THE MOLECULAR MECHANISMS UNDERLYING THE DEVELOPMENT OF PRIMARY VARICOSE VEINS

Cheuk Bong Tang BSc (Hons) MBBS MSc FRCS

The Academic Vascular Unit
Department of Surgery
The Royal Free and University College London Medical School

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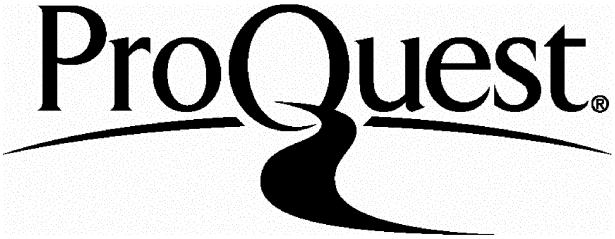

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Abstract

ABSTRACT

Of the theories regarding the aetiology of primary varicose veins (VVs), the 'Trendelenburg theory' is perhaps the most widely held, stating that VVs arise after failure of the sapheno-femoral valve (SFV). Although numerous additional factors are thought to contribute to VVs development, their true aetiology remains obscure.

It is proposed that a 'molecular' rather than 'physical' mechanism precipitates VVs development, as follows; venous stasis leads to localised venous hypoxia, stimulating the secretion of vascular endothelial growth factor (VEGF) and nitric oxide (NO) which act to increase permeability and dilatation. If the stimulus to dilate remains, but the vein is limited mechanically in the extent of dilatation it can undergo, the continued secretion of VEGF and NO may result in a sequence of molecular events leading ultimately, to a vein more prone to varicosity. Do, therefore, VVs reflect a defect in the balance between the production, release and response to these major agents mediating dilatation?

A clinical audit determined the incidence of primary VVs occurring in the presence of an intact SFV. Subsequently, a clinical trial examined the release of VEGF and NO (in patients with VVs, and in control subjects) in response to experimentally-induced venous stasis. Plasma was analysed for VEGF and NO, and peripheral blood mononuclear cells (PBMC's) for VEGF gene expression. Furthermore, to aid in the future investigation of these molecular events, an *in vitro* model of varicosity was developed.

Of all primary VVs assessed here, 43.5% co-presented with a competent SFV. The female:male ratio was 1.92:1, and there was no increase in incidence with age. Peak incidence in females was between 30-60 years, whilst in males this was later, being between 40-70 years. An increased incidence in females with an intact SFV was seen associated more with a 'pre-menopausal' state.

Baseline plasma VEGF was equivalent in VVs and controls. Induction of venous stasis stimulated an increase in plasma VEGF in controls ($P<0.025$), but not in VVs. Plasma NO was not affected by induced venous stasis but however, in all cases, was lower with VVs (all samples, $P<0.05$). In PBMC's from controls or VVs, baseline $VEGF_{165}$ and $VEGF_{121}$ gene expression were similar, and $VEGF_{165}$ was unaffected by induced venous stasis. In VVs however, there was a 91% increase in $VEGF_{121}$ gene expression ($P<0.08$), which was not seen in control subjects.

In explants of long saphenous vein in organ culture, a neo-intimal lesion developed in control ('normal') explants by day 7 ($P<0.006$), through the proliferation and migration of smooth muscle cells (SMC's). In VVs however, although the thickened intima seen at day 0 ($P<0.001$) remained largely similar, the thickness of the medial layer increased ($P<0.001$), suggesting SMC proliferation, but without migration.

In summary, the loss of release of VEGF, reduced plasma NO and changes in VEGF gene expression, combined with aberrant SMC function, may suggest a mechanism distinct from SFV failure and simple pressure for the aetiology of VVs.

Acknowledgements

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Statement of Originality

STATEMENT OF ORIGINALITY

The work reproduced in this thesis is the sole undertaking of Mr Cheuk Bong Tang, except for the histology and immunohistology procedures as outlined in Chapter 9. These were performed by the histology laboratory at Maidstone General Hospital by Ms Gill Howes under the supervision of Dr Steve Humphreys.

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Abbreviations

ABBREVIATIONS

ABC	Avidin:biotin complex
A-V	Arterial-venous
bFGF	basic Fibroblast Growth Factor
BM	Basement membrane
BMI	Body mass index
bp	Base pair
BSA	Bovine serum albumin
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
CVI	Chronic venous insufficiency
ECM	Extracellular matrix
EVG	Elastin Van Gieson
ELISA	Enzyme-linked immunosorbent assay
ET	Endothelin
FCS	Foetal calf serum
5'	Five-prime
H&E	Haematoxylin and Eosin
HIF-1	Hypoxia-inducible factor-1
HUVEC	Human umbilical vascular endothelial cells
LSV	Long saphenous vein
MMP	Matrix metalloproteases
NADPH	Nicotinamide adenine dinucleotide phosphate
NIH	Neointimal hyperplasia

NO	Nitric oxide
NOS	Nitric oxide synthase
cNOS	constitutive NOS
eNOS	endothelial NOS
iNOS	inducible NOS
nNOS	neuronal NOS
OD	Optical density
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PDGF	Platelet-derived growth factor
PKC	Protein kinase C
PlGF	Placental growth factor
PMN	Polymorphonuclear neutrophil
ROS	Reactive oxygen species
SFJ	Sapheno-femoral junction
SFV	Sapheno-femoral valve
SMC	Smooth muscle cell
SMA	Smooth muscle actin
SSV	Short saphenous vein
Taq	*Thermus aquaticus*
3'	Three-prime
TBE	Tris-borate
TBS	Tris-buffered saline
VEGF	Vascular Endothelial Growth Factor
VVs	Varicose veins

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INTRODUCTION

CHAPTER 1

Varicose Veins

CHAPTER 1

1.1 Definition of varicose veins

There are many different definitions as to what exactly constitutes a varicose vein (VVs). Arnoldi defined a VV as 'any dilated, elongated, tortuous vein, irrespective of size' (Arnoldi, 1957), whereas other definitions have tried to include an aetiological concept. The World Health Organization defines VVs as 'saccular dilatation of veins, which are often tortuous' (Prerovsky, 1964). Specifically, this excludes; dilatation of small, intradermal, subcutaneous veins (venectasis); tortuous, dilated veins that have formed secondary to previous thrombophlebitis, and any arterio-venous (A-V) fistulae. Further, VVs may be subdivided into dilated saphenous veins (trunk veins), dilated superficial tributaries (reticular veins) and dilated venules (hyphen-webs) (Widmer, 1978).

1.2 Clinical relevance of VVs

VVs are just one manifestation of the problems associated with chronic venous insufficiency (CVI). Venous system disease in the legs is a major problem affecting Western society. An early survey in the USA found that VVs were the seventh most common cause of medical referral, affecting between 30-60% of the adult population (US Department of Health, Education and Welfare, 1938). Not only are they unsightly, but they are a cause of considerable morbidity and suffering. An American survey by Wilder (1974), found that approximately 90% of people with VVs suffered occasional symptoms, while nearly 18% complained of frequent, or continuous symptoms. Aside from the cosmetic problems associated with varicosity, more serious symptoms include discomfort, which may range from some 'tenseness'

in the lower limbs (due perhaps, to dependent oedema), up to severe pain. These sensations are a result of several mechanisms such as nociceptor stimulation of the distended vein wall, accumulation of tissue metabolites, or an increase in interstitial pressure (Cockett, 1955). In addition, skin changes ranging from simple lower limb induration to frank and debilitating ulceration may occur, which can be extensive in nature. Venous ulcers are notoriously difficult to treat and provide the mainstay of attendees to vascular outpatient clinics, consuming both medical staff time and healthcare resources (Laing, 1992). More seriously, VVs are a known risk factor for the development of deep vein thrombosis, a potentially life-threatening condition. Therefore, VVs are responsible for a large part of any Western country's health budget. It is estimated that in the USA alone, up to $1.5 billion is spent per year treating the primary condition and its complications, while in the UK more than £450 million is spent.

1.3 Epidemiology of VVs

1.3.1 Problems encountered in correlating epidemiological surveys

There have been many epidemiological attempts to chart the prevalence of VVs in the general population. Unfortunately, they have all varied in the definition of the condition, the methodology and in the sample-population characteristics, giving rise to a varying assessment of VV prevalence with often, conflicting findings. Accurate figures are essential in order to plan for future health care needs and to aid debate over health care rationing. The problems encountered when assessing the results of differing surveys looking at the prevalence of VVs can be summarised as follows;

1.3.1(a) Epidemiological terms

Many differing epidemiological terms have been used in manifold studies. Terms such as incidence, prevalence, occurrence and frequency have all been cited. Incidence is the estimated number of patients in whom the condition has developed in a specified time period, whereas prevalence is the estimated number of patients in whom the condition exists at a specified time, or within a specified interval of time. More precise terms have included point prevalence, which is the number of patients with a condition at a single point in time and period prevalence, which is the number of patients with the condition over a period of time. Earlier studies have used less precise terms such as frequency and occurrence. This lack of consistency in terminology has hindered correlation of the various studies, so that elucidation of the actual number of affected individuals remains unclear.

1.3.1(b) Population sampling

Many studies have focussed on highly selected, or narrow, population groups, which do not represent adequately the general population. In addition, even within these unrepresentative samples, it has not been possible to assess everyone in the chosen group due to either lack of time, or unwillingness of the subjects to consent. It is important when interpreting the results of any one study to relate the findings to the sample group recorded.

1.3.1(c) Methodology

When designing any survey, there is always a compromise between accuracy, simplicity and size of sample. Questionnaires are favoured by surveyors as they are quick, simple and can attain large numbers of participants. However, as they are

either filled in by an interviewer, or by the subject themselves, they are open to gross error, being dependent on the subjectiveness of the interviewer, or limited by the understanding of the subject. More accurate methods of assessment are possible, but they may require a full physical examination, vascular assessment and/or photography by fully trained assessors. However, these more complex methods necessarily limit the population to be assessed, compared to the number that would be recruited by the use of a simple questionnaire.

1.3.1(d) Definition of venous disease

Unfortunately, the spectrum of venous disease has become broader, in part due to more effective methods of vascular assessment that can now identify deep and superficial vein incompetence. This has lead to the identification of subjects with no clinical signs of disease, but in whom there is measurable, venous, valvular incompetence. [Beneficially, it is in these subjects, with regular assessment, that both the true prevalence of the condition in the general population and the evolution of the condition in the individual may be assessed]. Initial problems have been encountered with clinical assessment of the subjects. VVs are more prominent when the subject is standing and the conditions in which the subjects have been examined have varied from study to study. Also, even when clinically obvious VVs are present, the manifold surveys have differed in their definition of VVs, ranging from 'any prominent superficial vein in the lower extremity' (Mekky *et al.*, 1969), to 'a vein which has permanently lost its valvular efficiency and as a result of continuous dilatation under pressure, in the course of time, becomes elongated, tortuous, pouched and thickened' (Dodd, 1971). Therefore, it is important to agree and stick rigidly to the definition to be used in the sample population. However, even when

the same definition has been used consistently in the same survey, it has been shown that there can be considerable intra-observer variation still, as to what constitutes VVs (Weddell, 1969).

1.3.2. A summary of the epidemiological data

(See Table 1.1 summarising the cited epidemiological studies). The first major epidemiological studies looking at VVs were carried out in the USA (US Department of Health, 1938). Between 1935 to 1936, a general health questionnaire was undertaken surveying 2.8 million households, the results of which were then extrapolated to the entire population. This found that 1.75 million of the population surveyed suffered from VVs and that it was the seventh most common medical condition. A further survey, the American National Health Survey (between 1959 and 1961), examining severe, chronic, disabling conditions, suggested a point prevalence of 2.25% for severe VVs (US Department of Health, Education and Welfare, 1962). Again in the USA, the Framingham study followed up middle-aged men and women (from the town of Framingham) over a 16 year period from 1948 to 1966, and revealed an annual incidence of VVs in women of 2.6%, while in men it was 1.9% (Brand *et al.*, 1988).

Since then, many local, regional and national studies have been performed in various countries in Europe and the Far East, revealing a broad range of prevalences in both sexes. At first inspection, it is difficult to draw any sensible conclusions from these surveys as the male prevalence varies from 0.6 - 56% while in women, it varies from 0.1 - 73% (Miyauchi, 1913; Lake *et al.*, 1942; Arnoldi, 1957; Weddell, 1969; Coon *et al.*, 1973; Stanhope, 1975; Beaglehole *et al.*, 1976; Widmer, 1978; Beaglehole,

Reference	*Year*	*Country*
Miyauchi	1913	Germany
US Dept. of Health	1938	USA
Lake *et al.*	1942	USA
Arnoldi	1958	Denmark
Weddell	1969	Wales
Mekkey *et al.*	1969	England, Egypt
US Dept. of Health	1962	USA
Coon *et al.*	1973	USA
Beaglehole	1975, 1986	Cook Is., New Zealand
Stanhope	1975	New Guinea
Widmer	1978	Switzerland
Abramson	1981	Israel
Brand	1988	USA
Evans *et al.*	1999	Scotland

Table 1.1 Table of cited VVs epidemiological surveys.

(1986). What these results do illustrate is the importance of study design. When examining these studies more closely, we see that the more extreme results are gleaned from those which have focussed on unrepresentative samples; *ie.* their sample populations have been weighted in either their age range, their occupations, their geographical location, their ethnicity, or they have been selected from a specific and defined group such as military personnel, or sourced from hospital outpatient departments.

However, if we collate the results from three studies which have examined specified samples from the general population, such as the data from Tecumseh in the USA (Coon *et al.*, 1973), data collected on Europeans living in New Zealand (Beaglehole, 1986) and information obtained from residents of West Jerusalem (Abramson *et al.*, 1981), we see that in a 'Western' population, there appears to be a female preponderance of somewhere between 2 - 3:1. Moreover, by analysing the results obtained from two epidemiological studies in Israel (Abramson *et al.*, 1981), and the USA (Coon *et al.*,1973), we see that the prevalence of all types of varicosity increases with age, reaching a peak between 45 - 65 years. However, other studies have disputed these two observations, such as the Framingham study from 1948 (Brand *et al.*, 1988) and more recently, the Edinburgh study in 1999 (Evans *et al.*, 1999).

The Framingham study showed no increase in the incidence rate of VVs development with age above 40 years, suggesting that any increase in VVs prevalence was due to the constant development of new cases with ageing. The Edinburgh study, had as its' aim, to study the prevalence of VVs and CVI in the general population. In all, 1,566 subjects were recruited between the ages of 18 - 64

years, selected randomly from age-sex registers of 12 local general practices. This study revealed that approximately one third of all men and women in the sample suffered from trunk varices, and that the prevalence of VVs and CVI did indeed, increase with age. However, more surprisingly, it found also that both VVs and CVI were more common in males than females, with an age-adjusted prevalence of VVs in men of 40% compared to 32% in women, whereas the age adjusted prevalence of CVI was 9% in males as compared to 7% in females.

Given that so much is still in dispute regarding the simple epidemiology of VVs, it is of no surprise that the establishment of risk factors for their development and their aetiology is still up for debate.

1.4 Risk factors

Numerous genetic, lifestyle and physiological characteristics have been suggested for the development of VVs, but they have been difficult to confirm without proper cross-sectional, randomised studies. Many lifestyle risk factors, such as a low fibre diet, lack of exercise and defaecation habit (Burkitt, 1972), may be responsible for the racial and geographical variations observed by many epidemiological studies. Of all the possible risk factors, it would appear that only two, pregnancy and obesity, have sufficient weight of evidence to support their influence on VVs development (see Table 1.2 summarising the risk factors for VVs development).

1.4.1 Pregnancy

It is widely believed that pregnancy coincides with the first appearance of VVs (Browse *et al.*, 1999), with many studies confirming this link. Again in the USA, a

study by Lake and co-workers in 1942, focussing on a study sample of 536 employees from a New York department store, showed an increased prevalence in multiparous (79.5%) versus nulliparous (66.9%) women (Lake *et al.*, 1942). The influence of pregnancy on the development of VVs has been suggested also by other studies, such as the Framingham study (Brand *et al.,* 1988), the Basle study (Widmer, 1978), and the more recent Edinburgh vein study (Evans *et al.*, 1999), although the precise risks are difficult to determine.

It would appear that the secundiparous have a 20-30% increased risk compared to primiparous, or nulliparous women (Widmer, 1978; Brand *et al.*, 1988). What could the possible mechanism be? The majority of VVs develop in the first trimester of pregnancy, with approximately 70 - 80% of subjects affected during this period (Rose and Ahmed, 1986; Mullane, 1952). In the second trimester, around 20 - 25% of patients will develop VVs while in the last trimester, 1 - 5% of patients will develop them (Mullane, 1952). If it is believed that VVs develop secondary to the obstructive effects of the uterus on venous return in the iliac veins, then why do the majority of VVs develop in the first trimester (when the uterus is too small to cause any significant obstruction)? It is possible that the increase in blood volume that occurs with pregnancy is a contributing factor (Fanfera and Palmer, 1968), but another more interesting possibility is the role played by the changing levels of hormones.

Both oestrogen and progesterone increase greatly during this period, (Marazita, 1946; McPheeters, 1949; Fried *et al.*, 1953; McCausland *et al.*, 1963; Sumner, 1981) and the increased levels of these hormones expressed as an oestrogen/progesterone

ratio, produces a relaxing effect on smooth muscle, with softening of collagen fibres, thus increasing the distensibility of veins (Wahl, 1977). This effect of oestrogen has been demonstrated and validated by various studies investigating the effect of the oral contraceptive pill. Pills containing high levels of progestogen appear to increase venous capacitance and induce venous stasis, while high oestrogen-containing pills increase coagulability (Fawer *et al.*, 1978). Further validation for this effect of hormones has come from examining the menstrual cycle, with increased venous distensibility occurring during the high hormone-output luteal phase compared to the follicular phase (Fawer *et al.*, 1978; McCausland *et al.*, 1963).

1.4.2 Obesity

Various studies have linked an increased prevalence of VVs with obesity. In a large case control study involving over 500 subjects by Sadick (1992), obesity (which was defined as greater than 20% ideal body weight) was more common in individuals with VVs (15%), than those without (3%). However, this relationship only held for females, as males did not seem to suffer from an increased risk of VVs development (an observation supported by a variety of population-based studies). A 1981 community survey performed in West Jerusalem showed that VVs were more common in females with a high body mass index (BMI), a relationship which did not hold true in males (Abramson *et al.*, 1981), while a study focussing on Europeans in New Zealand showed an association with the severity and incidence of VVs with weight, but again, only in women (Beaglehole, 1986). The Framingham study (Brand *et al.*, 1988) showed that over a 16 year period, females with a BMI greater than 27 kg/m^2 had a higher incidence of VVs development compared to females with a BMI less than 27 kg/m^2. This risk was calculated to be 1.4 in a retrospective,

longitudinal analysis in the Netherlands (Seidell *et al.*, 1986). Therefore, it would appear that obesity only plays a significant part in VVs development in females, and not in males.

1.4.3 Other risk factors

Heredity has been cited as a relative risk factor for VVs development. Few detailed studies have been performed looking at genetic predisposition and those that have, have suffered from a statistically significant lack of subjects. Gundersen and Hauge (1970) and Cornu Thenard and co-workers (1994), both showed an increased risk of VVs in those with affected relatives as compared to controls. Neither however, demonstrated a relationship with a known pattern of genetic inheritance.

Closely intertwined with heredity is racial predisposition. A comparison between Japanese and German soldiers in 1913 (Miyauchi, 1913), revealed a six-fold increase of VVs in the Germans. Abramson and co-workers (1981) showed that immigrant men from North Africa suffered less compared with men from either Europe, or the USA. It is suggested also, that black Africans suffer much lower rates of VVs compared to Europeans, supported by the observations of Dodd and Cockett (1956), who found only three cases of VVs out of 11,000 in-patient admissions in a Zululand tribal reserve. It has been proposed that black Africans are protected from developing VVs as their veins contain more valves per length when compared to Caucasians (Banjo, 1987). However, the prevalence of VVs is equal among black and white Americans (Nelzen *et al.*, 1991), suggesting that these differences in prevalence may not be due to genetic susceptibility, but either to simple physical, or lifestyle differences.

Risk factor	*Reference*	*Year*
Pregnancy	Lake *et al.*	1942
	Mullane	1952
	Fanfera and Palmer	1968
	Widmer	1978
	Rose and Ahmed	1986
	Brand *et al.*	1988
	Browse *et al.*	1999
	Evans *et al.*	1999
Obesity	Abramson *et al.*	1981
	Beaglehole	1986
	Seidell *et al.*	1986
	Brand *et al.*	1988
	Sadick	1992
Heredity	Gundersen and Hauge	1970
	Cornu Thenard *et al.*	1994
Ethnicity	Miyauchi	1913
	Dodd and Cockett	1956
	Abramson *et al.*	1981
	Banjo	1987
	Nelzen *et al.*	1991
Occupation	Lake *et al.*	1942
	Santler *et al.*	1956
	Mekky *et al.*	1969
	Weddell *et al.*	1969
Lifestyle	Cleave	1960
	Burkitt	1972

Table 1.2 Table of risk factors for VVs development

In the case of the Japanese, it has been suggested that the difference between them and the Germans may be because the Germans on average, are much taller than the Japanese and so, are more likely to develop VVs as a result of gravity (Miyauchi, 1913). In the case of black Africans, it has been suggested that their higher fibre intake (Cleave, 1960) and squatting position for defaecation (Burkitt, 1972), prevents straining at stool and so, reduces any possible compression on the iliac veins. However, other studies suggest that there may be no difference in prevalence between black Africa and the West, but that an apparent decrease in prevalence may be due to fewer complications, leading to a lower presentation rate. This theory is supported by data supplied by Rougemont (1973), who showed that women in Mali had a relatively high prevalence rate of 10.9% and by Daynes and Beighton (1973), who found a prevalence rate of 7.7% among women of the Transkei in South Africa. In the latter study however, none of the women complained of any symptoms.

As height, through gravity, is thought to have an influence on VVs development, it would be reasonable to suppose that occupation may also play a role. More specifically, those jobs that require the worker to stand for a considerable length of time, such as policemen, shop assistants and surgeons would be considered to be at high risk. Santler and co-workers (1956) reviewed 2,854 subjects with VVs and found that 6.3% held jobs that required them to walk, 29.2% spent much of their work-time sitting while 64.6% were required to stand. Further, Lake and co-workers (1942) when examining New York department staff, found that 74% of the employees that were required to stand had VVs as compared to 57% who sat. Other surveys have also demonstrated an association with standing and VVs, with an increased prevalence in cotton and metal workers (Mekky *et al.*, 1969) (although in

the case of the cotton workers there may also be a racial element) and in heavy manual occupations that require the lifting of heavy goods (Weddell, 1969).

1.5 Anatomy of the venous system

Before proceeding to examine the theories behind the aetiology of VVs, it is important to understand the anatomy of the venous system in the lower extremities (see Figure 1.1)

The lower limb venous system is divided into the deep (muscular) and the superficial systems. The two major superficial veins are the long saphenous vein (LSV), which lies in front of the medial malleolus running medially to the sapheno-femoral junction (SFJ); and the short saphenous vein (SSV), which lies superficial to the deep fascia on the lateral calf and lower third of the leg behind the lateral malleolus. Both saphenous veins are covered by, and supported by, the superficial fascia (Kubik, 1986), while their tributaries lie external to this, rendering them more susceptible to varicosity (Miller, 1974).

Approximately 90% of all venous blood returns from the lower extremities to the upper body utilising the 'calf muscle pump', actively pumping blood by intermittent contraction whilst walking (Kosinski, 1926; Klein Ronweler *et al.*, 1990). The remainder returns through a combination of hydrostatic pressure and negative intrathoracic pressure generated with respiration, with 'one-way' valves in the veins directing the blood from the superficial to the deep venous system. One such valve lies at the SFJ where the LSV enters the femoral vein. This is known as the sapheno-

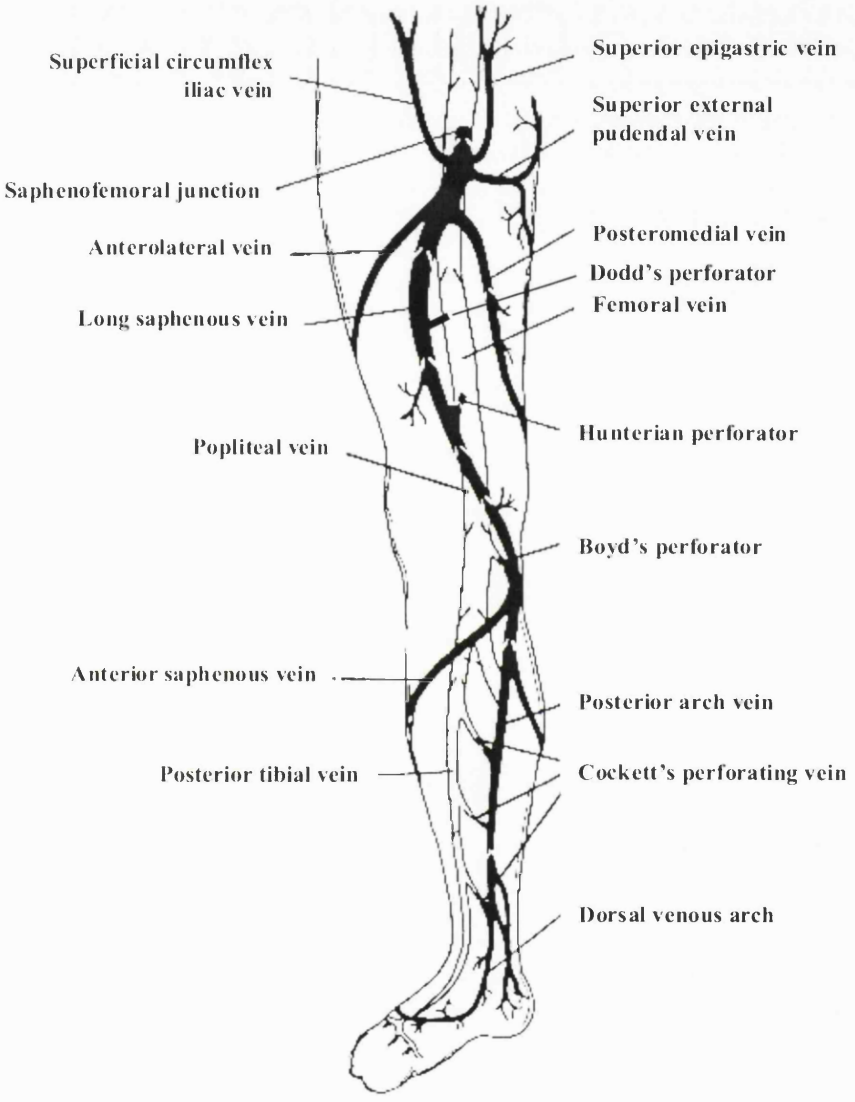


Figure 1.1 Figure demonstrating the venous anatomy of the lower limb

[excluding the short saphenous vein system]

femoral valve (SFV). The deep veins are contained within the muscular fascia and drain the superficial veins located outside, which serve primarily as a venous reservoir. Perforating veins serve to communicate between the two systems, directing blood from the superficial to the deep systems and therefore, provide alternative routes for venous return in case of blockage (Bouissou *et al.*, 1988). Their locations can vary from individual to individual, but there are four relatively constant perforating veins. Two of these are located in the thigh. The proximal one is the Hunterian perforator and the distal one is known as Dodd's perforator, both connecting the LSV to the superficial femoral vein (Dodd, 1959). The antero-medial Boyd's perforator acts to connect the LSV to the crural veins and is located distal to the knee, on the medial calf, while a group of perforating veins above the medial malleolus are known as Cockett's perforators (connecting the posterior arch vein to the posterior tibial veins) (Cockett, 1955).

1.6 The histology of large veins

Large veins such as the LSV consist of three identifiable layers. A thin, one cell thick inner intima, a thick, middle muscular media layer consisting of myocytes, collagen bundles and elastic fibres and an outermost, loose connective tissue adventitia. The function of the collagen in the vein wall is to prevent over-distension of the vessel, while the elastic fibres attempt to limit elastic recoil (Goldman *et al.*, 1994). However, with ageing, the intima thickens, thus disorientating the elastic fibres, which become irregular and dystrophic (Bouissou *et al.*, 1988). The media hypertrophies in its outermost layer with increasing disorganisation of the muscular bundles, and the adventitia becomes more fibrous. Without the elasticity provided by the elastic fibres and with the decrease in wall strength brought about by the

degeneration of the muscular media layer, aged veins are rendered more susceptible to pressure-induced distension, which may predispose to the development of varicosity (Goldman *et al.*, 1994).

1.7 Aetiological hypotheses for VVs

Several theories have been suggested for the pathogenesis of VVs. These are as follows;

- The valvular hypothesis
- The vein wall hypothesis
- The arterial-venous (A-V) anastomosis hypothesis
- The communicating vein hypothesis

1.7.1 The valvular hypothesis

This theory, as proposed by Trendelenburg (1890-1), suggests that VVs develop due to a sequential, descending failure of the venous valves from the SFJ above, downwards. These venous valves serve both to direct blood from the superficial to the deep venous system and to protect the vein below from the pressure in the vein above (Trendelenburg, 1890-1; Ludbrook, 1966; Schultz-Ehrenburg *et al.*, 1989). It is believed that failure of these valves leads to the exertion of pressure from the column of blood above, distending the vein below, which combined with reversed blood flow from the deep to the superficial vessels, transmits calf contraction pressures as high as 300 mmHg (Bjordal, 1970). This causes the vein to become dilated, distorted and tortuous. Therefore, it is believed that varicosity develops due to the inability of the vein wall to resist the unimpeded hydrostatic pressure exerted

by the blood in the venous system (Ludbrook, 1966; 1986). Although it has been proposed that this theory may explain the apparent preference of VVs to develop on the left (Cockett's syndrome), as the venous return in the left common iliac vein may be partially hindered by the right common iliac artery (as it compresses the left common iliac vein at the sacral promontory) (Cockett *et al.*, 1967), to date there has been little or no evidence published to support this.

However, if we examine the anatomy of the venous system, we see that above the SFV lies an external iliac valve which is absent in up to 40% of normal individuals (Eger and Casper, 1943; Basmajian, 1952). According to the 'incompetent valve' theory of varicosity, these individuals are therefore, at a greater risk of developing VVs as only the SFV exists to resist the hydrostatic pressure exerted by the column of blood extending from the SFV, to the right side of the heart. However, a survey by Basmajian (1952) failed to find an association between lack of these ilio-femoral valves and the development of VVs. That said, patients with congenital valve aplasia (who lack venous valves throughout the body) do develop severe VVs (Lodin and Lindvall, 1959; Lindvall and Lodin, 1962). Further, if incompetent SFV function is the cause of VVs, then by ligating the LSV we should observe a reversal of varicosity in affected veins, or at least cessation of the development of new VVs. Detailed investigations with varicograms and Duplex ultrasound scans show that this is not the case (Browse *et al.*, 1999). It would appear therefore, that despite the attractive simplicity of the descending theory of valvular incompetence, by itself it cannot be considered to be the sole factor contributing to the development of VVs.

1.7.2 The vein wall hypothesis

This hypothesis presumes that the underlying defect is a weakness in the wall of the vein, which then dilates, rather than hypertrophies, under pressure (Rose and Ahmed, 1986). The development of this theory was influenced by an article published by King in 1950 that noted several observations;

- VVs communicate with normal veins in which no valvular incompetence is noted
- The initial vein dilatation occurring before varicosity, forms distally to the SFV and not proximally which is what would be expected according to the incompetent valvular theory of VVs
- The extent and size of the varicosities vary from moment to moment without any apparent mechanical alteration in the vein wall
- There is hypertrophy of the vein wall muscle coat with increased vascularity of the adventitia

These observations led King to discard the incompetent valvular theory of VVs and instead, he proposed that venous dilatation was the initiating event for varicosity, due to what he believed were chemical stimulants. Central to this theory is that valvular incompetence follows rather than precedes varicose development.

