease, as outlined within the CEAP reporting standards, by standard air plethysmography (APG) and duplex-ultrasound evaluation (see below). Though limbs from which control veins were collected were not examined by APG or ultrasound, a careful visual examination allowed for exclusion of subjects with varicose veins.

Elastic tissue preparations

Vein specimens were immersion-fixed in fresh 10% phosphate-buffered formalin for at least 48 hours, embedded in paraffin (orientated transversely) and sectioned (5 μm) using a microtome. Sections were stained with Verhoeff’s elastic-tissue stain and Curtis’ modified van Gieson counterstain.

Endothelial cell (Häutchen) preparations

Specimens were rinsed in phosphate-buffered saline (0.001 M, pH 7.2) (PBS), then had loose periadventitial connective tissue carefully removed. Vessels were opened longitudinally and pinned out flat (using stainless-steel entomological pins), luminal surface up, on polythene strips. Vessels were fixed, immediately after pinning out, in fresh 10% phosphate-buffered formalin for 24 hours, then washed in running water for 1 hour. After staining with filtered Gill’s haematoxylin for 5 minutes and fixing and blueing the haematoxylin in Scott’s tap water to stain endothelial-cell nuclei, the tissue was dehydrated with increasing grades of ethanol, then processed by the modified Häutchen technique, to produce a single layer of endothelial cells mounted on a glass slide.

Physiological measurements

As part of their clinical examination, all patients with CVI (classes 2 and 4-6) had a full physiological (APG and duplex ultrasound) evaluation prior to surgery. Measurements included: 2-second outflow ratio, venous filling time (VFT), residual fraction (RF, representing the fraction of the venous blood volume remaining in the limb after 10 toe-stands) and the venous filling index (VFI). The ratio of VFT/RF was also calculated as a putative ulceration index (ulcer index). Unfortunately, physiological measurements were not available from the class 0 patients examined in this study.

Histological examination and analysis

All specimens (elastic-tissue and endothelial-cell preparations) were blinded and examined by bright-field light microscopy as previously described. Microscope
images were captured onto a computer-image analysis system and displayed on a monitor at 340-times magnification. Measurements were made using the public domain NIH Image program, version 1.57 (written by Wayne Rasband at the U.S. National Institute of Health and available from the Internet by anonymous ftp from zippy.nimh.nih.gov).

In elastic-tissue-stained transverse sections eight captures of the intimal–medial border were taken at pre-designated points of reference. The degree of elastic-tissue failure was assessed by two measurements: (1) percentage of elastic tissue (%ET), representing the fraction of the total intimal–medial border which contained elastic tissue along it and (2) average fragment length (AFL in μm), the average length of the elastic-tissue fragments present.

In endothelial cell (Häutchen) preparations eight randomly selected regions were captured and analysed. The endothelium was assessed by four measures: (1) endothelial cells density, in cells per 10^4 μm^2, (2) endothelial nuclei density, in nuclei per 10^4 μm^2, (3) endothelial giant cell (2 or more nuclei) density, in giant cells per 10^4 μm^2 and (4) mean number of nuclei per giant cell.

Results were statistically analysed using the Mann–Whitney U-test and correlation coefficients compared using Fisher's r to z transformations. Results were considered significant with a p value <0.05.

Results

Varicose veins (class 2) and venous ulcer (classes 4–6) specimens were often grossly dilated, thin-walled and tortuous. Occasionally CVI (classes 2 and 4–6) specimens contained focal saccular varices, but generally dilatation was more uniform within a given segment of vein. Veins from bypass patients (class 0) appeared grossly to be of lesser total diameter than either of the CVI groups. During opening for en face preparations it was noted that class 0 veins contained intact valve cusps. In both class 2 and class 4–6 specimens valve cusps were usually absent; however, the location of valve sites was identifiable by the presence of a raised ridge on the luminal surface of the vessel.

Measurements of elastic-tissue components of the vein wall (Fig. 1) revealed significant differences between classes (Table 2). There was significantly less elastic tissue along the intimal–medial border (ET%) of veins from patients with skin changes and/or ulcerated legs (classes 4–6) compared with normal (class 0) and primary varicose veins (class 2) (Fig. 2). This trend was also evident in the average elastic-tissue-fragment length (AFL); however, there was a considerable range in class 2 measurements (Fig. 3).

