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Causes and Effects of the Chronic Inflammation in Venous Leg Ulcers

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The pathogenesis of venous leg ulcers is multifactorial. In this review article new physiological, molecular and cellular abnormalities in venous ulcers related to the chronic inflammation are presented and discussed. Venous hypertension causes disturbed microcirculation and pathological changes of the capillaries, which eventually locks the condition in a self-amplifying, detrimental cascade with persistent elevated levels and activities of pro-inflammatory cytokines and proteases preventing progress into a healing phase. As a consequence fibroblasts senesce and become less responsive to growth factors the older the ulcers become. Current data imply there is no deficiency but rather an unfavorable distribution of growth factors in venous ulcers. An imbalance in proteolytic enzymes and their endogenous inhibitors is a common finding in chronic venous leg ulcers. Variation in disease severity and concomitant ailments in this heterogeneous patient group may explain the contradictory results in the literature. Thus, to advance the areas of research further, longitudinal studies involving larger number of patients are required to identify the major pathogenic factors. ***Key words: venous insufficiency; compression therapy; etiology; wound healing; fibroblasts; growth factors; cytokines; immunohistochemistry; in situ hybridization; matrix metalloproteinases; tissue inhibitors of metalloproteinases; neutrophil elastase; fibronectin; integrins; wound fluid.***

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INTRODUCTION

Whatever the cause of chronic wounds, being venous or arterial insufficiency, excessive pressure or diabetes mellitus, one hallmark is the presence of chronic inflammation (1, 2). Although the importance of the inflammatory reaction for an adequate wound repair response is established in acute wounds, little research effort has been devoted to the role of the extended inflammation in non-healing chronic wounds. A symposium was therefore organized to highlight the biological consequences of the chronic inflammation on cytokine levels and production, growth factor profiles, cell proliferation, and the proteolytic environment in chronic venous leg ulcers. The symposium was held on 28 August, 1998 as part of the 8th Annual Meeting of the European Tissue Repair Society in Copenhagen, Denmark and was chaired by Dr William H. Eaglstein. The speakers and topics presented were as follows: Drs Keith Harding, *Where are we today with regard to knowledge, where do we go and what is clinically usable?*; Mark W.J. Ferguson, *Tissue morphology and inflammatory reactions in venous ulcers*; Gregory S. Schultz, *Growth factor release and stability—trapping phenomena*; Keith Moore, *In vivo and in vitro lymphokine/cytokine response*; Magnus S. Ågren, *Fibroblast growth in acute and chronic wounds*; Ulpu K. Saarialho-Kere, *Matrix metalloproteinases in chronic wounds.*

This paper is a summary of the presentations together with current knowledge about the complexity of the pathogenesis of venous leg ulcers with emphasis on the role of the chronic inflammation.

EXTENT OF THE CLINICAL PROBLEM

Venous ulcerations of the lower extremities represent an increasing socioeconomic health care problem (3). It has been estimated that about 0.1% of the population of developed countries have venous leg ulcers and more than 90% of the patients are over the age of 60 years (4–8). The total annual cost for the treatment of leg ulcers probably amounts to over GBP 400 million in the UK (3). Although mortality is not increased, the condition is associated with high morbidity (9). Only about half of the patients heal their ulcers in a 5-year period and once healed, venous ulcers often recur (8–10).

CAUSE OF VENOUS ULCERS

The cause of venous leg ulcers is traditionally based on the underlying vascular pathology (11). About 50% of patients with venous leg ulcers have superficial and perforating vein incompetence primarily in the valves (9, 11, 12). Superficial venous reflux may be present in a large proportion of patients without visible varicose veins (11). Also, superficial venous reflux influences deep veins, which may aggravate the hemodynamic abnormality further (13, 14). A clinical history of deep vein thrombosis is associated with the poorest prognosis (15, 16). However, patients with documented previous deep venous thrombosis can subsequently have normal deep vein function (8, 11). It is unclear whether those patients are at the same degree of risk of developing ulceration as patients who have abnormal deep vein function. Furthermore, the role of arteriovenous shunting, calf-muscle pump function and edema need further clarification (17–20). However, not all patients with venous disease develop active skin ulcerations and the pathogenic steps leading from venous and capillary hypertension to ulceration and failure to heal are unknown (12, 21).

Inflammation has been implicated in the causation of

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chronic venous leg ulcers (22–24). The hypothesis suggests there is sustained venous pressure because the pressure differential is reduced between the arterial and venous systems effectively trapping or slowing polymorphonuclear leukocytes (PMNs) in the area where the skin eventually breaks down. Increased proteolytic activity in lipodermatosclerotic skin (25), a preulcer stage, may also predispose the skin to ulcerate (26). The circulatory PMNs are primed and can be activated more readily in patients with chronic venous insufficiency (21). In one study, PMNs derived from the legs and arms of 11 patients with active venous ulceration and lipodermatosclerosis were examined for their ability to generate oxygen free radicals. There was a marked elevation of the ratio of leg to arm oxygen free radical production of the PMNs from patients with active ulceration and in patients with underlying venous disease compared with healthy controls (27). The excess of iron in circumferential skin of venous leg ulcers may increase the generation of oxygen-derived radicals further by the Fenton reaction (28, 29). The activated PMNs also release proteases such as neutrophil elastase, cathepsins, collagenase and gelatinases in addition to the toxic oxygen metabolites that can damage the capillary endothelium (30). This results in leakage of macromolecules, including fibrinogen, which polymerizes on the outside of the vessels forming pericapillary fibrin cuffs (26, 31, 32). Fibrin cuffs have been suggested to prevent the diffusion of oxygen and nutrients to the skin (31–34). Plasminogen available to generate fibrin-degrading plasmin appears to be deficient in venous ulcers as well (35). It is still controversial, however, as to whether the pericapillary fibrin cuffs cause venous ulceration or if their presence is merely a consequence of the venous disease and/or the ulcer (36–38). An alternative/additional explanation for the local impaired skin oxygenation and nutrition leading to ulceration is the reduced capillary density in the gaiter area of the lower legs of patients with chronic venous insufficiency (39). Recently, Falanga and Eaglstein (40) proposed that macromolecules, such as fibrinogen and α_2-macroglobulin, leak into the dermis as a result of a venous hypertension and then bind or "trap" growth factors and matrix material which then become unavailable for tissue repair and the maintenance of tissue integrity. The validity of the hypothesis has, however, been questioned (41, 42).

COMPRESSION TREATMENT

In venous disease the standard conservative treatment is compression bandaging (43–45). The beneficial effects of compression therapy are well-documented (46). By applying external pressure the leakage of the dilated capillaries is prevented, the edema removed and the superficial disturbed capillary perfusion improved (47, 48). There are a variety of bandaging systems some producing up to 70% complete healing in 3 months (16, 44, 49–51); even up to 95% healed ulcers at 3 months has been reported with an individualized and optimized compression technique (52). The high compression systems such as the four-layer bandage system, consisting of, for example, a non-adherent contact material covered with sterile gauze, then orthopedic padding, crêpe, Elset® (Seton) and Coban® (3M), seem to yield better short-term results than the low compression systems (46, 49, 53). Do the differences in the results with different bandaging systems relate to the ability of them to modify the pathological inflammatory processes?

THE INFLAMMATORY REACTION IN NORMAL WOUND REPAIR

Wound healing is a complex biological phenomenon. The phases in the healing cascade overlap and the complexity of the repair process may explain the large variation in response in patients with leg ulcers. The important issue is to determine the factors responsible for such heterogeneity. Inflammation is traditionally thought to be over and done with in about 5 days, and has no role thereafter (54). In Fig. 1 the cell counts in open acute wounds healing by secondary intention (55) are presented for PMNs, eosinophils, macrophages, lymphocytes, plasma cells, fibroblasts and endothelial cells. As is evident from Fig. 1, the time scale appears very different in human wounds from the conventional scheme derived from standardized wounds in laboratory animals (56, 57). It is also well-documented that an attenuated inflammatory reaction is detrimental to wound repair as is an excessive inflammatory reaction (58, 59).

