

Expression of the Adhesion Molecules ICAM-1, VCAM-1, LFA-1 and VLA-4 in the Skin is Modulated in Progressing Stages of Chronic Venous Insufficiency

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In inflammation and wound healing, dynamic changes in cell adhesion and migration are fundamental properties of the cells involved. Disturbed interaction of leukocytes with microvascular endothelial cells has been proposed to be a central pathogenic factor in chronic venous insufficiency. This disease may therefore serve to elucidate dysregulated modulation of adhesion molecule expression in conditions of chronic inflammation and impaired wound healing. In this study, we determined how the expression of ICAM-1/VCAM-1 on endothelial cells and their ligands LFA-1/VLA-4 on leukocytes is modulated in skin of progressing stages of chronic venous insufficiency. Immunohistochemical staining of skin biopsies revealed an increase in the expression of ICAM-1 and VCAM-1 on endothelial cells in an early stage of venous disease such as stasis dermatitis. Such protein expression correlated with an increase of corresponding mRNA in skin biopsies. Expression of these CAMs on endothelial cells was accompanied by the occurrence of a marked perivascular infiltration of leukocytes, which expressed increased levels of LFA-1 and VLA-4. In progressing stages of chronic venous insufficiency, characterized by hyperpigmentation and lipodermatosclerosis, which precede skin ulceration, all these CAMs remained upregulated on endothelial cells and infiltrating leukocytes. Our findings indicate that following an initial peak expression during stasis dermatitis, vascular ICAM-1 and VCAM-1 expression is not downmodulated to baseline levels, but remains upregulated. This possibly promotes tissue damage by a perpetuated, upregulated influx of activated leukocytes, finally leading to skin ulceration. Key words: chronic venous insufficiency; adhesion molecules; endothelial cells; leukocytes.

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Chronic venous insufficiency (CVI) is characterized by a steady progression of skin inflammatory response, clinically resembling telangiectases, stasis dermatitis, hyperpigmentation and lipodermatosclerosis, and finally culminating in poor healing skin ulceration (1–3). CVI may therefore serve as a model system to study the factors involved in the different stages of a disturbed wound healing process. A central mechanism in the progression of uncontrolled inflammation is the dysregulation of leukocyte extravasation across the vascular endothelium infiltrating adjacent tissue (4, 5). Infiltrating leukocytes secrete high levels of inflammatory mediators, recruiting inflammatory cells into the site, perpetuating the

inflammatory response and finally leading to tissue damage instead of repair (5–8).

Decisive for the invasion of tissues by inflammatory cells is their transmigration across the microvascular endothelium, which is now known to be a complex process mediated by cellular adhesion molecules (CAMs), which are expressed on the surface of endothelial cells (ECs), and their corresponding receptors on leukocytes (4, 8, 9). The adhesion of the leukocytes to the endothelium is currently seen as divided into various stages, also termed the “adhesion cascade” (8). The first step is the reversible tethering and rolling interaction mediated by molecules of the selectin group, such as E-selectin and P-selectin, on the ECs, and their ligands sialyl Lewis X on leukocytes (10). In a further step, firm adhesion of leukocytes to the ECs is mediated by ICAM-1 and VCAM-1 and their corresponding ligands LFA-1 and VLA-4 on activated leukocytes. Such tight binding is a direct prerequisite for the transmigration of the leukocytes to the neighbouring tissue (4, 9).

Of central relevance in the late phase of the physiologic wound healing process is the downmodulation of EC CAM expression, terminating extravasation of inflammatory cells into the tissue (5, 11). Recently we have shown that the above-mentioned adhesion molecules are highly expressed on vascular endothelium and tissue invading leukocytes in the edges of leg ulcers (12). Dysregulation of EC CAMs, allowing chronic leukocyte influx and tissue damage, may be an important factor responsible for the continuing disease progression in CVI (13, 14). The aims of this study were to investigate the modulation of adhesion molecules ICAM-1, VCAM-1, LFA-1 and VLA-4 in different stages of progressing CVI and to define at what stage expression of these CAMs may become pathophysiologically decisive for the progression of the disease.

