The relationship between cytokine concentrations and wound healing in chronic venous ulceration

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Objective: **The importance of wound cytokine function in chronic venous leg ulcers remains poorly understood. This study evaluated the relationship between local and systemic concentrations of wound cytokines and wound healing in patients with chronic venous ulceration.**

Methods: **This prospective observational study was set in a community- and hospital-based leg ulcer clinic. Consecutive patients with chronic leg ulceration and ankle-brachial pressure index >0.85 were prospectively investigated. All patients were treated with multilayer compression bandaging. Wound fluid and venous blood samples were collected at recruitment and 5 weeks later. In the wound fluid and venous blood, cytokines and factors reflecting the processes of inflammation (interleukin 1β, tumor necrosis factor-α), proteolysis (matrix metalloproteinases-2 and -9), angiogenesis (basic fibroblast growth factor [bFGF], vascular endothelial growth factor), and fibrosis (transforming growth factor-β_1 [TGFβ_1]) were measured. Ulcer healing was assessed using digital planimetry at both assessments.**

Results: **The study comprised 80 patients (43 men, 37 women). Median (range) ulcer size reduced from 4.4 (0.1-142.4) cm^2 to 2.2 (0-135.5) cm^2 after 5 weeks ($P < .001$; Wilcoxon signed rank), although 17 of 80 ulcers increased in size. The volume of wound fluid collected strongly correlated with ulcer size (Spearman rank = 0.801, $P < .01$). Initial wound fluid concentrations of bFGF correlated with ulcer size (Pearson coefficient = 0.641, $P < .01$), and changes in wound fluid TGFβ_1 concentrations inversely correlated with changes in ulcer size (Spearman rank = −0.645, $P = .032$). There were no significant correlations between changes in other factors and ulcer healing. Wound fluid and serum cytokine concentrations correlated poorly.**

Conclusion: **Wound fluid collection volume correlates with ulcer size. Ulcer healing correlated with increased concentrations of TGFβ_1, possibly reflecting increased fibrogenesis in the proliferating wound. Aside from this, there was a large variation in wound and serum cytokine levels that largely limits their usefulness as markers of healing. (J Vasc Surg 2008; 48:1272-7.)**

The assessment and treatment of patients with chronic venous ulceration remains a massive clinical and economic burden for health care providers.[1,2] It is widely accepted that the skin changes that culminate in lower limb ulceration are a direct result of chronic venous hypertension,[3] although the precise pathophysiologic mechanisms are widely disputed. Theories describing fibrin cuff formation[4] and growth factor trapping[5] have failed to gain widespread support. Although favored, the white-cell trapping hypothesis proposed by Coleridge-Smith et al[6] fails to provide a complete explanation.

The complex and multiple processes leading to wound healing are controlled by cytokines and growth factors within the healing wound. Numerous researchers have speculated that further assessment of these factors may yield important clues regarding failed wound healing and indeed may indicate specific growth factor deficiencies. On the basis of such hypotheses, numerous topical growth factor preparations, including GM-CSF, bFGF (see Table I for abbreviation expansions), and others, have been proposed as potentially efficacious therapies, although reproducible studies demonstrating clinical benefits are scarce.[7-9]

The development and use of topical factors for therapeutic use has not been based on a sound understanding of venous ulcer pathophysiology or cytokine function. Inflammatory markers such as IL1 and TNFα have been shown to be present in significantly higher levels in chronic venous ulcers than in acute healing wounds.[10-12] Similar observations have been reported for angiogenic factors such

Table I. Abbreviations used in the text

Abbreviation	*Expansion*
ABPI	Ankle-brachial pressure index
bFGF	Basic fibroblast growth factor
ELISA	Enzyme-linked immunosorbent assay
GM-CSF	Granulocyte macrophage colony stimulating factor
IL1	Interleukin 1
MMP	Matrix metalloproteinase
TGFβ_1	Transforming growth factor-β_1
TNFα	Tumour necrosis factor-α
VEGF	Vascular endothelial growth factor

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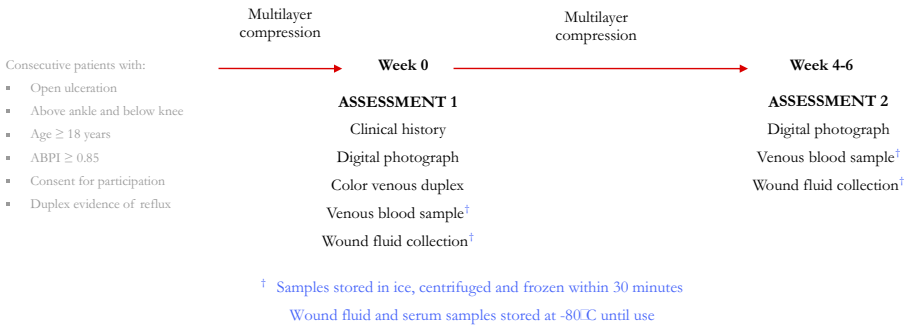

Fig 1. Flow diagram summarizes the study protocol. *ABPI,* ankle-brachial pressure index.