En face Häutchen examination of the endothelial lining of specimens (Fig. 4) also produced significant differences between classes (Table 2). The class 0 veins had significantly lower mononucleated endothelial-cell densities (ECD) (Table 2) and endothelial nuclei densities (END) (Fig. 5). There were no differences between classes in either giant-endothelial-cell density (GCD) or the mean number of nuclei per giant cell (MGN) (Table 2).

Significant correlations were observed between several of the histological and physiological measurements (Table 3). There was no correlation between

<table>
<thead>
<tr>
<th></th>
<th>ET%</th>
<th>AFL</th>
<th>ECD</th>
<th>END</th>
<th>GCD</th>
<th>MGN</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>60.3±23.6 (21)</td>
<td>20.5±15.4 (21)</td>
<td>12.3±2.3 (12)</td>
<td>15.8±3.8 (12)</td>
<td>1.0±0.5 (12)</td>
<td>3.1±1.0 (12)</td>
</tr>
<tr>
<td>2</td>
<td>51.3±20.2 (16)</td>
<td>41.4±36.8 (16)</td>
<td>19.6±4.9 (13)</td>
<td>23.9±5.7 (13)</td>
<td>0.9±0.6 (13)</td>
<td>4.1±1.8 (13)</td>
</tr>
<tr>
<td>4–6</td>
<td>21.0±15.6 (24)</td>
<td>12.9±12.7 (24)</td>
<td>20.5±7.7 (12)</td>
<td>25.5±4.8 (12)</td>
<td>1.0±0.5 (12)</td>
<td>3.8±1.9 (12)</td>
</tr>
</tbody>
</table>

Means (±1 standard deviation) for the 6 measurements made divided into CVI classes. Significant statistical differences were found between class 4–6 compared with classes 0 and 2 (*p<0.005) and between class 0 compared with classes 1 and 4–6 (**p<0.0005). Two measurements showed no significant differences between groups (N.S.D.).

Fig. 1. Histological section of long-saphenous-vein wall from a Class 2 CVI patient (primary varicose veins). It was by measuring the elastic tissue along the intimal–medial border region (arrows) that the elastic-tissue percentage (ET%) and the average fragment length (AFL) were made. Notice the intimal thickening (X) commonly observed in many vein specimens, including controls. Verhoeff’s elastic-tissue stain and von Gieson counterstain. Magnification ×185.
any of the endothelial-cell measures and the elastic-tissue measures, however. The mean number of nuclei within giant endothelial cells (MGN) significantly correlated with the residual fraction; however, this was the only correlation observed between endothelial cell and physiological measures. Interestingly, though age correlated with the venous filling time, the ulcer index and endothelial-cell densities, there was no correlation between age and elastic-tissue measures. Both measures of elastic-tissue disruption (AFL and ET%) had significant correlations with the ulcer index value for CVI patients. There was a highly significant correlation between AFL and ET% measures.

**Discussion**

In this study aspects of morphological alteration of veins subjected to venous reflux have been investigated. When deciding which features of vein morphology should be examined, several factors are worth...
Table 3. Significant correlations between measures of elastic tissue, endothelial cells, venous physiology and age.

<table>
<thead>
<tr>
<th>Measures pair (n)</th>
<th>Correlation</th>
<th>p-value</th>
</tr>
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<tbody>
<tr>
<td>ET%/AFL (61)</td>
<td>0.522</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>AFL/VFT (31)</td>
<td>0.447</td>
<td>0.011</td>
</tr>
<tr>
<td>ECD/Age (25)</td>
<td>−0.462</td>
<td>0.02</td>
</tr>
<tr>
<td>END/Age (25)</td>
<td>−0.395</td>
<td>0.05</td>
</tr>
<tr>
<td>VFT/Age (31)</td>
<td>−0.591</td>
<td>0.0003</td>
</tr>
<tr>
<td>Ulcer index/Age (31)</td>
<td>−0.611</td>
<td>0.0002</td>
</tr>
<tr>
<td>MGN/RF (11)</td>
<td>0.872</td>
<td>0.0002</td>
</tr>
<tr>
<td>ET%/Ulcer index (31)</td>
<td>0.355</td>
<td>0.049</td>
</tr>
<tr>
<td>AFL/Ulcer index (31)</td>
<td>0.405</td>
<td>0.023</td>
</tr>
</tbody>
</table>