Further to this, an anatomical investigation by Cotton in 1961, (using veins stripped at operation and veins from post-amputation limbs and cadaveric samples), examined resin casts of whole veins and, aided by the concomitant use of venograms, he drew the following conclusions;

- Varicose LSV have a lesser number of valves than normal LSV
- There is no gross evidence of primary valvular disease on naked inspection
- The number of valves does not diminish with age
- Vein dilations occur below the valve
- Varicose tributaries are elongated and tortuous, while the LSV is not
- Varicose tributaries have alternating sacculations along their length
- Tributaries joining communicating veins are also tortuous
- Deep veins of the leg show no varicosities

Again, this study demonstrated that the initial dilatation of the vein occurs below the valve. However, significantly, Cotton (1961), does not exclude valve incompetence as the preceding event to vein dilatation as he argues that this may occur as a result of regurgitant blood flow across an incompetent valve, putting pressure on the vein wall and so, causing it to weaken and dilate. Arguing against this possibility, the dilatations are found to be eccentric while the valve cusps are positioned concentrically in the vein.

Therefore, the vein wall hypothesis assumes that as vein dilatation proceeds, the valves stretch and expand to maintain valvular competence. However, as the dilatation increases, the valves cannot maintain their expansion and so, become incapable of closing the enlarged vein lumen leading to valvular incompetence (Rokitansky, 1852; Edwards and Edwards, 1940). As the dilatation progresses, the valves may become sclerotic and disappear altogether (Cotton, 1961). Indeed, many histological valve analyses have revealed morphological changes that support this theory. Among these features are depressed commissures, expansion of the valve

area, reversed and tortuous valve cusps and intimal thickening of the adjacent vein wall (Obitsu *et al.*, 1990).

What then is the underlying pathology which may be responsible for this vein wall defect?

First, we have to examine the composition of normal vein wall. Vessels need to withstand three different directional forces;

- Radial distension
- Longitudinal stretch
- Tangential shear stress, (Nerem and Girard, 1990; McIntire, 1994).

If the vessel is unable to withstand these stresses then vascular disruption, with dilatation and possible haemorrhage, will result. Each vein wall component has a specific role in helping vessels maintain their viability. Elastic fibres consist of an elastin and fibulin core associated with a fibrillin-1 microfibrillar sheath and are arranged radially, helping to both mitigate longitudinal stresses (Dobrin *et al.*, 1990) and to aid the vessel to recoil after stretch (Bush *et al.*, 1982). Modulation of both the distending and shear stresses is enabled by a complementary fibre arrangement in the media and interstitium consisting mainly of type III collagen (Dobrin *et al.*, 1990), while shear stresses are modulated by the endothelial cell (EC) cytoskeleton (Davies, 1995). There have been many analyses made of the vein wall composition of VVs which have produced as many different conclusions. In the early 1960's, Svejcar and co-workers (1962; 1963) examined normal veins, VVs and 'normal'

veins from varicose subjects, which were termed 'potential VVs'. They made the following observations;

- VVs and 'potential VVs' contain significantly less collagen compared to normal veins
- VVs contain significantly more muscle compared to normal veins
- VVs and 'potential VVs' contain significantly more hexosamine than normal veins
- VVs show more variation in both water and hexosamine content along their length compared to normal veins

Although the differences revealed in this study suggest that VVs may develop from an inherited abnormality of the vein wall, when this work was repeated by Barbaro and co-workers (1967), different results were reported. However, another study by Andreotti and co-workers (1978), reported a lower content of both collagen and elastin in VVs as compared to controls. Other studies have reported no change in either collagen, or elastin, but rather an increase in muscle cell density which would produce a relative decrease in the density of both collagen and elastin if not an actual decrease (Travers *et al.*, 1992). A change in the collagen:elastin ratio has however, been revealed in other studies, with an increase in collagen types I and III and a reduction in elastin content, with associated smooth muscle atrophy and collagen infiltration (Corcos *et al.*, 1989). It has been suggested that it is a change in venous elasticity, as marked by a change in the collagen:elastin ratio, which might be the final pathway in VVs development (Clarke *et al.*, 1989). In addition, the involvement of various other agents has been suggested. Studies have mentioned a

role for reactive oxygen species (ROS) (Farbiszewski *et al.*, 1996), mast cells (Yamada *et al.*, 1996), changes in anti-peroxidant activity (Deby *et al.*, 1989), anti-kinase activity (Garcia-Rospide *et al.*, 1991) and circulating adrenaline (Crotty, 1991).

1.7.3 The A-V anastomosis hypothesis

Microscopic A-V malformations are normal components of the circulation and play a role in temperature regulation (Kulka, 1966; Ryan and Copeman, 1969). The A-V anastomosis hypothesis proposes that multiple small A-V anastomoses in the subcutaneous tissues are responsible for the development of VVs, as they subject the much weaker venous system to both arterial pressures and pulsatility.

Using a thin-walled latex vein-model both Hasebroek (1916) and Cotton (1961) have demonstrated that ballooning of the latex tube can be produced using a pulsatile fluid pump that exerts pressures comparable to that found in the venous system. In addition, Nylander (1969) demonstrated that VVs form the shape of a sine wave, which is the waveform that results from an increase in blood flow. Pratt (1949) coined the term 'arterial varices' after claiming that arterial pulsations can be palpated over VVs, while Pigeaux (1843) observed that blood from VVs can be as red as that found in arteries. Furthermore, one study claims to demonstrate a higher pO_2 in blood taken from varices as compared to blood taken from the arm veins of the same subjects (Baron and Cassaro, 1986). However, a lower oxygen content has been found with blood samples taken from saphenous varicosities as compared with popliteal vein blood from the same level (Reikeras and Sorlie, 1983).

1.7.4 The communicating vein hypothesis

This suggests that the primary defect is incompetence of the communicating, perforating vein valves. Ludbrook (1966) and Fegan (1974), contend that this allows a jet of retrograde, turbulent and high-pressure blood flow to affect the vessel wall, producing dilatations and initiating varicosity. However, very little evidence exists to support this theory.

Of the four theories listed, only the valvular and vein wall hypotheses have much of a body of evidence to support their viability and there is much circumstantial clinical and experimental information to reject the valvular hypothesis as the 'sole' cause of varicosity. In 1986, Rose and Ahmed made a number of clinical observations that supported the vein wall hypothesis and argued against the valvular hypothesis. They noted that;

- 60% of VVs occur below a competent SFV
- A normal LSV used as an arterial conduit does not become varicose
- A varicose LSV used as an arterial conduit dilates and becomes aneurysmal
- Normal, *in situ* LSV bypass grafts, in which the valves have been previously destroyed, do not become aneurysmal once attached into the arterial system

Moreover, if a primary valvular defect were the sole responsible cause of VVs, it would be expected to produce changes along the entire length of the vein rather than at localised varicose segments, in continuity with normal segments of vein as typically seen. Also, examination of the vein valve cusps show that they have twice the strength of the vein wall and can withstand increased venous pressure more than

the vein wall itself, making it unlikely that valve incompetence is the precursor to varicosity.

Hypothesis	*Mechanism*	*Evidence for*	*Evidence against*
Valvular	Incompetent valve	VVs more common on the left due to compression of the left common iliac vein by the right common iliac artery	No association found between lack of ilio-femoral valves and development of VVs
Vein wall	Weakness of the vein wall predisposing to dilatation	Histological changes in the vein wall suggesting inherent structural weakness	Lack of unified demonstrable mechanism
Arterial-venous	Weaker vein wall subjected to greater, arterial pressures due to multiple, small A-V anastomoses	Experimental model suggests mechanism for VVs development	Contrasting venous O_2 measurements
Communicating vein	Incompetent communicating, perforating vein valves subject vein wall to turbulent, high pressure blood flow	Theoretical only	Little evidence to support this theory

Table 1.3 Summary of the aetiological theories for VVs development

CHAPTER 2

Molecular Considerations for Varicose Vein Development

CHAPTER 2

2.1 Background

The exact aetiology of VVs has yet to be defined.

Epidemiological studies have suggested a genetic and possible ethnic or gender predisposition, influenced by a multitude of environmental factors associated perhaps, with a 'Western' lifestyle. Moreover, experimental and clinical observations have suggested that an inherent defect in venous wall structure and/or function, rather than a 'strict' valvular incompetence, is a primary determinant in VVs development. As segments of varicosity are observed in continuity with normal vein, this suggests that any inherent 'defect' in vein wall is localised so that the remaining length of vein may not be prone to varicosity (Rose and Ahmed, 1986).

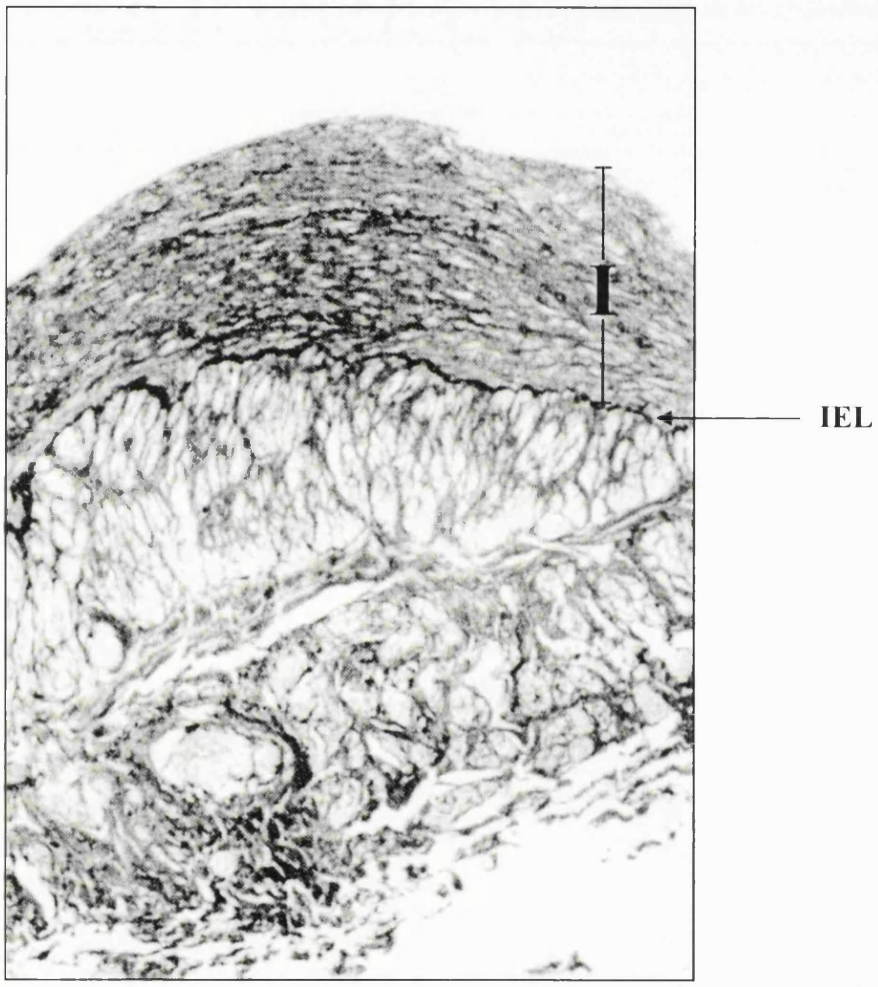
In order to formulate a new hypothesis for VVs aetiology, it is necessary to first examine the structural changes that occur in the wall of VVs and relate these changes with the known functions of vein wall.

2.2 Structural features of VVs

Histological analyses comparing these varicose segments to normal vein have demonstrated structural changes in vein walls. More specifically, in LSV there are reduced quantities of both laminin and type VI collagen with discontinuities observed in type XIV collagen layers in VVs segments as compared to normal vein (Lethias *et al.*, 1996). In addition, there is fragmentation and decrease of elastin fibrils, disorganised collagen fibrils and modifications in basement membranes

(BM). As types VI and XIV collagen are found in fibril networks and laminin is a major component of BM perhaps the modifications seen are representative of changes in the amounts of these compounds? Other features found in VVs include fibrosis invading the media and disturbing the smooth muscle cell (SMC) layers (with accumulation in the sub-intima), spreading throughout the vein wall and intimal thickening composed of SMC's, collagen and elastin (Charles and Gresham, 1993) (see Picture 2.1). The phenotypic modulation of SMC's (triggered by factors released by hypoxia-activated EC's) from 'contractile' to 'secretory' may well account for some of these observed modifications. 'Contractile' SMC's have a well developed contractile apparatus with a cytoplasm rich in both thin and thick myofilaments, whereas 'secretory' SMC's have few myofilaments but large numbers of rough endoplasmic reticulum, ribosomes and mitochondria enabling it to form elements of the ECM such as proteoglycan, collagen and fibronectin. Indeed, the reduced laminin demonstrated in VVs walls may be indicative of this phenotypic change. Alterations in vein wall structure and integrity could account for the localised development of varicosity seen. However, the process by which this occurs is unknown.

SMC phenotype	
Contractile	***Secretory***
Function: migration contraction	Function: secretory forms ECM
Contains large numbers of thick and thin myofilaments	Few myofilaments Large numbers of rough endoplasmic reticulum, ribosomes, mitochondria



Picture 2.1 Histological section of a primary VVs wall

Haematoxylin & Eosin (H&E) stain of a long saphenous vein. **I**: demonstrates the irregularly thickened intima commonly found in VVs. **IEL**: internal elastic lamina (adapted from Charles and Gresham, 1993).

Other substances surmised to play a role in VVs development include destructive ROS (Farbiszewski *et al.*, 1996) and mast cells (which produce a large number of vasoactive substances) (Yamada *et al.*, 1996) both of which, have both been found to be increased in VVs, perhaps acting to weaken vein wall strength. In addition, mast cells release histamine, which increases SMC proliferation leading to intimal thickening. Further studies have demonstrated enzymatic changes in the vein wall a decrease in collagen elastase, maleate dehydrogenase, non-specific esterases, adenosine triphosphate, 5' nucleotidases, lactic dehydrogenase, creatine kinase and tissue plasminogen activator. Correspondingly, increases in levels of neutrophil elastase, urokinase, acid phosphatase, lysozymes and an increase in tissue plasminogen activator at the level of the groin have also been revealed (Layer *et al.*, 1986; Urbanova *et al.*, 1972; Wolfe *et al.*, 1979; Garcia-Rospide *et al.*, 1991). In addition, there is reduction in inhibitory anti-peroxidant and anti-kinase activity (Deby *et al.*, 1989; Garcia-Rospide *et al.*, 1991). These changes in enzyme levels would suggest a role in VVs aetiology. For instance, neutrophil elastase is a highly destructive and potent serine protease stored in the azurophilic granules of mature neutrophils (Yoshimura and Crystal, 1992), responsible for the degradation of connective tissue and of fibrinogen. Lysozymes are another highly destructive enzyme, acting to dissolve ground substance and leading to structural degeneration of muscle and ECM. This degradation weakens the vein wall and may predispose to the formation of varicosity (Crotty, 1991).

Conversely, high levels of lactic dehydrogenase and creatine kinase are protective (Garcia-Rospide *et al.*, 1991), as they stimulate energy-rich phosphates to produce enough energy to maintain myofibrillar function and vein wall contractility. A

decrease in these enzymes will thus favour the 'dilated' state. This decrease in 'protective factors' is also evident in the loss of the anti-oxidant tocopherol (Deby *et al.*, 1989) which predisposes to increased depolymerisation of hyaluronic acid, a major component of the vein wall.

A more complicated situation arises when we examine the levels of tissue plasminogen activator and of tissue urokinase. Both of these substances are secreted by venous endothelium, acting as powerful fibrinolytics and thus helping to prevent the formation of thrombosis. Therefore, they comprise part of the normal 'protective' mechanisms essential for venous function. They act principally by converting plasminogen to plasmin, which in-turn degrades fibronectin (as well as a broad range of other matrix molecules) (Saksela and Rifkin, 1988). In addition, plasmin activates pro-collagenase to collagenase (Werb *et al.*, 1977). As expected, urokinase is said to be raised in VVs however, interestingly the level of tissue plasminogen activator is reported to be lowered in patients with severe CVI (Dormandy, 1996) although raised at the level of the groin (Layer *et al.*, 1986; Urbanova *et al.*, 1972; Wolfe *et al.*, 1979). Perhaps here, the loss of enzyme activity decreases the 'protection' afforded to venous endothelium, leading to increased damage and subsequent loss of function while at the groin, the increase demonstrated may exarcebate vein wall degradation. Furthermore, a role has been suggested for noradrenaline in VVs aetiology. Here, it is suggested that varicosities begin as acute dilator responses to noradrenaline (released from the vasa vasorum of the vein), accentuated by excessive, prolonged venous reflux which abolishes venous tone and thus promotes venodilatation (Crotty, 1991).

These findings suggest that VVs arise due to weakening of the vein wall, which is then predisposed to venodilatation. However, whether these changes are 'cause' or 'effect' is not known but their existence suggests an interplay of molecular mechanisms in VVs aetiology. What however, is the possible source of these changes? It would appear that venous endothelium plays a significant role in this process and that we must first examine its function in venous homeostasis.

Enzyme changes in VVs wall	
Decreased	***Increased***
Collagen elastase	Neutrophil elastase
Maleate dehydrogenase	Urokinase
Esterase	Acid phosphatase
Adenosine triphosphate	Lysozymes
Tissue plasminogen activator	Tissue plasminogen activator (at the groin)
5' nucleotidases	
Lactic dehydrogenase	
Creatine kinase	
Anti-peroxidant	
Anti-kinase	

2.3 The functions of venous endothelium

Previous studies have focussed on the macrocirculation and how haemodynamic changes affect the whole vein wall. However, the endothelial lining of the vein has

received much less attention. The venous endothelium has two important functions. First, it provides an optimum surface easing blood flow and second, it separates the intravascular and extravascular compartments (Dormandy, 1996). Dysfunction of the endothelium may give rise to the signs and complications of venous disease such as thrombosis, inflammation, oedema, tissue ischaemia, venous hypertension and ultimately, venous ulceration, many of which co-exist with VVs. Venous endothelium performs its' functions by both physical and chemical means. The former includes a surface negative charge, acting to prevent similarly charged blood cells from adhering to its' surface (Dormandy, 1996). The biochemical, secretory roles of the endothelium are manifold and have been investigated extensively. This includes a powerful anti-thrombotic action, requiring the synthesis and secretion of urokinase, tissue plasminogen activator and prostacyclin (Baillart *et al.*, 1994), and other substances involved in the normal homeostatic mechanisms of venous endothelium such as bradykinin, angiotensin, adenonucleotides, vascular endothelial growth factor (VEGF) and nitric oxide (NO).

2.4 The role of cytokines in vein wall homeostasis

One of the main homeostatic functions of vein (and particularly of the endothelium) is the ability to respond to changes in intraluminal pressure. Previous studies looking at the dilating and constricting ability of VVs segments have examined their response to physiological agents affecting venous tone. Lowell and co-workers (1992) using normal and primary varicose LSV segments (some with the endothelium scraped off), suspended in organ-baths, examined the effect of potassium chloride, norepinephrine and endothelin (ET) in affecting isometric tone, complemented by biochemical and structural studies. They found that both contraction and relaxation

were reduced in varicose as compared to control segments and that endothelium-independent relaxations in response to NO were further reduced compared to controls. Mangiafico and co-workers (1997) examined the plasma release of the vasoconstrictor ET-1, in VVs and controls before and after application of a sphygmomanometer cuff for 10 minutes to induce relative venous stasis. Radio-immunoassay of ET-1 plasma levels revealed that although both controls and VVs produced elevated ET-1 levels post-induced venous stasis, VVs demonstrated a significantly greater increase. Furthermore, Schuller-Petrovic and co-workers (1997) indirectly measured the release of NO and prostacyclin (both vasodilators) and the direct release of ET from cultured cells derived from VVs and controls with additional measurements of plasma ET and angiotensin II (vasoconstrictor) levels. They found that endothelial cells (EC) derived from VVs secreted less constrictor mediators compared to EC's derived from normal LSV and that in EC's derived from VVs the NO/cyclic guanosine monophosphate (cGMP) system is up-regulated which may predispose towards vasodilation thus contributing to the formation of varicosis. Indeed, it has been demonstrated that an abnormal distensibility of the venous wall characterises VVs and that all limb veins from subjects with primary VVs are more distensible than normal, again suggesting a genetic basis for VVs (Sansilvestri-Morel *et al.*, 1998).

So we see that the evidence thus far suggests that VVs aetiology may be due to an imbalance of vasoactive factors producing an over-active dilatory response. However, this theory would appear to be contradicted by the results produced by Mangiafico and co-workers (1997), whom demonstrated an increased capacity of VVs to secrete the vasoconstrictor ET-1. Here though, it is suggested that despite the

up-regulation of ET-1 there is a corresponding decrease in the number or density of its receptor, ET_B thus reinforcing the dilatory capacity of VVs.

2.5 VEGF and NO

The evidence from the previous studies is that VVs arise possibly, due to an awry and damaged homeostatic mechanism, originating from the venous endothelium, controlling venous tone. As stated earlier, one accepted definition of VVs is 'any dilated...vein' (Arnoldi, 1957). Could VVs result from a continued stimulus to dilate?

As mentioned previously, among the essential substances that are released by the endothelium, are VEGF and NO. Both of these agents mediate the venous response to venous 'hypertension' and stasis, by initiating vein dilatation. Previous observations have revealed both higher resting (84 mmHg in controls, and 90.5 mmHg in subjects with venous disease) and ambulatory (18 mmHg in controls, and 48.3 mmHg in subjects with venous disease) venous pressures in patients with venous disease, as compared to controls (Somerville *et al.*, 1974) predisposing to the development of venous stasis. This results in relative hypoxia, reduction in pH (Homans, 1917; Cheatle *et al.*, 1998) and an associated increase in oxidative metabolites and ROS, which may all damage the vessel wall (Michiels *et al.*, 1996; 1997; Lewis *et al.*, 1999; Wood *et al.*, 1999). Furthermore, involvement of other pro-inflammatory cytokines, activation of monocytes (Kalra *et al.*, 1996), macrophages (Lewis *et al.*, 1999) and neutrophils (Arnould *et al.*, 1993; Shields *et al.*, 1993), all combine to exacerbate vein wall damage and may interfere with endothelial function, off-setting its normal homeostatic mechanisms. Could one of

these affected mechanisms involve the control and release of VEGF and NO (Laitinen *et al.*, 1997; Servos *et al.*, 1999) leading to inappropriate plasma levels? The dilating ability of these two substances combined with the proposed down-regulation of ET-1 receptors may be the initiating event that leads to VVs formation. In order to understand how VEGF and NO may influence the development of VVs, it is necessary to examine them in more detail.

CHAPTER 3

Vascular Endothelial Growth Factor

CHAPTER 3

3.1 Background

VEGF was the first, selective angiogenic factor to be identified (in the 1980's) due to its ability to induce transient vascular leakage and endothelial cell (EC) mitogenesis (Leung *et al.*, 1989; Neufeld *et al.*, 1994).

VEGF is a homo-dimeric glycoprotein, approximately 34 - 46 kDa in size, which is secreted and exerts a direct effect on EC's via interaction with its' cellular receptors, kinase-domain receptor (KDR) (Terman *et al.*, 1992) and *fms*-like-tyrosine kinase-1 *(flt*-1) (de-Vries *et al.*, 1992). It belongs to the platelet derived growth factor (PDGF) family of growth factors and shares sequence homology with two other growth factors, PDGF and placental growth factor (PlGF). The amino acid sequence of VEGF is 20% identical with PDGF, including eight conserved cysteine residues located within the PDGF receptor-binding domain, defined by truncated forms of the v-*sis*-derived oncogenic protein (Keck *et al.*, 1989), (Houck *et al.*, 1991), (Tischer *et al.*, 1991). With PlGF, VEGF shares 53% amino acid homology including the same eight cysteine residues (Maglione *et al.*, 1991). The human VEGF gene is composed of eight polypeptide coding exons (Tischer *et al.*, 1991) and differential splicing of the gene produces five different isoforms as follows; exons 1-5 and 8 encode $VEGF_{121}$, the shortest form of VEGF. Inclusion of exon 7 generates $VEGF_{165}$ while the addition of exon 6 produces $VEGF_{189}$. $VEGF_{206}$ is identical to $VEGF_{189}$ but with the addition of a 17 codon chain following the 24 codon insertion in $VEGF_{189}$, and lastly $VEGF_{145}$ is identified by a 21amino-acid sequence encoded by exon 6 (Keck *et al.*, 1989; Leung *et al.*, 1989; Tischer *et al.*, 1989; Houck *et al.*, 1991; Poltorak *et al.*,

1997). The differential feature between these isoforms is their heparin and heparan-sulphate binding ability conferred by a 44 amino acid-long peptide sequence, and is primarily encoded by exon 7. $VEGF_{121}$ lacks this heparin binding ability, which is present with $VEGF_{165}$, enabling this isoform to bind to heparin (Park *et al.*, 1993; Cohen *et al.*, 1995).

As the extracellular matrix (ECM) contains a large amount of heparan sulphate, this binding ability enables complexing of VEGF isoforms in the vein wall. $VEGF_{145}$ contains a 21 amino acid sequence encoded by exon 6 that also contains elements conferring a heparin binding ability (Poltorak *et al.*, 1997). $VEGF_{189}$ and $VEGF_{206}$ contain peptide sequences encoded by both exons 6 and 7, and so, have a higher affinity for heparin than either $VEGF_{145}$ or $VEGF_{165}$, ensuring that they are more likely to be sequestered in the ECM attached to heparan sulphates. $VEGF_{189}$ is not secreted into the media of VEGF secreting EC's and is less active than either $VEGF_{121}$ or $VEGF_{165}$, although it can be cleaved by plasmin to produce a fragment ($VEGF_{110}$) that is both active and soluble (Houck *et al.*, 1991; Jonca *et al.*, 1997). The VEGF isoforms and their characteristics are summarised below;

VEGF isoform	***Heparan-binding***	***Secreted***
121	-	+
145	+	+
165	+	+
189	++	-
206	++	-

So, differential splicing of the VEGF gene determines the binding of VEGF to heparan sulphates in the ECM, thus controlling diffusion from cellular sites of synthesis and establishing the extent of local storage. These heparan-bound forms of VEGF can be released from the ECM by the action of heparinase and plasmin, which remove the C-terminal heparan-binding sequence encoded by exon 6 of the VEGF gene (Houck *et al.*, 1991). The three secreted and therefore, more active forms of VEGF, are $VEGF_{121}$, $VEGF_{145}$ and $VEGF_{165}$, with $VEGF_{189}$ and $VEGF_{206}$ acting mainly as a store within the vein wall (Jonca *et al.*, 1997). The predominant forms of VEGF produced by EC's are $VEGF_{121}$ and $VEGF_{165}$, with some $VEGF_{189}$. $VEGF_{145}$ is the predominant form produced by EC's derived from the reproductive system (Poltorak *et al.*, 1997).

3.2 Functions of VEGF

The functions of VEGF are summarised in the following table;

Functions of VEGF

Angiogenesis (EC mitogenesis and migration) – the 'foetal role'
Induces formation of fenestrations in the vessel wall
Increases expression of plasminogen activators
Increases expression of collagenases

However, VEGF has both a 'foetal' and an 'adult' role.

3.2.1 The 'foetal' role

VEGF plays a central role as a prime regulator of angiogenesis (Leung *et al.*, 1989; Carmeliet *et al.*, 1996). Targeted gene disruption has demonstrated that, with the loss of just one allele of VEGF mice foetus's are aborted, due to defects in the overall development of the cardiovascular system. Likewise, disruption of the genes encoding for VEGF receptors results in severe abnormalities of the vascular system, and have indicated that *flt*-1 is required for cell migration (Seetharam *et al.*, 1995; Barleon *et al.*, 1996), while KDR is required for both the efficient differentiation of EC's and migration of primitive precursors of EC's from the posterior primitive streak to the yolk sac (Shalaby *et al.*, 1997).

Although VEGF is essential for normal embryogenic development, it can have adverse effects. In the newborn, pathological VEGF production is evident in neonatal retinopathy. In this case, disordered VEGF secretion is iatrogenic, caused by the use of hyper-oxygenated chambers in the treatment of premature babies and their under-developed lungs. The high levels of oxygen inhibit the production of VEGF by astrocytes, which serve as the hypoxic sensors. This causes cessation of normal retinal angiogenesis. Then, as the baby is removed from the hyper-oxygenation chamber, the retinal cells experience severe hypoxia and produce large amounts of VEGF, leading to disordered angiogenesis of the retina and ensuing blindness in the newborn (Stone *et al.*, 1995; Pierce *et al.*, 1996).

3.2.2 The 'adult' role

In the mature organism, all normal, differentiated EC's throughout the vascular system retain the ability to respond mitogenically to VEGF. Using cells that

differentially lack either receptor *flt*-1 or KDR, stimulation by VEGF indicates that the mitogenic response appears to be mediated by the KDR receptor, while both KDR and *flt*-1 mediate cell migration and chemotaxis (Waltenberger *et al.*, 1994; Shalaby *et al.*, 1997). Other non-mitogenic responses elicited by VEGF include the expression of plasminogen activators (Mandriota *et al.*, 1995) and collagenases (Unemori *et al.*, 1992). Up-regulation of these substances, facilitate the penetration of capillaries in to tissues and combined with the increase in vascular permeability, enables vital nutrients to reach growing tissue.

Angiogenesis is an integral feature of normal tissue repair being maximally increased in rodent epidermal keratinocytes following dermal injury and in porcine coronary arteries, following myocardial ischaemia (Banai *et al.*, 1994). This function of VEGF may be aided by the heparin-binding isoforms as they can bind to cell surface and ECM-associated heparan-sulphate proteoglycans, releasing basic fibroblast growth factor (bFGF), which is stored as a complex with heparan sulphates in the ECM (Jonca *et al.*, 1997). bFGF is a 16 kDa monomeric peptide and is a potent SMC mitogen. In this way, both VEGF and bFGF can act synergistically to induce angiogenesis (Asahara *et al.*, 1995). The heparin-binding ability of VEGF may also help to regulate interaction with its own receptors. It has been demonstrated that heparin can enhance the binding of $VEGF_{165}$ to KDR, but not that of $VEGF_{121}$ (Gitay-Goren *et al.*, 1996). This may account for the observation that $VEGF_{121}$ is reported to be less potent compared to $VEGF_{165}$ (Keyt *et al.*, 1996).

The major stimulant for both VEGF and VEGF receptor production and secretion is hypoxia (Tuder *et al.*, 1995). As the main effect of VEGF is angiogenesis, it

provides an effective compensatory mechanism by which tissues can increase oxygenation and therefore plays an important role in wound healing. However, new vessel proliferation is not always beneficial as seen in neonatal retinopathy. In the adult it can specifically target EC's in tumour growth.

Tumour growth requires angiogenesis in order to progress. Indeed, VEGF and receptor messenger RNA (mRNA) has been found to be maximal in areas surrounding regions of necrosis and studies, using monoclonal anti-VEGF antibodies and a VEGF-binding construct which lacks the cytoplasmic tyrosine kinase domains found in the mouse KDR receptor, have been found to inhibit substantially, the vascularisation and growth of human tumours in nude mice (Kim *et al.*, 1993; Millauer B *et al.*, 1994). VEGF contributes also, to tumour growth because of its ability to increase vascular permeability (Roberts and Palade *et al.*, 1997; Esser S *et al.*, 1998). VEGF induces the formation of fenestrations in the vessel wall and vesiculo-vacuolar organelles, allowing the passage of blood-borne proteins, (which contribute to the formation of a matrix), that supports the growth of EC's and tumour cells thus allowing the invasion of stromal cells into the tumour.

