# Increased plasma vascular endothelial growth factor among patients with chronic venous disease

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Skin damage in the presence of chronic venous disease is partially mediated through leukocytes. The endothelium is activated and exhibits proliferation in the skin. Up-regulation of vascular endothelial growth factor (VEGF) expression in the skin of patients with chronic venous disease has been demonstrated with immunohistologic techniques. Abnormal VEGF expression can have local deleterious effects. The aim of this study was to determine whether patients with chronic venous disease have elevated plasma levels of VEGF.

We conducted a prospective study with 30 patients with varicose veins of clinical, etiologic, anatomic, and pathologic class C3 (normal skin, $n$ = 15) and C4 (trophic skin changes, $n$ = 15) and 25 control subjects with no clinical evidence of venous or arterial disease of the lower limb. Blood samples were collected from a foot vein of each subject before and after a period of experimental venous hypertension produced by means of standing. Assay of VEGF protein was performed with a sandwich enzyme-linked immunosorbent assay.

Plasma VEGF level was elevated in both groups of patients with venous disease compared with the control group. The median VEGF levels among patients were 81 pg/mL (interquartile range [IQR] 56 to 122) supine and 98 pg/mL (IQR 63 to 153) after standing for 30 minutes. Median VEGF levels among control subjects were 52 pg/mL (IQR 35 to 71) lying supine and 60 pg/mL (IQR 39 to 105) after standing for 30 minutes. Experimental venous hypertension caused a small rise in VEGF levels among the patients but not the control subjects. Further studies are required to determine whether increased VEGF expression contributes to tissue injury in chronic venous disease. (J Vasc Surg 1998;28:535-40.)

Venous ulceration is a serious health-care problem in western countries. In the United Kingdom alone 150,000 to 200,000 patients receive treatment at any one time from an estimated population at risk for ulceration of 1 to 2 million.[1-3] The cost of this problem is massive. Each ulcer requires frequent dressing changes, and community nurses spend 10% to 30% of their time attending to this problem. It is estimated that the Health Service in the United Kingdom spends £600 million per annum[4] on management of this problem,[4] which amounts to nearly 2% of the total health-care expenditure.

Among volunteers without venous disease, leukocyte sequestration in the lower limb and white blood cell activation have been produced by means of experimental venous hypertension lasting 30 minutes.[5] At the same time various endothelial factors are released, suggesting that this stimulus is enough to produce minor endothelial injury. Among patients with venous disease, plasma levels of neutrophil elastase and lactoferrin (a secondary granule enzyme) also are elevated, confirming the presence of a chronic inflammatory state.[6] However, there does not seem to be a great difference between the extent of this inflammatory response among patients with uncomplicated varicose veins and the extent among patients trophic skin changes. Many other factors are clearly involved. Inflammatory mechanisms play an important role in the pathogenesis of venous disease, but

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Table I. Distribution of subjects according to sex and clinical, etiologic, anatomic, pathologic (CEAP) stage

| *Stage* | *No. of patients* | *Age (y, mean value and range)* | *No. with superficial venous insufficiency* | *No. with deep and superficial venous insufficiency** | *No. with post-thrombotic damage* |
|---|---|---|---|---|---|
| C0 | 8F, 7M | 31 (23-42) | — | — | — |
| C3 | 12M, 3F | 48 (29-73) | 13 | 2 (2) | 1 |
| C4 | 7F, 8M | 51 (32-69) | 3 | 12 (10) | 10 |

*Value in parentheses is number of patients with superficial venous insufficiency.

the exact mechanisms producing lipodermatosclerosis and venous ulceration remain to be elucidated.[5]