HISTOPATHOLOGICAL STUDIES OF VENOUS LEG ULCERS

The chronic inflammatory cell infiltrate in the venous ulcer bed and surrounding tissue has been characterized using immunohistochemistry (60–64). Macrophages are the dominant cells comprising 50–70% of the inflammatory cells (63, 64). Although macrophages, detected as CD68+ cells, predominate at the wound edges and are more numerous than in normally healing wounds (63, 64), a large proportion of the macrophages show a low expression of certain activation markers (CD35, C3b receptor) (62). These findings indicate that macrophages within some chronic wounds are down-regulated in certain aspects and unable to direct the

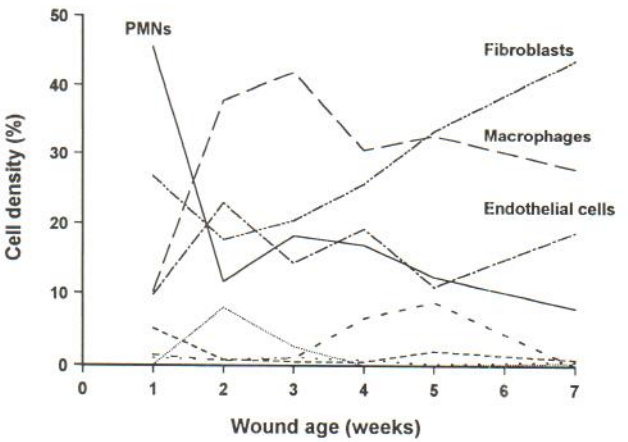


Fig. 1. Cellular composition, as determined histologically in biopsies, in acute human wounds healing by secondary intention (sacrococcygeal pilonidal sinus excisions) as a function of wound age. The wounds were initially 40 mm deep and completely closed by 10 weeks (55). PMNs are represented by a solid line, macrophages by a dashed 70/30 line, lymphocytes by a dotted line, plasma cells by a dashed line 30/70, eosinophils by a fine-dotted line, fibroblasts by a dash-dot-dotted line, endothelial cells by a dash-dotted line and other cell types by a dashed 50/50 line.

wound repair process appropriately (62, 63, 65, 66). The limited lymphocyte infiltrate (<20%) is predominated by T lymphocytes with a larger number of CD4+ than of CD8+ T lymphocytes although the CD4+/CD8+ ratio appears to be lower in non-healing venous ulcers compared to that of acute wounds (60–63, 67). Although only a few B lymphocytes are present in venous leg ulcers, Loots et al. (63) reported an increased number of B lymphocytes (CD20+) and plasma cells (CD79a+) in chronic venous leg ulcers compared with acute wounds. PMNs are only sparsely distributed in the ulcer base and surrounding tissue though at higher numbers than in mature acute wounds in age-matched patients (60, 63). After 2–4 weeks of effective compression therapy the inflammatory cell population infiltrate is converted into one more closely resembling the composition of a healing acute wound.

In venous leg ulcers, one of the prominent morphologic abnormalities is the pericapillary fibrin cuffs (Fig. 2). In histological cross-sections, the vessels appear clumped but when the ulcer heals with, for example, compression therapy the vessels are no longer clumped but more distributed throughout the ulcer stroma than in surrounding skin (1). The cuffs surrounding the capillaries, however, immunostain not only for fibrin but for most of the extracellular matrix molecules such as fibronectin, laminin, tenascin and collagen types I and III (1, 68). The type IV collagen layer and basal membrane of dermal capillaries are also thickened in venous insufficient legs (69). These "matrix" cuffs are present in the majority of venous ulcers and are persistent structures in non-healing ulcers but they resolve in the healing parts of most ulcers with optimal compression therapy (1, 26, 37, 70). Also, the cuffs contain high levels of growth factors and their cell receptors such as transforming growth factor-β1 (TGF-β1) and its receptor. Increased expression of the platelet-derived growth factor (PDGF) α-type and β-type receptors, and of vascular endothelial growth factor (VEGF) in endothelial cells of capillaries in venous leg ulcers has also been reported (71). Thus, there appears to be no deficiency but an aberrant distribution of growth factors in chronic venous ulcers (68). The vascularature in venous ulcers shows high angiogenic activity with virtually all pericytes and endothelial cells dividing. The high proliferative activity of capillaries appears to be linked to the skin ulceration rather than to the chronic venous insufficient state (72). The cuffs also show prominent staining for the vascular cell adhesion molecules intercellular adhesion molecule-1(ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) and their ligands leukocyte function-associated antigen-1 (LFA-1) and very late activated antigen-4 (VLA-4) on leukocytes, circumstances that facilitate emigration of leukocytes out of the blood vessels into the surrounding tissues (73, 74). The matrix cuffs also commonly contain numerous PMNs (75) as seen in Fig. 2. Typically one finds extravasated erythrocytes and deposits of hemosiderin extracellularly giving rise to "hyperpigmentation" (1).

CYTOKINE LEVELS AND GROWTH FACTOR STABILITY IN THE CHRONIC WOUND ENVIRONMENT

Mitogenic activity of chronic wound fluid

Do wound fluids collected from chronic wounds and acute wounds have comparable levels of factors that stimulate DNA synthesis? Addition of 10% of mastectomy fluid from the acute type of injuries to a chemical-defined medium stimulated proliferation of normal human fibroblasts at low passage (76). A mitogenic effect of wound fluid from donor site wounds has also been found previously (77). In contrast, if 10% volume of chronic wound fluids are added to the fibroblasts the effect is entirely opposite, that is they not only lack the ability to stimulate DNA synthesis but they actually suppress the limited amount of DNA synthesis that occurs, probably because the fibroblasts themselves are secreting

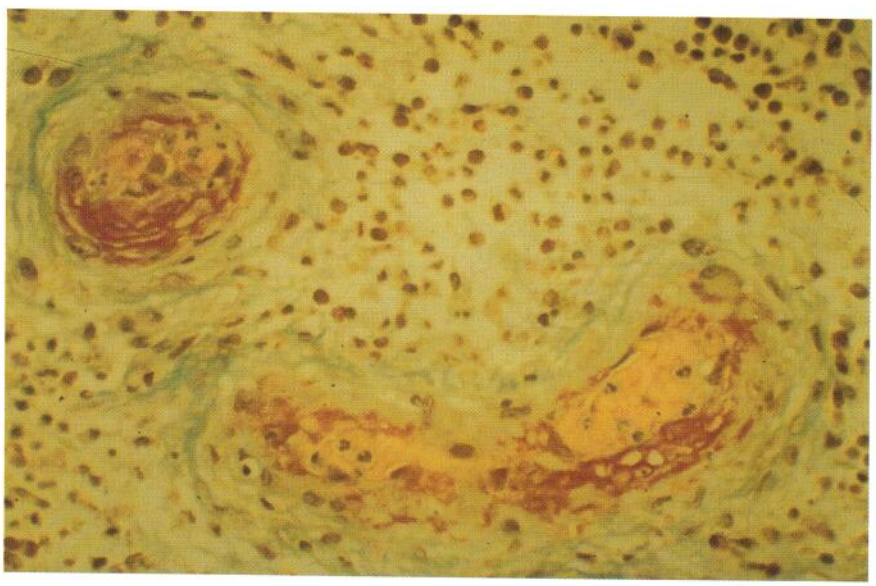


Fig. 2. Pericapillary fibrin cuffs in a non-healing venous leg ulcer of 3 months duration. Note the 2 clumped vessels surrounded by cuffs consisting not only of fibrin but also of other matrix molecules such as collagen, and the chronic inflammatory cell infiltrate composed of lymphocytes, plasma cells, macrophages and PMNs in vessel lumen and walls in edematous tissue. (Mallory's trichrome stain; yellow = erythrocytes, red = fibrin and blue = collagen.)

autocrine growth factors into the medium. The proliferative inhibitory effect of chronic wound fluid was found to be reversible, heat-sensitive and present in the low molecular weight fraction (<30 kDa) (78, 79). The opposite effects reported of chronic wound fluid on fibroblast proliferation, that is stimulation of proliferation, may be explained by differences in culture conditions, origin of fibroblasts and sampling techniques (79–81). Furthermore, it is uncertain whether the wound fluid precisely reflects the interstitial space fibroblasts are in. To resolve these apparent discrepancies, wound fluids from chronic venous leg ulcers and acute wounds in the same patient could be examined for their mitogenic activity. Epithelial cells cultured from human foreskins or endothelial cells cultured from large arteries react similarly to the different wound fluids (77, 82, 83).