3.3 Stimulants of VEGF production

The two main stimulants of VEGF production in EC's are hypoxia and oestrogen. Considering each factor in turn;

3.3.1 Hypoxia

Studies involving cultured cells have shown a 10-50 fold increase in VEGF mRNA as a consequence of lowering oxygen levels from 21% to 3% (Thomas, 1996). In

organ development, a hypoxic environment stimulates VEGF production in new cells, initiating a VEGF concentration gradient to which forming blood vessels grow towards (Stone *et al.*, 1995; Pierce *et al.*, 1996). As the hypoxic stimulus eases with the formation of the new blood vessels, VEGF production decreases, although a small background production is maintained to inhibit both apoptosis of the EC's and to stabilise the newly formed vessels (Alon *et al.*, 1995). How then does hypoxia serve to stimulate the production of VEGF? At present, the mechanism is only partially understood, but is believed to be similar to the process behind the stimulation of erythropoietin involving the binding of hypoxia-inducible factor 1 (HIF-1). This is a nuclear transcription factor, production of which is up-regulated by hypoxia (Beck *et al.*, 1993). It functions as a hetero-dimer consisting of the 120 kDa HIF-1α and the 91-94 kDa HIF-1β, both of which are basic-helix-loop-helix proteins (Wang and Semenza, 1995).

Hypoxic stimulation may involve $CoCl_2$, as this is known to stimulate erythropoietin and VEGF production by the replacement of iron with cobalt in the porphyrin ring of a putative haem-containing protein oxygen sensor, decreasing its affinity for O_2 and favouring the deoxy-conformation (Goldberg and Schneider, 1994). With regards to VEGF, HIF-1 is thought to bind to a HIF-1 binding site located in the VEGF promoter region. Recent evidence indicates that two potential regulatory DNA enhancer sequences (that are 90% homologous with the human erythropoietin hypoxia-response element) are located five-prime (5') to the transcriptional start site of the VEGF gene (Goldberg and Schneider, 1994). In addition, a 5' enhancer has been mapped to a 100-base pair (bp) segment, 800 bp upstream of the VEGF transcriptional start site (Minchenko *et al.*, 1994). Further, as well as inducing the

transcription of VEGF, hypoxia serves to stabilise VEGF mRNA by way of proteins such as HuR mRNA binding protein, that bind to sequences located in the three-prime (3') untranslated region (Levy *et al.*, 1998). Other studies using cerebellar haemangioblastomas and human renal carcinomas have implicated the involvement of the von Hippel-Landau gene and the tumour suppressor gene p53 (Mukhopadhyay *et al.*, 1995; Maher and Kaelin , 1997).

3.3.2 Oestrogen

Although many other promoters of VEGF have been identified, one requires special mention, as it relates to the apparent sex differential seen in VVs development. As expected, VEGF production is increased physiologically in organs undergoing vascularisation. This happens regularly in the uterus, under the influence of the steroid hormone oestrogen, which has been shown to stimulate VEGF mRNA expression in the rat uterus without prior protein synthesis (Cullinan-Bove and Koos *et al.*, 1993). This has been confirmed *in vitro*, using endometrial carcinoma cells that induce VEGF production with the addition of oestrogen. However, as the VEGF promoter lacks steroid responsive elements, the induction of VEGF must occur indirectly. Investigations looking into this possible mechanism have used cultured cancer cells, primarily breast and uterine. Oestrogen has been shown to increase cyclic adenosine monophosphate (cAMP) levels that then activate transcription of cAMP reporter genes (Aronica *et al.*, 1994). This may, in turn, induce the VEGF gene via AP-2 transcription factors. In addition, both oestrogen and progesterone can augment the production of phosphoinositides and phosphatidylcholine (Etindi *et al.*, 1992), (Krasil'nikov *et al.*, 1993), which act as precursors for 'second messenger' signalling molecules such as the protein kinase C (PKC) activator, di-acylglycerol,

which may trigger PKC-dependent VEGF induction (Cantley, 1994). Lastly, oestrogen can initiate tyrosine phosphorylation in breast cancer cells by activating c-Src tyrosine kinase, mediated by oestrogen receptors. Src is known also, to induce VEGF expression via generation of di-acylglycerol, which then activates PKC and its' subsequent signalling cascade (Migliaccio *et al.*, 1993).

3.4 VEGF receptors

The targets for VEGF are two cognate phosphotyrosine receptors, *flt*-1 (Shibuya *et al.*, 1990) and KDR (Terman *et al.*, 1992). The location of these two receptors is summarised in the following table;

Location of receptors:

flt-1	*KDR*
EC's	EC's
Trophoblasts	Haematopoietic stem cells
Monocytes	Megakaryocytes
Mesangial cells	Retinal progenitor cells

Predominantly, they are expressed by EC's but a few other cells have been shown to express either one, or both of them. *flt*-1 has been demonstrated on trophoblasts, monocytes and mesangial cells (Charnock-Jones *et al.*, 1994) (Takahashi *et al.*, 1995) (Barleon *et al.*, 1996), while KDR is expressed on haematopoietic stem cells, megakaryocytes and retinal progenitor cells (Yang and Cepko, 1996). Certain tumourigenic cell types are known to express both receptor sub-types (Gitay-Goren *et al.*, 1993). Both receptors are composed of seven extracellular, immunoglobulin-

like domains containing a ligand-binding region, a single short membrane-spanning sequence and an intracellular region that contains the tyrosine kinase domain (Shibuya *et al.*, 1990).

3.4.1 Receptor structure

Both receptors are approximately 1300 amino acid residues long, sharing 45% sequence homology with each other and are glycosylated, with only the final glycosylated form of KDR capable of undergoing autophosphorylation in response to VEGF (Takahashi and Shibuya *et al.*, 1997). In addition, the *flt*-1 receptor mRNA can be spliced to generate two forms of the receptor; one being the full length membrane-spanning receptor, and the other, a soluble form known as *sflt*-1, which is truncated on the C-terminal end of the sixth extracellular immunoglobulin-like domain (Kendall and Thomas, 1993). *sflt*-1 retains high affinity for VEGF, but has been shown to inhibit the expected mitogenic response (Kendall *et al.*, 1994). As previously mentioned, hypoxia has been shown to up-regulate the expression of both receptors although to a lesser degree compared to VEGF itself. The transcription of *flt*-1 is enhanced by hypoxia, but not that of KDR (Gerber *et al.*, 1997), although KDR production is up-regulated, indicating that this occurs via an as yet to be determined post-translational mechanism (Waltenberger *et al.*, 1996).

3.4.2 Receptor activity

As mentioned previously, studies using cells that differentially lack either VEGF receptor have indicated that they mediate different functions. This is summarised in the following table;

VEGF receptor	*Function*
KDR	Mitogenesis Migration Chemotaxis
flt-1	Migration Chemotaxis

3.4.3 Receptor binding and signal transduction

VEGF exists as a homo-dimer, consisting of two monomers attached via disulphide bridges in a 'head-to-tail' fashion, with a large overlap (Guberan *et al.*, 1973). Alanine-scanning mutagenesis has revealed that Arg, Lys and His amino-acids (Cohen *et al.*, 1995; Goad *et al.*, 1996), are essential for binding to KDR while Asp, Glu and Glu amino-acids (Siemeister *et al.*, 1998) are required for binding to *flt*-1. These binding domains are located at opposite ends of the VEGF monomer so that the main *flt*-1 binding domains are at opposite ends of the molecule to the main binding domains for KDR. Mutations of VEGF, which affect the binding domain for one receptor, do not affect binding to the other receptor, so preserving their differential activity (Keyt *et al.*, 1996). Like other growth factor transmembrane tyrosine kinase receptors, *flt*-1 and KDR can undergo ligand-induced dimerisation, thus triggering signal transduction by promoting either autophosphorylation, or transphosphorylation of the adjacent receptor subunit and by binding and phosphorylating downstream signal transduction protein mediators. 'Insert' sequences within the tyrosine kinase domains, containing tyrosine residues, aid this process by phosphorylating and generating docking sites for complexation with

downstream signal transduction proteins. The *flt*-1 kinase domain contains a 66 amino acid residue 'insert' (de-Vries *et al.*, 1992) sequence, while KDR contains a 70 amino acid sequence (Terman *et al.*, 1992), of which two tyrosine residues are subject to either auto- or transphosphorylation, the other two being located in the cytoplasmic domains.

In addition, several other cytoplasmic proteins, some of which containing the SH2 receptor phosphotyrosine-binding domain, are known to participate in signal transduction. These include phosphatidylinositol 3-kinase (which phosphorylates phosphatidylinositols at the 3-position of the inositol ring to produce second messengers) and phospholipase Cγ (which hydrolyses phosphatidylinositol 4,5-biphosphate to inositol 1,4,5-triphosphate and 1,2-diacylglycerol, stimulating Ca^{2+} release and activating PKC). In addition, phosphorylation of both the Ras GTP'ase-activating protein GAP and NcK, a protein containing 1 SH2 and 3 SH3 docking domains, may aid in coupling cell surface receptors to other downstream effectors (Guo *et al.*, 1995). As activation of *flt*-1 and KDR by VEGF produces different EC responses, this indicates that the signalling cascades for each are somewhat different. As mentioned previously, activation of *flt*-1 by itself does not result in EC mitogenesis and proliferation, an action that is reserved for the KDR receptor. Studies using porcine, aortic EC's that only express the KDR receptor have demonstrated binding and phosphorylation of the Shc and Nyc adapters, Grb-2 binding and MAP kinase activation. In contrast, cells that express the *flt*-1 receptor only do not activate MAP kinase (Kroll and Waltenberger, 1997), which indicates that MAP kinase is essential in EC mitogenesis. Furthermore, KDR associates physically with both SHP-1 and SHP-2 SH2 protein tyrosine phosphatases after

activation by VEGF (Seetharam *et al.*, 1995; Kroll and Waltenberger, 1997). The result is the generation of proteases which breakdown the BM of blood vessels (which is essential for the first stage of angiogenesis), the expression of specific integrins required for angiogenesis and finally, the initiation of cell proliferation and migration (Friedlander *et al.*, 1995).

However, despite the different actions of *flt*-1 and KDR, it is possible that one VEGF homo-dimer can bind to both *flt*-1 and KDR to form a hetero-dimer receptor complex and thus, activate both receptors (Fuh *et al.*, 1998). This is due to the previously mentioned overlap between the separate binding domains for *flt*-1 and KDR on the VEGF molecule. Further, there is some evidence to suggest that *flt*-1 and KDR can form hetero-dimers after VEGF binding (Kendall *et al.*, 1994). Both receptors contain a receptor dimerisation domain in their fourth immunoglobulin-like loop, providing a possible site for hetero-dimerisation to occur. As yet, however, no real experimental evidence exists to support this possibility.

3.4.4 Other VEGF receptors

In addition to the well characterised *flt*-1 and KDR receptors, EC's have also been found to express isoform-specific VEGF receptors, which are not related to either *flt*-1 or KDR (Gitay-Goren *et al.*, 1996). These receptors have an affinity for $VEGF_{165}$, but not $VEGF_{121}$ and using a combination of $VEGF_{165}$ chromatography and expression cloning, the genes encoding them have been identified as the same genes encoding for neuropilin-1 and neuropilin-2, receptors known to bind to semaphorins (Soker *et al.*, 1998). (Semaphorins are factors that act to inhibit nerve growth cones). However, neuropilins have short intracellular domains and so, are unlikely to act as

independent receptors themselves (although they may act as co-receptors). This is suggested by studies in which mouse embryos that lack neuropilin-1 are not viable, due to disordered development of the cardiovascular system and in studies of EC's that express neuropilin solely, which do not initiate a response to VEGF (Kitsukawa *et al.*, 1997). Furthermore, KDR has been shown to bind more effectively, and to produce a potentiated migratory response to $VEGF_{165}$ in cells that express neuropilin-1, compared to cells that do not. This specificity of neuropilin-1 for $VEGF_{165}$ may explain and determine why the isoform $VEGF_{121}$ is a less active mitogen than $VEGF_{165}$ (Soker *et al.*, 1998). It may provide also, another mechanism by which VEGF exerts its' effects, perhaps activating neurophilin receptors independently, or by interacting with semaphorins in angiogenesis. Furthermore, with the identification of another unrelated $VEGF_{165}$ specific receptor, the existence of other VEGF isoform specific receptors is a possibility.

CHAPTER 4

Nitric Oxide

CHAPTER 4

4.1 Background

The other factor with a potential role in VVs development is NO. NO was identified in 1980 by Furchgott and Zawadski (1980), based on its' ability to relax blood vessel walls. It was localised to the vascular endothelium and so, was termed endothelium-dependent relaxing factor. The exact identification of NO was accomplished by three different research groups acting independently of one another (Furchgott and Zawadzki, 1980; Palmer *et al.*, 1987; Ignarro *et al.*, 1987). NO is a stable, colourless gas, moderately soluble in water, with a multitude of biological effects.

4.2 NO formation

NO is formed directly from the guanidino nitrogen of L-arginine, in a two step process, by the oxidative enzyme nitric oxide synthase (NOS), resulting in the formation of L-citrulline and NO. Oxygen and nicotinamide adenine dinucleotide phosphate (NADPH) are co-substrates, with flavin nucleotides, haem and BH_4 as co-factors. The system is under the control of a negative feedback mechanism, whereby NO inhibits its' own production (Buga *et al.*, 1993; Davies *et al.*, 1995).

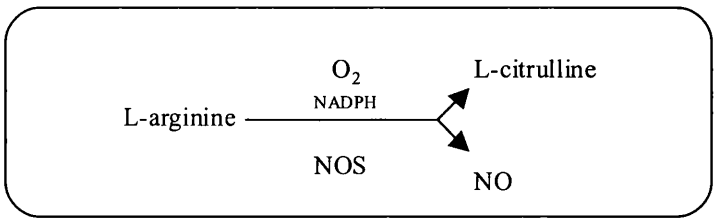


Figure 4.1 The NOS pathway

Illustration of the L-arginine/nitric oxide pathway. **NOS**: nitric oxide synthase, **NADPH**: nicotinamide adenine dinucleotide phosphate, **NO:** nitric oxide

4.3 NOS

NOS is a complex enzyme, 140 kDa in size and homologous to cytochrome P450, existing as three isoforms in mammals. Eight DNA sequences have been identified from three known NOS genes; neuronal (nNOS) and endothelial NOS (eNOS), both termed constitutive NOS (cNOS) and the third, known as inducible NOS (iNOS) (Davies *et al.*, 1995). Despite the existence of three different isoforms, they all share a common structure consisting of two domains; a N-terminal end consisting of a haem-oxygenase domain made up of tetrahydrobiopterin, haem and arginine binding sites, and a C-terminal end made up of a P-450 reductase domain containing recognition sites for NADPH, flavin mononucleotide and adenosine dinucleotide.

There are several important differences between cNOS and iNOS (see Table 4.1). cNOS is 'constitutive' in that it maintains background activity and does not require new enzyme protein synthesis in order to be initiated, but depends on a glutamate-induced, calmodulin-mediated calcium ion intracellular influx (Forstermann *et al.*, 1991). This induces a transient, short-lived response, producing NO in picomolar amounts. iNOS, on the other hand, is induced when required, by inflammatory stimuli such as endotoxins and cytokines and can be activated by the intracellular levels of Ca^{2+} found in resting cells. iNOS produces a more sustained (up to several days) and greater response of NO production, reaching the nanomolar range (Liu *et al.*, 1997). The different NOS isoforms differ also, in their subcellular distribution, in that cNOS is associated with the cellular membrane, while iNOS is a cytosolic enzyme. However, both cNOS and iNOS are inhibited by modified guanidino derivatives of L-arginine, while only iNOS is inhibited by glucocorticoids, cNOS being unaffected. The effects of NO are mediated through activation of the soluble

Characteristics of the NOS isoforms		
Characteristics	***Constitutive: neuronal endothelial***	***Inducible***
Subcellular location	Cellular	Cytosolic
Cell type	EC's	EC's
	platelets	SMC's
	neurons	leucocytes
		Kuppfer cells
		mesangial cells
		parenchymal cells
Activity	Permanent	Induced by inflammatory stimuli; endotoxin, cytokines
Calcium-influx	Dependent	Independent
Membrane binding	Yes	No
Release & activity	Short-lived & rapid	Delayed & sustained
Concentration	Picomoles	Nanomoles

Table 4.1 Table summarising the characteristics of cNOS and iNOS

haem-containing enzyme guanylate cyclase, producing guanosine 3', 5'-cyclic monophosphate (cGMP). Increases in cGMP then initiate a cascade of intracellular events that eventually results in a decrease in intracellular Ca^{2+}, thus mediating the effects of NO. Therefore, NO and its effects are strongly dependent on levels of Ca^{2+}, thus providing a possible method of NO manipulation (Kirkeboen *et al.*, 1994).

4.3.1 cNOS

cNOS or constitutive NOS is composed of two different enzymes, nNOS and eNOS.

4.3.1(a) nNOS

nNOS was first characterised and purified from rat and porcine cerebellum and has a widespread distribution in the neurones of both the central and peripheral nervous systems, as well as other non-neuronal tissues. Using a combination of Southern blotting and fluorescence *in situ* hybridisation, the precise location of the gene encoding for nNOS has been located to 12q24.2 and to 12q24.2-24.3 (Marsden *et al.*, 1993; Xu *et al.*, 1993). The gene consists of a single copy in the haploid human genome ranging over 200 kb, with a nucleotide sequence spanning 29 exons, coding for a protein of over 1400 amino acids (Schmidt *et al.*, 1991; Snyder and Bredt, 1991).

In neuronal tissues, NO acts as neurotransmitter and hormone, its' release stimulated by excitatory amino acids. In the brain, nNOS is located in 2% of cerebral cortical neurones and is present in dendrites and axons associated with the cerebral vasculature (Snyder and Bredt, 1991). Its' function in the nervous system ranges from peripheral non-adrenergic-non-cholinergic neurotransmission in contractile and

secretory tissues, to synaptic plasticity, learning and memory (Dawson and Snyder, 1994). Despite being a 'constitutive NOS', increasing evidence indicates that nNOS expression can be up-regulated by various physiological and pathological stimuli. Such stimuli include electrical, light, colchicine, formalin, phenobarbitone and hypoxia, indicating that nNOS expression is part of the cellular response to stress (Tsuchiya *et al.*, 1996). This up-regulation may be due to two different mechanisms, one being the general cellular stress response and the other, hypoxia-driven nNOS gene transcription (Kvietikova *et al.*, 1995). Several 'BACGTSSK' binding sites for HIF-1 have been identified along the nNOS genome indicating that this last mechanism may act in a similar fashion to hypoxia-driven VEGF gene transcription. It may involve HIF-1 binding to specific *cis*-acting elements. In addition, studies using rats have demonstrated that nNOS expression can be stimulated by sex hormones such as oestradiol and testosterone, in a variety of rat tissues (Ceccatelli *et al.*, 1996). Down-regulation of nNOS activity has been demonstrated in guinea pig skeletal muscle and rat brain *in vivo* (Gath *et al.*, 1997), with the addition of bacterial lipopolysaccharide and interferon-γ in rat brain, stomach, rectum and spleen (Bandyopadhyay *et al.*, 1997).

4.3.1(b) eNOS

eNOS was first identified in EC's using a specific antibody that localised the enzyme to various arterial and venous endothelium (Forstermann *et al.*, 1991). However, it has been demonstrated also, in human motor neurones (Marsden *et al.*, 1993; Dinerman *et al.*, 1994). Human eNOS mRNA is encoded by 26 exons spanning 21-22 kb of genomic DNA. The gene is present as a single copy in the haploid genome

and has been located to the 7q35-7q36 region of chromosome 7, producing a protein roughly 130 kDa in size (Marsden *et al.*, 1993).

eNOS has two major roles (see Table 4.2); the first is the physiological regulation of vascular tone (Kirkeboen *et al.*, 1994) and the second is its effects on platelets and leucocytes (Radomski *et al.*, 1987). Basal release into the circulation plays an important role in the regulation of blood pressure and blood flow distribution and is therefore, more pronounced in arteries compared to veins and in smaller vessels compared to the larger conductance vessels. eNOS-induced NO release is stimulated also, by both receptor-dependent and independent agonists, such as acetylcholine and free fatty acids respectively. In addition, as vascular EC's act as mechanoreceptors, NO production can be influenced by blood flow characteristics such as viscosity and pulsatility giving rise to 'flow-mediated vasodilation' (Davies, 1995). NO acts as an inhibitor of platelet aggregation, adhesion and activation and on occasion, can act synergistically with prostacyclin (Radomski *et al.*, 1990). Also, as platelets themselves are capable of synthesizing NO, they may find themselves to be under negative feedback control (Radomski *et al.*, 1990). Other effects that NO mediates in the vasculature includes interactions with leucocytes and monocytes/vascular SMC. NO modulates leucocyte-vessel wall adhesion and aggregation by down-regulating the adhesion glycoprotein complex CD11/CD18, and by inhibiting their release of superoxide anions (Kubes *et al.*, 1991; Clancy *et al.*, 1992). Proliferation of vascular SMC's is inhibited also, by both exogenous NO donors and endogenous NO production (Garg and Hassid, 1989). It can be seen therefore, that NO in the vasculature might act protectively as an anti-atherogenic compound.

Effects of NO synthesized by endothelial cells	
Target cells	***Effects***
Smooth muscle cells	Relaxation ⟶ vasodilation
Platelets	Inhibits aggregation, adhesion and activation
Leucocytes	Inhibits aggregation, adhesion and activation
Monocytes/smooth muscle cells	Inhibits mitogenesis

Table 4.2 Table summarising the effects of eNOS

Regulation of eNOS is possible via a multitude of factors. As mentioned previously, up-regulation can occur through changes in the blood flow dynamics within the blood vessel lumen, with the endothelial cells acting as mechanoreceptors. As with nNOS, hypoxia can also modulate NO production. However, the effects of hypoxia would appear to vary with the site of origin of the EC. In human pulmonary EC's, the consensus is that hypoxia down-regulates eNOS expression due perhaps, to both a decreased rate of transcription and an increased destabilisation of its' mRNA (Ziesche *et al.*, 1996). However, in EC's of vascular origin, the effects are inconclusive. Hypoxia-stimulated up-regulation of eNOS mRNA and protein expression in bovine aortic EC's and human vascular EC's, is mediated through enhancement of the 5' promoter sequence (Arnet *et al.*, 1996). However, the human promoter sequence lacks any site homologous to the HIF-1 binding site so it is uncertain how hypoxia exerts its effects. In addition, contradictory results have been obtained using human umbilical vein EC's (HUVECs) in which low oxygen tension resulted in a decreased stability of both eNOS mRNA and decreased promoter sequence activity (McQuillan *et al.*, 1994).

With regards to sex hormones, studies with guinea pigs indicated up-regulation of eNOS mRNA and protein with oestradiol, but not with progesterone (Weiner *et al.*, 1994). These results were duplicated in the aorta's of pregnant rats and in human EC's (Forstermann *et al.*, 1998). This effect was mediated through increased eNOS promoter activity, presumably from enhanced binding of transcription factor SV40 virus promoter specific transcription protein-1, as eNOS lacks the oestrogen-responsive element.

4.3.2 iNOS

iNOS has been located in neurones and EC's but is predominantly found in vascular SMC's and monocytes/macrophages (Denis, 1994). Southern blotting analysis has mapped the gene encoding iNOS to 17cen - 17q11.2, spanning a 40 kb genomic region over 27 exons (Xu *et al.*, 1994; 1996). In addition, multiple iNOS-like sequences have been found in the pericentric region of chromosome 17 and also, in chromosome 14 (Xu *et al.*, 1995).

Expression of this inducible NOS isoform is associated frequently with an inflammatory response resulting from infection, or tissue injury, and is induced by a wide range of microbes, their products and various cytokines (Morris Jr and Billiar, 1994). It plays both a beneficial and harmful role depending on the circumstances of its induction. In a number of clinical situations where there is vascular injury and concomitant endothelial damage such as in severe vascular trauma, or post-percutaneous transluminal coronary angioplasty, eNOS activity may be compromised, thereby requiring iNOS activity as a compensatory mechanism (Yan *et al.*, 1996). On the other hand, as iNOS induction is not dependent on Ca^{2+} intracellular influx, its action can be prolonged, releasing large amounts of NO over several days. This may give rise to a number of pathological conditions such as rheumatoid arthritis (Miyasaka and Hirata *et al.*, 1997), inflammatory bowel disease (Salzmann, 1995), atherosclerosis (Arthur *et al.*, 1997) and septic shock (Thiemermann , 1997).

The stimulation of iNOS activity is dependent on a number of factors. As described previously, NOS requires sufficient substrate, L-arginine and co-factor,

tetrahydrobiopterin (which facilitates dimerisation of two NO synthase monomers), to attain full enzymatic activity (Presta *et al.*, 1998). Thus, the co-ordinated expression of three enzymes, iNOS, GTP cyclohydrolase (the key enzyme in tetrahydrobiopterin synthesis) (Auerbach and Nar, 1997) and argininosuccinate synthetase (required to recycle L-citrulline back to L-arginine) (Xie and Gross, 1997) is required in order to generate the maximum amount of NO. However, once the enzyme is expressed, there seems to be little control over its activity. Moreover, there is a distinct lag phase in iNOS mRNA expression of approximately six hours (in rat aortic SMC's) with a further delay in nitrite production, although expression is sustained for several hours *in vivo* (Liu *et al.*, 1997).

The iNOS gene has been studied extensively in human, mouse and rat. In general, the promoter region of the iNOS gene has several binding sites for nuclear factor κB (NF-κB), AP-1, C/EBP, ATF/CREB and the STAT family of transcription factors (Xie *et al.*, 1994). NF-κB is present in the cytosol as an inactive hetero-trimer, activated by phosphorylation of the inhibitory IκBα subunit (Regnier *et al.*, 1997) and mediates enhanced iNOS expression in stimulated macrophages and SMC's. In addition, an increase in cAMP both increases iNOS expression directly, through stimulation of CREB and C/EBP (Hecker *et al.*, 1997) and indirectly, by potentiating the inductive effect of interleukin 1β on NF-κB (Hirokawa *et al.*, 1994). Protein kinase signalling cascades are thought to be involved including protein kinase A (Boese *et al.*, 1996), PKC (Hecker *et al.*, 1997) and other tyrosine kinases. In common with the other NOS enzymes, modulation of iNOS expression appears to be affected by a feedback loop involving NO. However, NO modulation of iNOS function may serve to either attenuate (Taylor *et al.*, 1997), or accentuate, (Muhl and

Pfeilschifter, 1996) NOS expression depending on the cellular type and species being investigated. This gives rise to the possibility of activated iNOS acting in a deleterious manner, potentiating and producing NO in amounts greater than required.

4.4 NO and its' products

NO and its' products are highly reactive ROS's, able to interact with many substances. In blood, NO is metabolised quickly via interaction with the haem group of haemoglobin, into both nitrite (NO_2^-) and nitrate (NO_3^-) and reacts also, with the thiol (-SH) groups contained in both amino acids and proteins, such as cytochromes, to form nitroso-thiols (-S-NO). In addition, NO will react with the haem-containing cytochromes, thus interfering with oxidative phosphorylation and with ROS to produce the extremely potent peroxynitrite ($ONOO^{2-}$), which is capable of causing cellular damage via lipid peroxidation, nitrosylation, sodium channel inactivation and transitional metal interaction (Kirkeboen *et al.*, 1994).

CHAPTER 5

Possible VEGF and NO Interactions in the Development of Varicose Veins

CHAPTER 5

5.1 Background

How is it proposed that both VEGF and NO are involved in VVs development? A study by Shoab *et al* in 1998, investigated the association of skin damage in the presence of chronic venous disease with plasma levels of VEGF. Histological analysis of the microcirculation in skin affected by lipodermatosclerosis has established that although the number of skin capillaries is not increased, they become elongated and more tortuous, taking on a glomerular rather than a (normal) pin-shaped capillary structure (Junger *et al.*, 1994). The impaired microcirculation therefore, develops an increase in the amount of capillary endothelium of disordered function, demonstrated by a diminished, cutaneous, hyperaemic response (Shields *et al.*, 1994).

Previous studies have indicated increased levels of both VEGF and PDGF in the skin of patients with venous disease and associated skin changes (Dvorak *et al.*, 1995). As VEGF is a known angiogenic agent, it was hypothesised that VEGF reached the main capillaries in the underlying papillary dermis, causing them to proliferate. By experimental induction of 'venous hypertension' in controls and patients with venous disease (with and without skin changes), they noted that patients with venous disease had a higher resting plasma level of VEGF compared to controls, which increased after induction of venous hypertension; the increase being greater in subjects with venous disease. This association was reinforced by another study from Shoab and co-workers (1999), which demonstrated a decrease in VEGF levels in patients with

venous disease and skin changes after treatment with the vaso-active flavonoid compound, Daflon (Servier Laboratories, France).

The association with NO levels and VVs has also demonstrated a similar relationship. A study by Schuller-Petrovic and co-workers (1997), investigated the levels of several vasodilators and vasoconstrictors released from cultured cells derived from controls and subjects with primary VVs. In addition, plasma levels of other veno-active substances were measured. They concluded that EC's derived from VVs secreted less venoconstrictor mediators than controls and that there was up-regulation of the NO/cGMP vasodilator system. Further, plasma levels of cGMP were also raised, while levels of the venoconstrictor angiotensin II were depressed in subjects with VVs.

So, it is suggested that both levels of VEGF and NO are raised in VVs subjects. Both substances are vasodilatory and are stimulated by EC's and other cell types in the vascular wall in response to venous stasis, venous 'hypertension' and venous hypoxia, situations that are all present in CVI (McEwan and McArdle, 1971). In fact, the raised levels of both these substances may explain other features found in subjects with VVs. VEGF is known to selectively promote extravasation of fibrinogen from the vessel and its deposition into the extra-luminal space as fibrin. This may account for the pericapillary fibrin cuffs found in venous ulceration that are thought to worsen/cause the resulting hypoxia (Burnand *et al.*, 1976; Montesano *et al.*, 1996). NO on the other hand, is capable of reacting with O_2^- to form peroxynitrite ($OONO^-$), which can then generate hydrogen peroxide (H_2O_2), hydroxide ions (OH^-) and nitrite (NO_2^-), all of which are highly reactive and destructive ROS. These

substances may account for the cytotoxicity displayed by macrophages and are capable of significant venous wall and EC damage. In addition, it has been demonstrated that the expression and activity of matrix metalloproteases (MMP) are increased in the wall of VVs (Parra *et al.*, 1998) and are able to degrade a number of vessel wall constituents such as collagen and elastin. Furthermore, hypoxia-activated EC's are hypothesised to promote SMC phenotype modification from 'contractile' to 'secretory', contributing to the thickened intima seen. Therefore, this combination of MMP's, ROS and modified SMC's may be responsible for the ECM changes noted on histological analysis of VVs. Activated polymorphonuclear neutrophils (PMN) are a potent source of further ROS release and hydrolases and as a result of venous stasis, become adherent to the surface of EC's, initiating endothelial activation that in turn, may lead to further VEGF and NO release. The combined destructive effects of ROS, hydrolases, MMP's and PMN activation may lead to altered endothelial function so that the EC's lose normal homeostatic control of venoconstriction and venodilatation, resulting in up-regulation of both VEGF and NO production and secretion.

Moreover, VEGF has itself been suggested as a stimulant of NO production (Liu *et al.*, 1994). Studies observing VEGF's effect on porcine myocardial function, indicate that it both induces NO-mediated hyperpermeability of coronary venules and NO-mediated hypotension, raising the possibility that the hyperpermeability seen with VEGF is in fact a function of NO release rather than being intrinsic to VEGF itself. VEGF is thought to increase NO via stimulation of iNOS, guanylate cyclase and cGMP-dependent protein kinase (Hariawala *et al.*, 1996; Wu *et al.*, 1996). Therefore, the situation could arise in which venous stasis, hypertension and hypoxia

stimulate production of VEGF and NO from EC's, SMC's and monocytes, potentiated by VEGF acting in a positive feedback loop, to further increase levels of NO.