PATIENTS AND METHODS

Patients

The study group comprised 10 healthy volunteers (average age 50.4 years, range 33–69 years, 5 females and 5 males) and 60 patients with 5 different clinical stages of CVI (mean age 62.7 years, range 44–93 years, 34 females and 26 males). The clinical diagnosis of CVI was confirmed by doppler sonography and plethysmography. A concomitant arteriosclerosis of the extremities was excluded by an ankle-brachial index >0.9. Six-mm skin punch biopsies from clinically affected skin of the lower limb were taken under local anaesthesia (1% Scandicain) following informed consent from all patients. The biopsies from controls were site-matched relative to patient biopsies. CVI patients were assigned to one of the clinical stages of CVI, which were defined as follows: telangiectases ($n=14$), stasis dermatitis ($n=10$), hyperpigmentation ($n=19$), lipodermatosclerosis ($n=13$) and venous leg ulcer ($n=4$). Immediately after biopsy, specimens were snap frozen in liquid nitrogen and stored at -80°C until further processing. For PCR examina-

tion, 5 specimens from healthy skin and 4 biopsies each, from skin with telangiectases, stasis dermatitis, hyperpigmentation, lipodermatosclerosis and venous leg ulcer were chosen. For immunohistochemistry, biopsies were obtained as follows: healthy skin ($n=5$), telangiectases ($n=10$), stasis dermatitis ($n=6$), hyperpigmentation ($n=15$) and lipodermatosclerosis ($n=9$).

Immunohistochemistry

Frozen skin specimens were embedded in Tissue Freezing Medium (Fa. Jung, Nussloch, Germany) and 5 μ m serial cryostat sections were prepared (Cryocut 1800, Fa. Reichert & Jung, Germany). Frozen sections were stained with one of the following mAbs (RT, 30 min): ICAM-1 (Clone 84 H 10, IgG 1 mouse), VCAM-1 (Clone 1 G 11, IgG 1 mouse), LFA-1 (Clone 25.3, IgG 1 mouse) and VLA-4 (Clone HP 2.1, IgG 1 mouse) (all Dianova, Hamburg, Germany) (12) using a 4-step immunohistochemical staining protocol (ABC technique, Dako, Hamburg, Germany) as described previously (12, 15). Slides were evaluated by two independent observers in a blinded fashion using a Zeiss Axioskop, equipped with an MC 100 camera system. In each specimen, epidermis, endothelial cells of capillary loops and pericapillary infiltrate were evaluated separately for the expression of ICAM-1 and VCAM-1 or VLA-4 and LFA-1, as described elsewhere (12). The expression of adhesion molecules on ECs was determined by counting all capillary loops of a specimen. Thereafter, the percentage of capillary loops that expressed ICAM-1 and VCAM-1 was calculated. Furthermore, pericapillary infiltrating leukocytes and lymphocytes were assessed for VLA-4 and LFA-1 positive staining, and the percentage of capillary loops with LFA-1 and VLA-4 positive infiltrate was determined.

Oligonucleotides

The transcript-specific primers were selected using the oligo program of Rychlik & Rhoads (16) based on the sequences from an EMBL Nucleotide Sequence Database. The primers were synthesized on an automated 394 DNA DNA/RNA Synthesizer as follows: ICAM-1, 5'-ACTGGCACCCCTCCCCTCTT-3', 5'-GAACCCCATTCAGCGTCACC-3' (61°C annealing, EMBL AC J03132); VCAM-1, 5'-GGAAGTCCCTGGAAACCAAG-3', 5'-CTGGGGGCAACATTGACATA-3' (55°C annealing, EMBL AC X53051); LFA-1, 5'-GCTGGGGGCTTCTTGACCT-3', 5'-GCCTTGGAACTGGGGGTAGA-3' (59°C annealing, alpha-subunit, CD11a EMBL AC Y007969); VLA-4, 5'-GAGTGCCCCCTGATTTACGA-3', 5'-TCAACGAGGTTTGTTCCTCA-3' (54°C annealing, alpha-subunit EMBL AC X16983); β -actin (5'-TGAAGTACCCCATCGAGCAC-3', 5'-AAGGGTGTAACGCAACTAAG-3' (57°C annealing). The corresponding amplified fragments were ICAM-1 686 bp, VCAM-1 1027 bp, LFA-1 876 bp, VLA-4 581 bp and β -actin 960 bp.