Thus, the basic difference between chronic venous ulcers and acute wounds in this *in vitro* model is that wound fluids from chronic ulcers may have detrimental biological effects on wound healing.

Cytokine levels in chronic wound fluid

The most important early events in wound healing is the pro-inflammatory cytokine cascade (84, 85). A series of pro-inflammatory cytokines and their naturally occurring inhibitors were measured in chronic and acute wound fluids. Although mastectomy wound fluids have immunologically detectable levels of tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) using enzyme-linked immunosorbent assays (ELISA) the chronic wound fluids tend to have substantially and significantly higher levels of immunoreactive TNF-α and IL-1β (85). Furthermore, the ratio of the inhibitor p55 (soluble TNF receptor protein) to TNF-α and the ratio of IL-1ra (IL-1 receptor antagonist) to IL-1 are consistently higher in acute wounds than the in chronic wounds (Table I). Theoretically then, increased amounts of unbound cytokines may exert increased bioactivity in chronic wounds. These results should, however, be interpreted with caution because of the anatomical differences between a cutaneous chronic wound and a surgical drain placed subcutaneously in the chest region. Furthermore, leg ulcer wound fluid contains micro-organisms while the mastectomy wound fluid is sterile. The presence of micro-organisms might initiate a subclinical pathological inflammatory response (86). Harris et al. (87) were unable to show differences in the levels of granulocyte/macrophage colony-stimulating factor (GM-CSF), IL-1α, IL-1β and IL-6 between healing and non-healing venous leg ulcer, whereas Wallace and Stacey (88) found elevated levels of immunoreactive TNF-α in non-healing as opposed to healing venous leg ulcers.

Table I. *Ratios of inhibitor/cytokine in acute and chronic wound fluids*

	Acute wounds[a] (n = 30)	Chronic wounds[b] (n = 14)
p55/TNF-α	12:1 (0.84:0.07 ng/ml)	4:1 (4.93:1.23 ng/ml)
IL-1ra/IL-1β	480:1 (8.68:0.018 ng/ml)	7:1 (19:3 ng/ml)

[a] Mastectomy drain wound fluid.
[b] The chronic wound group comprised 4 venous leg ulcers, 4 diabetic ulcers and 10 pressure sores.

In conclusion, cytokine activities and cytokine protein levels in terms of agonisms and antagonisms in wound fluid appear to reflect the differences in healing stage of acute and chronic wounds.

Is there "trapping" of growth factors in venous ulcers?

The "trap" hypothesis (40) was tested in an *in vitro* experiment. Biopsies from venous leg ulcers and normal skin were first incubated with radiolabeled growth factors and then the release of the growth factors from the tissue explants was measured *in vitro*. If there are trapping components present in the chronic wounds then, theoretically, a delay in the release kinetics of the growth factor back into the medium from the biopsies would be anticipated. Basically, no significant amount of trapping of the growth factors by the chronic ulcer bed was detected because the growth factors were liberated at the rate of normal skin (Fig. 3). The growth factors were found intact and biologically active after the incubation period and measured radioactivities were therefore not due to proteolytic fragmentation of the growth factors.

Although no trapping of growth factors by venous leg ulcer tissue was observed in this *in vitro* experiment, it might not mimic correctly the *in vivo* circumstance in which growth factors are produced within the pericapillary cuffs or taken up from the vascularature.

Stability of growth factors in chronic venous ulcers

What happens to the growth factors once released to the chronic wound environment? This question has been approached by several researchers. The ability of chronic wound fluids to degrade growth factors was examined using epidermal growth factor (EGF) as one growth factor that is involved in healing. There was essentially no measurable degradation of EGF by the acute wound fluids, obtained from surgical drains or open cutaneous wounds, but by the chronic wound fluids (85). Other investigators found similar detrimental effects of chronic wound fluid on PDGF-AB, PDGF-BB and TGF-β1 (89, 90). Degradation of the growth factors was prevented by the addition of inhibitors of serine proteinases, but not by inhibitors of matrix metalloproteinases, indicating the detrimental effects of excess protease activity such as that of neutrophil elastase in chronic wounds (89, 91). In contrast to these findings, the mitogenic activity of relative high concentrations of PDGF-BB, measured with bioassays, remained undisturbed after having been pre-incubated in chronic wound fluid (80, 92). The apparently contradictory results may reflect the heterogeneity among chronic venous leg ulcer patients.

PRODUCTION AND EFFECTS OF PRO-INFLAMMATORY CYTOKINES *IN VITRO*

Cytokine production in venous ulcer tissue

To characterize the pro-inflammatory cytokines IL-1β, IL-8 and TNF-α being produced by wound tissue, explants from the base and margin of venous leg ulcers were incubated *in vitro* for up to 24 h, and the cytokines in the conditioned medium quantified by immunoassay or bioassay (93, 94). Large amounts of human IL-1β was produced in this system.

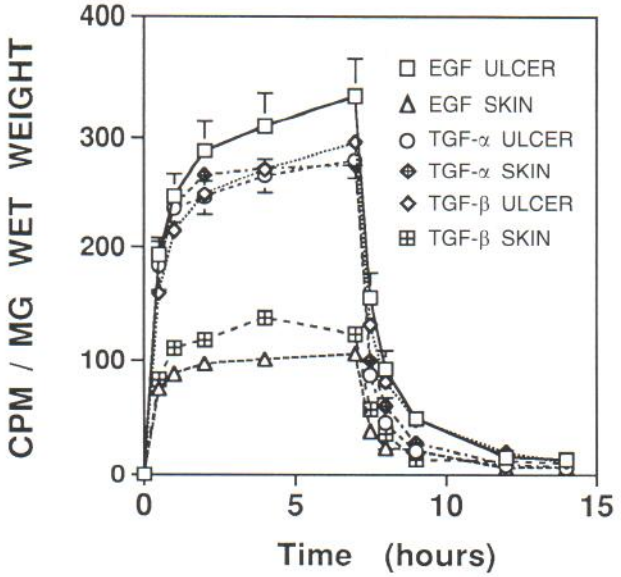


Fig. 3. Kinetics of absorption and release of radiolabeled growth factors by tissue from venous leg ulcers and normal skin. Four-mm punch biopsies were obtained from the center of a venous leg ulcer and from mammary normal skin, respectively. Biopsies were transferred into a culture medium (Medium 199) where they remain viable for at least 24 h. Human recombinant growth factors (EGF, TGF-α and TGF-β) were radiolabeled with ^{125}I which emits gamma rays that penetrate easily through a few mm of punch biopsy tissue. Biopsies were then incubated in culture medium at 37°C containing the radiolabeled growth factors in separate plastic vials, and at each time point from 0 to 8 h the biopsies were removed from the culture medium containing the radiolabeled growth factors, rinsed in fresh medium devoid of radiolabeled growth factors and the radioactivity of radiolabeled growth factors were measured in the biopsies using a Wallac gamma counter. The biopsies were then returned to the culture medium containing radiolabeled growth factors and incubated until the next time point when radioactivity was measured again. This procedure was repeated until the uptake of radiolabeled growth factors had reached a plateau at approximately 8 h, at which time the biopsies were transferred to a large volume of fresh culture medium at 37°C lacking radiolabeled growth factors. At the indicated time points, the biopsies were removed from the culture medium, rinsed quickly and the radioactivity measured with the Wallac gamma counter. The biopsies were then again placed in a large volume of fresh culture medium lacking radiolabeled growth factors until the next time point when the radioactivity in the biopsy was measured again. Almost all radioactivity that was taken up by the biopsies was released during incubation of the biopsies in culture medium lacking radiolabeled growth factors. Thus, the amount of radioactivity that was taken up and released from each biopsy was similar indicating that there was no "trapping" of growth factors in biopsies from the ulcer bed or in biopsies from normal skin.