5.2 Hypothesis

(Figure 5.1 summarises the potential pathways involved in VV's aetiology). As both VEGF and NO are vasodilatory, it is proposed here that VVs develop as a result of aberrant dilatory responses as part of the normal vein homeostatic mechanism, in response to either changes in venous blood chemistry, or intraluminal pressure. If the conditions that initiated the homeostatic response are not resolved, despite venodilatory stimuli, or if the vein wall has been so damaged by ROS, hydrolases and MMP's rendering it incapable of mediating an appropriate response, an excess of both VEGF and NO production results (with the involvement of both activated PMN's and macrophages) due to a positive feedback loop (Dembinska-Kiec *et al.*, 1997). The end result is a weakened, thinned and dilated vessel wall unable to resume its previous tonic state, prompting further dilatation and possible VVs development.

In order to investigate this hypothesis, it is necessary to first establish evidence that refutes the 'descending valvular incompetence' theory as the sole cause of varicosity. The Middlesex hospital contains a vascular Duplex laboratory to assess the valvular state of all referrals with VVs. A detailed audit of subjects with primary VVs only, and noting the state of their SFV will hopefully provide enough information in order for us to proceed.

Next, a clinical trial that includes controls and subjects with primary VVs is required in which experimental venous stasis is induced in the lower limbs with measurements of plasma VEGF and NO levels pre- and post-inducement of venous stasis. This will provide us with information regarding the basal levels of these two substances and of any changes in their production following venous stasis. Furthermore, evaluation of VEGF gene induction can be performed on the cDNA of PBMC's isolated from subject venous plasma to assess the effect of venous stasis on gene expression.

Lastly, to aid in further investigation of the possible molecular mechanisms involved in varicose aetiology, the development of an *in vitro* model that more closely mimics the expected *in vivo* cellular interplay of the vein wall layers of intima, media and adventitia, is required.

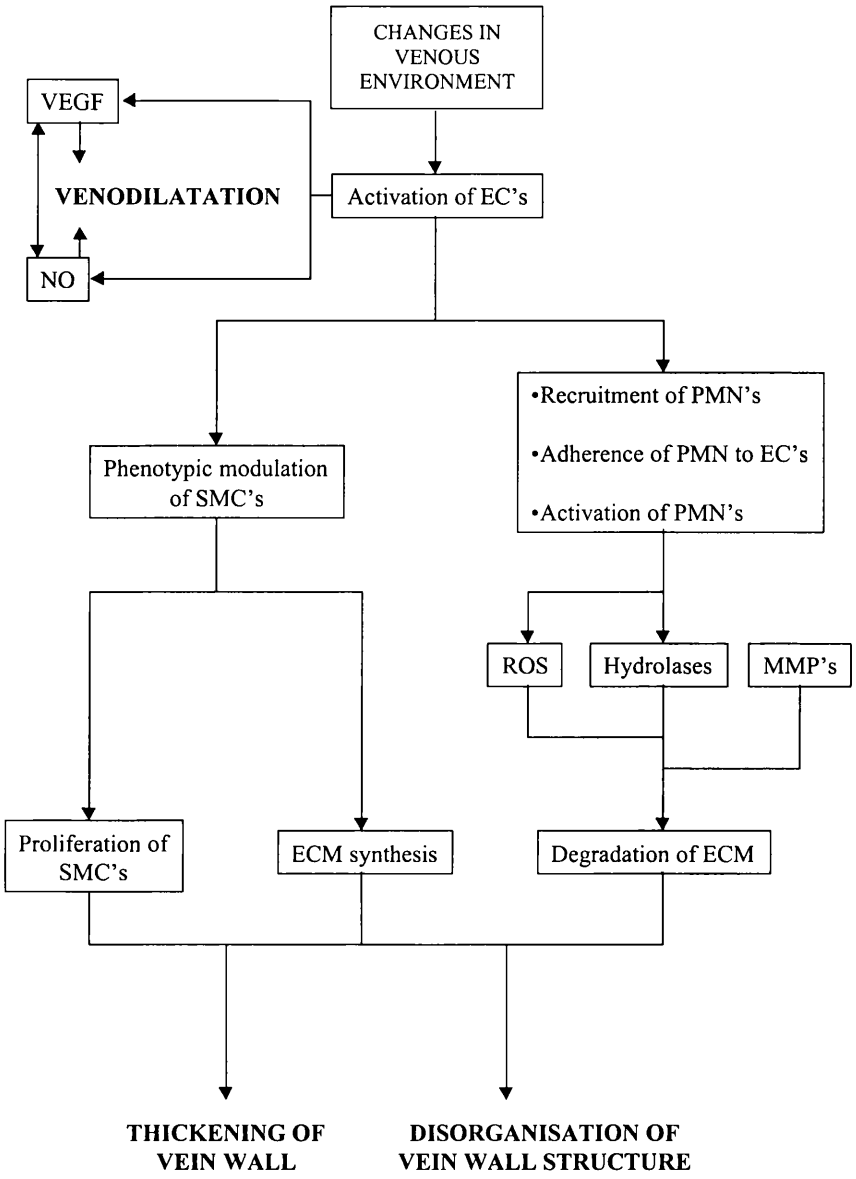


Figure 5.1 Schematic of hypothesis

Schematic representation of the proposed biochemical mechanism linking venous stasis in the lower limbs to VVs aetiology and alterations in vein wall. Bold type represents the end result. **EC**: endothelial cell, **VEGF**: vascular endothelial growth factor, **NO**: nitric oxide, **PMN**: polymorphonuclear neutrophils, **ROS**: reactive oxygen species, **MMP**: matrix metalloprotease, **ECM**: extracellular matrix, **SMC**: smooth muscle cell.

MATERIALS AND METHODS

CHAPTER 6

Primary Varicose Veins in the Presence of an Intact Sapheno-Femoral Valve

CHAPTER 6

6.1 Background

The theory of an 'incompetent valve' is probably the most widely taught and believed theory of VVs causation (Lake *et al.*, 1942; Burkitt, 1972) however, studies have been performed disputing this (Basmajian, 1952; Browse *et al.*, 1999). In addition, it has been suggested that VVs are more common in females compared to males (Brand *et al.*, 1988) and that the prevalence increases with age (Abraham *et al.,* 1981; Coon *et al.*, 1973) although here again there is much dispute (Evans *et al.*, 1999).

In order for us to proceed with our investigation and to relate any results to the local population, it is necessary to perform an audit to determine the incidence of VVs in the presence and absence of a competent SFV and to examine how this may be affected by both age and gender.

6.2 Audit methods

A retrospective analysis was undertaken on all venous, colour Duplex ultrasound scans performed on patients referred by both general practitioners and hospital-based doctors for assessment of their primary VVs. Duplex ultrasound combines echo-pulsing with Doppler velocity recording and allows both direct visualisation of the veins and identification of blood flow (although strictly, velocity) through venous valves. Scans were performed in the Vascular Laboratory, at the Middlesex Hospital, London over an 18 month period during 1998 to 2000. Experienced technicians, using a General Electric duplex machine (GE Medical Systems, PO Box

414, Milwaukee, Wisconsin, 53201, USA), were able to pinpoint areas of venous reflux associated with incompetent venous valves, aided by pressure-induced colour-coded flow analysis, where red areas were associated with laminar blood flow (or flow away from the heart) and blue areas were associated with turbulent blood flow (or flow towards the heart). Scans reporting VVs associated with the short saphenous system only or in conjunction with other valvular abnormalities, were excluded.

6.3 Analysis of results

A record of primary VVs presenting in the presence, or absence, of a competent SFV was made. Female:male ratios were determined. All data was subsequently re-assigned in to '10 year' age bands, to enable both the incidence of VVs with overall increasing age, and the incidence of VVs with overall increasing age either in the presence, or absence, of a competent SFV, to be analysed.

6.4 Statistics

Data was summarised by descriptive statistics only and the results presented as ratios and percentages and for age, as medians, with an inter-quartile range. No further statistical comparisons were made.

CHAPTER 7

VEGF and NO Plasma Levels with Experimentally-Induced Venous Stasis

CHAPTER 7

7.1 Background

The lower limbs in humans are subjected continuously to variations in intra-luminal venous pressure during the course of a normal day (Somerville *et al.*, 1974). These changes, ranging from venous hypo- or hypertension are actively countered by the homeostatic mechanisms of the vein wall, which attempt to minimise changes either by veno-constriction or dilatation, respectively. As dilatation is intrinsic to the definition of VVs, could VVs in fact result from a damaged, deficient, or variant vein wall homeostatic mechanism?

VEGF, an important factor in maintaining and preserving vascular integrity and responsivity, works in concert with NO to mediate vascular permeability and dilatation (Hariawala *et al.*, 1996; Wu *et al.*, 1996). The aim here was to assess the ability of primary VVs to produce and release these two important factors in response to induced hypoxic stress.

7.2 Local research ethics

Application was made to the Joint University College London and University College London Hospitals Committees on the Ethics of Human Research for approval to perform the following study. Ethics committee approval was granted before commencement of the study.

7.3 Recruitment of subjects

Individuals with primary VVs were recruited from among those attending the vascular outpatients clinic at the Middlesex Hospital, London. Normal, 'control' subjects were recruited amongst the staff and medical students of the Royal Free and University College London Medical School. All participants were given detailed information leaflets that stated clearly the purpose of the study and which explained the bloodletting procedure.

7.3.1 Subject consent

Following a full explanation of the experimental protocol and reasons for performing the research study, each participant was invited to volunteer and hence, to sign an informed consent form. All subjects were allowed to withdraw from the study, at any point and for any reason, if they so wished.

7.3.2 Inclusion and exclusion criteria

Subjects were carefully selected to fulfill the study's criteria. A complete medical history was acquired. Further information from hospital notes and general practitioners was sought, when needed, to complete the subjects' history. All the participants had to be in good health. A list of exclusion criteria was followed, when recruiting, for every volunteer.

The criteria for exclusion in the study were;

- established cardiovascular disease
- a history of cancer

- an invasive procedure, or trauma, in the last three months
- a recent infection
- being a smoker
- having a coagulopathy
- administration of any regular medication that might affect vascular tone
- pregnancy

Established cardiovascular disease was defined as evidence of one or more of the following;

- hypertension
- previous coronary episode
- angina
- coronary bypass graft surgery
- cerebrovascular episode

7.4 Subject groups

The volunteers were divided into four groups according to sex and the presence, or absence, of primary VVs. The groups were as follows;

Group	*Subject*
1.	Female with primary VVs
2.	Female without primary VVs
3.	Male with primary VVs
4.	Male without primary VVs

7.5 Collection of peripheral blood samples

All subjects were rested supine on a couch and allowed to relax for 10 minutes. The LSV at the ankle was identified and cannulated with a disposable 18 French size butterfly needle. In VVs subjects, this was positioned on the leg with the varicosity. 5 mls of blood was drawn in to EDTA bottles (Vacutainer®) from the LSV, along with 5 mls of blood from a peripheral vein in the antecubital fossa. A sphygmomanometer was then applied 'below-knee' at a pressure of between 90 - 95 mmHg, for 10 minutes, to produce a relative venous stasis, after which a further 5 mls of blood was drawn from the same butterfly needle at the ankle. All venous blood samples were kept in an ice bath until processed in the laboratory. Although, it would have been beneficial to obtain samples from the unaffected limbs of VVs subjects as a comparison given the limitations of a busy out-patient department this was not practical.

7.6 Processing of peripheral blood samples

Processing of the samples involved separating the serum and the mononuclear white blood cells from the rest of the blood constituents. All blood samples were brought to room temperature and layered on to an equal volume of density gradient (Histopaque 1077; Sigma, UK) in a clean, sterile, 50 ml Bluemax tube (Becton Dickenson, UK). The tubes were then spun at 400 x *g* for 30 minutes at room temperature, with the brake off. Four phases were obtained; the uppermost phase consisted of serum, the second phase contained lymphocytes and monocytes ('buffy coat') localised to a 'milky', thin layer lying just below the serum. A thick layer of density gradient then interposed, under which was found the third phase, consisting of a thin, white layer containing the polynuclear white blood cells. The remaining lower fraction consisted of the remaining granulocytes and erthyrocytes.

7.6.1 Collection of peripheral blood plasma

Using a clean pipette, the uppermost serum phase was aspirated carefully, so as not to include any of the underlying buffy coat and transferred to a fresh 15 ml Bluemax junior tube (Becton Dickenson, UK), where it was spun at 250 x *g* for 10 minutes (at room temperature) to collect any cells by centrifugation. Again, using a clean pipette, the serum was aspirated, making sure that the cell pellet was not disturbed. This was then transferred to 2 ml Cryo Vials (NUNC, UK), where they were immediately frozen at –20 °C.

7.6.2 Isolation of peripheral blood mononuclear cells (PBMC's)

From the centrifuged-separated blood, the 'buffy coat' layer was transferred using a clean pipette, to a fresh 15 ml Bluemax junior tube (Becton Dickenson, UK).

Phosphate buffered saline (PBS) was added to a total volume of 10 ml and the sample centrifuged at 250 x *g*, for 10 minutes (at room temperature). Following centrifugation, the PBMCs formed a pellet at the bottom of the tube. The supernatant was discarded and the cell pellet re-suspended in 2 mls PBS. This washing procedure was repeated twice, with the pellet being re-suspended in a final volume of 1 ml of PBS.

The PBMC cell pellet was then lysed by the addition of 1 ml of Tri® reagent (T9424; Sigma, USA); a mixture of guanidine thiocyanate and phenol in a mono-phase solution - this effectively dissolves the DNA, RNA and protein. The sample was aspirated gently to ensure total cell lysis and allowed to stand at room temperature for 10 minutes. Samples, at this stage, were either frozen at –20 °C or processed for the next step.

CHAPTER 8

Laboratory Molecular Studies

CHAPTER 8

8.1 Background

In order to determine the relative importance and involvement of either VEGF or NO in the aetiology of VVs, it is necessary to examine the levels of both factors in the serum of subjects enrolled in the study. An enzyme-linked immunosorbent assay (ELISA) was used to detect VEGF, while the Griess reagent was used specifically, to detect NO. However, establishing the levels of these factors in the serum tells only one part of the story. To evaluate properly any possible role that they may play in the development of VVs, the source of these changes must be established. One way in which levels of any factor can be modified, is by gene induction. This can be investigated using techniques to isolate mRNA, produce complementary DNA and run polymerase chain reactions (PCR), looking for specific DNA sequences and then running the PCR products on an electrophoretic gel to produce fluorescent bands which can be analysed subsequently. In this case, the gene for VEGF was isolated and any 'up' or 'down' turn in its' induction was established by reading changes of the optical density (OD) values for its' respective PCR bands, using a gel spectrophotometer.

8.2 Analysis of plasma levels of VEGF

Measurement of plasma VEGF was performed using an established protocol.

8.2.1 Basics

Measurement of the levels of VEGF in the plasma of blood samples collected from the peripheries, were performed using an indirect ELISA method. The VEGF

ELISA is a 'sandwich' enzyme-linked immunosorbent assay. It uses enzyme-conjugated antibodies to detect and quantitate the concentration of soluble cytokines, or chemokine proteins, with high specificity and sensitivity. The 'sandwich' consists of 'capture antibodies' (anti-VEGF antibodies) which are 'coated' (non-covalently adsorbed) on to the sides of plastic well plates. These 'capture antibodies' bind to their antigen (VEGF), thus immobilising them to the sides of the well. 'Detection antibodies' (which are biotin-conjugated anti-cytokine antibodies) are then added, binding both to the 'captured' VEGF and to the addition of a chromogenic substrate-containing solution (horseradish peroxidase-labelled streptavidin) which produces a colour change. The intensity of the colour change is proportional to the amount of bound antigen (VEGF). This change can then be measured spectrophotometrically, using a microplate reader set to read light at an appropriate wavelength, to give an individual value of OD. However, this reading can only be interpreted when compared against a 'standard curve' (calibration curve). This is a plot of known concentrations of the antigen in pg/ml (produced by serial dilutions of a known 'standard') against the mean OD values, so that the OD readings obtained from the ELISA assay can be converted into concentrations by referring to the 'standard curve'. Since conditions may vary, a separate 'standard curve' must be produced for each ELISA plate.

A commercially available kit from R&D Systems, UK was used according to the manufacturer's instructions.

8.2.2 Reagents

All the reagents, unless specified, were supplied by R&D Systems, UK. PBS, carbonate/bicarbonate, TWEEN 20, Bovine Serum Albumin (BSA) and sucrose were purchased from SIGMA, UK (see the following table). In this assay, all the solutions used were made fresh, each time, unless stated otherwise and kept at room temperature for the duration of the assay.

Reagent	***Preparation***
Carbonate/bicarbonate	0.5M pH=9.6
PBS	1 litre de-ionised water, 5 tablets of PBS
Washing Buffer	PBS with 0.05% TWEEN 20
Blocking Buffer	PBS with 1% BSA, 5% Sucrose
TBS	100 ml de-ionised water (pH 7.3), 0.24g of Trizma base, 0.8g of NaCl
Diluent	TBS with 0.1% BSA, 0.05% Tween 20

8.2.3 Indirect ELISA for VEGF

All samples and reagents were first defrosted and brought to room temperature before use. The capture antibody, goat anti-human clonal $VEGF_{121}$ and $VEGF_{165}$ antibody (AF-293-) (stock concentration 100 μg/ml) was diluted to a concentration of 0.4 μg/ml in carbonate/bicarbonate buffer (40 μl stock solution in 10 mls carbonate/bicarbonate buffer) and 100 μl was pipetted into each well of a plastic Maxisorb® micro-titre, flat bottomed, 96-well plate (NUNC, UK), covered with

plastic cling film and left overnight for 16 hours at 4 °C. The antibody was then forcibly discarded and the plate washed 3 x with 250 µl wash buffer per well, carefully tapping the excess on to a paper towel. Following this, 250 µl of blocking buffer was added to each well, left covered in cling film for two hours (at room temperature) and then washed a further 3 x with wash buffer as above.

The recombinant human VEGF (293-VE) standard serial dilution was set up in pairs in the top two rows of the Maxisorb® plate ranging from 4000 pg/ml to 7.75 pg/ml. The VEGF standard stock solution was supplied at a concentration of 10 µg/ml. The method of dilution was as follows; 4 µl of the stock VEGF was added to 96 µl of diluent to produce a concentration of 0.4 µg/ml. 10 µl of this solution was pipetted and added to 990 µl of diluent, to produce the required concentration of 4000 pg/ml. Using a multi-channel pipette, 100 µl was pipetted into the wells of the first and second column (two wells in each column). 100 µl of diluent was then drawn up, again using a multi-channel pipette and the tips placed into the two wells of the second column, being careful to position them so as not to touch the sides or bottom of the wells which could dislodge the capture antibodies. The wells were then mixed carefully in a volume of 200 µl thus diluting the concentration of VEGF standard to 2000 pg/ml. 100 µl (half of 200 µl) was pipetted back with the multi-channel and re-pipetted into the two wells of the adjacent third column (2000 pg/ml). Again, 100 µl of diluent was drawn up in a multi-channel pipette and positioned into the two wells of the third column (2000 pg/ml), mixed as before and 100 µl taken up and re-pipetted into the next 2 wells of the fourth column (1000 pg/ml). This process was repeated until the concentration of VEGF standard was 7.75 pg/ml in the wells of the tenth column. The four wells of the eleventh and twelfth columns contained diluent

only (0 pg/ml). The serum samples were defrosted and pipetted, diluted 1:1 in diluent, in to the plate in pairs. This procedure was performed as follows; 100 µl of serum was pipetted into a well containing 100 µl of diluent and mixed gently. 100 µl was redrawn and pipetted into the adjacent, inferior well. In this way, all 96 wells of one plate were filled. The plate was again covered with cling film and left for two hours at room temperature, then washed a further 3 x with wash buffer.

100 µl of 0.2 µg/ml biotinylated anti-human VEGF detection antibody (BAF293) (stock concentration 50 µg/ml) was added per well (40 µl of stock solution in 10 mls of diluent), the plate again covered and left for a further two hours (at room temperature). The plate was washed 3 x with wash buffer and then 100 µl of 1:50000 ExtrAvidin® conjugated to Alkaline Phosphatase (E2636; Sigma, UK) (serial dilutions with 5 µl of Streptavidin in 95 µl diluent (1:20 dilution), then 20 µl of the 1:20 was added to 80 µl of diluent (1:100), followed by 20 µl of 1:100 added to 9980 µl of diluent (1:50000)) was added per well and left for 30 minutes at room temperature. The plate was washed a final 3 x with wash buffer following which, 100 µl of 1-p Nitrophenyl Phosphate (pNPP) (Sigma Fast®, UK) substrate solution was added per well (one tablet of pNPP and one tablet of TBS in 20 mls of de-ionised water). The plate was re-covered with cling film and left for 60 minutes, at room temperature, in the dark, after which the absorbance (OD value) in each well was read using a spectrophotometric plate reader (Dynex) set at 405 nm. The mean of at least two separate experiments (each with duplicate wells/sample or standard) was calculated and used for further analysis.

8.3 Analysis of levels of plasma NO

The levels of NO in the plasma of all subjects participating in the study was measured using a commercially available assay that measured total nitrites, from R&D, UK.

8.3.1 Basics

Direct measurement of NO is difficult, as it is a transient and volatile gas. As most of the NO is oxidised to both nitrite (NO_2^-) and nitrate (NO_3^-), measurements of these anions can be used as indirect measurements of NO production. The conversion of NO into nitrate and nitrite is via the following reactions:

Eq 1. $\cdot NO + \cdot O_2^- \rightarrow ONO_2^- \rightarrow NO_3^- + H^+$

Eq 2. $2 \cdot NO + O_2 \rightarrow N_2O_4 \rightarrow NO_2^- + NO_3^- + 2\ H^+$

Eq 3. $\cdot NO + \cdot NO_2 \rightarrow N_2O_3 \rightarrow 2NO_2^- + 2\ H^+$

The total NO assay involves the enzyme nitrate reductase, converting all of the nitrate to nitrite and then via the Griess reaction, which allows the spectrophotometric measurement (OD) of nitrite at 540 nm. The Griess reaction is based on a two step diazotization reaction in which acidified NO_2^- produces a nitrosating agent, which reacts with sulfanilic acid to produce the diazonium ion. This ion is coupled to *N*- (1-naphthyl) ethylenediamine, to form the chromophoric azo-derivative which absorbs light at 540 nm. The intensity of the colour change is proportional to the amount of nitrite in the system. In order to interpret the results, a nitrate standard 'calibration curve' is produced in each plate. This consists of a dilution series of a known concentration of nitrate, ranging from 100 μmol/l to 3.12

μmol/l, plotted against their respective OD readings. In this way, OD readings can be compared against the OD values produced by the 'calibration curve' and a concentration measurement made.

8.3.2 Samples

All plasma samples were diluted two-fold in Reaction Buffer (30 mls of 10 x Reaction Buffer concentrate diluted with de-ionised water to produce 300 mls of 1 x Reaction Buffer). 100 μl of serum was mixed with 100 μl of Reaction Buffer in a 10,000 molecular weight (MILIPORE, UK) cut-off filter and spun at 400 x *g*, for 40 minutes at 4°C to eliminate proteins.

8.3.3 Reagents

All reagents, unless specified, were provided by the manufacturer and prepared according to their instructions. All reagents were kept at room temperature, for the duration of the assay, except the reconstituted beta-Nicotinamide adenine dinucleotide (NADH) and Nitrate Reductase that were kept on ice.

8.3.4 Greiss reagent determination of total nitrites

A total NO Assay kit (R&D Systems, UK) was used. The procedure was performed using a 96 well plate (supplied) with the nitrate standard arranged in pairs. The nitrate standard was supplied as a 1,000 μmol/l concentration. To produce the dilution series with 100 μmol/l as the high standard and 0 μmol/l as the zero standard, six tubes were labelled 100, 50, 25, 12.5, 6.25 and 3.12 μmol/l. 900 μl of reaction buffer was pipetted into the 100 μmol/l, tube while 500 μl of the reaction buffer was pipetted in to each of the remaining tubes. Using the 1,000 μmol/l stock,

100 µl was pipetted in to the 100 µmol/l tube. It was mixed thoroughly and after changing the pipette-tip, 500 µl was drawn up from the 100 µmol/l tube and mixed in the 50 µmol/l tube. The pipetting action was repeated with a fresh pipette-tip each time until the final mix in the 3.12 µmol/l tube. Reaction buffer by itself served as the 'zero standard'. The first two rows of the 96 well plate were set aside for the 'nitrite standard'. In descending concentration, starting with 100 µmol/l, 50 µl of each concentration was pipetted in to the two wells of each column as a pair, the last column containing 50 µl of reaction buffer representing the 'zero standard'. 200 µl of reaction buffer was pipetted into the remaining wells of the first two rows. 50 µl of the already diluted and filtered serum was added in duplicate in to the remaining wells.

The next step required the addition of NADH reagent to all wells. This had been reconstituted with 1 ml of de-ionised water, mixed and allowed to sit for three minutes, on ice. Immediately before use, 900 µl was diluted with 1.8 mls of de-ionised water and 25 µl added to each well.

The addition of nitrate reductase involved its reconstitution in 1 ml of nitrate reductase storage buffer followed by two rounds of vortexing and sitting at room temperature for 15 minutes, with a last vortexing after which it was kept on ice. Before use, the nitrate reductase was to be diluted according to the following protocol;

Step	*Process*
1.	nitrate reductase(μl) = (number of wells + 5) x 5 μl
2.	reaction buffer (μl) = (volume from 1) x 4 μl
3.	volumes from (1.) and (2.) were added in a tube and vortexed
4.	final volume was placed on ice and 25 μl was added to each well within 15 minutes of its, dilution

The wells were mixed by tapping gently on the side of the plate and then covered with an adhesive strip for 30 minutes (at room temperature). 50 μl each of both Griess Reagent I (sulfanilamide in 2N Hydrochloric acid) and II (*N*-(1-naphthyl) ethylenediamine in 2N Hydrochloric acid) were added to each well, except the blank wells, mixed by gentle tapping on the side of the plate and then, left to incubate for 10 minutes at room temperature. Finally, after 60 minutes, the plates were read using a microplate reader (Dynex) set at 540 nm and OD values recorded.

Standard curves were performed for each experiment and for analysis the mean of at least two separate experiments, each with duplicate standard/sample wells, were calculated and used for further analysis.

8.4 Preparation of PBMC's

PBMC's were isolated and prepared as described in section 7.6.2.

8.4.1 Isolation of total cellular mRNA

The RNA isolation from the solubilised mononuclear white blood cells was performed following a protocol proposed by SIGMA, UK for use with the lysing agent Tri® reagent (Chomczynski, 1993). All work was carried out on ice, to inhibit any activity by contaminating RNA'ases.

The solubilised PBMC nucleoprotein in Tri® reagent was left at room temperature for five minutes to ensure complete dissociation of any nucleoprotein complexes. The purification of nucleic acids required the removal of proteins and was accomplished with the addition of chloroform. In this case, 0.2 ml of chloroform was added, the sample shaken for 15 seconds and then allowed to stand for 10 minutes, at room temperature. This combination of chloroform, with the phenol contained in the Tri® reagent, has several advantages. First of all, de-proteinisation is more efficient when two different organic solvents are used instead of just one. Second, the combination of the two solvents inhibits RNA'ase activity. Third, it prevents the localisation and exclusion of RNA molecules containing long tracts of poly-(A) sequences and fourth, the chloroform helps to remove all trace of the phenol from the nucleic acid isolate. Centrifugation at 12,000 x *g* for 15 minutes, at 4 °C, separated the Tri® reagent/choroform mixture into three phases; an uppermost, colourless aqueous RNA phase, a narrow middle DNA interphase, and a lower, red organic protein phase. The aqueous RNA phase was transferred, being careful not to aspirate any of the DNA containing interphase, using a Gilson's pipette to a fresh Eppendorf tube (Sarsted, UK), containing 0.5 ml of isopropanol (0.5 ml per ml of Tri® reagent used). The sample was allowed to stand for 10 minutes at room temperature, followed by centrifugation at 12,000 x *g* for 10 minutes at 4°C. The

RNA precipitated as a pellet on the side and bottom of the tube. The supernatant was discarded and the RNA pellet washed with 1 ml of 75% ethanol, followed by a final spin at 7,500 x *g* for 10 minutes at 4°C. The supernatant was again discarded and the RNA pellet allowed to air dry for 10 minutes after which it was re-suspended in 30 µl of water and immediately stored at –20°C.

8.4.2 Preparation of complimentary DNA (cDNA)

cDNA was prepared from the isolated RNA following a standard protocol as proposed by GIBCO BRL. The protocol was optimised for the specific reactions performed.

When working with RNA it is important not to introduce contamination with RNA'ases, which could degrade the RNA and reduce the yield. Therefore, sterile, disposable, plastic gloves were worn at all times and periodically rubbed with 70% alcohol during use. All surfaces were sprayed with 70% alcohol and all plastics/glassware autoclaved.

The total PBMC RNA had already been isolated in Section 8.4.1 and suspended in 30 µl of de-ionised water. Reverse transcription is the production of cDNA from RNA. Synthesis of the first strand of cDNA requires the action of RNA-dependent DNA polymerase (reverse transcriptase) which itself requires the presence of primers to initiate DNA synthesis. These primers are present in large molar excess to enable each molecule of mRNA to bind to several molecules of primer, with priming of cDNA synthesis occurring initially from the most proximally attached primer. Synthesis of the second strand of cDNA is initiated with self-priming by the first

strand. This process begins with the formation of a hairpin structure at the 3' end of the single stranded cDNA. To enable this hairpin structure to form, it is necessary to first denature the cDNA:mRNA construct by boiling, which allows the action of reverse transcriptase to synthesise the complementary cDNA strand. The resulting construct consists of a double-stranded cDNA molecule joined at what was the 5' terminus of the previous mRNA strand.

A volume of 40 µl of cDNA was prepared for each sample, which allowed enough quantity for repeat PCR reactions. All the following procedures were performed on ice to prevent any possible degradation of RNA by RNA'ase contamination. In an Eppendorf tube (Sarsted, UK), 1 µl of random primers (500 ng/µl) (C1181; Promega, USA) and 3 µl of de-ionised water were added to 20 µl of the RNA solution, making a total volume of 24 µl. Random primers were stored at a concentration of 3 µg/µl in 3 mM Tris-HCL (pH=7), 0.2 mM EDTA and therefore, a dilution of 1:6 in sterile water was used for the reaction. This was mixed gently with a pipette, placed in a thermal cycler (Techne Progene), heated to 70 °C for 10 minutes and then chilled to 4 °C. Next, 8 µl of 5 x buffer, 4 µl 0.1M DTT and 2 µl of 10mM dNTP (10 µl each of 100 mM ATP, TTP, GTP and CTP, 60 µl de-ionised water, all Promega, USA) were added and mixed gently, making a total volume of 38 µl. This was placed in a thermal cycler, incubated at 42 °C for two minutes. Then, 2 µl of Moloney Murine Leukaemia Virus reverse transcriptase (M-MLV RT, Promega, USA) was added, with a further incubation at 42 °C for 50 minutes. Finally, the reverse transcriptase was inactivated by heating to 70°C for 15 minutes and the now cDNA solution, chilled to 4°C.

8.5 Molecular analysis of PBMC's

The cDNA prepared, as described in section 8.4.2, from the mononuclear PBMC's, was used for the study of the VEGF and Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH-3) genes by polymerase chain reaction.