Skin damage caused by chronic venous insufficiency is associated with severe microangiopathic changes in the skin. This was first recognized histologically by Burnand et al.[7] Normal skin has occasional capillaries on histologic sections, and the number of capillaries does not increase among patients with varicose veins. However, in the presence of lipodermatosclerosis, there is great proliferation of the skin capillaries. Microscopic examination of the capillaries has been used to study the microcirculation of the skin in the presence of venous disease.[8] This has shown that the capillaries are elongated and tortuous. In advanced stages they look glomerular compared with the pin-shaped capillary loops normally found.[9] There appears to be an increase in the amount of capillary endothelium but not in the number of capillaries. The highly tortuous capillaries are cut many times on histologic sections, and this accounts for the apparent increase in numbers reported by Burnand et al.[7] The resulting microcirculation is abnormal and shows many features of impaired efficiency, including a diminished cutaneous hyperemic response.[10] The proliferation of the capillary endothelium has not been fully explained, although production of vascular endothelial growth factor (VEGF) in the epidermis has been demonstrated (H. Pardoe, unpublished data, 1996). A possible explanation of the microangiopathic changes is that VEGF reaches the main capillaries in the underling papillary dermis and causes them to proliferate. Many growth factors have been identified that might be responsible for the angiogenic response in chronic venous disease.[11,12] The aim of our study was to measure plasma VEGF levels among control subjects and patients with chronic venous disease before and after a period of experimental hypertension to investigate the role of this angiogenic factor in development of chronic venous disease.

## METHODS

Ethical consent to undertake this study was obtained from the committee for medical ethics at our institution. Patients attending the vascular clinic at the Middlesex Hospital for management of lower limb venous problems were asked to participate. Twenty-five persons volunteered to act as control subjects. This group comprised members of the staff of the department of surgery and patients being treated for unrelated conditions. They had no symptoms or signs of venous disease of the lower limb. Volunteers with a history or clinical evidence of arterial disease, diabetes mellitus, connective tissue disorders including rheumatoid arthritis, blood disorders, infection within the previous 6 weeks, or use of medication known to alter white blood cell activity were excluded from the study. Patients and controls who gave informed, written consent were considered for inclusion in this study. The demographic details on subjects included are shown in Table I. All subjects were examined clinically for signs of venous disease by a surgeon trained in the management of vascular disease. Patients were divided into the two groups on the basis of clinical, etiologic, anatomic, and pathologic (CEAP)[13] class determined at clinical examination. There were 15 patients in the C3 group (varicose veins and edema) and 15 patients in the C4 group (trophic skin changes). Patients underwent color duplex ultrasonography by skilled vascular technologists and photoplethysmography. The extent of venous valvular incompetence and post-thrombotic venous damage in the deep and superficial venous systems was established and recorded systematically.

A foot vein or the long saphenous vein at the ankle was cannulated with an 18-gauge cannula (Vasculon 2; Viggo-Spectramed, Helsingborg, Sweden). Blood samples were obtained from each subject before and after a period of experimental venous hypertension. The subject first lay supine for 20 minutes to minimize venous pressure in the leg. The subject then stood supported with minimal movement for 30 minutes. This was followed by a 10-minute period of lying supine. Blood was collected at the end of each period; ethylenediametetraacetic acid (EDTA) was used as the anticoagulant. Plasma was separated by

Table II. Median plasma levels of vascular endothelial growth factor (pg / mL)

| *Study period* | *Control subjects* | *Class C3 patients* | *Class C4 patients* | *All patients* |
|---|---|---|---|---|
| Supine (*P* = difference between control subjects and patients)* | 52 (35-71) | 92 (70-108) *P* = 0.01 | 82 (47-157) *P* = 0.072 | 81 (56-122) *P* = 0.004 |
| Standing (*P* = difference between control subjects and patients)* | 60 (39-105) | 102 (60-118) *P* = 0.066 | 99 (48-200) *P* = 0.14 | 98 (63-153) *P* = 0.03 |
| *P* = difference between supine and standing periods† | *P* = 0.08 | *P* = 0.17 | *P* = 0.19 | *P* = 0.008 |

Values in parentheses are interquartile range.
*Mann-Whitney test.
†Wilcoxon signed rank test.

means of spinning the blood sample at 2000 rpm for 10 minutes. We showed previously that standing produces pressures of 70 to 80 mm Hg in the foot veins.[14] Because this pressure is generated hydrostatically, it is irrespective of the presence of venous insufficiency, which would be important if the patients were exercising during the standing period. It has long been recognized that abnormalities in venous pressure among patients with lower limb venous disease become apparent only during exercise.