Interestingly, repeated medium changes every 2 h with cultured wound bed tissue decreased IL-1β production whilst at the same time IL-8 production increased. These *in vitro* findings are in accordance with recent *in vivo* findings where Fivenson et al. (95) noticed an increase in IL-8 concentration in both wound fluid and tissue extract from venous leg ulcers, which healed 4 weeks after compression therapy was initiated. Surprisingly, in the tissue explant system only low levels of TNF-α were produced although there were many CD68+ macrophages in the ulcer bed. The culture system results were confirmed by immunostaining for TNF-α and the only positive staining seen was in endothelial cells and basal cells of the epidermis at the ulcer margin. However, when the macrophages present in the explant tissue were stimulated by bacterial lipopolysaccharide (LPS), TNF-α production was induced within 2 h after adding LPS. Thus, the culture system could support the production of TNF-α. In leg ulcers infected with *Pseudomonas aeruginosa* many macrophages stain for CD68 and a large proportion are immunopositive for TNF-α. Exposure to *Pseudomonas aeruginosa* activates the macrophages and induces production of TNF-α.

In conclusion, in a non-infected chronic venous leg ulcer there is a selective lack of production of TNF-α compared with the production of IL-1β and IL-8.

Abnormal keratinocyte phenotype

Keratinocyte migration is one proposed dysfunction in chronic venous leg ulcers while the proliferation of keratinocytes at the margins of venous ulcers appear unimpeded (96, 97). In a normally healing wound, migrating basal keratinocytes express α5β1 integrin. In chronic wounds, the α5β1 expression is only faint on the basal keratinocytes of the wound edge, indicative of non-migratory keratinocyte phenotype. In a series of experiments, it was examined whether the non-migratory phenotype of the keratinocytes of the margin of the chronic wound could be converted into a migratory phenotype of an acute wound. Biopsies from the margin of venous leg ulcers were then compared before and after culture with or without LPS to determine whether an increase in TNF-α production correlated with an increase in α5β1 keratinocyte phenotype in the wound margin. Tissue before culture, as predicted, had a low level of α5β1 keratinocyte expression. After culture for 24 h in control medium, a low level of α5β1 expression and a relatively low level of TNF-α production were again found. However, both α5β1 expression and TNF-α production increased when cultured in the presence of LPS (Table II). If a pro-inflammatory signal is delivered, TNF-α production is induced. Induction of TNF-α is concomitant with an increase

Table II. *Lipopolysaccharide (LPS) induces TNF-α production and keratinocyte phenotype changes in chronic wound margin tissue explants*

	TNF-α production (pg/mg tissue)[a]	Keratinocyte α5β1 expression[b]
Fresh tissue	–	±
24 h control (no LPS)	34	±
24 h with LPS	144	+++

[a] Punch biopsies (15–100 mg wet weight) of marginal venous leg ulcers were fragmented and incubated for 24 h at 37°C in air/5% CO_2 in RPMI medium with 10% fetal calf serum, penicillin (100 units/ml), streptomycin (100 μg/ml) and fungizone (2 μg/ml). The tissue slices were incubated either without or with LPS (10 ng/ml) in the control medium to induce the production of TNF-α. After 24 h incubation the level of TNF-α was measured with an ELISA immunoassay (Biosource cytoset).

[b] α5β1 was detected in frozen sections with a monoclonal antibody (Dako A/S, Glostrup, Denmark) at 1:250 dilution.

in α5β1 although LPS by itself can induce α5β1 expression directly, though at considerably higher concentration of LPS than used in the present investigation. Our results may, at a first inspection, contradict those of Wallace and Stacey (88) who found elevated levels of immunoreactive TNF-α, albeit not of bioactive TNF-α, in non-healing as opposed to healing venous leg ulcers. TNF-α has, however, very diverse effects depending on cell type, and wound fluid levels reflect the average of the whole wound tissue and not local levels of TNF-α. Furthermore, toxins produced by bacteria together with the increased level of TNF-α might contribute to the non-healing state of some venous leg ulcers (98).

In conclusion, in the tissue culture system used, a local deficit of a particular cytokine, namely TNF-α, could explain that the non-migratory keratinocyte phenotype in chronic venous ulcers fails to convert to a migratory keratinocyte phenotype.

FIBROBLASTS IN VENOUS LEG ULCERS

Fibroblasts are crucial in wound repair for synthesizing and remodeling extracellular matrix molecules such as collagen but also for producing mitogens for keratinocytes, endothelial cells and fibroblasts (99–101). What irreversible effects do the micro-environment of chronic wounds have on the fibroblasts? To answer this question fibroblasts were cultured from chronic venous leg ulcers and also, importantly, from acute wounds as an appropriate control (100), and from normal dermis under optimal *in vitro* conditions without the influence of the wound environment. The cell populations were characterized regarding growth, morphology, growth factor mitogenic response and the number of growth factor receptors (102).

The growth of the different fibroblast strains was expressed as the population doubling times, that is the time it takes for the number of fibroblasts to double during the logarithmic growth phase. For acute wound fibroblasts, the population doubling time was about the same as for the normal dermal fibroblasts. In sharp contrast, the population doubling time was increased four-fold for the chronic wound fibroblasts compared with acute wound and dermal fibroblasts (Table III). However, the fibroblast cultures from the different venous ulcers showed great variability. The 5 fibroblast strains, out of a total of 8, that displayed a senescent growth behavior, that is irreversible arrest of cell proliferation, were cultured from ulcers older than 3 years. The population doubling time was accordingly increased for the fibroblasts isolated from the older ulcers (Table III). These fibroblasts were larger with an abnormal morphology resembling senescent fibroblasts (103). These cell characteristics could be one of the reasons chronic venous ulcers heal slowly, if at all, that is the poorer proliferative capacity of fibroblasts the slower the healing rate of the ulcers. In support of these *in vitro* findings are the clinical results by Falanga et al. (104). They observed that transplanting old venous ulcers, with a skin equivalent populated with allogeneic fibroblasts beneath a compression bandage, was superior to the same compression therapy alone (104). Also, old venous ulcers are less likely to heal within 24 weeks than ulcers of shorter duration (105). Experimentally, there is also a good correlation between the rate of replication of fibroblasts *in vitro* and the corresponding wound healing rates *in vivo* (106). The chronic wound fibroblasts also grew slower than those from adjacent lipodermatosclerotic and normal skin (102), also found by Stanley et al. (107). Senescence has been verified in cultured fibroblasts from chronic venous leg ulcers using the biochemical marker, beta-galactosidase, at pH 6.0 (108, 109).