8.5.1 Polymerase chain reaction (PCR)

PCR is used to isolate and amplify a specified sequence of DNA defined with the use of short complementary sequences of DNA known as primers, directing the action of DNA polymerase. These primers are designed to be exactly complementary to the template required so that efficient hybridisation to the target sequence occurs with negligible hybridisation to other related sequences in the sample. They exist as a 'pair', known as 'forward' and 'reverse' primers, consisting of slightly different sequences, binding at opposite ends of the DNA strand and flanking the desired DNA sequence between them. PCR consists of three steps;

Step	*Condition*
1.	Denaturation
2.	Primer annealing (hybridisation)
3.	Elongation (extension)

In the above steps, it is important to keep the time spent for each step to a minimum, as too much time may cause deterioration of the DNA polymerase. Optimisation of these times and temperatures is the main difficulty in producing an efficient PCR.

Denaturation is required to 'unwind' and separate the two strands of the cDNA molecule, thus exposing the nucleotide sequences to the action of DNA polymerase and allowing primer annealing. A typical denaturation step would require incubation for more than two minutes, at a temperature of 94 °C.

Primer annealing occurs at a lower temperature than that for denaturation, the efficiency of which is affected by the length of the primers and their concentration. An estimation of the annealing temperatures required for each primer can be made by counting the number of double hydrogen bonds (between nucleotides adenine and thymidine) and triple hydrogen bonds (between guanidine and cytosine) contained within the primer sequence. The annealing temperature (T_a) in degrees centigrade, can then be estimated using the following formula;

$$T_a = (2\ (A+T) + 4\ (G+C)) - 4$$

However, this is only an estimate as the T_a actually used in the PCR must take into account the different individual T_a for both the forward and reverse primers, the concentration of both the template DNA and primers and the exact buffer conditions.

Elongation of the annealed primers is the last step in the PCR cycle and relies on the activity of a thermostable DNA polymerase purified from the thermophilic bacterium *Thermus aquaticus* (*Taq*). This enzyme is stable at 95°C and so, will not be degraded in the heat denaturation step and therefore, will not need to be replenished during the cycle. In addition, as annealing can occur at higher temperatures, mis-priming is reduced, thus improving both the specificity and yield of the reaction.

Elongation is traditionally performed at 72°C and as approximately 60 bp are synthesized every second, the time taken is dependent on the length of the desired DNA sequence. In practice, this is usually around 1-2 minutes. The cycle of denaturation, annealing and elongation is repeated many times, with the amount of product increasing exponentially from the third cycle onwards. This increase accumulates according to the formula;

$$N_f + N_o (1 + Y)^n$$

Where N_f = final copy number of target DNA sequence

N_o = initial copy number

Y = the efficiency of primer extension per cycle

^{n} = number of PCR cycles under conditions of exponential amplification

However, this exponential amplification is not an unlimited process as once the number of copies reaches around 10^6, the enzymes become rate limiting and continuation beyond 30 cycles of amplification will result in a decrease in the specificity, efficiency and fidelity of the process. This can result in the amplification of un-specified bands, the appearance of small deletion mutant bands, or even the disappearance of the desired sequences. If more product is desired, aliquoting and diluting a small amount of the DNA sample up to 10,000, fold which then undergoes a fresh PCR cycle, can increase the yield up to 10^{10} copies over the course of 60 PCR cycles.

In addition to the above steps, further optimisation of the PCR can be achieved by initial incubation without DNA polymerase. This is performed at 94 °C, or at a temperature above the T_a of the primers and can aid in reducing the degree of primer dimerisation and non-specific priming, which may occur during heating of the reactants from cold. This stage, known as 'Hot Start', occurs only once and typically lasts for four minutes, at the end of which the DNA polymerase is added.

Therefore, it is impossible to have a standard set of conditions that will always guarantee the successful amplification of DNA fragments required. The PCR reaction used in this study was developed in order to be able to amplify all the secreted subtypes of VEGF. Experiments were performed (with the aid of Mr Michael Dialynas) in order to optimise the conditions. Different annealing temperatures, incubation timing and magnesium chloride ($MgCl_2$) concentration titrations were tried until the optimal conditions were achieved. VEGF (as described in the 'Introduction') exists as five subtypes; 121, 145, 165, 189, and 206 amino acids. The 121 and 165 (secreted) subtypes constitute the main focus for this project. In each reaction, a positive and a negative control were used for quality assessment. A pool of cDNA prepared from HUVEC lysate was used as a positive control for all assays. HUVECs were shown to express both GAPDH-3 and VEGF. Therefore, they were considered suitable for use as positive controls. GAPDH-3 served as a control for the PCR, as this glycolytic protein is involved in a number of critical cell functions such as glycolysis, endocytosis, DNA replication and repair. Therefore, it is expressed by all living cells and lack of GAPDH-3 expression can be inferred to mean that either the cells are dead, or that the PCR has failed. Replacement of cDNA with sterile water served as a negative control. All PCR reactions were

performed twice, on different days, using the same cDNA. All the assays were performed in a thermocycler provided by Techne, Progene, USA.

8.5.2 Primers for PCR

Careful design of the primers used was sought to ensure specificity of the fragments amplified. The designed primers were not complementary at the 3' end to avoid formation of primer dimers, whilst both had the same annealing temperature (T_a). All primers were manufactured by GIBCO BRL and were provided in desiccated, lyophilised form. They were reconstituted with sterile water to a stock concentration of 100 pmoles/ml. The reconstituted primers were aliquoted and stored at –20 °C.

8.5.2(a) VEGF

The primer sequences used were;

Forward primer 5'-CAT CCT GTG TGC CCC TGA TG-3'

Reverse primer 5'-TTC CTC CTG CCC GGC TCA C-3'

The final protocol is now described. A 50 µl reaction was performed for each sample in sterile microcentrifuge tubes. All the following procedures were performed wearing gloves, on ice, to inhibit the action of any contaminating RNA'ases and with the judicious use of 70% ethanol to provide a 'clean' work-field. In an Eppendorf tube (Sarsted, UK), containing 33 µl of de-ionised water, 4 µl of 10 x Buffer and 4 µl of 25 mM $MgCl_2$ were mixed. Next, 1 µl of 10 mM dNTP and 0.5 µl each of the forward and reverse primers were added, followed by 2 µl of the

cDNA (all chemicals Promega, USA). This gave a total volume of 45 µl. The Eppendorf was placed in to the thermal cycler, which had been programmed with the following cycle;

Step	*Stage*	*Temperature*	*Time*
1.	'Hot start'	94 °C	4 minutes
2.	Denaturing	94 °C	45 seconds
3.	Annealing	60 °C	45 seconds
4.	Extension	72 °C	60 seconds

After Step 1, 5 µl of *Taq* DNA polymerase mix (M1661; Promega, USA) (44 µl de-ionised water, 5 µl of 10x buffer and 1 µl of *Taq* DNA polymerase) was added into the Eppendorf.

Taq DNA polymerase mix

44 µl de-ionised water

5 µl 10x buffer

1 µl *Taq* DNA polymerase

Steps 2 - 4 were repeated 35 times, followed by cooling to 4 °C.

8.5.2(b) GAPDH-3

For the quantitation of the VEGF gene levels and comparison between samples, a housekeeping gene was amplified to serve as a standard control for each sample. The gene selected was GAPDH-3, which was expressed in all samples. A separate PCR reaction was developed. The primers for the PCR reaction were designed to have the same annealing temperature as the VEGF gene, to facilitate the working conditions. All the PCR reactions for VEGF and GAPDH-3 were performed in the thermocycler simultaneously.

The primer sequences for GAPDH-3 used were;

Forward primer 5'- CTC ATG ACC ACA GTC CAT GC -3'

Reverse primer 5'- TGA CAA AGT GGT CGT TGA GG -3'

The protocol described in section 8.5.2(a) was used for the amplification of the GAPDH-3 gene.

8.5.3 Agarose gel electrophoresis of PCR products

The specificity of the PCR was analysed by evaluating the production of the target fragments relative to other products by gel electrophoresis. The size of the VEGF PCR reaction products amplified were;

PCR Product	*Size*
$VEGF_{121}$	225 bp
$VEGF_{165}$	307 bp
GAPDH-3	400 bp

The standard method used to separate and identify DNA fragments is electrophoresis, through either polyacrylamide, or agarose gels. Both media enable the direct visualisation of the DNA to be performed under ultraviolet light with the use of the fluorescent, intercalating dye, ethidium bromide. The choice of using either polyacrylamide, or agarose gels depends on the size of the DNA fragments that need to be identified. Polyacrylamide gels are more effective in separating short DNA segments from 5-500 bp and have an extremely high resolving power enabling them to separate fragments differing from each other by as little as 1 bp. However, polyacrylamide gels are more difficult to prepare and more fragile than their agarose counterparts. Furthermore, agarose gels have a lesser resolving power than polyacrylamide gels but a greater separation range, being able to deal with fragments from 200-50,000 bp in length. Given the relative complexity of the polyacrylamide gels and the fact that the DNA fragments to be identified vary significantly in size, agarose gels were used.

Agarose is a polymer extracted from seaweed, made up of D-galactose and 3,6-anhydro L-galactose, forming a matrix gel, the density of which is dependent on its' concentration. Agarose gels are run horizontally in an electric field, so that the

negatively charged DNA migrates towards the anode. The rate of migration is dependent on a number of factors;

- Molecular size of the DNA

Linear, double-stranded DNA migrates through agarose gels at a rate that is inversely proportional to the $\log_{10}$ of the number of bp, as larger molecules migrate more slowly due to increased frictional drag and have a lesser ability than that of smaller molecules to move through the gel matrix pores.

- Agarose concentration

The equation:

$$\text{Log } \mu = \text{Log } \mu_0 - K_r^T$$

Where μ = electrophoretic mobility of DNA

μ_0 = free electrophoretic ability

K_r = retardation coefficient related to the properties of the gel and size and shape of the migrating molecule

^{T} = gel concentration

describes the movement of a DNA fragment through an agarose gel. From this equation, it can be seen that as the concentration of the gel increases, there is more resistance to the movement of the molecule and therefore, the distance it will migrate will be less.

- Conformation of the DNA

Three forms of DNA exist. Superhelical (form-I), nicked circular (form-II) and linear (form-III). DNA molecules with the same molecular weight, but of different conformations, will migrate through agarose gels at different rates so that those molecules with a larger radius will migrate more slowly than the linear forms.

- The applied current

If the applied voltage is low, then linear DNA migrates at a rate proportional to the voltage applied. However, as the applied voltage is increased, the mobility of higher molecular weight DNA increases differentially so that the range of separation decreases as the voltage is increased.

- Composition of the electrophoresis buffer

Ions are required in the buffer to enable electrical conductance to exist. Too few and the DNA will not migrate. Too many and too much heat may be generated, enough to melt the gel and denature the DNA. Many different buffers, exist but they all contain EDTA (pH 8.0). Here, we have used 1 x Tris-borate (TBE) as a compromise between buffering capacity and cost.

The gel was made fresh each time and the density chosen was 1.6% as this was considered to be the optimal density to obtain maximum clarity in the visualisation of the products.

The two opposite, open ends of a readily bought 20 cm x 20 cm plastic tray were sealed with autoclave tape, to form a shallow box. 5 x TBE solution was made up

(54 g TRIZMA base (Sigma, UK), 27.5 g boric acid and 20 ml 0.5 M EDTA (pH 8.0)) and diluted to 1 x strength with de-ionised water. To produce a 1.6 % agar gel, 1.6 g of RNA quality agar powder was added to 100 mls of 1 x TBE in a glass conical flask and heated in a 800 W microwave, at full power, for 90 seconds. This dissolved the agar powder to produce a clear solution. 3.5 µl of ethidium bromide was added to the agar solution, mixed and then poured into the plastic electrophoresis tray. Any bubbles formed on the surface of the liquid were popped with a pipette-tip and then a 12-tooth plastic comb was positioned at one end of the gel to produce 12 wells. The gel was left to cool in a fridge until set.

After the gel had set completely, the autoclave tape and comb were removed carefully so as not to damage the gel. The gel was placed in the electrophoresis tank and covered to a depth of 1mm with 1 x TBE buffer. Next, 5 µl of bromophenol blue, a gel-loading buffer (0.25 % bromophenol blue and 40 % (w/v) sucrose in water) was mixed with 5 µl of the PCR product and pipetted in to one of the wells. The gel-loading buffer has several uses. It serves to increase the density of the sample, so ensuring that the DNA settles evenly at the bottom of the well. It aids in visual identification of the sample and thus, eases the loading of the sample. Lastly, it migrates at a known constant towards the anode, enabling the separation process to be tracked. In addition to the PCR products, 2.5 µl of a 100 bp DNA molecular weight marker (GIBCO, UK) was loaded in to the wells at the side of the gel to help size the PCR products.

The lid was then placed on to the electrophoresis tank, making sure that the anode was positioned at the opposite end from the wells and the gel was left to run for 90

minutes, at 100 V and 75 mA. After the running time, the gel was then viewed on a shielded, ultraviolet light illuminator (which emits light at 302 nm) and a Polaroid camera was used to take an instant photograph of the bands produced.

8.5.4 Scanning densitometric analysis of PCR products

The gel photograph was scanned in to a computer and the separated product bands were analyzed using a scanning densitometer (Epson GT-9500, Japan). OD readings for each PCR band were determined with a computerised analysis program (Labworks™ Image Acquisition and Analysis Software, Ultraviolet Products, Cambridge, UK). The specificity of the products was determined by the size of the product bands as compared with the molecular weight markers run in the same gel. Absence of any band in the negative controls and presence of the correct size band products in the positive controls were the criteria of acceptance of the assay.

8.6 Statistics

Distribution of experimentally obtained values was determined first using a one-sample Kolmogorov-Smirnov test for normality. Results for plasma levels of VEGF and NO have been presented as mean values with 95% confidence intervals (95% CI) in parenthesis, and for Figures, mean values ± s.e.m. Results for OD ratios of VEGF to GAPDH-3 have been presented as mean values ± s.e.m. Comparisons of 'before cuff' and 'after cuff' samples, and between controls and experimental groups were then made using the Student's *t*-test, for paired or un-paired samples, as appropriate. All tests were two-tailed.

CHAPTER 9

Development of a Model of Varicose Veins

CHAPTER 9

9.1 Background

The molecular biology of primary VVs remains largely unknown. If, as we believe, this may involve the activation of the genes encoding for VEGF and NO, it would be advantageous for a stable, reproducible and closely physiological vein model to be developed that could be utilised to study the possible underlying molecular mechanisms of development. Several models do exist for cardiovascular research, focussing mainly on the pathogenesis of SMC-neointimal hyperplasia (NIH), which affects the results of interventional surgery. As yet, no suitable experimental model, either *in vivo* (animal), or *in vitro* (cell culture-based) model has been developed specifically to study the aetiology of primary VVs.

9.1.1 Existing in vitro vein models

As animal models can be both time consuming and expensive, often producing variable results, interest has focused on producing suitable *in vitro* models. *In vitro* techniques include cell culture models, which allow the investigation of growth factors and hormones on SMC's isolated from either animals or humans. It has been particularly useful in the study of growth kinetics of cells derived from stenotic lesions (Dartsch *et al.*, 1990). Two methods are available for the culturing of SMC's. In direct-trypsinization and dispersed-cell culture, the vascular tissue is minced and trypsinized immediately with 0.2% trypsin solution. SMC's are released which are then centrifuged, collected and explanted on to either prosthetic materials (eg. nylon, velour), or on to natural materials (such as omentum, or granulation tissue). Another method is to sub-culture vascular tissue fragments. By this method,

tissue is incubated for up to three weeks to allow SMC's to migrate from the tissue fragments and on to the culture flask, forming a monolayer. Cells are again centrifuged, collected and explanted on to a suitable material (Adachi *et al.*, 1973). Although beneficial in examining SMC-NIH, such techniques deal with cells in isolation and vascular re-modeling *in vivo* represents an interplay between many cell types and all layers of the vessel wall. What is required is a sustainable vein model which allows interaction between all the existing vein wall layers.

9.1.2 Explant tissue culture model

Originally described by Soyombo and co-workers (1990) and subsequently modified by Holt and co-workers (1992), "explant tissue culture" is a system whereby vessel samples (explants) are maintained in culture. This is considered to be more physiological than other *in vitro* techniques as the vessel wall layers (of tunica intima, media and adventitia) are still intact. It is thought therefore, to mimic more closely the *in vivo* situation. Originally, this model used LSV obtained from patients undergoing coronary artery, or femoral-popliteal bypass grafting. The veins were stripped of excess fat and opened up longitudinally. 0.5 cm fragments were formed, which were then pinned on to a mesh, resting on a layer of Sylgard resin. Cultures were maintained in Roswell Park Memorial Institute (RPMI) 1640 media, supplemented with 30% foetal calf serum (FCS), at 37°C in a humidified atmosphere of 5% CO_2 in air and the culture media was changed every two to three days. Porter and co-workers (1996) sought to validate this model of intimal hyperplasia and compared the formation of a neointima in the superficial layers of the explant vein intima, to samples of vein graft stenosis. Marked similarities were found in both the cellular and ECM composition, with an abundance of secretory SMC. The SMC

proliferation rate and neointimal thickness was observed to be maximal by day 14 in culture, and neointimal thickness was maintained despite down-regulation of the proliferation rate beyond this point. This method of tissue culture using normal LSV has therefore, been shown to be a valid model of SMC-NIH development. In this study, a similar technique of explant tissue culture has been developed but using human, primary varicose LSV with comparisons made with explant cultures of human, normal LSV. (It should be noted that there were differences in the manner of tissue retrieval between the two groups in that the varicose LSV underwent inversion stripping with possible intimal trauma, while the normal LSV were obtained without damaging the intimal layer). Objective histological analyses have been made of changes seen in the intima and media of the vein wall, with immunohistochemical techniques employed to identify the nature of the cells present.

9.2 Preparation of isolated vein explant cultures

Normal segments of LSV were obtained from three patients undergoing coronary artery bypass grafting (two female and one male). Varicose LSV's were obtained from 17 patients (14 females and three male) undergoing inversion stripping for varices. [These tissues would normally be discarded after surgery and their use conformed to the guidelines of the Ethical Committee at University College London Hospitals NHS Trust]. On removal, tissue samples were placed immediately into "ice-cold" Soltran (Baxter) kidney perfusate solution (1 litre contains potassium citrate 8.6 g, sodium citrate 8.2 g, mannitol 33.8 g, magnesium sulphate 10.0 g and potassium 80 mmol/l, sodium 84 mmol/l, magnesium 41 mmol/l, citrate 54 mmol/l, sulphate 41 mmol/l, osmolarity 480 mOsm/l and pH 7.1), packed on ice, and taken to

the laboratory to be prepared for explanting. All further procedures were performed using standard sterile techniques in a Class II safety cabinet. The veins were handled carefully, always on ice, and kept viable by being immersed in Soltran solution. The traumatised ends of each vein sample were trimmed with scissors and the peri-vascular fat removed. Veins were opened out by slicing longitudinally, divided into 10 mm sections and pinned, using stainless steel "Minuten" pins (Watkins and Doncaster, UK), endothelial side uppermost, under tension, on to silicone filter paper (Whatmann, UK) resting on cellulose pads (Tesco, UK), cut to fit each well of a 6-well culture plate (Falcon, UK) (See Figure 9.1). Next, 5 mls of media (RPMI 1640 with glutamax), supplemented with 30% FCS and 100 IU/ml each of penicillin and streptomycin (all Gibco, UK) was pipetted in to each well, to which an additional 5 mls of RPMI 1640/30% FCS (at twice the desired concentration was added). The plates were incubated at 37°C, in 5% CO_2 humidified air, and every two to three days, 5 mls of media was removed and replaced with 5 mls of fresh medium. For analysis, samples were removed carefully from the wells and fixed by placing in 4% formal saline, storing at 4°C until further processed.

9.3 Histology

Fixed vein segments were dehydrated in alcohol and cleaved in xylene before being embedded in paraffin wax. Serial sections, 3 μm in thickness, were cut on a microtome and stained with H&E and Elastin Van Gieson (EVG) stains.

9.3.1 Staining with H&E

Sections were de-waxed in xylene and hydrated through graded alcohols to distilled water. Slides were then immersed in Mayer's Haematoxylin (a nuclear dye) for five

minutes. After washing in alkaline water (rendering the Haematoxylin staining a deep blue), slides were examined by light microscopy. If over-staining was present, 1% acid alcohol was added for a few seconds to differentiate the stain from the

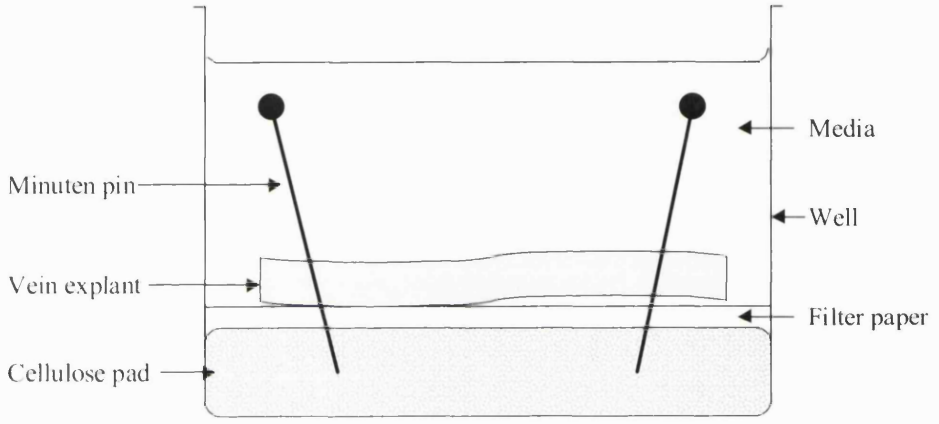


Figure 9.1 Isolated explant culture

Figure showing an isolated explant culture set up in one well of a six-well plate according to a protocol described by Soyombo *et al.* (1990).

background. Slides were counter-stained in 1% eosin for two minutes. The slides were dehydrated through graded alcohols, before clearing in xylene. Finally, slides were mounted in resinous mountant.

9.3.2 Staining with Miller's Elastin and Van Gieson

[For selective demonstration of elastin fibres, identifying the internal elastic lamina, thus determining the intima and media borders]. Sections were de-waxed in xylene and hydrated through graded alcohols. 0.5% potassium permanganate was added for five minutes, followed by 5% oxalic acid for two minutes. Slides were then immersed in Miller's elastin stain for three hours. The counter-stain was with Van Gieson's stain (picric acid nine parts and 1% acid fuschin one part) which was added for three minutes. Slides were blotted dry and cleared in xylene. The results demonstrated elastin fibres a deep purple; collagen fibres were red and muscle fibres, yellow.

9.4 Immunohistochemistry

The protocol used is explained as follows.

9.4.1 Principles

Immunohistochemistry is an 'indirect' method used specifically to demonstrate expressed proteins within cells by using antibodies, raised to localise to the required antigen. Several layers of agents are involved in this process. The first layer consists of primary antibodies that bind to their respective antigens. In this case, anti-human smooth muscle actin (SMA) was used, which reveals the presence of SMC's. The second layer consists of a biotinylated secondary antibody (link

antibody), which binds to the primary antibody. The biotin is strongly attached to the secondary antibody by covalent bonds and is required for its known high affinity for avidin. Avidin, which contains four biotin-binding sites, is found on the avidin-biotin-peroxidase complex and binds strongly to the secondary antibody. This constitutes the third layer. The attached peroxidase induces a colour change to an applied chromogenic substance, enabling the identification of the target antigen to take place.

9.4.2 Antibodies

The antibodies used are detailed in the following table;

Primary antibody	***Secondary antibody***
Mouse anti-human SMA	Biotinylated goat anti-mouse IgG2a

9.4.3 Protocol

After the slides had been de-waxed and washed in absolute alcohol, 12 mls of methanol with 200 µl 30% hydrogen peroxide was added for 10 minutes to block endogenous peroxidase activity. The slides were then washed with water and rinsed in TRIS buffered saline (TBS). Normal goat serum (Serotec, UK) was added, diluted 1 in 10 with TBS, for 10 minutes. The serum was then 'flicked off' and primary antibody (mouse anti-human SMA) was applied (optimally diluted), for one hour, in a damp chamber at room temperature. The anti-human SMA was specific for human SMC and therefore, was used to confirm that lesions were SMC derived.

The slides were then washed three times, for two minutes, in TBS and TWEEN (detergent). A secondary antibody was added (biotinylated goat anti-mouse IgG2a; Serotec, UK), for 35 minutes, optimally diluted. The slides were washed again in TBS/TWEEN. Purified avidin/biotin complex (ABC), with conjugated peroxidase (Serotec, UK), was added for 35 minutes followed by a further wash in buffer.

Diaminobenzidine (the chromogen), and hydrogen peroxide (the substrate for peroxidase) was added for 10 minutes, which turned brown due to the presence of horse radish peroxidase. The slides were washed in water and the nuclei counter-stained with Mayer's Haematoxylin. They were dehydrated through graded alcohols, cleared in xylene and finally, mounted in a resinous mountant.

9.5 Measurement of neointimal thickness

The neointima was defined as; that new intima developing immediately beneath the vein endothelium and above the luminal side of the prime internal elastic lamina. Following collection and preparation, all samples were cultured (in the presence of 30% FCS) for up to 14 days. At 0, 7 and 14 days following culturing, samples were collected and prepared for analysis. Development of SMC-NIH was assessed by analysis of intima:media ratios determined from at least four serial H&E-stained (to differentiate nuclear structures) sections. EVG staining (to differentiate elastin fibres) was used to help determine the intima:media borders. Using a computerised image analysis system (Aequitas IA, Dynamic Data Links Ltd, Cambridge, UK) a minimum of four measurements of intima and media thickness were taken per section, away from the sample edges, at sites where the vein was intact and free from artefactual spaces. Mean ± s.e.m. values were calculated. Further, serial sections of

each explant were stained using standard immunocytochemical techniques in conjunction with monoclonal antibodies specific for human SMA, to determine the cell types present within the lesions that developed.

Comparisons between controls at differing time points were made using the raw results for intima:media ratios. In all cases, the results were further analysed by deducing a 'factor change' in intima:media ratio (either increase, or decrease) as compared to time point by using the following;

$$\textit{'factor change'} = \frac{\text{'experimental value + (-control value)'}}{\text{'control value'}}$$

where the control value is mean intima:media ratio at day 0.

9.6 Statistics

Distribution of experimentally obtained values was determined first using a one-sample Kolmogorov-Smirnov test for normality. In all cases, the results are presented as mean values ± s.e.m. Comparisons between samples at days 0, 7 and 14 were made using the Student's *t*-test, for paired or un-paired samples, as appropriate. All tests were two-tailed.

RESULTS AND DISCUSSIONS

CHAPTER 10

Primary Varicose Veins in the Presence of an Intact Sapheno-Femoral Valve

CHAPTER 10

10.1 Background

As discussed previously, it is thought that primary VVs may result from an incompetent SFV leading to a progressive, descending, valvular incompetence of the LSV (Trendelenburg, 1890-1; Burkitt, 1972). However, varicosities in the presence of a competent SFV are well documented. In addition, it has been suggested that the early 'swelling' of the vein which occurs in varicosity is located distal and not proximal to the SFV as would be expected with the Trendelenburg theory of progressive valvular incompetence (King, 1950).

10.2 Aim

The aim here was to determine the incidence of VVs in the presence, or absence, of a competent SFV and how this may be affected by both age and gender.

10.3 Methods

Over an 18 months period between the years 1998-2000, 1,911 subjects with primary VVs in the distribution of the long saphenous system attended the vascular laboratory, at the Middlesex Hospital, for colour duplex assessment. They were referred by both general practitioners and hospital-based doctors. VVs in the presence, or absence, of a competent SFV were recorded as well as the sex and age of the subjects. (materials and methods, section 6.2 – 6.3)

10.4 Sample size and demographics

The median age of all subjects was 52 years (interquartile range [39-64] years). 1,257 cases, or 65.8%, were female (50 [38-62] years) and 654, or 34.2%, male (55 [40-66] years), with a resultant female to male ratio of 1.92:1. The results are summarised in Table 10.1.

10.5 Primary VVs with age

Stratification of all VVs into '10 year' age-bands revealed that there was no increase in incidence of VVs with age and that incidence peaked in both sexes between the ages of 31-70 years (although females peaked earlier from 31-60 years, while males peaked between 51-70 years). The results are presented graphically in Figure 10.1.

10.5.1 Incompetent SFV and VVs with age

The pattern of age-band incidence for these subjects showed a different pattern to that seen for total primary VVs with age, as both sexes peaked 10 years earlier. Females peaked between 21-70 years, while males peaked between 41-70 years. The female disposition for developing VVs was evident with the percentage of affected females per 10 year age-band, representing 78.8% (at 21-30 years), 64.2% (at 31-40 years) and 63.5% (at 41-50 years) of total primary VVs. Between 51-100 years, the percentage of affected females and males was roughly equivalent. The results are presented graphically in Figure 10.2.

10.5.2 Competent SFV and VVs with age

The pattern of age-band incidence here, unlike that for an incompetent SFV, was similar to that for total VVs, differing only in that the numbers of affected males was

reduced. Females again, were disproportionately affected per 10 year age-band at 70.6% (21-30 years), 79.1% (31-40 years), 81.9% (41-50 years) and 69.1% (51-60 years). Thereafter, between 61-100 years, the percentages of affected females and males, was similar. The results are presented graphically in Figure 10.3.

10.6 SFV status with age and gender

Considering incompetent and competent SFV status;

10.6.1 Incompetent SFV and VVs

Of the 1,911 scans, 56.5% (n=1,079) revealed VVs in the presence of an incompetent SFV, of which 62.4% were female (n=673) (52 [38-64] years) and 37.6% male (n=406) (54 [40-64] years), giving a female to male ratio of 1.66:1. The results are summarised in Table 10.2.

10.6.2 Competent SFV and VVs

Of the 1,911 scans, 43.5% (n=832) revealed VVs in the presence of a competent SFV, of which 70.2% were female (n=584) (49 [38-60] years), while 29.8% were male (n=248) (57 [41.25-67] years), giving a female to male ratio of 2.35:1. The results are summarised in Table 10.3.

10.7 Gender differences in presentation with an incompetent and competent SFV

Between the ages of 31-60 years, the numbers of females presenting with either an incompetent, or competent SFV were roughly equivalent. Percentages of females

with a competent SFV, as a percentage of total females with primary VVs, was 49.2% (31-40 years), 50.7% (41-50 years) and 49.6% (51-60 years).

However, a different picture emerges when we analysed these numbers for males. There was found to be a marked reduction in males presenting with a competent SFV and VVs as a percentage of total males with primary VVs. The percentages in this case were 31.4% (31-40 years), 28.3% (41-50 years) and 36.5% (51-60 years).

10.8 Discussion

The principal aim of this study was to examine the incidence of VVs in the presence of a competent SFV and so, produce a body of evidence to negate the proposal of a progressive, descending valvular incompetence being the sole aetiological factor behind the development of VVs. Also, we examined how this incidence varied with both gender and age. It is appreciated here that although the sample population may not have been matched for age, gender or ethnic group and that other possible contributory factors such as pregnancy and obesity were not noted the results obtained do represent a random selected population.

Overall, for total primary VVs, there was a female to male ratio of 1.92. This figure is consistent with figures published from a number of studies (Coon *et al.*, 1973; Widmer, 1978; Abramson *et al.*, 1981) and in fact, is similar to the figure recently presented at the Venous Forum of the Royal Society of Medicine, in Chester, UK in 2000 (Coleridge Smith, 1999) of between 2-2.5:1. However, this ratio is at odds with the figure published from the Edinburgh Vein Study in 1999 (Evans *et al.*, 1999). In addition, our study showed no increase in the incidence of total primary

VVs with increasing age but instead, revealed an incidence peaking in females between the ages of 30-60 years and in males between 40-70 years, decreasing thereafter (see Figure 10.1), again a finding which is at odds with expected wisdom (Coon *et al.*, 1973; Widmer, 1978; Abramson *et al.*, 1981) but in agreement with others (Da Silva *et al.*, 1974). More interestingly, this study revealed that nearly 50% of total primary VVs co-existed with competent SFV's 43.5%, (see Table 10.3). This figure by itself provides valuable impetus to question the incompetent valve theory of VVs development as it irrefutably demonstrates that VVs can develop in the absence of SFV incompetence.