Assay of $VEGF_{165}$ protein was performed with a sandwich enzyme-linked immunosorbent assay. A Quantikine kit supplied by R&D systems (Oxon, United Kingdom) was used. Plasma levels were read from standard curves obtained by means of plotting mean absorbance for standards. The kit used enables detection of 25% of VEGF protein activity in the plasma. In contrast, detectable levels in serum are 100%. Thus absolute levels of $VEGF_{165}$ are about four times the levels depicted herein. However, detection in both serum and plasma is equally reliable and the manufacturer confirms that this kit is suitable for use with plasma samples. There are no reports to indicate significant differences among healthy persons in the respective age groups.

The test is used to measure levels of $VEGF_{165}$. This is one of the four transcripts that encode mature monomeric VEGF ($VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$). $VEGF_{121}$ and $VEGF_{165}$ are diffusible proteins secreted into the medium. The other two are mostly bound to heparin-containing proteoglycans in the matrix. Results obtained for naturally occurring human VEGF and the recombinant $VEGF_{121}$ showed linear curves parallel to the standard curves obtained with the Quantikine kit standards.

## RESULTS

The median plasma $VEGF_{165}$ levels for controls and patients before and after induction of venous hypertension are shown in Table II. There have been no large population studies of plasma levels of VEGF measured with this assay. However, data on record in the supplier's laboratory show a median plasma level of 62 pg / mL (R&D Systems) for control subjects, which is very close to that for our control subjects (52 pg / mL lying, 60 pg / mL standing). In our study, levels of VEGF were higher among patients than among controls in both supine and standing positions. Because of the large scatter of the data, statistical significance was not reached until the results for all patients with venous disease were considered together. Levels of VEGF were approximately 60% higher for the patient groups than for the control group in both lying and standing positions (Fig. 1). The two patient groups showed similar levels of VEGF (Fig. 2). There was a small rise in VEGF level after 30 minutes of venous hypertension in the control group, which did not reach statistical significance. The patient groups had a similar modest rise in plasma VEGF level, which did reach significance because of the larger numbers when all patients were considered together.

## DISCUSSION

VEGF (vascular permeability factor) is a member of the platelet-derived growth factor (PDGF) family. It is a disulphide linked homodimeric glycoprotein. It is a potent mitogen for endothelial cells even at very low concentrations and markedly increases vascular permeability.[15] VEGF is important in both angiogenesis and edema formation.[16] VEGF acts through two different tyrosine kinase membrane receptors, which have been identified on vascular endothelial cells.[17] Its angiogenic role in neoplasia is well established. Cells that produce VEGF include vascular smooth muscle cells, fibroblasts, keratinocytes, and histiocytes.[18] VEGF is ubiquitous in wound repair, and the inflammatory process has an important part in the healing process. However, abnormally increased levels over a prolonged period can have local deleterious effects.

In chronic venous disease there is proliferation of

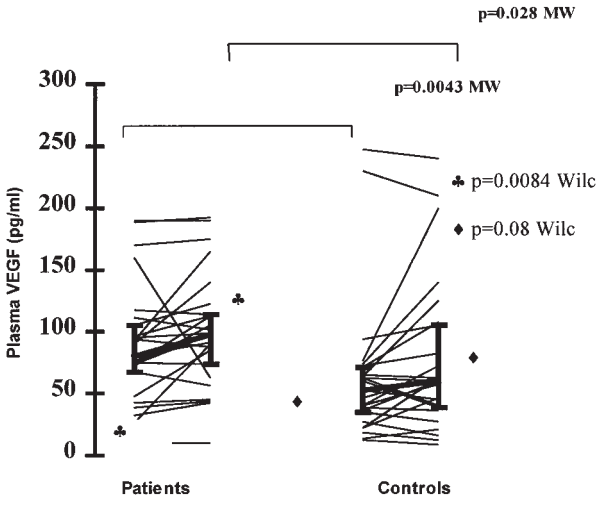


Fig. 1. Plasma levels of vascular endothelial growth factor (VEGF). Comparison between patients and controls. *Horizontal lines* at the top compare VEGF levels in the supine and standing positions between the two groups. *P* values indicated by special symbols are respective values for differences between supine and standing positions within the same group.