Table III. *Growth rate, expressed as population doubling time (h), is decreased for fibroblasts cultured from old chronic venous ulcers (102)*

Dermis (n=5)	Acute wounds (n=10)	Chronic venous leg ulcers (n=8)	
28 h	33 h	119 h	
		<3 years (n=3)	>3 years (n=5)
		39 h	167 h

The ability of the chronic wound fibroblasts to synthesize DNA was also severely depressed as demonstrated by decreased incorporation of the thymidine analogue 5-bromo-2′-deoxyuridine (BrdU). In an attempt to rescue the proliferative capacity of the chronic wound fibroblasts, human recombinant polypeptide growth factors were added to the fibroblast cultures. Although some mitogenic response to the growth factors was observed in the chronic wound fibroblasts, it was reduced markedly compared to acute wound and dermal fibroblasts (Fig. 4). Notably, the fibroblasts from the oldest ulcers were completely unresponsive to any of the tested growth factors. The reduced mitogenic

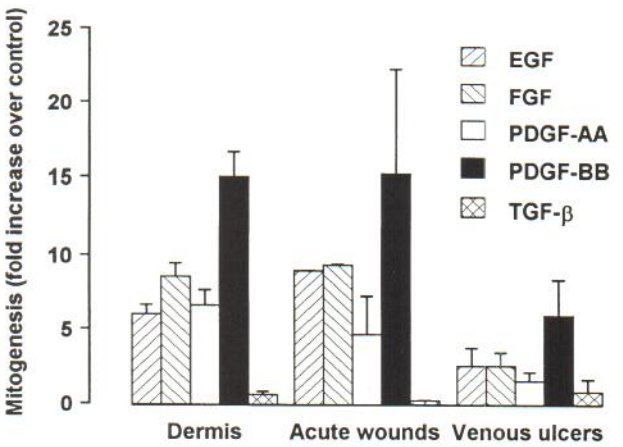


Fig. 4. The maximal mitogenic response to human recombinant growth factors of fibroblasts cultured from dermis of healthy volunteers, acute wounds and chronic venous leg ulcers (102). Growth factors or the vehicle for the growth factors (control) were added to confluent and growth-arrested fibroblasts grown in fibroblast basal medium (Clonetics) containing 2.0% fetal calf serum in 96-well plates. The final concentration of EGF was 50 ng/ml, basic fibroblast growth factor (bFGF) 5 ng/ml, PDGF-AA and PDGF-BB 20 ng/ml, and the final concentration of TGF-β1 was 10 ng/ml. Fibroblasts were incubated with or without growth factors for 20 h with 5-bromo-2′-deoxyuridine (BrdU) present (10 μM, final concentration) during the last 2 h of incubation. The incorporated BrdU, measured colorimetrically with an ELISA reader, was severely depressed in the venous leg ulcer fibroblasts. The bars represent the mean+SEM (standard error of mean). Data from Table III in Ågren et al. (102) were used to generate the figure.

response of chronic wound fibroblasts may provide one explanation for the marginal efficacy of the topical application of recombinant growth factors (PDGF-BB and EGF) to chronic wounds (110, 111). One possible explanation for the reduced mitogenic response to growth factors could be a lower number of cell surface receptors for the growth factors. However, no difference was observed regarding the amount of PDGF-α receptors, PDGF-β receptors or EGF receptors among the different fibroblast strains. Thus, it seems that the decreased response to growth factors is not due to fewer cell surface receptors but possibly to dysfunctional intracellular signal pathways in the chronic wound fibroblasts.

In conclusion, fibroblasts cultured from chronic venous leg ulcers show a pronounced diminished growth capacity and mitogenic response to growth factors compared with fibroblasts from normally healing wounds. Duration of ulcers was an important determining factor for the function of the fibroblasts with a seemingly declining proliferative ability and growth factor response with increasing duration of the ulcers. The phenotypic changes of fibroblasts from old venous leg ulcers might have been caused by repetitive cell division due to the chronic inflammation (112). Decreased fibroblast activity appears to be a common feature of other types of non-healing chronic cutaneous wounds such as diabetic ulcers and pressure sores (112, 113).

PROTEASES AND PROTEASE INHIBITORS IN LEG ULCERS

Recent studies suggest that the main groups of proteases in repair processes of skin wounds are the serine proteinases (e.g. plasmin, neutrophil elastase, cathepsin G) and the matrix metalloproteinases (MMPs) (114–118). By acting on the various matrix molecules, proteases not only remodel structural proteins but indirectly alter cell functions such as migration and proliferation. Proteases are regulated both by soluble factors (cytokines and growth factors) and by matrix molecules via integrins.

There is an age-dependent change in the levels of endogenous proteases in the skin which may be one of the reasons for skin elastosis and skin atrophy in the aged population. Interestingly, the major protease up-regulated in the skin of older women, in particular, is neutrophil elastase (91). Thus, there may also be superimposed an age- and gender-dependency of the proteolytic environment in venous ulcers.

Extracellular matrix in venous ulcers

The stromal architecture and the immunostaining for the extracellular matrix molecules collagen types I and III, laminin and tenascin appear rather normal in venous leg ulcers (1). The glycoprotein fibronectin is, however, virtually deficient in the margin and base of venous ulcers in contrast to the prominent reaction to fibronectin in normal skin. Fibronectin is an essential component of the provisional matrix deposited during early wound repair and serves as a substrate for migrating keratinocytes (119). Fibronectin reappears as the ulcers heal (1). Why is there no fibronectin in the non-healing venous ulcer? Fibroblasts from venous leg ulcer patients can synthesize the full range of extracellular matrix molecules including fibronectin *in vitro* compared with normal fibroblasts indicating that the lack of fibronectin in the base of venous ulcers is related to degradation rather than lack of synthesis (120). Degradation products of fibronectin have also been found in wound fluid from venous ulcers (121–123).

Neutrophil elastase

Grinnell and Zhu (123) were able to prevent degradation of fibronectin by wound fluid from chronic venous ulcers by the addition of specific inhibitors of neutrophil elastase *in vitro*. Neutrophil elastase is made by PMNs and many PMNs immunostain for neutrophil elastase in venous ulcers (60). In addition to elastin, neutrophil elastase cleaves a variety of matrix components such as collagens and fibronectin. Neutrophil elastase activity is complexed and neutralized by α_1-antiproteinase and α_2-macroglobulin. The inhibitory activity of α_1-antiproteinase was recently, however, reported to be counteracted by the syndecans (124). Neutrophil elastase activities were detected in wound fluid from about half of 15 patients with venous ulcers using a synthetic peptide substrate. In patients with a high neutrophil elastase activity before treatment, it decreased as the ulcer healed. However, Weckroth et al. (125) found no difference in the activity of neutrophil elastase in wound fluids from chronic venous ulcers and acute cutaneous wounds. This discrepancy might be due to degradation of the synthetic peptide substrate despite being bound to the inhibitor α_2-macroglobulin (123). Furthermore, extensive degradation of the inhibitors occurs in chronic venous leg ulcers (123, 126).

Neutrophil elastase also appears to be responsible for excessive local degradation of tenascin-C, another important extracellular matrix component for keratinocyte migration, in chronic venous leg ulcers (127).

Matrix metalloproteinases (MMPs)

MMPs are a family of proteolytic enzymes with at least 14 members, the collagenases (MMP-1, MMP-8, MMP-13), gelatinases (MMP-2, MMP-9), stromelysins (MMP-3, MMP-10, MMP-11), macrophage elastase (MMP-12), matrilysin (MMP-7) and membrane-type MMPs (MMP-14, MMP-15, MMP-16, MMP-17) (128). MMPs can degrade most of the extracellular matrix components *in vitro*. Inflammatory cells appear to be the predominant source of MMPs, mostly MMP-8 and MMP-9, in acute wounds, although some MMPs are also expressed by wound edge epithelium (129–131). As the acute inflammatory response resolves and healing commences MMP activities drop to the low levels of uninjured skin (132, 133). Analogously, chronic inflammatory conditions of the skin and other organs are associated with increased MMP activities (134–138). The activity of MMPs in wounds is largely regulated by the tissue inhibitors of metalloproteinases (TIMPs), which exist in 4 subtypes (TIMP-1, -2, -3 and -4). Ratios of MMPs and TIMPs are also altered in normal skin with intrinsic aging, favoring the catabolic processes with increased age (139, 140).

Wound fluid analyses.