VEGF, bFGF, and other cytokines.[13,14] Unfortunately, no consensus exists for the optimum technique for sampling or measuring these cytokines, and the huge heterogeneity of cytokine levels has limited the benefit of previous research. Nevertheless, improved understanding of cytokine and growth factor dysfunction in chronic venous ulceration may inspire new therapeutic pathways. The aim of the present study was to test the hypothesis that local and systemic cytokine concentrations relate to wound healing in patients with chronic venous ulceration.

METHODS

Patients and setting. The study was performed within the setting of an established nurse-led specialist leg ulcer service in Gloucestershire, United Kingdom. Consecutive new and follow-up patients with open ulceration between the ankle and knee and an ABPI ≥0.85 were targeted. Patients with duplex evidence of venous reflux were included, and a goal of 80 patients was planned. The study was granted full approval by the Gloucestershire Research Ethics Committee.

All patients were treated with nonadhesive dressings (NA, Johnson & Johnson, New Brunswick, NJ) and multilayer compression bandaging (Profore, Smith & Nephew, Hull, UK) providing 40 mm Hg of pressure at the ankle and 17 mm Hg at the calf. For patients with bilateral leg ulceration, the limb with the largest ulcerated area was studied. All bandaging was applied by trained staff, and no treatments were altered or withdrawn on account of participation in this study. Patient compliance with compression therapy was recorded.

Assessments. A relevant medical history and clinical examination were performed in all patients. No assessments of leg edema were performed. Venous reflux was assessed using a Sonos color venous duplex scanner (Hewlett Packard MPG, Andover, Mass) performed by accredited vascular technicians.[15] Wound fluid and venous blood samples were collected and standardized digital photographs were taken. Ulcer areas were calculated from digital photographs using planimetry software (Mouseyes, Dr R. J. Taylor, Salford, UK). In cases where wound edges were difficult to define, the planimetry process was independently verified by a second researcher. Wound fluid sampling, venous blood tests, and digital photography were repeated after 5 weeks. The study protocol is summarized in Fig 1.

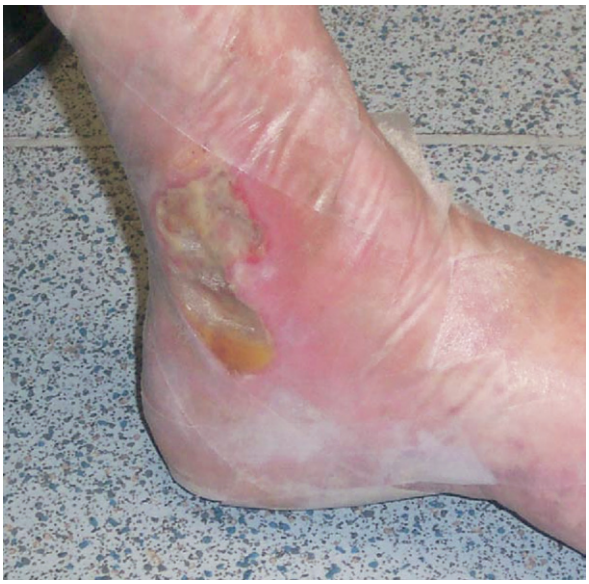

Fig 2. Photograph demonstrates wound fluid sample collected under transparent adhesive dressing.

Venous blood sampling. Venous blood samples were taken from the antecubital fossa with standard antiseptic precautions. Samples were immediately centrifuged at 13,000g for 10 minutes to obtain the serum fraction, and serum aliquots were stored at −80°C until use. In addition to cytokine and growth factor assays, levels of renal function and inflammatory markers were assessed.