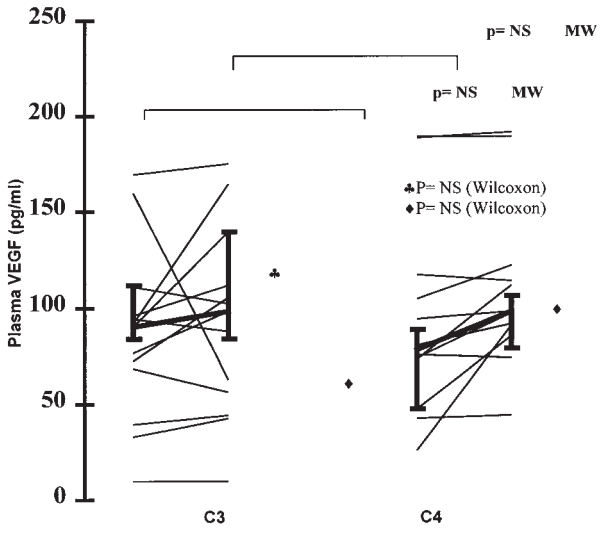


Fig. 2. Plasma levels of vascular endothelial growth factor (VEGF). Comparison between patients with and without skin changes. *Horizontal lines* at the top compare VEGF levels in the supine and standing positions between the two groups. *P* values indicated by special symbols are respective values for differences between supine and standing positions.

vessels in the skin.[19] This correlates with the severity of clinical skin damage. Until recently the growth factors, which mediate this process, have remained unknown. It has been reported that VEGF and platelet-derived growth factor expression is increased in the skin of patients with venous disease and may be the cause of vascular proliferation.[16]

In this study our aim was to assess the association of the development of skin changes with plasma VEGF levels. We therefore used the CEAP system to divide the patients into those with no clinically detectable skin changes and those with lipodermatosclerosis. This allowed us to investigate the factors responsible for development of the skin changes, which are not necessarily the factors that cause venous valvular incompetence of the deep or superficial veins. It is recognized that physiologic indices of venous function, such as air plethysmographic findings, are predictive of the severity of venous disease but that there is considerable overlap between the impairment of venous function among patients from different clinical groups. This simply reflects the fact that the development of skin changes and venous ulceration is not simply a matter of ambulatory venous hypertension–producing leg ulcers but that the response of the tissues is an important determinant of the severity of the clinical syndrome. It is specifically this point that we wanted to investigate, because it reflects a poorly understood aspect of the disease that might eventually be a useful target for drug treatment.

Our control subjects were somewhat younger than either of the study groups. We investigated the influence of age on VEGF levels among the control subjects and could find no correlation. A Medline search of the world literature revealed no studies of the influence of age on plasma VEGF levels. The median VEGF levels among our control subjects were similar to those measured by the manufacturer of the analysis kit with human control subjects, and we believe that these levels reflect those in a population of healthy subjects. Nevertheless we admit that the age difference between the patient and control groups leaves the possibility that some of the differences we observed may be explained with age differences.

VEGF is strongly expressed by epidermal keratinocytes during wound healing, in psoriasis, in skin with ultraviolet burns, and in bullous diseases such as erythema multiforme and bullous pemphigoid. All these disorders are characterized by increased microvascular permeability and angiogenesis. A large increase in VEGF messenger ribonucleic acid (mRNA) and protein levels is seen after irradiation of quiescent keratinocytes with physiologically rele-

vant doses of ultraviolet B light. Overexpression of VEGF is considered dependent on de novo protein synthesis.[20] Tissue damage in general, especially hypoxic damage, can cause up-regulation of VEGF expression.[7,21,22]

The data from this study show that both groups of patients had similar elevations in plasma VEGF level compared with controls. This may reflect the fact that this growth factor is induced in patients with venous disease to repair tissue damage caused by venous hypertension. However, measurement of plasma levels may not reliably reflect the actual tissue levels expressed in the region of tissue repair. It is unlikely that VEGF as reflected by these plasma levels is an important factor in explaining the differences between C3 and C4 patients.