However, whenever we undertake to proceed with any population-based study we must consider any bias that may have a bearing on the results. Our study examined the results of duplex scans performed at The Middlesex Hospital, of patients with primary VVs whom had attended their general practices seeking treatment. As it is commonly supposed that females are more likely to seek treatment for cosmetic reasons than for males, this self-selection may explain the female preponderance found in our study. Many studies have suggested that the female:male ratio is approximately 2.1 (Coon *et al.*, 1973; Beaglehole *et al.*, 1976; Abramson *et al.*, 1981; Coleridge Smith, 1999) which is close to our figure of 1.92. However, this figure differs significantly from that published by Evans and co-workers (1999), whom performed a cross-sectional study of 12 general practices in the city of Edinburgh. A total of 1566 subjects of both sexes, between the ages of 18-64 years were randomly selected and examined for varying degrees of trunk varices. No sex difference was found in the prevalence of total VVs, producing a female:male ratio of 1:1 (although there was a female preponderance in those affected with grade 2

'hyphen-web' varices and reticular varices). In addition, we must also take into account variations in the referral patterns of the general practices in the local catchment area (as the General Practitioners will not necessarily refer all cases for further investigation), the ethnic mix and the socio-economic status of the local population. Previously, the prevalence of VVs has been shown to vary with both ethnicity (Miyauchi, 1913) and profession (Lake *et al.*, 1942; Santler *et al.*, 1956; Mekky *et al.*, 1969; Weddell, 1969) and we have not been able to determine accurately the effect of these variables in our study.

The female preponderance evident in the figures for total primary VVs is even more pronounced when we examine the numbers broken down into 10 year age bands. In females, there is an earlier peak incidence of between 30 - 60 years, whereas in males, the peak occurs 20 years later between 50 - 70 years (see Figure 10.1). If we look at the 20 - 60 years age band, we can see that the female:male ratio has increased to 2.23 (n=920 female, n=412 male). Beyond 70 years, the numbers of males and females is roughly equal. What can explain this significant sex difference? The earlier female peak may be due to a number of factors. Again the desire for 'cosmetic perfection' may be an important factor accounting for this difference in females. More interestingly, this difference may represent the female 'pre-menopausal' state, with higher levels of circulating oestrogens (which can encompass the taking of the oral contraceptive pill and pregnancy themselves recognised risk factors for the development of VVs) (Lee, 1999), as opposed to the 'post-menopausal' state and a corresponding decrease in oestrogen levels. Moreover, the greater incidence seen in the younger age group may have been emphasised as during pregnancy, women are placed under greater medical scrutiny.

When examining our results in relation to SFV status, we can show that 43.5% of total primary VVs co-exist with a competent SFV (see Table 10.3). Although this value by itself refutes the 'descending valvular incompetence' theory as the sole cause of VVs, it is less than the figure reported by Rose and Ahmed in 1986 and Abu-Owen and co-workers in 1994, which was in the region of 60%. This difference, although unsubstantiated, may be due to differences in the population examined. More significantly, when we break this figure down in to both 10 year age bands and with gender, we see that in females, a more narrow age band incidence is apparent of between 30-60 years for a competent SFV (see Figure 10.3), whereas for an incompetent SFV, the peak age band is slightly broader, ranging from 20-60 years (see Figure 10.2). Also, it can be shown that the numbers of females in the 30-60 years age band can be divided, roughly equally, in to competent and incompetent SFV status (see Figures 10.2 and 10.3). Why this should be so is unknown, but it may be reflective of the 'menopausal' status.

In males, the incidence of primary VVs in the presence or absence of a competent SFV, above the age of 60 years, is roughly equal. Again, as for females, a narrower and much later peak incidence age band is seen with VVs and a competent SFV (50 - 70 years), compared with VVs and an incompetent SFV (30 - 70 years). However, whereas in females the proportion of females in the 30-60 years age band was divided roughly equally between VVs and the presence or absence of a competent SFV, in males this was not the case. The number of males with VVs in the 30-60 years age band co-existing with a competent SFV was only 30% that for males with an incompetent SFV (see Figures 10.2 and 10.3) which, all factors being the same, we would have expected again to be equally divided.

Is this increased incidence of VVs and competent SFV in females before the age of 60 years indicative of a mechanism related to the pre-menopausal state? If so, it may help to explain the difference seen in males with competent SFV and suggests that the increased incidence in females may be as a result of an increased incidence of VVs co-existing with a competent SFV. This apparent association of VVs in the presence of a competent SFV and 'pre-menopausal' status suggests a hormonal mechanism in their development.

10.9 Summary

In summary, this study appears to demonstrate that;

- Nearly 50% of all primary VVs co-exist with a competent SFV
- There is an apparent female preponderance
- Females appear more likely than males to develop VVs at a younger age
- Females appear more likely than males to develop VVs in the presence of a competent SFV

Our findings suggest that the aetiology of VVs is multifactorial and that the female preponderance may be related to hormonal status.

All primary VVs	Female	Male	Total
Median (IQR) age	50 (38-62) years	55 (40-66) years	-
Percentage	65.8%	34.2%	-
n (number)=	1,257	654	1,911
Ratio (total)	1.92	1.0	-

Table 10.1 Sample size and demographics

Table summarising the total number of individuals with primary VVs in the presence and absence of a competent SFV. **n** is the number of individuals, **Ratio** is the female to male ratio.

Incompetent junction	Female	Male	Total
Median (IQR) age	52 (38-64) years	54 (40-65) years	-
Percentage	62.4%	37.6%	56.5%
n (number)=	673	406	1,079
Ratio (incompetent)	1.66	1.0	-

Table 10.2 Primary VVs in the presence of an incompetent SFV

Table summarising the total number of individuals with primary VVs in the presence of an incompetent SFV. **n** is the number of individuals, **Ratio** is the female to male ratio.

Competent junction	Female	Male	Total
Median (IQR) age	49 (38-60) years	57 (41.25-67) years	-
Percentage	70.2%	29.8%	43.5%
n (number)=	584	248	832
Ratio (intact)	2.35	1.0	-

Table 10.3 Primary VVs in the presence of a competent SFV

Table summarising the total number of individuals with primary VVs in the presence of a competent SFV. **n** is the number of individuals, **Ratio** is the female to male ratio.

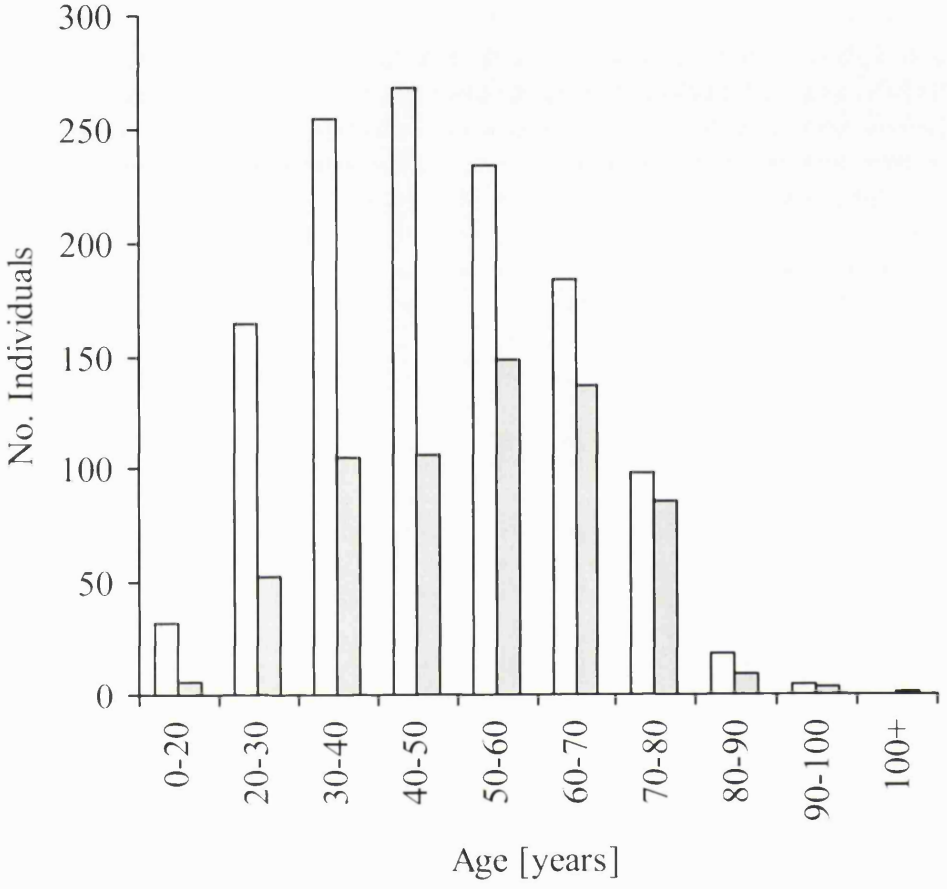


Figure 10.1 Incidence of primary VVs with age

Histogram demonstrating the incidence of primary VVs with each 10 year age band.

Clear bars represent females and shaded bars represent males.

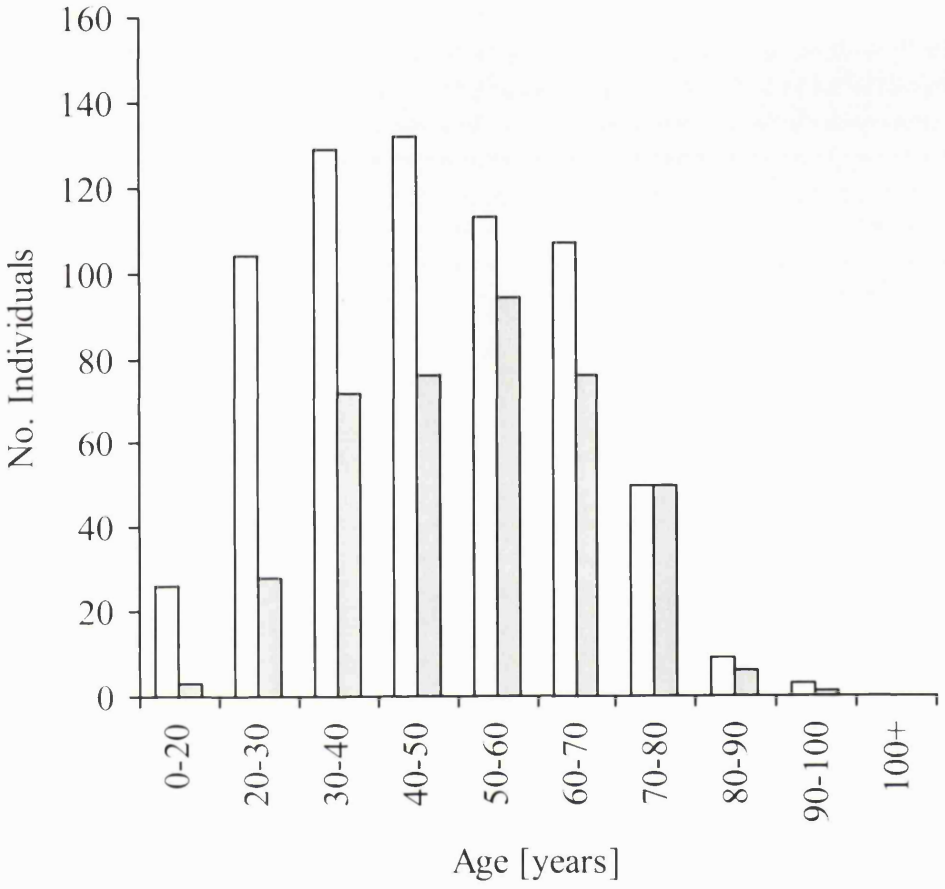


Figure 10.2 Primary VVs in the presence of an incompetent SFV

Histogram demonstrating the incidence of primary VVs in the presence of an incompetent SFV, with each 10 year age band. Clear bars represent females and shaded bars represent males.

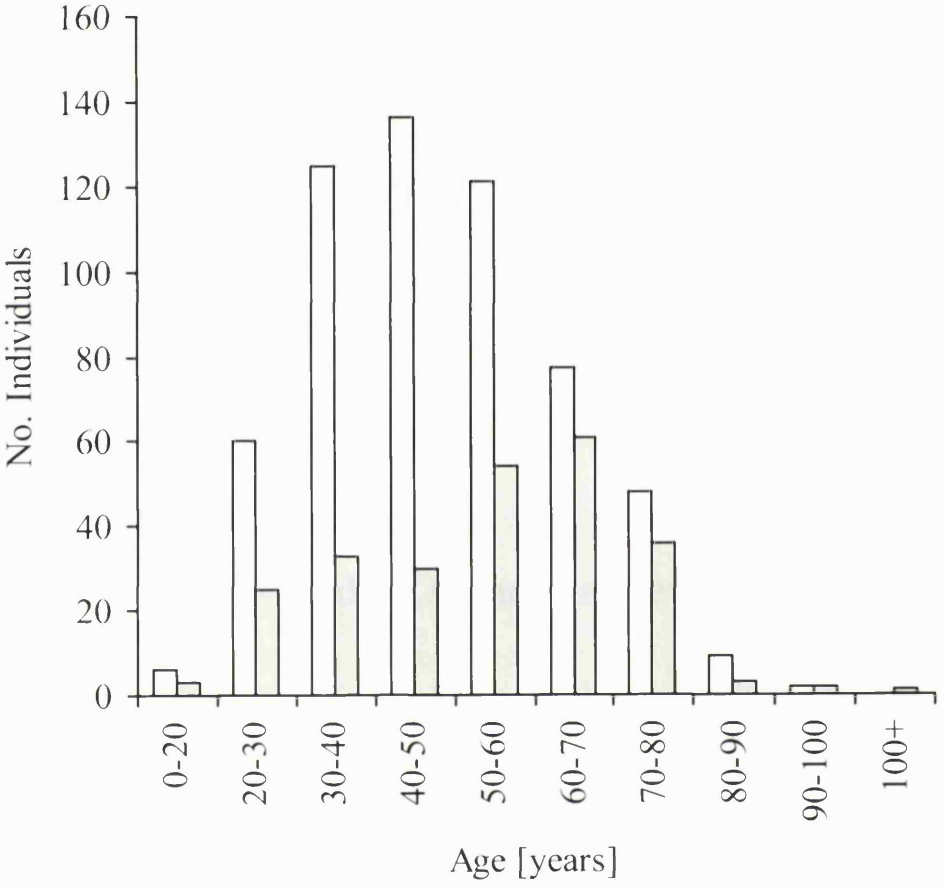


Figure 10.3 Primary VVs in the presence of a competent SFV

Histogram demonstrating the incidence of primary VVs in the presence of a competent SFV, with each 10 year age band. Clear bars represent females and shaded bars males.

CHAPTER 11

VEGF and NO Plasma Levels with Experimentally-Induced Venous Stasis

CHAPTER 11

11.1 Background

As previously mentioned, the lower limbs are subjected continuously to considerable variations in intra-luminal pressure during the course of a normal day (Somerville *et al.*, 1974). The ability of the vessels to adapt to these variations in pressure depends on normal homeostatic mechanisms, which tend to minimise these pressure changes by dilating, or constricting, the vessels in turn. VEGF and NO play an important role in this process and alterations from 'normal' plasma levels may be involved in the aetiology of varicosity.

11.2 Aim

The aim here was to assess and compare the ability of both normal and primary VVs to produce and release these two important factors in response to induced hypoxic stress, or venous stasis.

11.3 Methods

Plasma samples were obtained and processed from the antecubital fossa, the foot (after resting supine for 10 minutes) and the foot after application of a below-knee sphygmomanometer (at a pressure of 90-95 mmHg for 10 minutes - materials and methods chapter, section 7.5 - 7.6.1). Plasma VEGF levels were determined by VEGF ELISA (ng/ml) (material and methods, section 8.2 - 8.2.3) and plasma NO levels were determined by the Griess reagent (pmol/L) (materials and methods chapter, section 8.3 - 8.3.4). The results obtained experimentally were of a normal

distribution and have been presented as mean values with 95% confidence intervals (95% CI) in parenthesis, and for Figures, mean values ± s.e.m.

11.4 Sample size and demographics

21 subjects with primary VVs were enrolled in this study (six males and 15 females; median age, 46 (range, 21 - 78 years)) and 11 controls without VVs (seven males and four females; median age, 32 (range, 22-55 years)).

11.5 Levels of plasma VEGF

The analysis of the levels of plasma VEGF proved variable dependent on the quality of the sample obtained; for example, where blood samples obtained were limited in volume, or had coagulated to some degree, both of which occurred associated with the difficulty in obtaining blood from the foot veins of some individuals. Hence, samples from individuals were included for analysis here, only if they produced on (three) consecutive and separate analyses by ELISA, results with less than a 10% inter-experimental variation. The results are summarised in Table 11.1 and presented graphically in Figures 11.1 and 11.2.

The baseline levels of plasma VEGF from the arm, or foot after rest but before application of the sphygmomanometer, were similar in control subjects, primary VVs subjects, and also, between the two experimental groups (all, $P>0.1$; Table 11.1, Figures 11.1 and 11.2).

In control subjects, following application of the below-knee sphygmomanometer for 10 minutes, there was a clear increase in the levels of plasma VEGF with an overall

increase in the mean level of plasma VEGF of 8.46 (-3.37 – 20.29) % (95 % CI, $P<0.025$, Student's paired t test; Table 11.1, Figure 11.1).

In contrast, in those with VVs, application of the below-knee sphygmomanometer had little or no effect on the levels of plasma VEGF detected; a change in the overall mean level of plasma VEGF of 5.85 (-13.75 – 25.44) % (Table 11.1, Figure 11.2).

11.6 Levels of plasma NO

The results are summarised in Table 11.2 and presented graphically in Figures 11.3 and 11.4.

As with plasma VEGF, the baseline levels of plasma NO from the arm and foot, after rest but before application of the cuff, were similar in either the control subject group, or the group with primary VVs (all, $P>0.1$; Table 11.2, Figures 11.3 and 11.4). In contrast to VEGF however, cuff application for 10 minutes had little or no effect on the levels of plasma NO detected in either controls of those with primary VVs (all, $P>0.1$; see Table 11.2, Figures 11.3 and 11.4).

Furthermore, and in contrast to the levels of plasma VEGF, in all samples from patients with primary VVs (from the arm, or foot, before or after cuff application), the levels of NO detected in the plasma were reduced as compared to the control subjects (all, $P<0.05$; for arm, or foot, before or after cuff application; all, Student's unpaired t test; see Table 11.2, Figures 11.3 and 11.4).

11.7 Discussion

VVs are 'dilated, tortuous veins' with alterations in their vessel wall constituents and aberrant SMC growth and distribution. These include a lower collagen content, relatively more muscle and a higher hexosamine content than for normal veins (Svejcar *et al.*, 1962; 1963). In addition, there is interruption of the smooth muscle layer by elastin fibres. These findings would seem to suggest that there is both a loss of structural integrity and of physiological functionality of the vein wall (Rose and Ahmed, 1986; Travers *et al.*, 1992). It is vitally important that the vein wall can respond to the changes in intra-luminal pressure that occurs throughout the course of a normal day and to changes in the chemical environment, such as pH and oxygenation. In order to minimise these changes, it must be able to both constrict and dilate in measured response (with the vein wall playing a central role in this process). Therefore, could VVs arise as a result of alterations in the vessel wall's ability to respond to these constantly fluxing conditions?

Organ bath experiments using isolated ring sections of VVs have recently been used to investigate the constricting and dilating ability of VVs (Schuller-Petrovic *et al.*, 1997). Known constrictors, such as norepinephrine, angiotensin II, KCl and ET-1 were added to the organ bath and elicited a reduced ability of the VVs ring sections to constrict (as measured by induced maximal tension). This reduced constriction was related to the degree of varicosity (Rizzi *et al.*, 1998) and for ET-1, was shown to be due to the decreased level of expression of its' receptors in the vein wall (Barber *et al.*, 1997). However, the dilating capacity of the VVs ring sections was found also, to be reduced as shown by assessing the response to the known dilators calcium ionophore, NO and forskalin (Lowell *et al.*, 1992). Other studies looking at the

plasma levels of these agents in the VVs subjects, showed little change in the levels of ET-1, cAMP or bradykinin, when compared to the plasma of normal individuals (Schuller Petrovic *et al.*, 1997). However, the levels of cGMP (via which NO induces relaxation) were raised and angiotensin II, a constrictor, reduced (Schuller-Petrovic *et al.*, 1997). These results, when examined together, would suggest that the dilatory state is favoured as EC's derived from VVs, secrete fewer mediators of constriction, whilst increasing cGMP/NO, which promotes dilation.

A contrasting result was seen in a separate study using the application of a below-knee cuff. Here, application of the cuff for 10 minutes induced a significant increase in ET-1 in both controls and VVs subjects, but with a greater increase seen in the VVs subjects (Mangiafico *et al.*, 1997). More importantly, the baseline ET-1 plasma levels in VV subjects were shown also, to be raised compared to controls. It is possible therefore, that higher plasma ET-1 levels 'down-regulate' the expression and number of ET-1 receptors, leading to a reduced response to ET-1 as shown by the organ bath experiments using isolated VVs ring sections (Mangiafico *et al.*, 1997).

The main agents involved in vessel dilatation and permeability are VEGF and NO, which can act both independently and synergistically with one another (Laitinen *et al.*, 1997; Servos *et al.*, 1999). These two factors play a central and important role in the homeostatic mechanisms of the vessel wall, not only in regulating vessel reactivity, but also in vessel repair and growth (Servos *et al.*, 1999). A detailed investigation of these two factors as they relate to varicosity has yet to be undertaken. Here, we have sought to evaluate the changes in their serum levels in both VVs and controls in response to induced venous stasis.

11.7.1 VEGF

The baseline levels of VEGF, as shown in the arm and foot before sphygmomanometer application, were similar in varicose and control subjects (see Table 11.1, Figures 11.1 and 11.2). Previously, higher levels of VEGF have been detected in conjunction with VVs but only with co-existing skin changes (Shoab *et al.*, 1992). Since it has been shown previously, that VVs have a decreased ability to respond to dilatory stimuli, how does this explain the 'normal' baseline levels of VEGF in the serum of VV subjects? It would seem to suggest that it is not lack of availability of VEGF that leads to a decreased dilatory response, but that the veins have somehow lost the ability to react to it. This may reflect 'physical' factors for example, if the vein wall is dilated maximally (as is often suggested with VVs) it may not be able to dilate further, due to the physical constraint of the wall itself, or because of opposition from the surrounding structures. Alternatively, a 'molecular' mechanism may be responsible. Perhaps, as for ET-1, there is decreased expression, or changes in the distribution and density of VEGF receptors, *flt*-1 and KDR within the vein wall of VVs, or more interestingly, there may be receptor redundancy subsequent to a repeated stimulus to dilate, as might be the case with prolonged standing and the resultant pooling of venous blood.

Following sphygmomanometer cuff application to the lower limb to induce mild venous stasis, in control subjects there was a significant increase in the serum levels of VEGF ($P<0.025$; see Table 11.1, Figure 11.1). This was to be expected, as the chemical conditions induced by venous stasis should stimulate an increase in VEGF levels, leading to dilatation and increased permeability of the vessel wall. However, in VVs subjects (see Table 11.1 and Figure 11.2), no such increase was observed.

This lack of VEGF induction correlates with the results previously published showing a decreased responsiveness to dilatory stimuli in isolated VVs ring sections (Lowell *et al.*, 1992) and loss of vein reactivity.

11.7.2 NO

In contrast to the situation found with VEGF, baseline serum levels of NO in VVs subjects were much reduced compared to controls (all $P<0.05$; see Table 11.2, Figures 11.3 and 11.4). This, at first, seems to be at odds with the accepted synergistic interplay between VEGF and NO, as we would expect an equivalent NO level in VVs subjects compared to controls. However, this finding may fit with the manner in which VEGF and NO interact (Servos *et al.*, 1999). Increased levels of VEGF should act to increase NO levels however, if there is an inability of VVs to respond to the initial rise in VEGF (perhaps due to a decrease in the number, or sensitivity, of VEGF receptors in the vessel wall), then it is reasonable to presume that there would not be a corresponding increase in NO. In addition, there may be an inherent inability of VVs to stimulate NO production due perhaps, to a lack of, or defective, cNOS or there may be damage to the endothelium (essential in vascular reactivity). Last of all, the methodology used to measure NO levels is extremely labile, as it is both under a time constraint and sensitive to temperature changes. Therefore, an actual change may not have been detected. Other methods, which determine changes in NO by measuring cGMP, may be more illuminating.

Again, post-application of the sphygmomanometer cuff showed no increase in NO in either controls, or VVs subjects (see Table 11.2 and Figure 11.3, 11.4). In controls, despite an induced increase in VEGF, NO was not raised in sympathy. This may be

explained as detailed previously, or perhaps there was only a slight rise in NO, undetectable by our methodology. It may be more useful therefore, to examine changes in cGMP (a more sensitive assay) as performed in previous studies (Schuller-Petrovic *et al.*, 1997). Further, the post-induced samples were obtained after 10 minutes of venous stasis. cNOS activity is short-lived, so any increase in NO might only occur within the first few minutes. Therefore, it may be more beneficial to examine samples taken at intervals up to 10 minutes. However, more relevant in this pathologically simulated situation is the activity of iNOS. The activity of this enzyme shows a distinct lag phase of between four to six hours, with a further delay in NO production, although expression of this enzyme can be sustained for several days (Liu *et al.*, 1997). However, in this clinical study it was not possible to simulate this time course in our subjects and any increase in iNOS activity would not have been detected. In VVs subjects, as there was no corresponding increase in VEGF, we would not expect to see any increase in NO.

Our hypothesis states that venous stasis (as occurs with prolonged standing and immobility) stimulates production and release of VEGF (as demonstrated in our study) and of NO by the vein wall, increasing both vascular permeability and diameter and so acting to ameliorate the conditions initiating their production. However, if the response is insufficient or absent then their production continues unabated, leading to excessively high levels. This excessively high VEGF level is the stimulus for the development of VVs, as it could lead (through stimulated production of NO) to a progressively thinned and weakened vein wall, more prone to tortuosity and varicosity. Here, we have demonstrated equivalent levels of VEGF in controls and VVs and a loss of VEGF reactivity in VVs which would appear to

contradict our hypothesis. However, the hypothesis supposes the conditions present in the initiation of varicosity whereas the results obtained refer to the established varicose state. As explained previously, the over-stimulated VEGF levels (in a manner similar to that of ET-1), in the absence of an appropriate response could lead to a compensatory 'down-regulation' of VEGF receptors and subsequent loss of vein reactivity. This loss of VEGF receptors coupled with damage and subsequent loss of EC function (as a result of stress brought about by repeated or prolonged dilatation of the vein wall) may lead to an inability of the vein to react to further induction of venous stasis and subsequent VEGF production.

11.8 Summary

In summary, we have demonstrated;

- equivalent baseline levels of VEGF in VVs subjects as compared with controls,
- no increase in VEGF brought about by induced venous stasis in VVs
- controls demonstrated the expected, induced increase in VEGF.

This loss of induced reactivity of VVs (and of VEGF release) is in line with previously published reports and can be explained within our hypothesis.

	Control veins (ng/ml)	Varicose veins (ng/ml)
Arm	6.07 (1.44 – 10.69)	6.16 (1.47 – 10.85)
Before cuff	6.10 (1.87 – 10.32)	6.06 (1.52 – 10.61)
After cuff	6.67 (2.10 – 11.24)†	5.97 (1.70 – 10.23)
***n =**	8	12

Table 11.1 Levels of plasma VEGF

Levels of plasma VEGF (ng/ml) in peripheral blood samples obtained from the arm and foot veins, before and after cuff application, in control subjects and patients with primary VVs. Values are presented as means with 95% confidence intervals (95% CI) in parenthesis. *n, is the number of individuals whose results were used for analysis, those in whom repeat ELISA analysis produced consecutive results with <10% inter-experimental variation; each value represents the mean of at least triplicate experiments, each of at least duplicate wells per experiment. 'Before cuff' and 'after cuff' are samples obtained from foot veins. †, $P<0.025$, 'before' versus 'after' cuff application, Student's paired *t* test.

	Control veins (pmol/L)	Varicose veins (pmol/L)
Arm	46.9 (28.5 – 65.3)	30.1 (25.2 – 35.1) §
Before cuff	47.8 (30.2 – 65.4)	32.4 (26.7 – 38.0) §
After cuff	48.0 (29.4 – 66.7)	32.8 (27.3 – 38.2) §
***n=**	11	20

Table 11.2. Levels of plasma NO

Levels of plasma NO (pmol/L) in peripheral blood samples obtained from the arm and foot veins, before and after cuff application, in control subjects and patients with primary VVs. Values are presented as means with 95% confidence intervals (95% CI) in parenthesis. *n, is the number of individuals whose results were used for analysis, those in whom levels of plasma NO were determinable; each value represents the mean of duplicate experiments each of duplicate wells. 'Before cuff' and 'after cuff' are samples obtained from foot veins. §, $P<0.05$, 'normal' versus 'varicose' for all samples (arm, leg before and after), Student's unpaired *t* test.

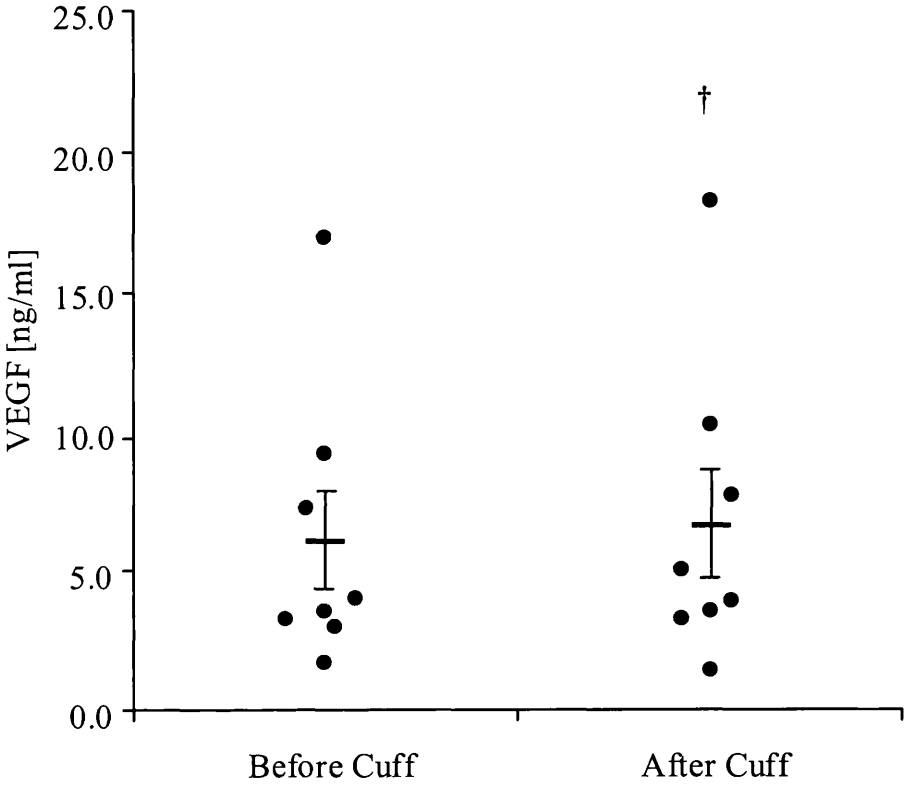


Figure 11.1. Levels of plasma VEGF in controls

Levels of plasma VEGF (ng/ml) in peripheral blood samples obtained from the foot veins, before ('Before Cuff') and after ('After Cuff') cuff application, in control subjects. Individual values are presented as filled circles; bars represent the mean value ± s.e.m. [Results included are only from those individuals in whom repeat ELISA analysis produced consecutive results with <10% inter-experimental variation; each point represents the mean of at least triplicate experiments, each of at least duplicate wells per experiment]. †, $P<0.025$, 'before' versus 'after' cuff application, Student's paired *t* test (see Table 11.1.)

