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A Reduction in Serum Cytokine Levels Parallels Healing of Venous Ulcers in Patients Undergoing Compression Therapy

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Introduction: *vascular endothelial growth factor (VEGF) and tumour necrosis factor alpha (TNFα) have been specifically implicated in the tissue damage associated with chronic venous disease (CVD). Furthermore, production of both factors is known to be upregulated in vessel wall cells subject to hypertension. The aim of this study was to determine the local venous levels of VEGF and TNFα in limbs with venous ulcers before and after treatment with graduated compression.*
Patients and methods: *eight patients with venous ulcers and 8 patients with varicose veins only were included in the study. For ulcer patients, serum samples were taken from the superficial veins in lower limbs and repeated after 4 weeks of treatment with 4-layered graduated compression. Serum from the arms of the same patients served as controls. Determination of the concentrations of VEGF and TNFα proteins were performed with sandwich enzyme-linked immunosorbent assays.*
Results: *both groups of patients had elevated levels of VEGF and TNFα. In patients with venous ulcers there was a reduction in the levels of both cytokines to below control values with treatment. These changes correlated with healing of the ulcers as determined by reduction in ulcer size.*
Conclusion: *these data, for the first time, suggest a central role for both TNFα and VEGF in the pathogenesis of venous ulceration which may constitute a causative link between venous hypertension and tissue pathology.*

Key Words: Chronic venous disease, VEGF, TNFalpha, Cytokines.

Introduction

Chronic venous disease is the commonest but least understood cause of lower leg ulceration. The tissue changes and ulceration which develop in patients with chronic venous disease (CVD) are strongly correlated with elevated ambulatory pressures (AVP) in the superficial veins.[1] A number of theories have attempted to explain this association. Accumulation and activation of sequestered white cells in affected limbs forms the basis of the currently favoured theories.[2,3] None of these, however, explain the relationship between elevated AVP and tissue pathology in CVD. There is as yet no established causative link between venous hypertension and tissue disease.[2]

Compression treatment is the first line therapy for venous ulcers and is effective in achieving healing of ulcers in the majority of patients. Patients with smaller ulcers present for shorter durations have the greatest response to this treatment and healing rates of up to 95% have been reported in these groups.[4] Graduated compression is thought to promote ulcer healing by acting to restore venous tone, reducing reflux and ambulatory venous pressures and normalising the venous haemodynamics.[5] Multilayer bandaging systems based on the Charing Cross model achieve the greatest level of sustained graduated pressure and are accepted as effective methods of promoting healing of venous ulcers.[6]

Recently, *in vivo* studies of systemic essential hypertension,[7] pre-eclampsia[8] and pulmonary hypertension[9] have shown an associated elevation in serum cytokines, notably vascular endothelial growth factor (VEGF) and tumour necrosis factor alpha (TNFα). Elevated serum levels of VEGF have also been demonstrated in the limbs of patients with chronic venous disease (CVD).[10] It has further been shown, using *in-vitro* models, that endothelial cells subject to abnormal sheer forces and hypertension display altered function and cytokine secretion.[11–15] This is accomplished through a process of mechanotransduction, the mechanism whereby cells transduce mechanical stimuli into an intracellular signal and biological response.[13] There is as yet no published data on the effect of venous hypertension on cytokine secretion from the vascular wall.

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Table 1. Patient characteristics.

	Median (range)
Number/sex	8 (5 M/3 F)
Age (years)	67 (62–82)
Duration of ulcer (months)	6 (1–12)
Ulcer size (T0) (square centimetres)	2.9 (1–5)
Ulcer size (T1) (square centimetres)	1.4 (0–3)
Weeks to complete healing from T0	10 (4–12)
Duplex reflux (no./percentage)	
Superficial	8 (100%)
Deep	2 (25%)
ICP	8 (100%)

It is our hypothesis that superficial venous hypertension produces in a local alteration in cytokine production resulting in an alteration of the cytokine content of the microcirculation, tissues and wound microenvironment in limbs with CVD. We further hypothesise that these changes are reversible upon reduction of venous hypertension with compression treatment.

A number of cytokines with established roles in angiogenesis and wound healing are among those whose production have been shown to be stimulated by elevated pressures. From the currently available data those cytokines which are likely to be of importance in CVD include VEGF and TNFα.

The aim of this study was to determine the serum levels of VEGF and TNFα in the superficial veins of patients with venous ulceration at first presentation and again after a period of treatment with 4-layer graduated compression.

Methods

Patient selection

Ethical approval for this study was granted by the North Eastern Health Board of Ireland. Eight patients with non-healing ulcers were selected from a specialised ulcer clinic before institution of treatment. Patients were excluded if they evidence of peripheral arterial disease as determined by ankle brachial pressure indices (ABIs) of <0.8. Patients with diabetes or rheumatoid disease were also excluded.