Enzymatic activity, measured with the substrate Azocoll (140), in wound fluid from chronic wounds was almost

completely blocked by the metal chelator EDTA and by the hydroxamate synthetic MMP inhibitor GM 6001 indicating that the majority of enzymes belong to the MMP family (84, 140). The effect of hospitalization on protease activity was studied in patients with venous leg ulcers that had failed conventional out-patient therapy. Wound fluid was obtained from the patients at admittance and 2 weeks after treatment in the hospital. In 12 of the 15 patients proteases were lowered in collected wound fluid after 2 weeks of treatment (Fig. 5). The Azocoll assay does not discriminate among the MMPs and therefore it is not possible to deduce which of the MMPs contributed to the total enzymatic activity. In another study, pro MMP-9 (92-kDa gelatinase or gelatinase B) was found to be closely associated to the progression of wound healing and appears to be a reliable prognostic marker of wound repair (130, 143). Persistently elevated levels of MMP-9 were also found in venous leg ulcers with no healing tendency over a 3-week period in contrast to healing acute wounds where the MMP-9 level decreased during the course of healing (Fig. 6). The non-healing state of the venous ulcers was indicated by the constant wound size and the low total protein level in wound fluid (142). However, the absolute levels of MMP-9 in wound fluids were similar for the chronic and acute wound types (Fig. 6). Because MMP-9 is mostly derived from PMNs and macrophages, the MMP-9 level in wound fluid might reflect the intensity and extension of inflammation in wounds. Furthermore, in many of the venous leg ulcer patients there was an inverse relationship between TIMPs and MMPs as the ulcers began to heal, the TIMP level increased and the MMP level decreased as found by other research groups (125, 131, 144).

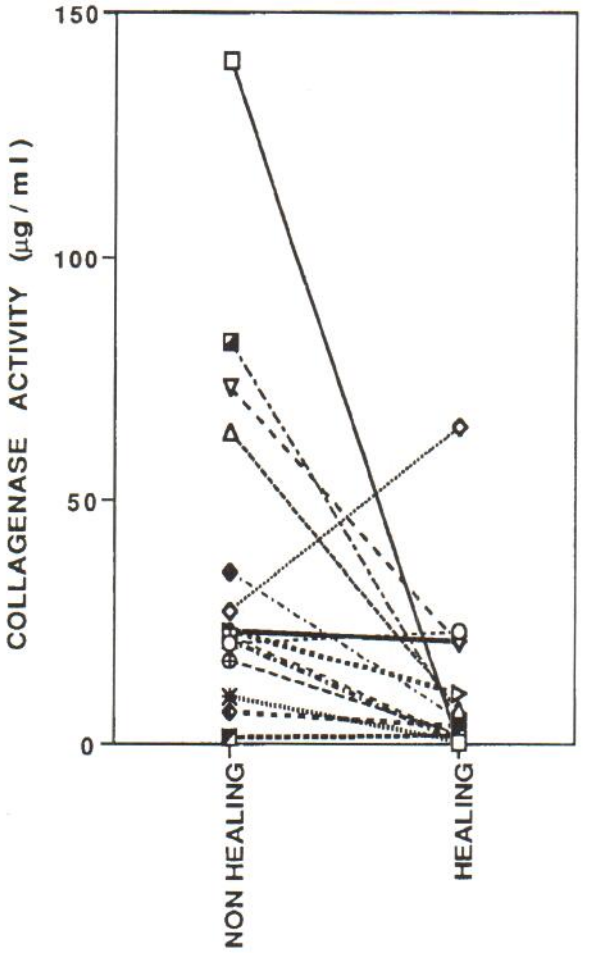


Fig. 5. Net activity of endogenously activated and inhibited proteases in paired wound fluid samples in patients with venous ulcers before (non-healing state) and after (healing state) 2 weeks of hospitalization. Patients were held overnight without liquid and then given 1 liter of water to drink. The ulcer was covered with a polyurethane film dressing (Op-Site, Smith & Nephew, UK), the leg put into dependent position and wound fluids were collected for 1 h according to the protocol of Trengove et al. (142). Protease activity, detected in the wound fluids using Azocoll, a collagen-derivatized-substrate which preferentially records MMPs, decreased significantly ($p<0.05$, paired students' t-test) after the hospitalization period.

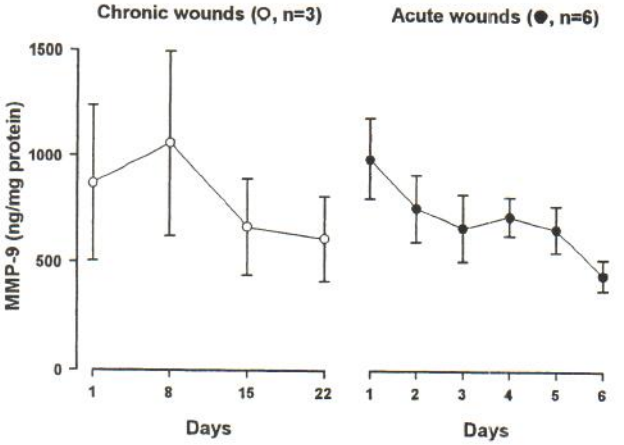
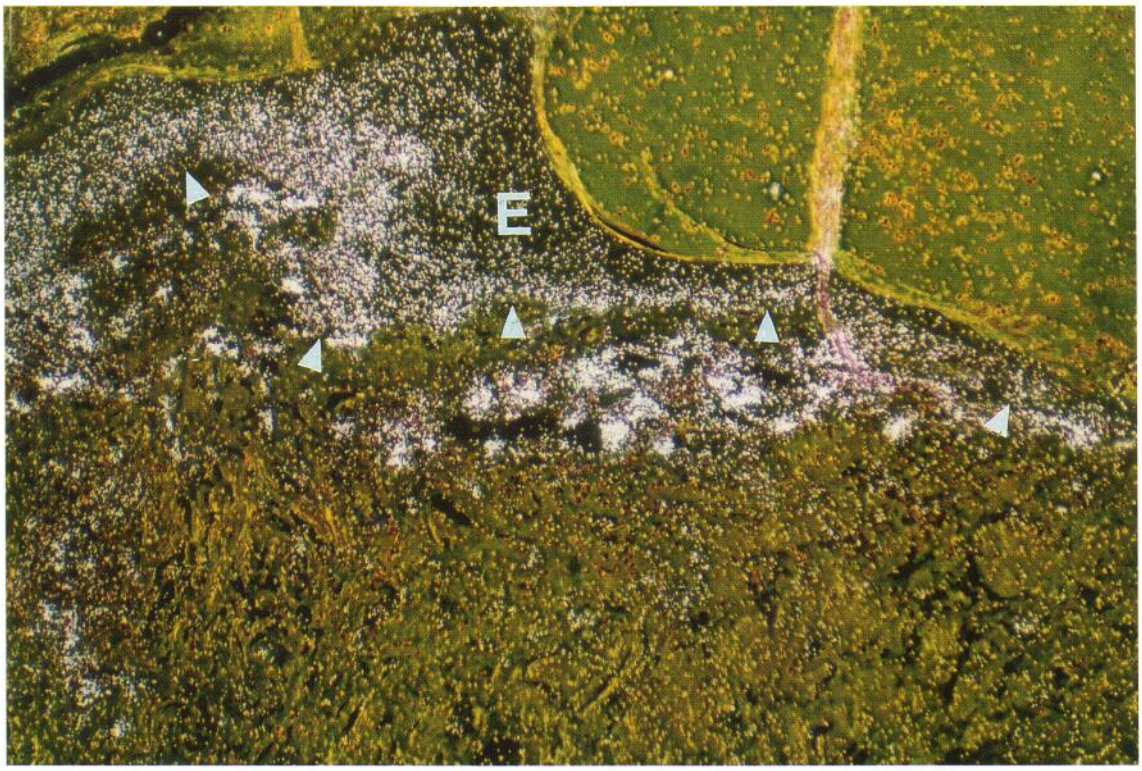
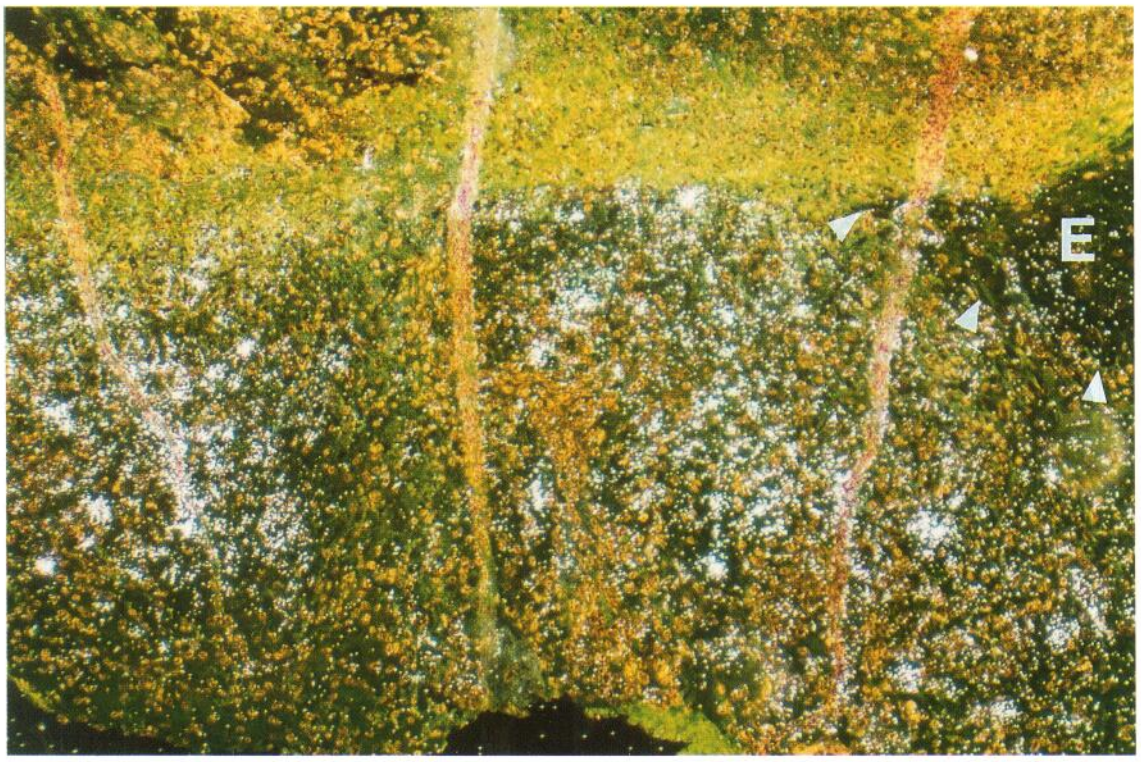