Wound fluid collection. Wound fluid collection was performed using a standard protocol, as described previously.[12,16] In brief, the ulcerated area was covered by Tegaderm, a clear adhesive dressing (3M, St. Paul, Minn). The patient was asked to keep the leg dependent in the seated position, and any fluid accumulated under the dressing after 90 minutes was aspirated using a hypodermic needle and syringe (Fig 2). The collected fluid was kept under ice and centrifuged within 30 minutes at 13,000g for 10 minutes to separate particulate matter. Aliquots were stored at −80°C until use.

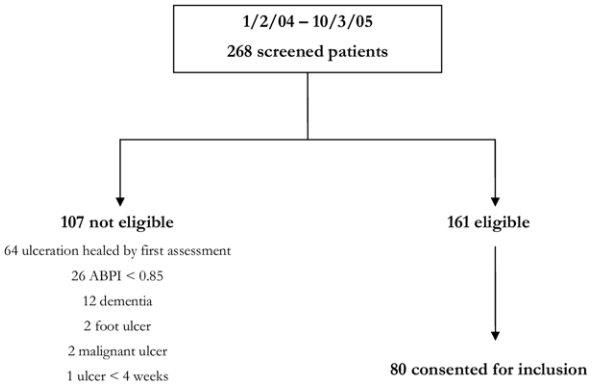

Fig 3. Details of patients screened, excluded, and recruited for the study. *ABPI,* ankle-brachial pressure index.

Assaying details. For this study, cytokines and factors widely believed to reflect the processes of inflammation (TNFα, IL1β), angiogenesis (bFGF, VEGF), fibrogenesis/fibroblast proliferation ($TGF\beta_1$), and proteolysis (MMP2, MMP9) were chosen to give a broad assessment of wound biological activity. With the exception of MMP2 and MMP9, all assays were performed using a sandwich ELISA technique (R & D Systems, London, UK). MMP2 and MMP9 were measured using gelatin zymography, as described previously.[17] In brief, appropriately diluted samples were loaded on a polyacrylamide gel copolymerized with gelatin and electrophoresed at 100 V. Gels were stained with Coomassie blue, and bands were quantified using digital software.

Data analysis. All data were stored on an Access database (Microsoft Corp, Redmond, Wash) and analyzed using SPSS 13.0.1 software (SPSS Inc, Chicago, Ill). Correlations between ulcer healing and changes in cytokine levels were tested using Pearson and Spearman rank tests for parametric and nonparametric data, respectively.

RESULTS

Between February 1, 2004, and March 10, 2005, 268 consecutive patients were screened for this study, of which 80 consented to inclusion (Fig 3). The median age was 74 years (range, 36-93 years), and ulcers were present for median of 3 months (range 1-180 months). The CEAP grades (level II) for the 80 patients were C6sEp in 68 and C6sEs in 12. Other demographic variables for the study population are presented in Table II. Both assessments were attended by 74 (93%) of the 80 patients. No patients in the study had signs of soft tissue infection, and all were compliant with compression bandaging for the duration of this study.

Clinical progression. Complete ulcer healing was seen in 12 of 74 (16%) by the 5-week assessment, and a further 45 (61%) reduced in size during the study period. Ulcer size increased in 17 participants (23%).

Collection of wound exudates. Successful wound fluid collection was achieved in 52 of 80 patients (65%) at assessment 1 and 34 of 74 (46%) at assessment 2. Failure to collect wound fluid was caused by a healed or dry ulcer in 65 of 68 occasions, although in three cases fluid was present but leaked out under the dressing. The median (range) volume of wound exudates collected was 0.23 (0-2.0) mL at assessment 1 and 0.23 (0-6.5) mL at assessment 2. The size of the ulcer correlated strongly with the volume of exudate collected (Spearman $\rho = 0.686$, $P < .001$).

Table II. Demographic variables and details of venous reflux for study population

Variable	*No. or median (range)*
Patient, No.	80
Age, years	75 (40-93)
Sex	
Male	43
Female	37
Ulcer chronicity, months	3 (1-180)
BMI, kg/m^2	27.6 (19.3-64.9)
Diabetes mellitus	10
Previous DVT	12
Ulcer size, cm^2	4.7 (0.1-142.4)
Venous reflux	
Isolated superficial	
GSV only	27
SSV only	12
GSV and SSV	6
Perforators only	5
Isolated deep	4
Deep and superficial	
Deep and GSV	13
Deep and SSV	4
Deep and GSV and SSV	9

BMI, Body mass index; *DVT,* deep venous thrombosis; *GSV,* great saphenous vein; *SSV,* small saphenous vein.