All patients had duplex confirmed CVD. Venous reflux was determined using a Hewlett Packard Sonos 500 scanner with venous imaging software. Reflux was defined as reversed flow lasting greater than 0.5 s in the superficial, deep or perforator veins according to recent consensus guidelines.[16]

Ulcer size ranged from 1–5 cm^2 and had been present for a median of 6 months (Table 1).

A further group of 8 patients presenting with varicose veins but without ulceration (CEAP clinical class 2) were included for comparison. All had Duplex confirmed long saphenous vein (LSV) incompetence.

Serum sampling

Serum samples were taken from the superficial veins in the lower limb in the ulcer vicinity (immediately proximal to the ulcer). Samples were repeated once during the treatment period. The repeat samples were taken after between 4 and 6 weeks of treatment with 4-layered graduated compression when the ulcers exhibited healing. This time point was chosen, as it is the timeframe in which a positive response to therapy has been shown to manifest as a reduction in ulcer size.[4] Patients who demonstrate reduction in ulcer size at this point are those in whom compression therapy is effective as a method of treatment. This is presumably because this group of patients represents those who are compliant and whose venous haemodynamics are beneficially altered by compression. Healing was measured as a reduction in ulcer size using computerised planimetry. Patients with varicose veins only had single samples taken. In these patients serum samples were taken from superficial varicosities in the distribution of the LSV in the lower leg.

In all sampling groups control samples from the patients' median cubital vein were taken simultaneously to serve as controls. Blood samples were collected in plain tubes and centrifuged at 2000 rpm for 10 min to obtain serum fraction. Samples were then stored at −80 °C until assayed.

Cytokine ELISA

Determination of the concentrations of VEGF and TNFα proteins were performed with sandwich enzyme-linked immunosorbent assays (R&D Systems, U.K.). The assays were performed according to the manufacturer's protocol. Results were obtained from an ELISA reader and calculated using statistical software programme "Analyse-it for Microsoft Excel", Leeds, U.K. Statistical analyses were carried out using the Wilcoxin Signed Ranks test. p values of <0.05 were considered significant.

Results

All eight patients (5 male/3 female) with venous ulcers completed the study. In all cases patients exhibited healing as determined by a reduction in the ulcer at the time of repeat sampling. All ulcers had completely

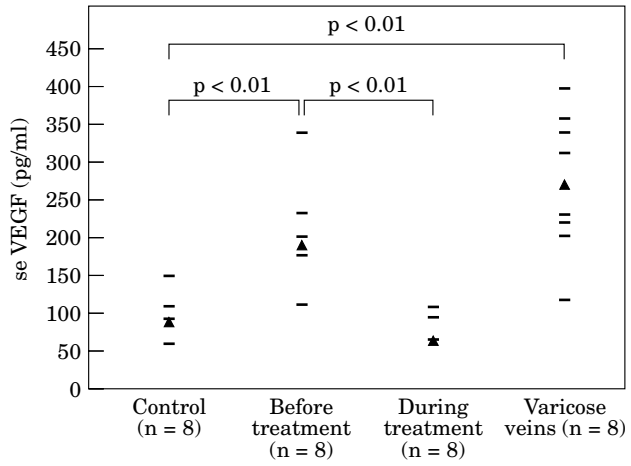

Fig. 1. Serum concentrations of VEGF in pg/ml. Values for each patient are shown as (-) with median values marked as ((). Difference between T0 samples and controls are significant at $p=0.0078$, T1 <T0, $p=0.005$; V Veins>control, $p=0.0078$. Wilcoxin signed ranks test.

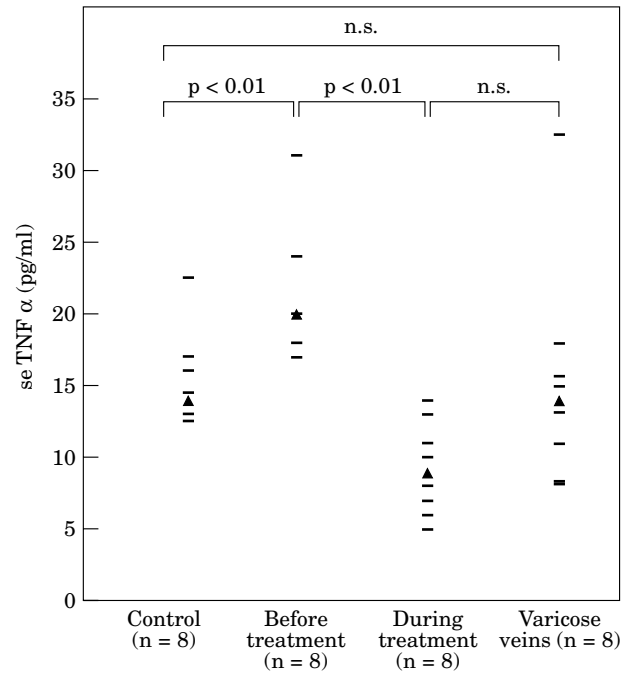

Fig. 2. Serum concentrations of TNFα in pg/ml. Values for each patient are shown as (-) with median values marked as ((). Difference between T0 samples and controls are significant at $p=0.0078$, T1 <T0, $p=0.005$; TNF in varicose Veins> Control but this is not significant at $p=0.84$. Difference between TNFα in varicose veins and treated ulcers did not reach significance, $p=0.078$ using Wilcoxin signed ranks test.

re-epithelialised within 8 weeks of samples taken in the healing phase (Table 1).