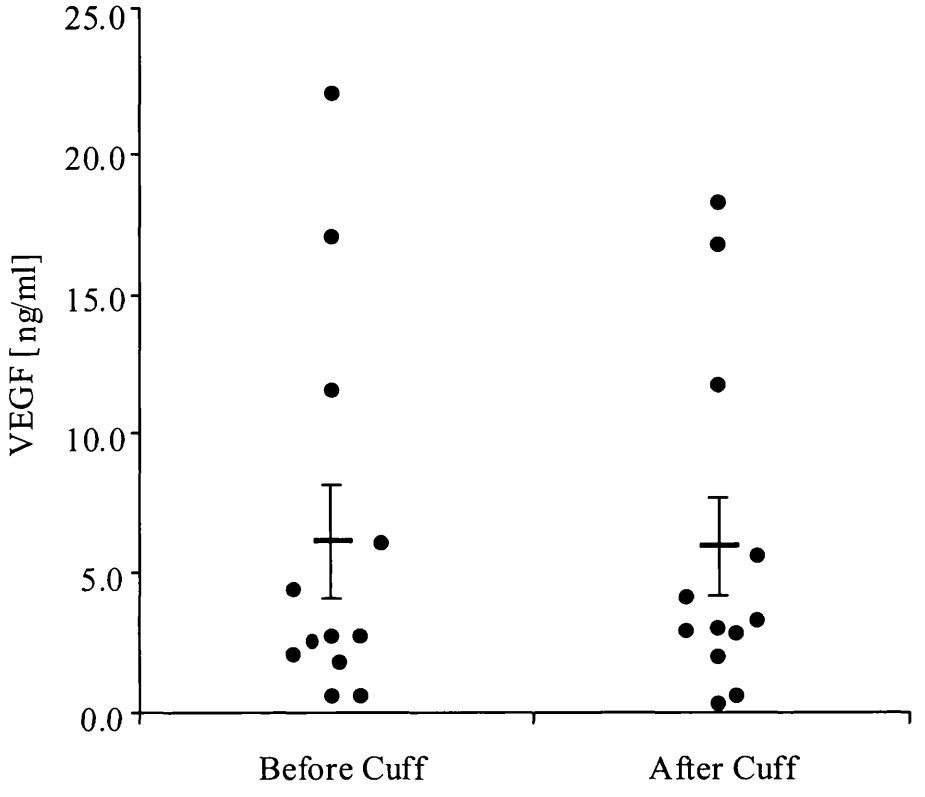


Figure 11.2 Levels of plasma VEGF in VVs

Levels of plasma VEGF (ng/ml) in peripheral blood samples obtained from the foot veins, before ('Before Cuff') and after ('After Cuff') cuff application, in patients with primary VVs. Individual values are presented as filled circles; bars represent the mean value ± s.e.m. [Results included are only from those individuals in whom repeat ELISA analysis produced consecutive results with <10% inter-experimental variation; each point represents the mean of at least triplicate experiments, each of at least duplicate wells per experiment].

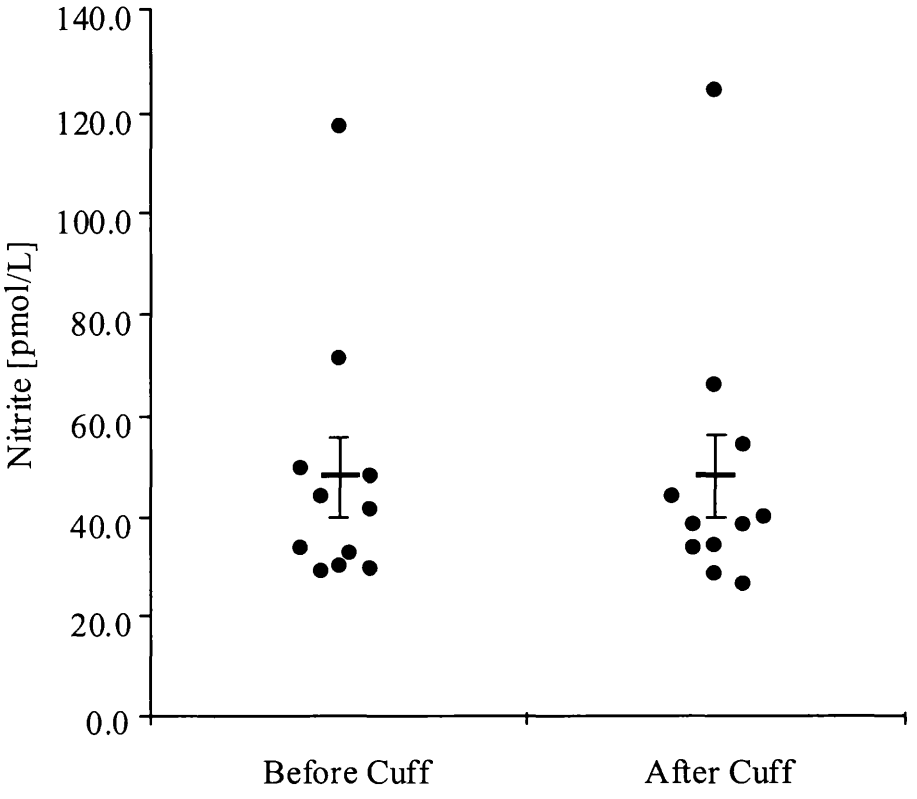


Figure 11.3 Levels of plasma NO in controls

Levels of plasma NO (pmol/L) in peripheral blood samples obtained from the foot veins, before ('Before Cuff') and after ('After Cuff') cuff application, in control subjects. Individual values are presented as filled circles; bars represent the mean value ± s.e.m. [Results included are only from those individuals in whom levels of plasma NO were determinable; each value represents the mean of duplicate experiments each of duplicate wells].

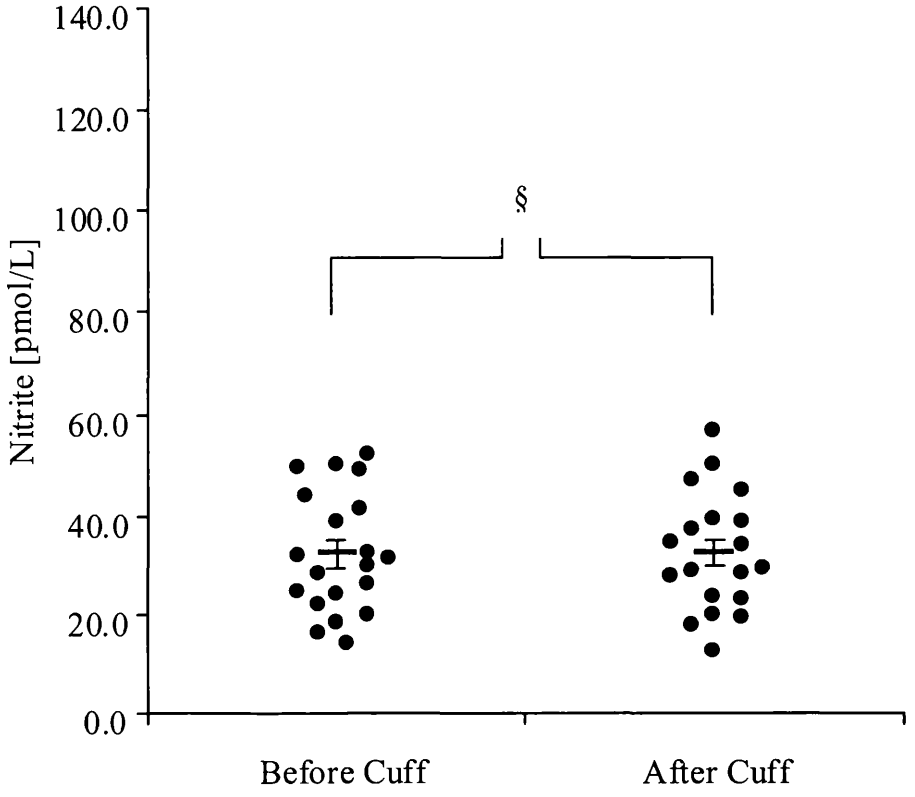


Figure 11.4 Levels of plasma NO in VVs

Levels of plasma NO (pmol/L) in peripheral blood samples obtained from the foot veins, before ('Before Cuff') and after ('After Cuff') cuff application, in patients with primary VVs. Individual values are presented as filled circles; bars represent the mean value ± s.e.m. [Results included are only from those individuals in whom levels of plasma NO were determinable; each value represents the mean of duplicate experiments each of duplicate wells]. §, $P<0.05$ for either 'before' or 'after' cuff as compared to control subjects (presented in Figure 11.3.), Student's unpaired *t* test (see Table 11.2.).

CHAPTER 12

Changes in PBMC Gene Transcription of VEGF Following Experimentally-Induced Venous Stasis

CHAPTER 12

12.1 Background

We have hypothesised that the levels of both VEGF and NO may play an important role in the development of varicosity, by modulating the reactivity of veins in response to intra-luminal pressure and venous oxygenation, resulting in an adverse effect on the vein wall structure. These effects may well weaken the vein wall and predispose to the development of varicosity. However, changes in their respective plasma levels gives no indication as to their source of production and although endothelial NO production is stimulated by VEGF via a calcium-dependent pathway (Hariawala *et al.*, 1996; Wu *et al.*, 1996) VEGF is produced *de novo* by a variety of cell types, such as SMC's, macrophages and EC's. In addition, it is possible that the increased serum level of VEGF may originate from stores in the vein wall itself, bound to heparan sulphate proteoglycan, a major constituent of both the ECM and of the BM. This provides a readily available source, which can then be released into the circulation when needed.

Previously, it has been shown that white blood cells are activated during experimental venous stasis, in both controls and varicose vein subjects and that this activation is associated with EC activation (Shoab *et al.*, 1998). Furthermore, it is supposed that the phenomenon of 'white cell trapping' is involved in the activation of granulocytes and EC's, with subsequent skin damage. Perhaps, it is these white blood cells which provide the source of increased serum VEGF?

12.2 Aim

Here, we have focussed on PBMC's as a possible source of VEGF, by investigating changes in VEGF gene induction.

12.3 Methods

cDNA produced from the PBMC's of blood samples, obtained from the foot at rest and after induction of venous stasis (by application of a sphygmomanometer cuff at 90-95 mmhg, for 10 minutes), was used to produce the PCR products for GAPDH-3 and VEGF (materials and methods chapter, section 8.4 - 8.5.4.). Only subjects with expression of both GAPDH-3 and VEGF in both blood samples were analysed. The PCR products GAPDH-3 and VEGF of each subject, for each separate sample, were run on a fluorescent agarose electrophoretic gel. A Polaroid photograph was taken of the resultant PCR bands and analysed on a scanning densitometer, to produce individual OD values for each PCR band (materials and methods chapter, section 8.5.5 - 8.5.6). To normalise the results and to ascertain relative changes in gene activation, the OD ratios of VEGF to GAPDH-3 were calculated for each VEGF isoform.

All values represent the mean of duplicate results and have been presented as mean ± s.e.m

12.4 Sample size and demographics

Eight subjects with primary VVs were enrolled in this study (three males and five females; median age, 48 (range, 27 - 59 years)) and six controls without VVs (four males and two females; median age, 33 (range, 22 - 55 years)).

12.5 Changes in VEGF gene transcription following experimental venous stasis

We shall consider the results for controls and VVs separately.

12.5.1 Changes in VEGF gene transcription following experimental venous stasis in controls

The results of the six control subjects were grouped and analysed together. Two isoforms of VEGF were identified; $VEGF_{165}$ and $VEGF_{121}$. The ratio obtained for $VEGF_{165}$ to GAPDH-3 showed little difference before (0.26 ± 0.03) and after (0.24 ± 0.03) application of the sphygmomanometer cuff to the foot. This situation was mirrored in the values obtained for the $VEGF_{121}$ isoform. Mean OD ratios obtained for before (0.27 ± 0.04) and after (0.23 ± 0.04) application of the sphygmomanometer cuff to the foot were again equivalent (see Table 12.1 and Picture 12.1.)

12.5.2 Changes in VEGF gene transcription following experimental venous stasis in primary VVs subjects

In primary VVs subjects, the results were somewhat different. As for control subjects, there was little difference in the ratios obtained for the $VEGF_{165}$ isoform before (0.17 ± 0.04) and after (0.22 ± 0.06) application of the sphygmomanometer cuff to the foot. However, the values obtained for the $VEGF_{121}$ isoform showed a significant increase in gene activation. Application of the sphygmomanometer cuff to the foot showed a 91% increase in gene activation ($P<0.08$, Student's paired *t* test) compared to 'before' (0.22 ± 0.04) and 'after' (0.42 ± 0.11) (see Table 12.2. and Picture 12.1.)

12.6 Discussion

(The results are summarised in Table 12.3.) The two main isoforms of VEGF secreted into the serum by EC's are $VEGF_{121}$ and $VEGF_{165}$ (Jonca *et al.*, 1997) and these results suggest a selective activation of the $VEGF_{121}$ isoform, in PBMC's isolated from patients with VVs following induced venous stasis. Further, transcription of the $VEGF_{165}$ isoform was seemingly unaffected by either induced venous stasis, or by varicosity.

Why is the $VEGF_{121}$ isoform raised? Does it have a separate function to that of $VEGF_{165}$? We know that $VEGF_{121}$ lacks the amino acids encoded by exons 6 and 7 of the VEGF gene and so lacks heparin and heparan sulphate binding ability (Park *et al.*, 1993; Cohen *et al.*, 1995). Therefore, it cannot bind to and be stored in the ECM, unlike $VEGF_{165}$, which contains a 44 amino acid-long peptide encoded by exon 7 of the VEGF gene, conferring heparin and heparan sulphate binding ability (Park *et al.*, 1993; Cohen *et al.*, 1995). Both isoforms of VEGF induce proliferation of EC's and *in vivo* angiogenesis (Leung *et al.*, 1989) and are the predominant isoforms secreted by most VEGF-producing cell types (Jonca *et al.*, 1997). However, it has been suggested that $VEGF_{165}$ is more potent than $VEGF_{121}$ (Keyt *et al.*, 1996), although it is unclear why this is so. It is thought that the heparan sulphate binding ability confers a protective advantage to $VEGF_{165}$, helping to restore its' VEGF receptor binding ability following oxidative damage, whereas $VEGF_{121}$ is immune to this effect. In addition, several studies have indicated that heparan sulphates may bind to VEGF receptors perhaps modulating, or helping to regulate, their $VEGF_{165}$ binding ability. Again, $VEGF_{121}$ is seemingly unaffected. Moreover, the heparin binding ability of $VEGF_{165}$ enables it to bind to cell-surface, ECM-associated, heparan

sulphate proteoglycans and so, can release angiogenic factors such as bFGF, which is stored in the ECM and has a synergistic action with VEGF in angiogenesis (Jonca *et al.*, 1997). Therefore, it would appear that an increase in the more active $VEGF_{165}$ would be expected. Nonetheless, its increased potency lies in its proliferative and angiogenic functions, which are not the properties of VEGF in which we are interested. Our hypothesis believes that it is the NO stimulating ability of VEGF, through an as yet undetermined signalling cascade involving iNOS, guanylate cyclase and cGMP-dependent protein kinase, that is involved in the aetiology of VVs. Could $VEGF_{121}$ have a more specific NO stimulating ability?

For many years it was thought that VEGF receptors were confined to the surface of EC's only, but for our theory to have any credence, VEGF receptors must be present on the surface of NO-producing cells such as monocytes. Recently, several studies have indeed demonstrated that this is the case (Barleon *et al.*, 1996). The gene for the VEGF receptor *flt*-1 has been found to be expressed on the surface of human monocytes and although it is postulated here that VEGF is involved in the chemotaxis of monocytes as part of inflammation, it is conceivable that VEGF may also, in some manner, up-regulate or modulate the monocyte's NO-producing ability. If this were the case, the increase in $VEGF_{121}$ gene expression would fit well into our hypothesis. At present, there is no experimental evidence that $VEGF_{121}$ binds preferentially to *flt*-1. In fact, it would appear that $VEGF_{121}$ binds selectively to the KDR rather than the *flt*-1 receptor (Gitay-Goren *et al.*, 1996). However, even if this were the case, there are a number of ways in which $VEGF_{121}$ may activate the *flt*-1 receptor. The spatial arrangements of the receptor binding sites on VEGF allow it

theoretically, to form hetero-dimers of KDR and *flt*-1 (Kendall *et al.*, 1996), thus providing a possible mechanism by which $VEGF_{121}$ may activate *flt*-1.

In addition, a soluble form of *flt*-1, named *sflt*-1, has been identified which is truncated on the C-terminal side of the sixth extracellular Ig-like domain. This has been shown to inhibit VEGF-stimulated EC mitogenesis, presumably via a 'dominant negative' mechanism (Kendall *et al.*, 1996). For this to occur, it must be capable of undergoing dimerisation with both *flt*-1 and KDR receptors thus forming inactive receptor dimers which do not trigger intracellular, tyrosine kinase, dimerisation-dependent signal transduction. As theoretical hetero-dimerisation of VEGF receptors is possible, perhaps a function of a *sflt*-1/KDR hetero-dimer activated by $VEGF_{121}$ is to increase NO production? This though, is mere speculation and in both the above cases, the presence of the KDR receptor would still be required.

Of greater interest are the identification of the $VEGF_{165}$ specific receptors, neuropilin-1 and 2 (Soker *et al.*, 1998) found on non-EC's and on breast and prostate cancer cells. These were originally identified as receptors for semaphorins, which inhibit nerve growth cones but are here hypothesised to act as co-receptors in angiogenesis (Kitsukawa *et al.*, 1997). Their existence not only raises the possibility of identifying $VEGF_{121}$ specific receptors which may have a NO-producing specific function but also suggests the interaction of as yet unknown factors in VEGF function.

However, when interpreting these results we must consider the limitations of the study. We know that the mean resting venous pressure in the lower limbs of patients

with CVI is approximately 85-90 mmHg and here, have presumed that the application of a cuff to the lower limb at a pressure of between 90-95 mmHg for ten minutes is sufficient for the induction of venous stasis (and possible hypoxia). Although on visual inspection, the limbs do attain a bluish discolouration suggestive of some degree of venous stasis and relative hypoxia (followed by rebound erythema on release of the cuff), confirmation of this was not made by objective measurement. One way in which this could be done would be to examine venous flow in the lower limb before and after application of the cuff using a venous Duplex. Also, confirmation of relative hypoxia could be confirmed by venous blood analysis. However, as the subjects were recruited from the vascular clinic, neither was performed due to lack of both equipment and clinic time.

12.7 Summary

In summary this study has demonstrated that;

- There was no change in transcription of the genes for either $VEGF_{121}$, or $VEGF_{165}$, in the PBMC's collected from normal, control subjects following venous stasis
- That there is selective activation of the gene encoding for the $VEGF_{121}$ isoform in the PBMC's collected from varicose subjects following venous stasis

	$VEGF_{165}$ control PBMC (OD units)	$VEGF_{121}$ control PBMC (OD units)
Before cuff	0.26 ± 0.03	0.27 ± 0.04
After cuff	0.24 ± 0.03	0.23 ± 0.04
***n=**	6	6

Table 12.1 $VEGF_{165}$ & $VEGF_{121}$ / GAPDH-3 OD ratios in PBMC's obtained from controls

OD ratios of $VEGF_{165}$ & $VEGF_{121}$ / GAPDH-3 RT-PCR bands in controls. Each value represents the mean of triplicate experiments. *n, represents the number of subjects with complete values available for analysis. Results are presented as mean values ± s.e.m. 'Before cuff' are OD ratios obtained pre-application of the sphygmomanometer cuff to the leg. 'After cuff' are OD ratios obtained post-application of the sphygmomanometer cuff to the leg.

	$VEGF_{165}$ varicose PBMC (OD units)	$VEGF_{121}$ varicose PBMC (OD units)
Before cuff	0.17 ± 0.04	0.22 ± 0.04
After cuff	0.22 ± 0.06	0.42 ± 0.11†
***n=**	8	8

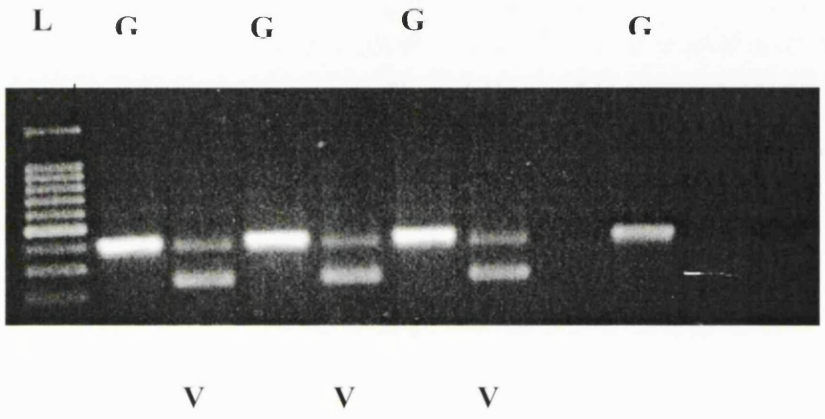
Table 12.2 $VEGF_{165}$ & $VEGF_{121}$ / GAPDH-3 OD ratios in PBMC's obtained from subjects with primary VVs

OD ratios of $VEGF_{165}$ & $VEGF_{121}$ / GAPDH-3 RT-PCR bands in subjects with primary VVs. Each value represents the mean of triplicate experiments. *n, represents the number of subjects with complete values available for analysis. Results are presented as mean values ± s.e.m. 'Before cuff' are OD ratios obtained pre-application of the sphygmomanometer cuff to the leg. 'After cuff' are OD ratios obtained post-application of the sphygmomanometer cuff to the leg. †, $P<0.08$.

	$VEGF_{121}$ control PBMC (OD units)	$VEGF_{121}$ varicose PBMC (OD units)
Before cuff	0.27 ± 0.04	0.22 ± 0.04
After cuff	0.23 ± 0.04	0.42 ± 0.11†
***n=**	6	8

Table 12.3 $VEGF_{121}$ / GAPDH-3 OD ratios in PBMC's obtained from controls and subjects with primary VVs

OD ratios of $VEGF_{121}$ / GAPDH-3 RT-PCR bands in controls and subjects with primary VVs. Each value represents the mean of triplicate experiments. *n, represents the number of subjects with complete values available for analysis. Results are presented as mean values ± s.e.m. 'Before cuff' are OD ratios obtained pre-application of the sphygmomanometer cuff to the leg. 'After cuff' are OD ratios obtained post-application of the sphygmomanometer cuff to the leg. †, $P<0.08$, Student's paired t test compared to 'before' (0.22 ± 0.04) and 'after' (0.42 ± 0.11) for varicose PBMC's.



Picture 12.1 Agarose gel RT-PCR bands

Polaroid photograph of a typical electrophoretic agarose gel viewed under ultraviolet light. Eight columns are visualised. From the left, the first column **(L)** represents bands produced from a commercially available 100bp DNA ladder to aid in identification. The next seven columns demonstrate the RT-PCR bands for GAPDH-3 **(G)** and VEGF **(V)**. The first pair of columns represents the GAPDH-3 and VEGF bands obtained from PBMC's isolated from blood drawn from the arm. The next pair of columns represents the GAPDH-3 and VEGF bands obtained from PBMC's isolated from blood drawn from the foot, pre-application of the sphygmomanometer cuff. The next pair of columns represents the GAPDH-3 and VEGF bands obtained from PBMC's isolated from blood drawn from the foot, post-application of the sphygmomanometer cuff. The last column represents the GAPDH-3 internal control for the PCR reaction. The GAPDH-3 bands consist of a single band, while VEGF consists of two bands representing the soluble isoforms $VEGF_{165}$ (upper) and $VEGF_{121}$ (lower).

CHAPTER 13

Development of a Model of Varicose Veins

CHAPTER 13

13.1 Background

Given that the 'descending, incompetent valve' theory of primary VVs aetiology is unlikely to be the sole cause of VVs and that molecular mechanisms are very likely to play an important role, it is necessary for a sustainable and reproducible vein model to be developed to aid in further investigation of this pathology. Cardiovascular literature cites many models, both animal (*in vivo*) and cell culture-based (*in vitro*) systems, to aid in the investigation of SMC-NIH. However, many of these models are either time-consuming and produce variable results, or only study cellular systems in isolation, ignoring the involvement of the different vein wall layers in re-modelling. The 'isolated explant cell culture' technique, as developed by Soyombo and co-workers (1990), uses full-thickness, human, normal LSV explants incorporating all the vein wall layers and thus, provides a more physiological model of vascular remodelling. Here, we have used a similar method but adapted it for ourselves by using human, primary VVs, LSV explants.

13.2 Aim

To develop a valid, reproducible, *in vitro* vein model to aid in the investigation of primary VVs.

13.3 Methods

Human, normal LSV was obtained from patients undergoing cardiac bypass procedures and human, varicose LSV's, were obtained from patients undergoing inversion vein stripping for varicosity, both at the Middlesex Hospital, London. All

veins were processed to explant culture (as described in the materials and methods section 9.2) and stained (as described in the materials and methods section 9.3 - 9.4.3). The explants were then analysed at days 0, 7 and 14 for assessment of SMC-NIH lesions (materials and methods, section 9.5).

13.4 Sample size and demographics

Human, normal LSV's were obtained from patients undergoing cardiac bypass procedures, at the Middlesex and University College Hospitals, London. Three separate samples were obtained (from one male and two females: median age 63.5 years, range 59 - 68 years), from which a total of 13 explants were cultured. Five control samples were fixed at day 0 (control for lesion development), four explants at day 7 and four at day 14. (See Table 13.1).

Human, varicose LSV's were obtained from patients undergoing inversion vein stripping for varicosity, at the Middlesex and University College Hospitals, London. 17 samples were obtained in total (from three males and 14 females: median age 29.5 years, range 25 - 54 years) from which 60 explants were cultured. 22 control samples were fixed at day 0 (controls for lesion development), 20 explants at day 7 and 18, at day 14. (See Table 13.2)

13.5 Statistics

In all cases, the experimental values obtained were distributed normally as determined by a one-sample Kolmogorov-Smirnov test for normality. Comparisons were made using the Student's *t*-test for un-paired samples. For clarity and due to the number of comparisons made, statistical values for significance have been

included within the tables summarising the results and have not been incorporated within the figures.

13.6 Explants of control LSV

A total of 13 samples were explanted into tissue culture and maintained for up to 14 days.

Explants were harvested at days 0, 7 and 14, followed by fixing in 4% formal saline. They were processed to slides and then stained with H&E and EVG stains. Samples were stained further, using standard immunocytochemical techniques, in conjunction with monoclonal antibodies specific for human SMA to confirm that the developed lesions were SMC-derived. It was found that control explants of normal veins developed a significant SMC-NIH by day 7 (intima:media ratio of 0.052 ± 0.010; mean ± s.e.m.; $P<0.006$; factor increase 1.887 ± 0.54 versus control at day 0). This change however, reflects an increase in the intimal layer ($P<0.01$, Student's unpaired *t* test) and a decrease in the media ($P<0.01$, Student's unpaired *t* test). Immuno-histochemistry confirmed that the lesion was SMC-derived, which interestingly, had regressed by day 14 (intima:media ratio of 0.020 ± 0.001; mean ± s.e.m.; $P<0.06$; factor increase 0.116 ± 0.048 versus control at day 0).

Further, histological analysis of all samples confirmed tissue viability, thus demonstrating that changes in vein wall thickness was indicative of tissue re-modelling rather than loss of viability during culture.

The results obtained are summarised in the following graphs and tables. Table 13.1. shows the mean intima:media ratios for the control explants and these are depicted graphically in Figure 13.1. Picture 13.1 (A) shows a SMA section of a control explant at day 0. Picture 13.2 (A) demonstrates a SMA stain of a representative intimal lesion produced by day 7 (with immunohistochemical confirmation of the presence of SMC's). Picture 13.2 (B) demonstrates an EVG stain of the same section as in 13.2 (A), highlighting the internal elastic lamina.

13.7 Explants of varicose LSV

A total of 60 samples were explanted into tissue culture and maintained for up to 14 days.

Explants were harvested at days 0, 7 and 14, followed by fixing in 4% formal saline. They were processed to slides and then stained with H&E and EVG stains. Samples were further stained using standard immunocytochemical techniques in conjunction with monoclonal antibodies specific for human SMA, to confirm that developed lesions were SMC-derived. It was found that the results from varicose veins contrasted with that from normal veins. At day 0, varicose explants demonstrated a larger intima:media ratio compared to normal explants ($P<0.001$, Student's t test), which was reflective of a substantially thicker intima ($P<0.001$, Student's t test). At day 7, there was a trend for a reduction in the intima:media ratio, reflective of a significant increase in medial thickness ($P<0.001$, Student's t test), which produced a factor reduction compared to controls at day 0 of -0.103 ± 0.102. By day 14, there was a reduction in the intima:media ratio from 0.067 ($\pm$ 0.005) to 0.052 ($\pm$ 0.004); mean $\pm$ s.e.m.; $P<0.03$; a factor decrease of -0.229 ± 0.055 versus control at day 0).

As with the normal explants, histological analysis of all the samples revealed them to be viable at day 14. Any changes therefore, in intima:media ratios is indicative of vein wall re-modelling and not due to loss of sample viability during culture.

The results obtained are summarised in the following graphs and tables. Table 13.2. shows the mean intima:media ratios of varicose veins which is graphically depicted in Figure 13.2. Picture 13.1 (B) shows a SMA section of a VVs explant at day 0 demonstrating the thickened intima.

13.8 Discussion

How then do primary, varicose LSV's differ to normal LSV's? Primary VVs demonstrate SMC hypertrophy, with thicker SMC layers in both the intima and media when compared to normal veins (Travers *et al.*, 1992). In addition, there is invasion of fibrous tissue throughout the media, disturbing the SMC layers and accumulating in the sub-intima, while elastic fibres extend from the internal and external elastic laminae to spread throughout the vein wall (Rose and Ahmed, 1986; Travers *et al.*, 1992). These disturbances in vein wall structure and integrity weaken the vein wall and may act as initiating foci for the development of varicosity. Factors that may aid in this process include physical 'stressors', such as pressure and turbulent haemodynamic flow and molecular mechanisms, including ROS damage, mast cell infiltration and activation, an increase or decrease in MMP enzymatic activity and the involvement of both VEGF and NO (Deby *et al.*, 1989; Garcia-Rospide *et al.*, 1991; Farbiszewski *et al.*, 1996; Yamada *et al.*, 1996). It is because of the possible involvement of molecular mechanisms in primary VVs aetiology that a suitable *in vitro* model is required to aid in its investigation. Here, we have

modified a technique to suit our own purposes that has been used previously to examine the development of SMC-NIH.

The original isolated vein explant culture method using human, normal LSV, demonstrated SMC-NIH by day 14. This model was validated by Porter and co-workers (1996), in which comparisons were made of the lesions produced in this model of SMC-NIH and pathological vein-graft stenoses obtained from below-knee and femoral-popliteal arterial bypass grafts. Application of H&E stain, immunohistochemistry and transmission electron microscopy, revealed the presence of "secretory" SMC's in both samples, while alcian blue/PAS staining showed that these cells were surrounded by similar mucopolysaccharides in the ECM. This model has been studied extensively using both veins and arteries and has been found to be reliable and reproducible.