Epidermally derived VEGF is likely to be a factor in the angiogenesis of lipodermatosclerosis. This increased expression of VEGF is seen even before any skin changes develop. We found increased serum levels of VEGF in our study with patients with venous disease. In our study the changes in serum levels were actually more prominent among the group with C3 disease. Studies that measure the expression of VEGF mRNA levels might more conclusively prove the actual site of origin of the protein.

VEGF seems to act both in the short term (over a few minutes) and in the long term (over a few hours) to increase microvascular permeability.[23] VEGF promotes extravasation of fibrinogen and deposition in the tissues as fibrin. This might represent a mechanism for formation of the fibrin cuffs characteristically found in chronic venous disease.[24]

Our previous work showed that leukocyte activation occurs within 30 minutes of experimental induction of venous hypertension with the same model as used in this study. This can be shown for both control subjects and those with venous disease. This leukocyte activation is associated with evidence of endothelial activation, and we believe these are the mechanisms that initiate the skin damage caused in chronic venous disease. This may initiate a repair process that involves increased expression of VEGF by keratinocytes and vascular smooth muscle cells. This causes the neovascularization essential to any tissue repair process. The neovasculature is permeable to large molecules compared with normal capillary endothelium and allows perivascular accumulation of large molecules, accounting for the fibrin cuff originally reported by Burnand et al.[7] Such perivascular cuffs are of course common in many inflammatory conditions.

It has been demonstrated that hyaluron oligosaccharides (OHA) in the intercellular matrix modulate the invasive and proteolytic properties of bovine microvascular endothelial cells and synergize specifically with VEGF in induction of angiogenesis in vitro. The synergism between OHA and VEGF probably plays a role in regulation of angiogenesis. This might be exploited therapeutically in situations that would benefit from modulation of growth of new blood vessels.[22,25]

VEGF has been associated with stimulation of nitric oxide production. The signaling cascade is believed to involve inducible nitric oxide synthase, guanylate cyclase, and cyclic guanosine monophosphate–dependent protein kinase. Experimental administration of VEGF has been found to cause severe hypotension among animals. The hypotension is thought to be mediated by production of nitric oxide and is reversible with appropriate blocking therapy with *N*-(G)-monomethyl-L-arginine.[21,26] The presence of nitric oxide also can explain the hyperpermeability induced by VEGF. Excessive release of nitric oxide can be caused by this mechanism. in contrast to its usual beneficial role, this free radical may contribute to local tissue damage.

To summarize all these observations, a possible scenario for the sequence of events in skin injury in the presence of chronic venous disease is as follows. Damage to the tissues is caused by abnormal leukocyte activation. The injury itself may cause either an increase in VEGF protein synthesis or release of VEGF from depot sites. Activated platelets or macrophages at the site of injury may release peptide factors that in turn stimulate VEGF release. The abundant macrophages in skin exposed to chronic venous disease also are a potential source of VEGF. These macrophages can be present within the basement membrane or extracellular matrix. Components of the extracellular matrix, such as heparan proteoglycan, also are exposed by injury. These are known to facilitate migration and tube formation by endothelial cells.[7,27] Resynthesis of components of the extracellular matrix and migration of pericytes might represent a mechanism of quenching the angiogenic stimulus.[28]

## CONCLUSIONS

In our study plasma VEGF levels were elevated in both groups of patients with venous disease compared with control subjects. Experimental venous hypertension caused a further statistically significant rise in VEGF levels. Increased VEGF expression may play a role in causing tissue injury in the presence of chronic venous disease. Leukocyte-mediated tissue

damage might directly release VEGF from depots in the intima or intercellular matrix. Excessive neovascularization with hyperpermeable vessels might contribute directly to the skin changes that occur with chronic venous disease. Increased production of nitric oxide is a possible mechanism of local tissue damage. The source of the VEGF detected in this study is unclear. We are undertaking studies to determine localization of VEGF mRNA expression to confirm the origin of the protein. It remains to be seen whether an early rise in plasma VEGF level will serve as a marker for development of skin changes. Although VEGF may well be the cause of cutaneous capillary proliferation in venous disease, whether pharmacologic inhibition of this process would achieve a clinically useful effect is very much a matter for debate.

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