Initial serum and wound cytokine concentrations. Concentrations of IL1β, TNFα, and bFGF were not detected in serum samples and were excluded from further analyses. Concentrations of the other cytokines are reported in Table III. In general, the range of cytokine levels was large within the study population. A significant positive correlation between ulcer size and initial concentration of wound fluid bFGF was present (Fig 4). However, there was no relationship between the initial concentrations of any of the measured factors and subsequent wound healing.

Changes in serum and wound cytokine concentrations. Changes in the serum levels of VEGF negatively correlated with patient age, meaning that older patients had the lowest increases (or greatest decreases) in serum VEGF from 0 to 5 weeks (Spearman $\rho = -0.321$, $P < .01$). When the wound fluid cytokine changes and wound healing over the duration of the study was compared, only $TGF\beta_1$ changes correlated significantly with changes in ulcer size (Table IV). Healing ulcers were associated with an increase in wound fluid concentrations of $TGF\beta_1$.

DISCUSSION

This study confirmed that wound fluid collection is often unsuccessful and is significantly more likely in larger

Table III. Cytokine concentrations at assessment 1 for the study population

Cytokine[a]	*Initial level (range)*
Serum	
proMMP9, % std[b]	51.3 (7.2-449.9)
proMMP2, % std[b]	28.7 (0-114.7)
VEGF, pg/mL	165 (21.2-1315.0)
Wound fluid	
proMMP9, % std[b]	280.1 (33.4-829.1)
proMMP2, % std[b]	21.1 (2.7-193.4)
IL1β, pg/mL	17,877 (864-85,848)
TNFα, pg/mL	767 (92-26,160)
BFGF, pg/mL	228 (65-1370)
VEGF, pg/mL	2950 (130-18580)
$TGF\beta_1$, pg/mL	114 (33-771)

[a]See Table I for abbreviation expansions.
[b]MMP concentrations expressed as a percentage of an MMP2 standard sample used for all assays.

Table IV. Relationship between changes in wound cytokines and ulcer healing from 0 to 5 weeks

Cytokine[a]	*Correlation of cytokine and ulcer size changes*	P[b]
Serum		
proMMP9	−0.193	.110
proMMP2	−0.201	.098
VEGF	−0.131	.304
Wound fluid		
proMMP9	−0.194	.296
proMMP2	−0.151	.462
IL1β	0.028	.931
TNFα	−0.100	.713
bFGF	−0.127	.726
VEGF	−0.146	.565
TGFβ1	−0.645	.032

[a]See Table I for abbreviation expansions.
[b]Spearman rank test.

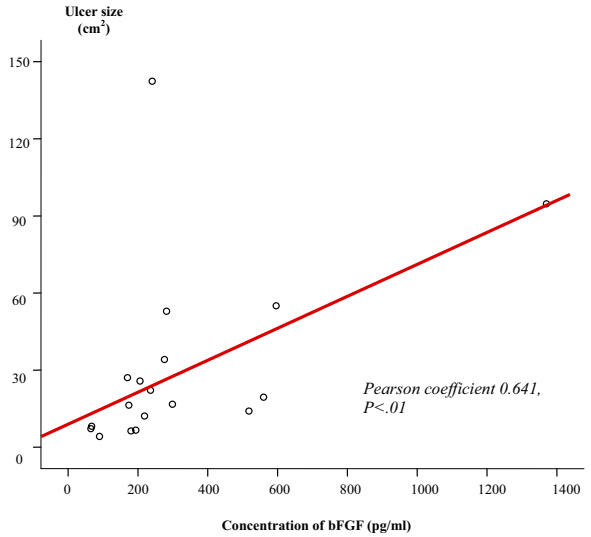

Fig 4. Scatter plot demonstrates the significant correlation between wound fluid levels of basic fibroblast growth factor *(bFGF)* and ulcer size.

venous ulcers. Initial bFGF levels in the wound fluid were higher in the larger ulcers. Concentrations of VEGF correlated inversely with patient age, and $TGF\beta_1$ levels in the wound fluid increased during venous ulcer healing, possibly reflecting the greater fibrogenesis, matrix deposition, and proliferation in the healing wound. Whether extrinsic $TGF\beta_1$ administration would enhance venous ulcer healing requires further investigation.