Fig. 6. Matrix metalloproteinase-9 (MMP-9) levels in wound fluid from non-healing venous leg ulcers (<4% reduction in wound surface area/week) and healing acute wounds (>55% reduction in wound surface area/week) (143). Standardized acute partial-thickness wounds (3.2 ± 0.3 cm^2, mean$\pm$SEM) were made on the posterior lower leg of healthy male volunteers using a hand-held dermatome. Wound fluid was collected with a hydrophobic polyurethane foam applied to the wound and covered with a polyurethane film dressing (Tegaderm®, 3M) over a 24-h sampling period. Wound fluids were squeezed out of the polyurethane foam in a plastic syringe, clarified by centrifugation at 3000 r.p.m. and stored at -70°C until analyzed for total protein and immunoreactive MMP-9. Latent MMP-9 levels were measured with a specific ELISA assay (130) and normalized for the total protein content. The concentration of total protein was significantly lower ($p<0.01$, unpaired t-test) in chronic wound fluid (55 ± 4 mg/ml) than in acute wound fluid (86 ± 7 mg/ml) without a concomitant significant ($p=0.56$) difference between the two groups in the concentration of total proteins in serum. This finding emphasizes the unfavourable micro-environment in chronic venous leg ulcers and a possible local protein deficiency rather than a general protein malnutrition in venous ulcer patients. At the first day of sampling, the MMP-9 levels (mean$\pm$SEM) of wound fluids were similar in acute wounds (986 ± 192 ng/mg) and in chronic venous leg ulcers (866 ± 363 ng/mg) although they were substantially elevated compared with the baseline level represented by the serum MMP-9 level (3.6 ± 0.5 ng/mg in the acute wound group and 5.5 ± 1.7 ng/mg in the chronic ulcer group). But subsequently, MMP-9 levels decreased significantly ($p=0.02$; paired t-test) in the acute wounds whereas they remained fairly constant ($p=0.49$) in the venous ulcers over the examination period. Note the different time scales of the x-axis for the 2 wound types.



A



B

Fig. 7. TIMP-3 expression in acute wounds and chronic venous ulcers. A. A dark-field view of TIMP-3 mRNA expression in the epithelium and stroma of a 5-day-old full-thickness acute wound on the anterior thigh (original magnification × 100). B. A dark-field photo of a chronic venous ulcer of 2 years duration with prominent stromal signal for TIMP-3 mRNA but with no signal for TIMP-3 in the epidermis of the ulcer (original magnification × 100). E marks the epidermis; white arrowheads depict the epithelial edge.