However, in our study, normal control explants developed a lesion at day 7, which had regressed by day 14 (see Table 13.1, Figure 13.1 and Picture 13.2). This contrasts with the result demonstrated by Soyombo and co-workers (1990), in which a definite lesion was produced by day 14. This regression of SMC-NIH needs to be explained. The lesion produced by day 7 in our study reflected an increase in the intima and a slightly smaller decrease in the media. We know that the lesions of NIH are derived from SMC's, suggesting that the increase in intimal thickness is due to SMC migration from the media to the intima, with associated SMC proliferation. The regression of this lesion by day 14 contrasts with the results expected according to the original protocol. This regression reflected both a decrease in the intima and an increase in the thickness of the media. As histological analysis has confirmed that

there has been no loss of explant viability, sample deterioration cannot account for this regression. However, previous studies examining the formation of rabbit SMC-NIH arterial lesions have demonstrated similar findings (Booth *et al.*, 1989), although here regression of the lesion was over a 4 - 6 week period. As the tissue remains viable, it is more likely that with the lack of an appropriate pathological stimulus, the sample has managed to repair the lesion and that the regression seen merely demonstrates normal vascular repair and remodelling. This lesion regression may be due also, to an insufficient initial stimulus in first setting up the culture. In other explant models, a stimulus for SMC-NIH has been provided in the form of scraping off the entire endothelial surface, revealing the rich source of SMC's in the media below (Caplice *et al.*, 1994). Obviously, this supplies a potent boost to lesion development and may aid in prolonging its' existence.

This situation contrasts with that found in varicose explants. At day 0, consistent with previous reports on varicose vein histology, the samples demonstrated a significantly thickened intima compared to the media, being three times thicker in this study (Travers *et al.*, 1992) (see Table 13.2, Figure 13.2 and Picture 13.1(B)). The aetiology of this is unclear. No lesion development was seen at all, up to and including day 14. More specifically, at day 7, a decrease was noted in the intima:media ratio. Here, although there was a slight increase in the thickness of the intimal layer, there was a greater increase in the medial layer, resulting in an overall decrease in intima:media ratio. Given that there was an increase in intimal thickness, it would appear that as with normal, control explants there had been SMC proliferation, although in the absence of SMC migration from the media to the intima. Perhaps this alteration in SMC activity contributes to the aetiology of

primary VVs? The chemical environment found in varicosity may contribute to this altered function, or perhaps, this difference in activity is reflective of a change in SMC phenotype from 'contractile' to 'secretory', affecting also the ultrastructure of VVs ?

By day 14, the intima:media ratio in the varicose explants, similar to that demonstrated by normal, control explants, had returned to a level comparable to the ratio seen at day 0. Again, histological analysis confirmed that this was not due to a loss of tissue viability, but was a reverse of the effects seen by day 7. Moreover, as with the normal, control explants, it demonstrates that primary VVs tissue may retain the ability to repair itself in the absence of a continuing pathological stimulus.

However, one crucial difference between normal and varicose explants is that on retrieval, varicose explants suffer inversion stripping with possible intimal denudation. Could this affect their activity in culture? As mentioned previously, we would expect that intimal denudation (not affecting the viability of the explant) to provide a potent stimulus to intimal proliferation (Caplice *et al.*, 1994). However, here this would not appear to be the case. In this study, although varicose explants show some intimal proliferation it is the medial layer (which does not suffer from direct trauma) that shows an apparent proliferative boost. Is this again an indication of a differing cellular response between normal and varicose veins?

The apparent prevention of NIH development in VVs samples may reflect another crucial difference between normal and primary, varicose LSV's. CVI and associated varicosity is linked with an increase in the plasma level of VEGF (Shoab *et al.*,

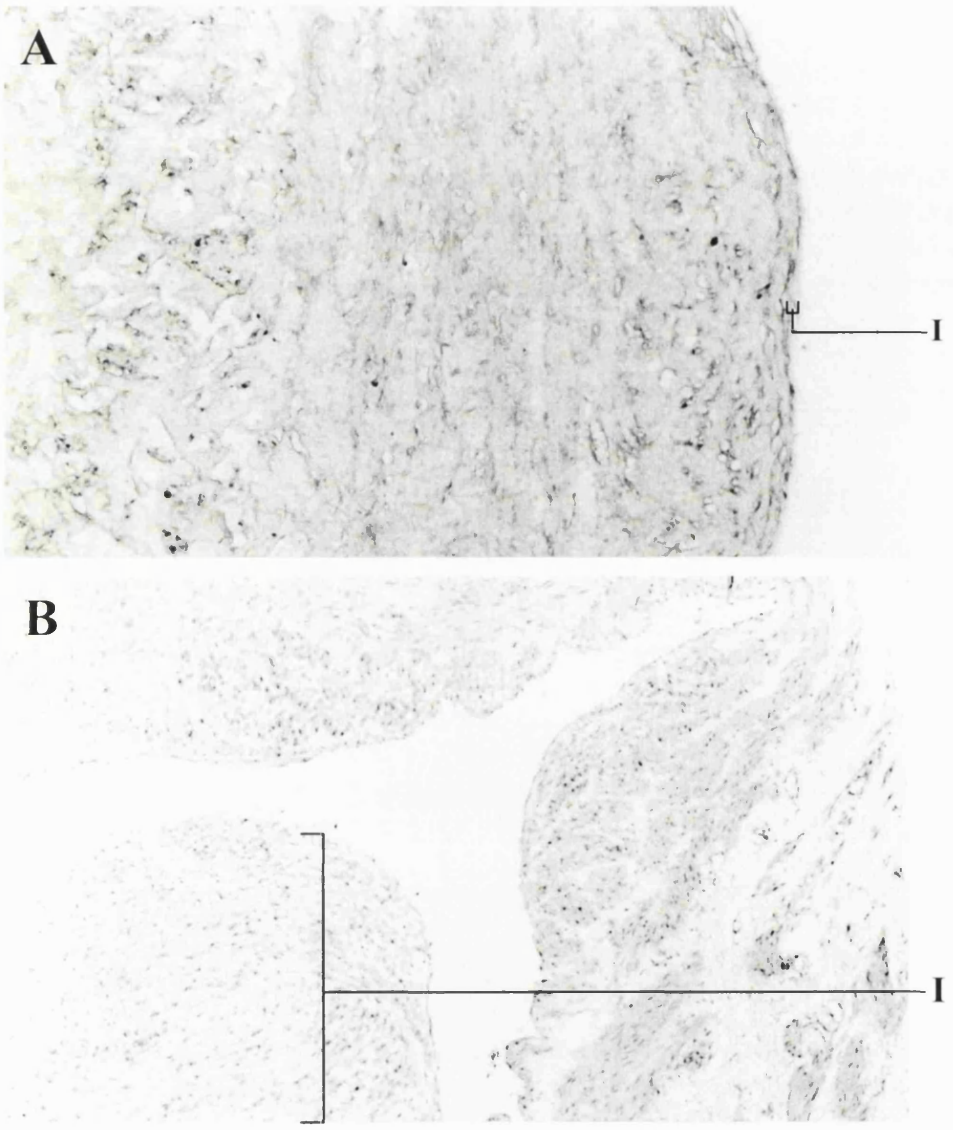
1998). In a rabbit model, VEGF has been shown to repair endothelial damage induced by balloon denudation, therefore preventing the development of NIH (Luo *et al.*, 1998). In addition, it stimulates EC production of NO, which initiates vasodilation and which inhibits also, the formation of NIH. Therefore, VEGF appears to act as an anti-NIH agent, first by stimulating the repair of damaged endothelium and therefore, reducing the reactive proliferative response and second, by stimulating the production of the anti-hyperplastic NO. A source of VEGF is from SMC's. As it has been suggested that SMC function may be altered in VVs, accounting for the differences seen in NIH lesion development between normal, control and varicose explants, perhaps it is the VEGF secretory function of SMC's in the varicose explants which is awry. It is feasible that the SMC's, or a subset of SMC's in VVs, continue to secrete high levels of VEGF, thus inhibiting lesion development for the duration of culture.

This study has demonstrated that an *in vitro* model of varicosity can be developed from an established model of SMC-NIH, using varicose explants of LSV. Furthermore, as a result of this study, it has been suggested that dysfunction of SMC's may contribute to the aetiology of primary VVs. Lastly, it has been shown that as with normal LSV explants, primary varicose LSV explants retain the ability for repair and re-modelling following a pathological stimulus.

13.9 Summary

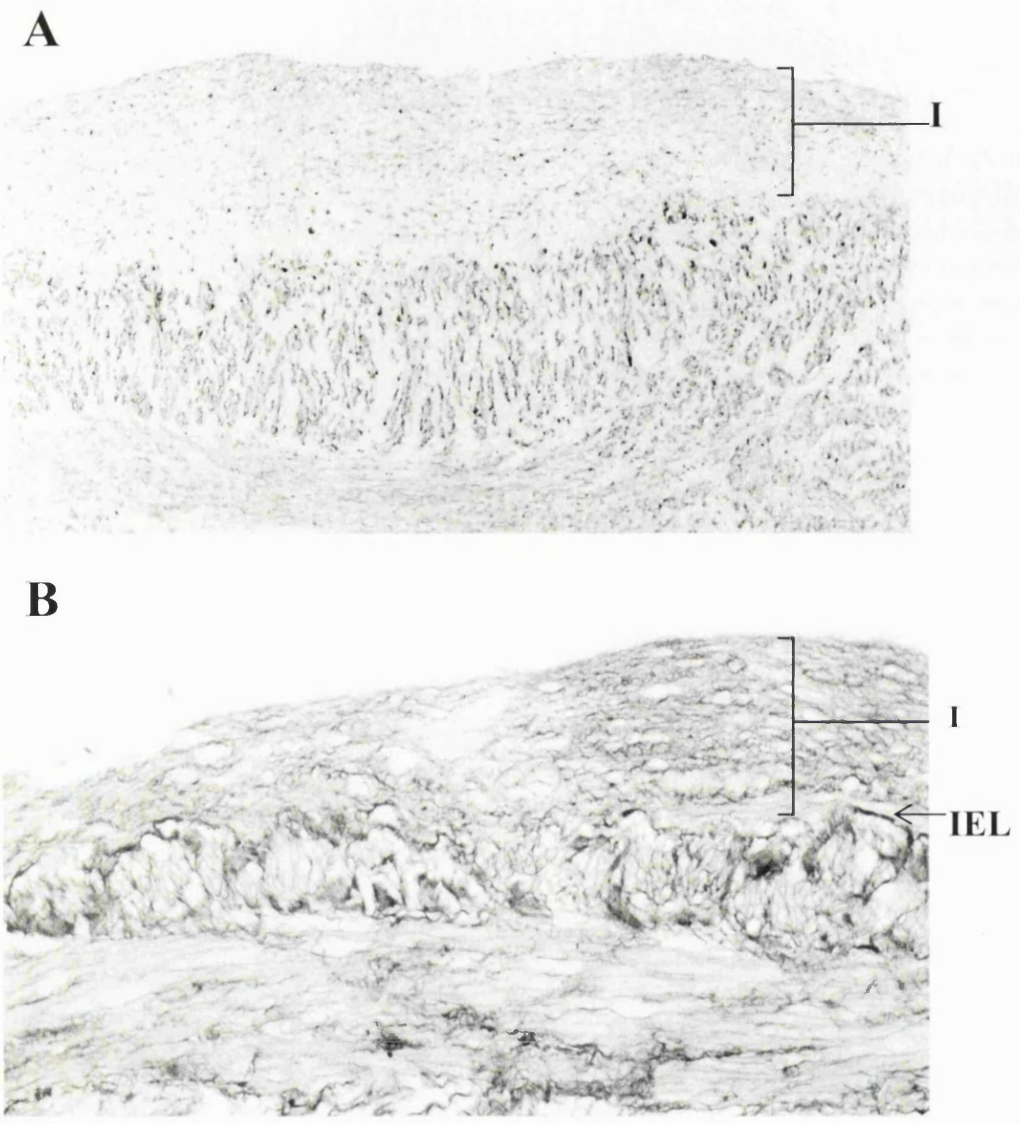
In summary, this study has demonstrated that;

- The original isolated vein explant model using human, normal LSV, as described by Soyombo and co-workers (1990), has been adapted and validated with human, varicose LSV
- Normal, control explants developed a lesion by day 7 of culture (produced by an increase in the intima and a decrease in the media), which had regressed (due to a decrease in the intima and increase in the media), by day 14
- Varicose explants at day 0 demonstrated a thickened intima compared to the media. By day 7, a decrease in the intima:media ratio was observed in the presence of a slight increase in the intimal thickness. This was due to a greater increase in the medial layer. By day 14 of culture, the intima:media ratio had returned to a level comparable to that found at day 0
- Variations in the intima:media ratio may reflect differences in medial SMC's activity or phenotype which may contribute to the structural features seen in VVs
- Varicose explants may retain the ability to remodel themselves after injury



Picture 13.1 Day 0 control and VVs explants - histology

Photograph **(A)** is a SMA section of a control explant at day 0 demonstrating the normal thin intima (magnification x 100). Photograph **(B)** is a SMA section of a VVs explant at day 0 demonstrating the irregularly, thickened intima (magnification x 40). **I**: intima.



Picture 13.2 Day 7 control explants – histology

Photograph **(A)** is a SMA section of a day 7 control explant demonstrating the intimal lesion. Photograph **(B)** is an EVG section of the same intimal lesion highlighting the darkly-stained, internal elastic lamina which separates the intima from the media. Magnification x 100. **I**: intima, **IEL**: internal elastic lamina.

	Day 0	Day 7	Day 14
Intima (μm)	0.010 (± 0.000)	0.023 (± 0.004)†	0.010 (± 0.000)
Media (μm)	0.556 (± 0.010)	0.462 (± 0.013) †	0.528 (± 0.026)
Intima:media ratio	0.018 (± 0.001)	0.052 (± 0.010)‡	0.020 (± 0.001)§
Factor change in intima:media ratio compared to day 0	-	+ 1.887 (± 0.54)	+ 0.116 (± 0.048)
***n (explants) =**	5	4	4

Table 13.1 Mean intima:media ratios of control explants

Table summarising the changes in the intima and media thickness of normal LSVs over time (0-14 days), with culturing. Values are expressed as the mean with s.e.m. in parenthesis. Intimal and medial thickness' are in μm. Factor changes in intima:media ratio compared to day 0 are calculated as described in 'Methods'. *n (explants), represents the total number of individual explants set up in culture (from which results are calculated). †, $P<0.01$; ‡, $P<0.006$; §, $P<0.06$; all versus values at day 0. Results are expressed graphically in Figure 13.1.

	Day 0	Day 7	Day 14
Intima (μm)	0.035 (± 0.003)†	0.042 (± 0.005)	0.029 (± 0.002)
Media (μm)	0.588 (± 0.018)	0.744 (± 0.022)‡	0.606 (± 0.024)
Intima:media ratio	0.067 (± 0.005)†	0.060 (± 0.007)	0.052 (± 0.004)§
Factor change in intima:media ratio compared to day 0	-	- 0.103 (± 0.102)	- 0.229 (± 0.055)
***n (explants) =**	22	20	18

Table 13.2. Mean intima:media ratios of varicose explants

Table summarising the changes in the intima and media thickness of varicose LSVs over time (0-14 days), with culturing. Values are expressed as the mean with s.e.m. in parenthesis. Intimal and medial thickness' are in μm. Factor changes in intima:media ratio compared to day 0 are calculated as described in 'Methods'. *n (explants), represents the total number of individual explants set up in culture (from which results are calculated). †, $P<0.001$ versus 'normal' veins at day 0. ‡, $P<0.001$ and §, $P<0.03$; both versus values at day 0. Results are expressed graphically in Figure 13.2.

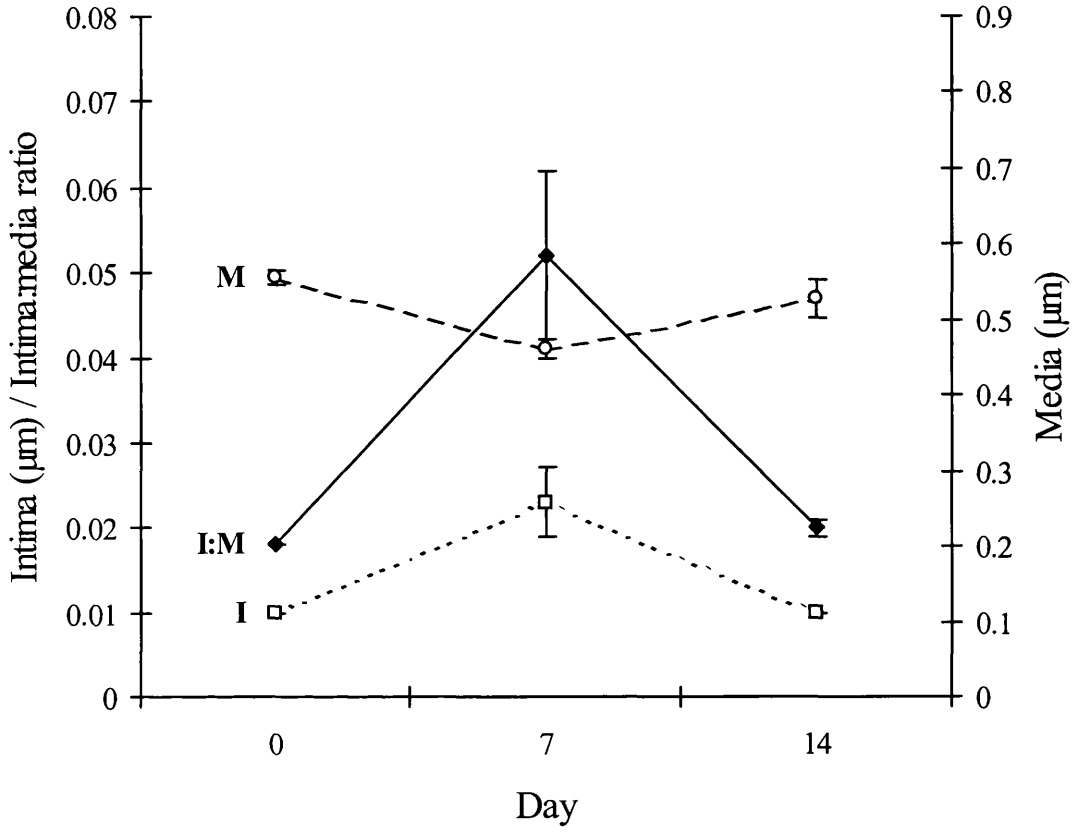


Figure 13.1 Graphical representation of the mean intima:media ratios of control explants

Graph representing changes over time, with culturing, in the thickness of the intima (I) and media (M), and the change in intima:media ratio (I:M). Results are presented as mean values ± s.e.m. Actual values and levels of statistical significance are summarised in Table 13.1.

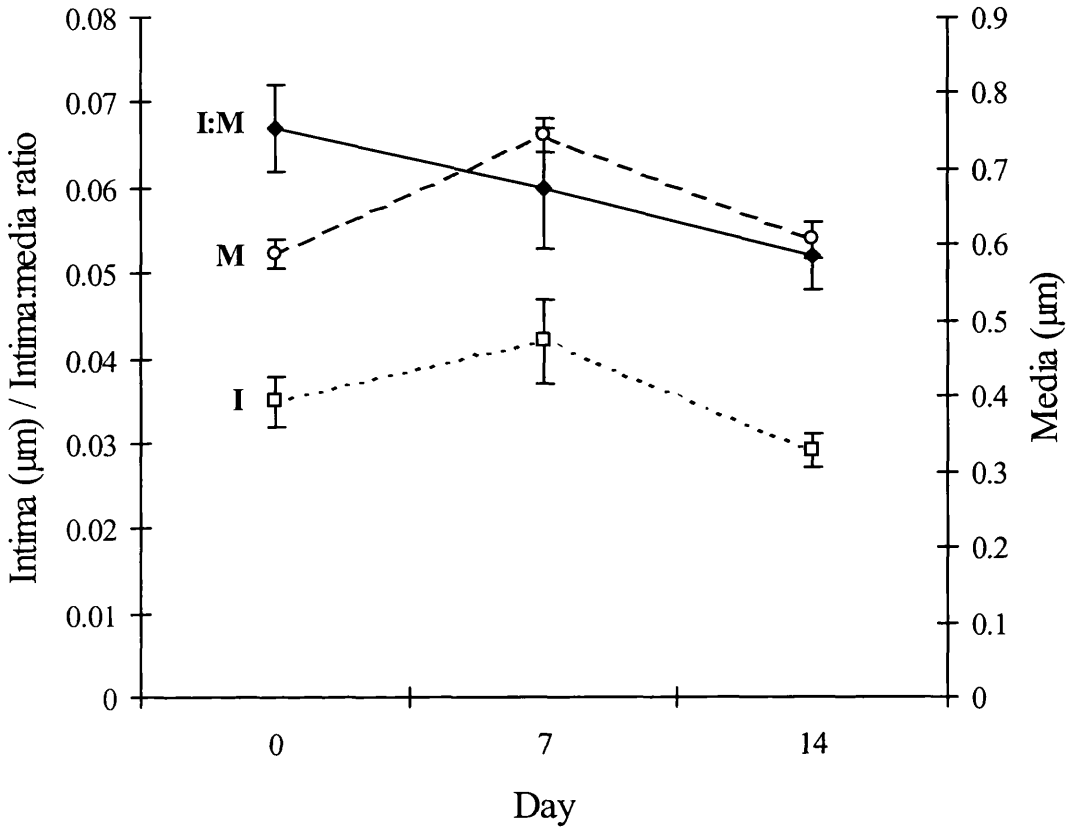


Figure 13.2 Graphical representation of the mean intima:media ratios of varicose explants

Graph representing changes over time, with culturing, in the thickness of the intima (I) and media (M), and the change in intima:media ratio (I:M). Results are presented as mean values ± s.e.m. Actual values and levels of statistical significance are summarised in Table 13.2.

CHAPTER 14

Final Conclusion

CHAPTER 14

So how do these results support our hypothesis regarding the aetiology of primary VVs? Here, it is proposed that primary VVs develop due to the interaction and effect of the venodilatory substances VEGF and NO on the vein wall. In summary, the higher VEGF and NO plasma levels found in subjects with CVI and VVs (Shoab *et al.*, 1998; Schuller-Petrovic *et al.*, 1997) act in synergy to potentiate their venodilatory effect on vein wall. This prime effect, coupled with both the weakening of the vein wall and alterations to the normal homeostatic control of venoconstriction and venodilatation (due to the combined destructive actions of ROS, MMP's and activated leucocytes) results in up-regulation of both VEGF and NO production and secretion. Moreover, VEGF and NO are proposed to act in a positive feedback loop augmenting each other's production (Liu *et al.*, 1994). However, if either the conditions which initiated the homeostatic response are not resolved, despite venodilatory stimuli, or if the vein wall has been damaged by the combined actions of ROS, MMP's and activated leucocytes, to the extent that it is unable to mediate an appropriate response, an excess of both VEGF and NO may result, potentiated by a positive feedback loop between these two substances. Therefore, the vein wall becomes thinned, dilated and weakened, unable to return to its previous tonic state, thus predisposing to further dilatation and possible VVs development.

The results of this study are as follows:

The clinical audit of primary VVs subjects, whom underwent a colour Duplex examination at the Middlesex hospital revealed that;

- Nearly 50 % of all primary VVs co-exist with a competent SFV
- Females outnumber males 2:1
- Females are perhaps more likely to develop VVs at a younger age
- Females are perhaps more likely to develop VVs in the presence of a competent SFV

Trendelenburg's theory of 'descending, sequential, valvular incompetence' being the sole cause of VVs is refuted, as nearly 50 % of patients with primary VVs in the clinical audit were demonstrated to have a competent SFV. Therefore, other mechanisms (possibly molecular) must play a role in VVs aetiology. In addition, VVs in the presence of a competent SFV were more likely to develop in younger females, indicating that there is something peculiar to the female sex that predisposes to the development of varicosity. This 'pre-menopausal' association may be associated with the sex hormone oestrogen.

In controls and subjects with VVs, plasma levels of VEGF and NO and of PBMC VEGF gene induction following induced venous stasis, revealed that;

- VVs subjects have equivalent baseline plasma levels of VEGF compared to controls, with no increase brought about by induced venous stasis
- Controls demonstrate the expected increase in plasma VEGF due to induced venous stasis
- There was no change in transcription of the genes for either $VEGF_{121}$, or $VEGF_{165}$, in the PBMC's collected from normal, control subjects following venous stasis

- There was selective activation of the gene encoding for the $VEGF_{121}$ isoform in the PBMC's collected from varicose subjects following venous stasis

Subjects with VVs demonstrated equivalent baseline plasma levels of VEGF compared to controls, which did not increase following induced venous stasis. However, PBMC gene transcription for the $VEGF_{121}$ isoform was increased. From our original hypothesis we would expect VVs subjects to exhibit both higher baseline levels of plasma VEGF and to demonstrate a greater response following induced venous stasis compared to controls. How can this be explained? Previous studies have demonstrated a decreased dilatory response in VVs segments (Schuller-Petrovic *et al.*, 1997). This may represent an end-stage in VVs development in which the vein wall has been so damaged by the combined effects of VEGF, NO, ROS, MMP's and leucocytes, that it no longer retains the ability to respond to either VEGF or NO, due perhaps, to a down-regulation in the expression of the VEGF receptors, *flt*-1 and KDR. It would explain also, the lack of VEGF production following induced venous stasis and correlate with the observation of decreased NO plasma levels in varicose subjects when compared to controls. If it is accepted that there is a down-regulation in VEGF receptors, then despite the baseline level of VEGF, an appropriate response will not be initiated and NO levels will fail to show a corresponding increase.

However, despite the lack of VEGF production post-induced venous stasis in varicose subjects, there was a 91% increase observed in PBMC gene expression for the $VEGF_{121}$ isoform. Both $VEGF_{165}$ and $VEGF_{121}$ are secreted into the media of VEGF secreting cells and it is commonly held that $VEGF_{165}$ is the more active of the two (Keyt *et al.*, 1996). However, does the $VEGF_{121}$ isoform display additional

functions separate to its known mitogenic and migratory activities? To recap, it is proposed that high levels of VEGF stimulate the production of NO, potentiating the dilatory effect on vein wall. It has been shown that monocytes, which are NO-producing cells, express the VEGF receptor *flt*-1 on their cell surfaces (Barleon *et al.*, 1996). Could $VEGF_{121}$ have a preferential ability in stimulating monocytes to produce NO and thus lead to the development of a positive feedback loop involving NO and VEGF? Of greater interest, the existence of other VEGF receptors (or co-receptors) such as the $VEGF_{165}$ specific co-receptors neuropilin-1 and 2 (Soker *et al.*, 1998) gives rise to the possibility of $VEGF_{121}$ specific receptors which may exihibit a NO-producing specific function.

The above results can be explained within the original hypothesis, but it is apparent that more research is needed to investigate and substantiate this theory. What is required is a reproducible, *in vitro* model of varicosity, which can be used to investigate possible molecular mechanisms theorised to produce VVs. A study was undertaken to adapt an established and validated vein model, which originally used human, normal LSV to investigate SMC-NIH and examine the possibility of using human, varicose LSV instead. The results were as follows;

- The original isolated vein explant model using human, normal LSV as described by Soyombo and co-workers (1990), is validated for the use of human, varicose LSV
- Control explants developed a lesion by day 7 of culture which had regressed by day 14

- Varicose explants at day 0 demonstrated a thickened intima compared to the media. By day 7, a decrease in the intima:media ratio was observed in the presence of a slight increase in the intimal thickness. This was due to a greater increase in the medial layer. By day 14 of culture, the intima:media ratio had returned to a level comparable to that found at day 0
- Medial SMC's may contibute to the structural features seen in VVs
- Varicose explants may retain the ability to remodel themselves after injury

This study has not only demonstrated that an *in vitro* model for varicosity can successfully been established but has revealed also, an insight into the molecular biology of VVs. Culturing of the VVs explants over 14 days suggests that a dysfunction in SMC activity may account for the varying intima:media ratio observed in the explants and changes to VVs wall ultrastructure. It is known that SMC's can secrete VEGF. Moreover, SMC's contain oestrogen receptors (Karas et al., 1994). As this sex hormone has been implicated already in the pathogenesis of VVs (possibly related to the 'pre-menopausal' state) and is a known promoter of VEGF production (Cullinan-Bove and Koos *et al.*, 1993), it is compelling to consider the possibility of it influencing both the function of SMC's and in turn, the structure of VVs walls.

The results of this study can be explained within the confines of our hypothesis regarding VVs pathogenesis. There is strong evidence produced here to suggest the involvement of factors VEGF and NO in VVs aetiology, coupled with the activity of medial SMC's in the vein wall, influenced perhaps by the sex hormone oestrogen.

Additional work is required to deal with the issues revealed by the results of our study. Confirmation of the 'induced venous state' in the lower limbs should be performed with the use of a Duplex machine while blood gas measurements can be used to confirm the presence of relative venous hypoxia. Further research is required to measure the plasma levels of NO as part of a new clinical study, using the same protocol (or perhaps by measuring cGMP levels) but performed over a more protracted time-scale to take into account the delay in NO production via iNOS. Histological analysis of the vein wall will aid in the identification and localisation of VEGF receptors (*flt*-1 and KDR) and reveal any differences in SMC phenotype between controls and VVs. Finally, the *in vitro* model of varicosity (developed within this thesis) can be utilised to investigate any differences in function of $VEGF_{121}$ and $VEGF_{165}$. In addition, (using the same model) changes in VEGF and NO production and of SMC phenotype/activity can be examined with the addition of varying concentrations of oestrogen.

List of Publications

PUBLICATIONS

1. Hollingsworth SJ, Tang CB, Barker SGE (2001). Primary varicose veins in the presence of an intact sapheno-femoral junction. *Phlebology*; **16**: 68 – 72.

2. Hollingsworth SJ, Tang CB, Dialynas M, Barker SGE (2001). Varicose veins: loss of release of vascular endothelial growth factor and reduced plasma nitric oxide. *European Journal of Vascular and Endovascular Surgery*; **22**: 551 – 556.

3. Hollingsworth SJ, Tang CB, Barker SGE (2001). An *in vitro model* for the molecular investigation of varicose veins: use of cultured explants of human long saphenous vein. *Phlebology*; **16**: 53 – 59.

4. Hollingsworth SJ, Tang CB, Barker SGE (2001). The effects of heparin on cultured explants of varicose long saphenous vein. *Phlebology*; **16**: 60 – 67.

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List of Presentations

PRESENTATIONS

National

The Royal Society of Medicine Venous Forum, Chester, UK October 2000

1. Tang CB, Hollingsworth SJ, Barker SGE (2001). Activation of VEGF-121 transcription in blood mononuclear cells from patients with varicose veins in response to hypertension.

2. Tang CB, Hollingsworth SJ, Barker SGE (2001). Primary varicose veins in the presence of an intact sapheno-femoral junction.

Hospital

The Middlesex Hospital: The Pearce Gould Visiting Professor March 2000/1

1. Tang CB, Hollingsworth SJ, Barker SGE (2001). From vein to valve, the aetiology of primary varicose veins.

2. Tang CB, Hollingsworth SJ, Barker SGE (2001). Primary varicose veins in the presence of an intact sapheno-femoral junction.

List of Abstracts

ABSTRACTS

1. Tang CB, Hollingsworth SJ, Barker SGE (2001). Primary varicose veins in the presence of an intact sapheno-femoral junction. *Phlebology*, **15**: 89-90.

2. Tang CB, Hollingsworth SJ, Barker SGE (2001). Activation of VEGF-121 transcription in blood mononuclear cells from patients with varicose veins in response to 'hypertension'. *Phlebology*, **15**: 95.

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