Wound cytokine concentrations have been assessed previously by a number of researchers, and comparisons between acute and chronic wounds have clearly demonstrated a massive increase in proinflammatory cytokines (TNFα and IL1β).[12,18] Moreover, the levels of MMP2, MMP9, and other proteases have consistently been shown to be higher in chronic wounds compared with acute wounds.[19-21] Although protease changes were mapped over time for the patients with venous ulcers in one study,[17] few studies have assessed changes in other cytokines and factors over time in chronic wounds. Studies by Trengove et al[16] assessed wound fluid protease concentrations before and after 2 weeks of inpatient leg elevation and demonstrated lower levels of MMP2 and MMP9 and proinflammatory cytokines in healing ulcers. These findings were not reproduced in our study; however, the influence of outpatient compression therapy may differ from inpatient elevation.

Although patient recruitment for clinical trials for venous ulceration is notoriously challenging, the sample size for this study was significantly larger than other comparable studies. The inclusion criteria were deliberately very open, and consecutive eligible patients were approached to ensure that the target population was truly representative of the entire leg ulcer population.

A number of previous studies have investigated cytokines in venous ulcers, but few have evaluated the practical difficulties and reliability of such assessments. In this study, the success of sample collection, reasons for failure, and comparisons between different sampling modalities were primary objectives in addition to cytokine measurements. Despite practical limitations, the trial methodology was carefully planned to ensure a scientifically robust study design.

Unfortunately, paired wound fluid analysis was only possible in 43% of recruited patients. Studies by Trengove et al[12] and Murphy et al[18] did not report any difficulties with wound fluid collection, although ulcer sizes were larger than in the current study population. Most of these failures in sample collection were due to practical factors: Ulcers were often dry and did not produce fluid. The effectiveness of compression therapy may have contributed to failed wound fluid collection. However, successful fluid collections were achieved in healing ulcers, where compression therapy was presumably effective. An alternative method of wound fluid collection, such as the use of filter

paper discs, may result in a greater proportion of successful fluid collections,[17] although the tiny volumes collected would preclude many of the cytokine assessments performed in this study. It should be recognized, however, that wound fluid assessment is more likely to be representative in patients with larger venous ulcers rather than for the entire ulcer population.

Cytokine levels and changes in cytokine levels varied enormously between different patients. In most cases, the huge range in measurements did not relate to clinical criteria, meaning that same level of a specific cytokine might be normal for one individual but highly abnormal for another. General conclusions are therefore difficult to draw, although paired assessments were used to partly combat these population variations. In view of the heterogeneity among the study patients, the failure to study both ulcerated legs in patients with bilateral ulcers may represent a missed opportunity in this study.

An assumption of the study is that measured cytokines directly reflect the chronic wound, whereas the influence of other illnesses or nonpathologic behaviors on cytokine concentrations is unclear. Cytokine analysis was performed using venous blood and wound fluid samples in this study, but whether these samples are actually representative of the wound bed is simply not known. Nor is it clear if cytokine or factor secretion into the wound fluid or absorption into the blood stream is consistent over time. Some authors have suggested that that protease release may vary in different parts of the same wound, but this has not been widely substantiated.[17]

Wound fluid $TGF\beta_1$ was the only measured cytokine that mirrored venous ulcer healing. Unfortunately, paired analyses were only possible for the 46% of participants with successful paired wound fluid collection, meaning that these changes might only be applicable to patients with larger ulcers. A negative correlation with ulcer size was identified, although these findings may simply reflect the changes of the healing and deteriorating venous wound. An alternative explanation is that the lack of progression in the deteriorating wounds is secondary to a deficiency in $TGF\beta_1$. Whether the relationship between $TGF\beta_1$ and ulcer healing is causal or consequential remains unknown. A clinical trial investigating the influence of extrinsic $TGF\beta_1$ supplementation may resolve this point. Unfortunately, such a study is unlikely to be supported in the near future because this type of product may take years to develop and trials of other extrinsic growth factors have yielded little clinical benefit.[9]

CONCLUSIONS

This study demonstrated that cytokine assessment in chronic venous ulcers is associated with practical sampling difficulties and huge interpatient variation in cytokine concentrations. In this study population, concentrations of $TGF\beta_1$ in wound fluid mirrored ulcer healing. The relationship between wound cytokines and venous ulceration was otherwise poor. Further studies are required to confirm the validity of these findings and explore potential clinical applications.

AUTHOR CONTRIBUTIONS

Conception and design: MG, JT, MY, KP
Analysis and interpretation: MG, RW
Data collection: MG, RW, JT
Writing the article: MG, MW, KR
Critical revision of the article: MS, RW, JT, MW, KP
Final approval of the article: MS, RW, JT, MW, KP
Statistical analysis: MG
Obtained funding: Not applicable
Overall responsibility: MG

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