All patients had significantly elevated levels of VEGF in samples during the non-healing phase (Fig. 1). Similarly, TNFα was elevated in non-healing samples (Fig. 2).In each case there was a reduction in the levels of both VEGF to below control values with treatment (Figs 1 and 2).

Serum from patients with varicose veins but no ulceration (CEAP 2) also showed elevations in the levels of VEGF cytokines in all cases (Fig. 1). TNFα levels in varicose vein patients were also elevated but failed to reach statistical significance compared to the systemic controls at the start of treatment (Fig. 2).

Discussion

This study, for the first time, demonstrates a significant elevation in VEGF and TNFα in patients presenting with venous ulceration and shows that these levels are reduced with effective treatment.

The significance of elevated VEGF concentrations in limbs with CVD is more readily appreciated when it is considered that this cytokine was originally named Vascular Permeability Factor because of its potent effect on the permeability of the vascular wall. Increased vascular permeability may play an important role in tissue oedema, fibrin deposition and lipodermatosclerosis in CVD.

TNFα is a well-characterised cytokine which has been strongly implicated in tissue damage associated with CVD.[17] Endothelial cells can be stimulated to produce TNF.[18] Serum TNFα is increased in hypertensive states including essential hypertension and pre-eclampsia. TNFα not only inhibits the growth of endothelial cells *in vitro* but has been demonstrated to selectively inhibit the pro-angiogenic effect of VEGF and synergistically increase its effect on permeability.[19,20] Furthermore, it inhibits anti-coagulatory mechanisms and promotes thrombotic processes and therefore plays an important role in pathological processes such as venous thromboses.

The study demonstrates an abnormal cytokine environment in the serum of patients with venous ulceration and a reversal with successful therapy. The findings are consistent with those of a number of recent studies in patients with various hypertensive states and particularly with the data presented by Shoab *et al.*[10] who showed a small but significant rise in VEGF with experimental hypertension in patients with CVD. *In vitro* work, showing cytokine production as an intracellular biological response to altered mechanical forces, suggest hypertension induced mechanotransduction as a potential source of cytokines in these conditions. Our conclusions are based on venous ulceration occurring as a consequence of increased pressure in the superficial veins and healing of ulceration being promoted by effective reduction of these elevated pressures. The study hypothesis at-

tempts to provide an explanation of how the hypertension might cause inflammatory changes and ulceration in the tissues. Both of the cytokines measured in this are recognised as having important effects on mediating inflammation and in the regulation of wound healing. Release of vasoactive and inflammatory mediators such as VEGF and TNFα in response to local venous hypertension may provide an explanation for the "missing link" in the pathogenesis of tissue manifestations of venous disease.

Although this study involved only a small number of selected patients and may be regarded as preliminary, the clear difference in the cytokine levels before and after treatment does show a statistically significant change in the levels of detectable cytokines. Examination of two groups at the extremes of the spectrum of venous disease suggests that elevations in these factors are a cause rather that result of tissue inflammation and ulceration. The use of control samples from the arms of the same patients supports the generation of cytokines at a local level in the lower limb. A control group of patients with normal lower limbs might further strengthen the data. Such a group was not recruited in this study due to the methodological difficulties of performing duplex scanning in a group of asymptomatic people, necessary to demonstrate normal venous haemodynamics. VEGF and TNFα levels previously taken from a small number of individuals in our laboratory have shown low or undetectable levels consistent with published series.

This study examined a group of patients with ulcers which were predicted to heal in compression therapy. Patients with larger ulcers present for greater durations are less likely to heal in compression treatment. From the current data, it is not known what effect compression treatment has on serum cytokine levels in these patients. It is possible that the degree of tissue damage in such cases is refractory to healing even when underlying abnormalities are corrected. This is an important clinical question as it is these patients who pose the largest burden to clinical resources. The sequential changes in cytokine levels in this group and a correlation with healing forms the basis of current investigations in our laboratory. Resistance to healing as a consequence of persistent elevation in cytokine levels may suggest the use of targeted anti-cytokine agents as potential future therapies.

In conclusion, VEGF and TNFα are implicated in the pathogenesis of venous ulceration. Release of these cytokines in response to elevated venous pressures may constitute a causative link between venous hypertension and tissue pathology. However, further work, including *in-vivo* demonstration of intracellular up-regulation of cytokine production in hypertensive veins will be necessary to establish this mechanotransduction as a causative factor in the tissue damage associated with CVD.

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