Elastic-tissue percentage (ET%), average elastic-fragment length (AFL), endothelial-cell density (ECD), endothelial-cell-nuclei density (END), mean number of nuclei per giant cell (MGN), venous filling time (VFT), residual fraction (RF) and a putative ulceration index representing VFT/RF (ulcer index). p-values were calculated with Fisher’s r to z transformation.

noting. Firstly, the development of lipid-containing phlebosclerotic lesions within veins of the lower limb is rare in man, even in those with severe venous reflux.6 Secondly, fibromuscular alteration (primary intimal phlebosclerosis) is commonly recognised in both varicose and non-varicose veins of both sexes.10 Primary intimal phlebosclerosis also tends to be rather heterogeneous in thickness and may be influenced by external features such as proximity to arteries.10 Moreover, since these specimens could not be perfusion-fixed, it was deemed inappropriate to measure intimal and medial area. Because of these factors, it was decided to investigate elastic tissue at the intimal–medial boundary and endothelium-lining vessels. Elastic tissue has a relatively low turnover in vascular tissues, even once it has been disrupted or broken,11 and may therefore reflect the effect of cumulative disruption. Furthermore, reduction in the content of structural proteins such as elastin and collagen has been reported previously in varicose compared with normal veins.12 Endothelial-cell measures were investigated because, as these cells line the lumen of blood vessels, they may reflect any haemodynamic/mechanical disruption,13–15 such as may be caused by venous reflux, within the vessel.

A recent study examining therapeutic arterio-venous-fistulae veins from haemodialysis patients has shown that arterialismed veins have considerably more disrupted elastic tissue and increased endothelial-cell packing compared to normal veins.7 Incorporation of a vein into the arterial circuit results in alteration to the structure of the vessel,16 the extent of which appears to correlate with the magnitude of the local haemodynamic disturbance.17 Though the nature, in terms of pulse-wave form, and magnitude of the haemodynamic disturbance found in refluxing lower-limb veins is significantly different to that of arterialised veins, the trends in terms of elastic-tissue failure and endothelial-cell measures appear remarkably similar.7 Most studies of venous morphological change are described in varicose veins with no account of either the degree of reflux or the severity of clinical disease.17 This is pertinent in view of the fact that some patients with long-standing varicose veins progress to develop class 4 or greater insufficiency while others do not. Differences in vein morphology may help explain why eventual clinical outcomes can be so different. It is also conceivable, however, that differences in vein-wall morphology may simply reflect differences in the present grade of reflux within any given specimen.

Physiological measures of reflux such as the venous filling index18 and the ulcer index (VFT/RF)2 have been shown to correlate with the severity of chronic limb-venous disease. In this study we demonstrate that vein-wall alterations parallel the associated haemodynamic disturbance present in the refluxing limb. Though age-correlated with some physiological and endothelial-cell measures, the lack of correlation between age and elastic-tissue measures indicates that elastic-tissue alteration represents a pathophysiological change and is not simply a reflection of senescence.

By using the histological measurements of elastic tissue and endothelial-cell densities, it was possible to identify a stratification of specimens by disease severity. For example, normal veins from patients, of a similar age to other patient groups, could be identified by high measures of elastic tissue and low endothelial-cell densities. Primary varicose veins (class 2) had similar elastic measures but significantly higher endothelial-cell measures, while veins from limbs with skin changes (classes 4–6) had similar endothelial-cell measures to varicose veins but significantly lower elastic-tissue measures.

In conclusion, both elastic-tissue and endothelial-cell measurements may represent subtle indicators of the pathophysiological state of veins subjected to chronic venous insufficiency. Understanding the interaction of such alterations and their effects on the pathogenesis of CVI may eventually aid in determining which patients are most prone to progression of this disease.

Acknowledgements

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