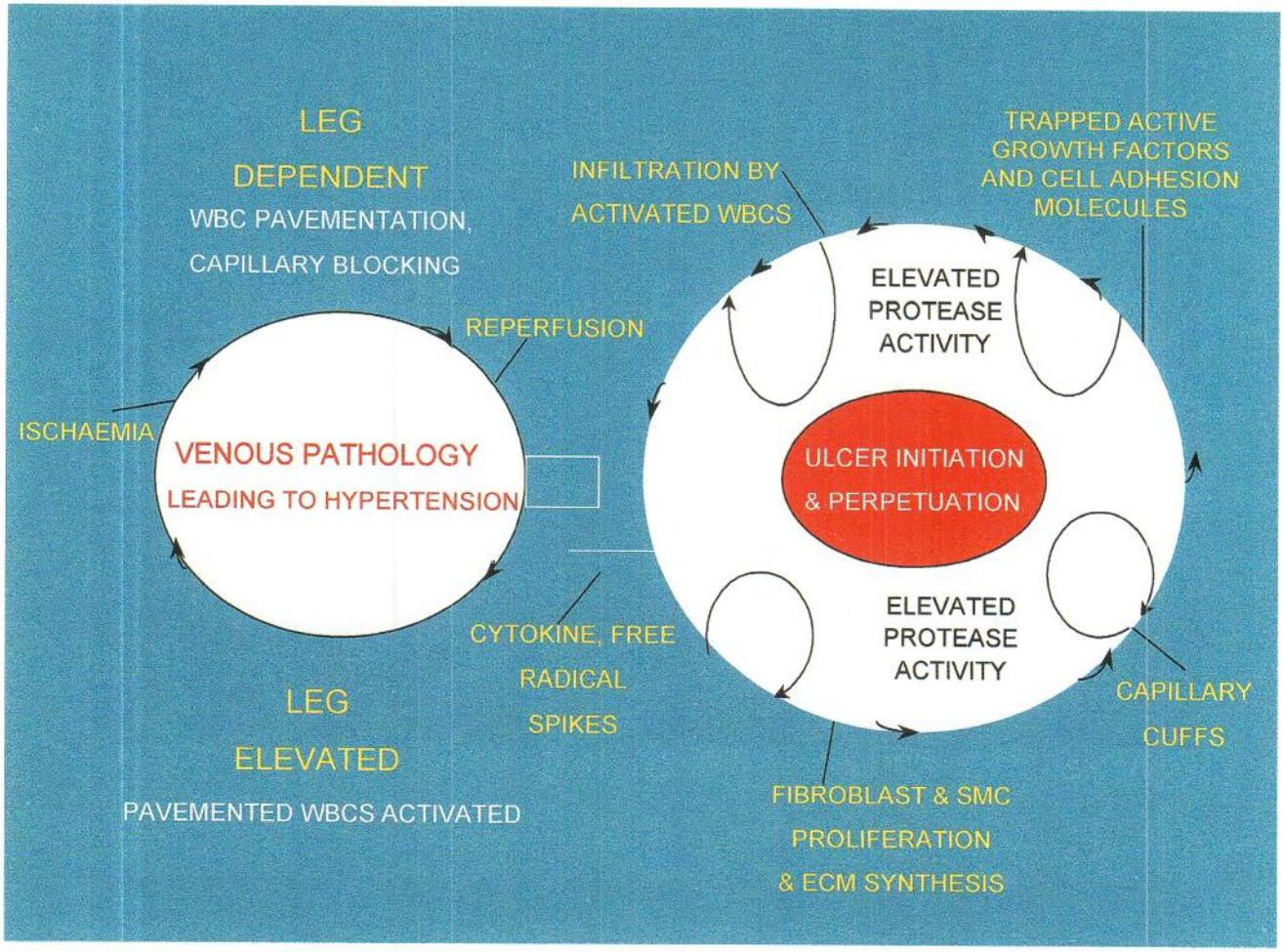


Fig. 8. A proposed mechanism for the cause and persistence of venous ulcers focusing on the pathology and activity of the pericapillary cuffs. Leukocytes (WBCS) pavement the endothelium of capillaries in venous incompetent legs in a dependent position, a blocking which leads to ischemia. When the leg is elevated the pavemented leukocytes are activated resulting in a chronic ischemic–reperfusion injury. This causes spiking of cytokines and oxygen-derived free radicals. These spikes of cytokines cause fibroblasts and smooth muscle cells (SMC) to proliferate and to synthesize extracellular matrix (ECM) molecules which give rise to capillary cuffs. The pericapillary cuffs trap cell adhesion molecules and growth factors, which are active, causing more cuffing around the vessels and the infiltration by activated leukocytes leading to more proliferation. The dynamic cuffs with trapped cell adhesion molecules and active growth factors greatly enhance the extravasation of activated leukocytes, particularly PMNs, into the surrounding tissues. The PMNs in turn release numerous proteases, particularly neutrophil elastase, resulting in a selective degradation of extracellular matrix molecules, e.g. fibronectin (1, 91). The dermis of the legs of elderly patients already contains higher proteolytic activity, particularly of neutrophil elastase, than the leg dermis of younger subjects, rendering the elderly susceptible to dermal proteolytic destruction (91). Repeated daily bouts of ischemia–reperfusion result in repeated activation of this autodestructive cascade, such that eventually protease levels from the chronic inflammatory cell infiltrate reach a threshold level so that tissue destruction exceeds synthetic capability, resulting in tissue breakdown. The initial trigger for ulcer formation may be a minor traumatic incident but the non-healing is perpetuated by the above ischemia–reperfusion cascade—a pathology that is only corrected by therapeutically treating the underlying vascular disease, e.g. by adequate compression therapy.

In situ hybridization and immunolocalization of MMPs and TIMPs in acute wounds and chronic venous leg ulcers.

MMP-1, or interstitial collagenase 1, appears to be particularly important for keratinocyte migration (145), although overexpression of MMP-1 is associated with impaired re-epithelialization in mice (146). MMP-1 is abundantly expressed in migrating keratinocytes at the edge of both chronic venous ulcers and acute wounds, although a greater number of basal keratinocytes are MMP-1 positive in chronic than in normally healing wounds (129). There is also abundant MMP-1 messenger RNA in the stroma of both chronic and acute wounds. The novel MMP-13 or interstitial collagenase 3 shows no hybridization signal in the epidermis but it is expressed in granulation tissue and in the areas of neoangiogenesis of chronic venous leg ulcers. However, no expression of MMP-13 in either epidermis or stroma was detected in acute cutaneous wounds (147). Information is lacking about the differential expression of MMP-9 in venous ulcers. Although the MMP-3 (stromelysin 1) and MMP-10 (stromelysin 2) have about the same substrate specificities, they are expressed in different locations in chronic venous ulcers (148). MMP-3 is expressed more distal to the migrating epidermal tip than MMP-10. A similar expression pattern of the stromelysins is seen in migrating epithelium of normally healing wounds (129). There is stromal expression of MMP-3,

but not of MMP-10, in cutaneous wounds regardless of etiology. In MMP-3-deficient mice, wound contraction was impaired whereas re-epithelialization proceeded at a normal rate (117). MMP-7, or matrilysin, does not appear to be linked to cutaneous wound healing. MMP-12, or macrophage elastase, is expressed occasionally by macrophages in chronic and acute cutaneous wounds.

The activity of MMPs in wounds is largely controlled by the TIMPs. In acute, normally healing cutaneous wounds, TIMP-2 is localized at the migrating epidermal tip, whereas TIMP-1 and TIMP-3 are expressed more distal in the proliferating keratinocyte compartment as shown by co-immunolocalization of the proliferation marker Ki67 (129, 149, 150). In sharp contrast, the expression of the 3 TIMPs is absent in the epidermis of chronic venous ulcers (Fig. 7). TIMP-3 is not induced by TNF-α or IL-1β but by TGF-β2 in cultured human keratinocytes. Stromal expression of the TIMPs shows a strong hybridization signal in both chronic and acute wounds (Fig. 7).

To conclude, the expression of MMPs is qualitatively similar, whereas expression of the TIMPs is different in chronic and acute wounds. MMP-1, MMP-3 and MMP-10 are closely linked to re-epithelialization. In normally healing, acute wounds, TIMP-2 is found at the tip of migrating epidermis, and TIMP-1 and TIMP-3 in the proliferating keratinocyte compartment. TIMP expression is absent in the epidermis of chronic venous ulcers. Taken together, these results suggest that an imbalance in the levels of MMPs and TIMPs rather than overexpression of a specific MMP contributes to detrimental wound healing in chronic venous leg ulcers.

POSSIBLE MECHANISM FOR THE CAUSE AND PERSISTENCE OF VENOUS LEG ULCERS

One of us (MWJF) has presented a hypothesis as to what causes and maintains venous leg ulcers in a non-healing state based on the dynamic and active matrix pericapillary cuffs (Fig. 8). Largely due to the ischemia–reperfusion injury at elevation of the diseased leg, cytokines and oxygen-derived free radicals are liberated locally around pericapillary cuffs locking the condition into an autodestructive cascade. The hypothesis was tested in patients with venous leg ulcers and as predicted significantly increased levels of neutrophil superoxide, indicative of increased oxygen stress, was found in the diseased and ulcerated legs upon elevation from a dependent position compared to healthy control legs. An important feature of this hypothesis is that it indicates that the venous ulcer is not a static "deficiency state" but rather a high turnover pathology with high levels of cellular proliferation, matrix synthesis and growth factor activity. However, these high anabolic events occur in a sea of proteases induced by the repeated ischemia–reperfusion. Minor adjustments to the balance of synthesis versus degradation, e.g. by compression therapy or by normalization of the proteolytic environment pharmacologically, may allow the ulcer to heal properly.

CONCLUSIONS

There are several pathophysiological factors contributing to the non-healing state of venous leg ulcers (96). This symposium has highlighted new physiological, molecular and cellular abnormalities in venous ulcers. Venous hypertension results in a preferential accumulation of activated leukocytes in the diseased leg resulting in oxygen stress and vessel injury (21). Congested capillaries become cuffed with different matrix molecules, highly proliferative and more adhesive for leukocytes. This aggravates the condition further and it becomes locked in a self-amplifying, detrimental cascade with persistent elevated levels and activities of pro-inflammatory cytokines and proteases preventing progress into a healing phase (84). As a consequence, fibroblasts senesce and become less responsive to growth factors the older the ulcers become (102). Current data imply there is no deficiency but rather an unfavorable distribution of growth factors in venous ulcers (68, 85). An imbalance in proteolytic enzymes and their endogenous inhibitors contributes to inappropriate matrix remodeling for optimal keratinocyte migration (150). It was recognized that venous leg ulcers are heterogeneous and the severity of individual ulcers fluctuate profoundly over time. Therefore, to advance the areas of research on chronic venous leg ulcers further longitudinal studies involving larger number of patients are required.

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