Reverse transcription-PCR (RT-PCR)

Total cellular RNA was extracted from frozen skin specimens by guanidine thiocyanate lysine buffer, followed by CsCl gradient purification, as described elsewhere (17). cDNA was prepared using 1 μ g of total cellular RNA, 6 μ M random hexamer primer (New England Biolabs, Beverly, MA, USA), 63 μ M each of dNTP and 150 units superscript reverse transcriptase (Gibco, Gaithersburg, USA) in a final volume of 50 μ l. Respective DNA was amplified by PCR from total cDNA with the primer pairs specified above. PCR reaction was performed applying the hot start technique in Taq-polymerase buffer (Gibco) containing 63 μ M each of dNTP, cDNA corresponding to 0.1 μ g of total RNA (0.01 μ g for amplification of actin), 0.5 μ M transcript-specific primers and 2.5 units of Taq DNA-polymerase (Amersham, Braunschweig, Germany). Thermocycling conditions were as follows: 30 cycles/1 min strand separation at 94°C, 2 min at primer-specific annealing temperature and 3 min extension at 72°C.

Analysis of PCR reaction products

Amplified fragments were separated in parallel with the coamplified actin reference fragment and with 1.0 μ g of 100 bp-ladder DNA reference (Pharmacia, Uppsala, Sweden) by electrophoresis on a 1.5% agar-

ose gel (Seakem LE-agarose) for 90 min at 60 V and stained for 60 min in ethidium bromide. Fluorescence was visualized at 312 nm UV, digitalized with a high-resolution CCD camera (Herolab, Wiesloch, Germany) and the individual fragments were quantitated with E.A.S.Y. software (Herolab). Variance of fluorescence intensities between different gels was normalized against the corresponding 800 bp signal of the 100 bp reference, and variance between separate sets of RT-PCR was normalized by reference to the fluorescence intensity of the corresponding actin fragment.

RESULTS

Vascular endothelial cells and epidermal basal keratinocytes in early stages of CVI express increasing amounts of ICAM-1 and VCAM-1

Immunohistochemistry in healthy skin ($n=5$) (Figs. 1A, B) and biopsies of telangiectases ($n=10$) showed no epidermal expression of ICAM-1 and VCAM-1. In contrast, in specimens of stasis dermatitis ($n=6$), lipodermatosclerosis ($n=9$) (Figs. 1E, I) and hyperpigmentation ($n=15$), a positive staining pattern for ICAM-1 on basal keratinocytes was observed. On dermal ECs in healthy volunteers, weak to moderate ICAM-1 and VCAM-1 expression was detected by immunohistochemistry (37% ICAM-1-positive and 14% VCAM-1-positive capillary loops) (Figs. 1A, B and 2). In biopsies from skin with telangiectases, there was no significant increase in surface expression of these molecules (Fig. 2). However, in stasis dermatitis, an increased expression of ICAM-1 and VCAM-1 was detected on dermal capillary loops (53% ICAM-1-positive and 47% VCAM-1-positive capillary loops) (Figs. 1E, F and 2). Cell surface expression decreased in the later CVI stage of hyperpigmentation, again showing an increased expression in lipodermatosclerosis (Figs. 1I, J) with 50% of all capillary loops staining positive for ICAM-1 and 40% for VCAM-1 (Fig. 2).

LFA-1 and VLA-4 are strongly expressed on infiltrating leukocytes in stasis dermatitis and hyperpigmentation

As a further step, we were interested in whether infiltrating leukocytes would express corresponding ICAM-1 and VCAM-1 ligands LFA-1 or VLA-4. In specimens from early CVI stage telangiectases, only a slight perivascular infiltration of LFA-1- and VLA-4-positive leukocytes was observed when compared to normal skin (Figs. 1C, D and 2). Expression of LFA-1 and VLA-4 peaked in stasis dermatitis, with 62% and 73% of capillary loops surrounded by a positive infiltrate (Figs. 1G, H and 2). However, a marked perivascular accumulation was observed in patients with hyperpigmentation (LFA-1: 50% and VLA-4: 56% of capillary loops surrounded by a positive infiltrate). Less expression of these molecules was detected in lipodermatosclerosis (LFA-1: 45% and VLA-4: 42% of capillary loops surrounded by positive infiltrating cells). However, the difference was not statistically significant compared to hyperpigmentation (Figs. 1K, L and 2).

High levels of mRNA of ICAM-1, VCAM-1, LFA-1 and VLA-4 are detected in stasis dermatitis, lipodermatosclerosis and leg ulcers

To determine if modulation of CAMs in CVI was regulated at the mRNA level, we examined the pattern of mRNA levels of ICAM-1, VCAM-1, LFA-1 and VLA-4 in progressing CVI.

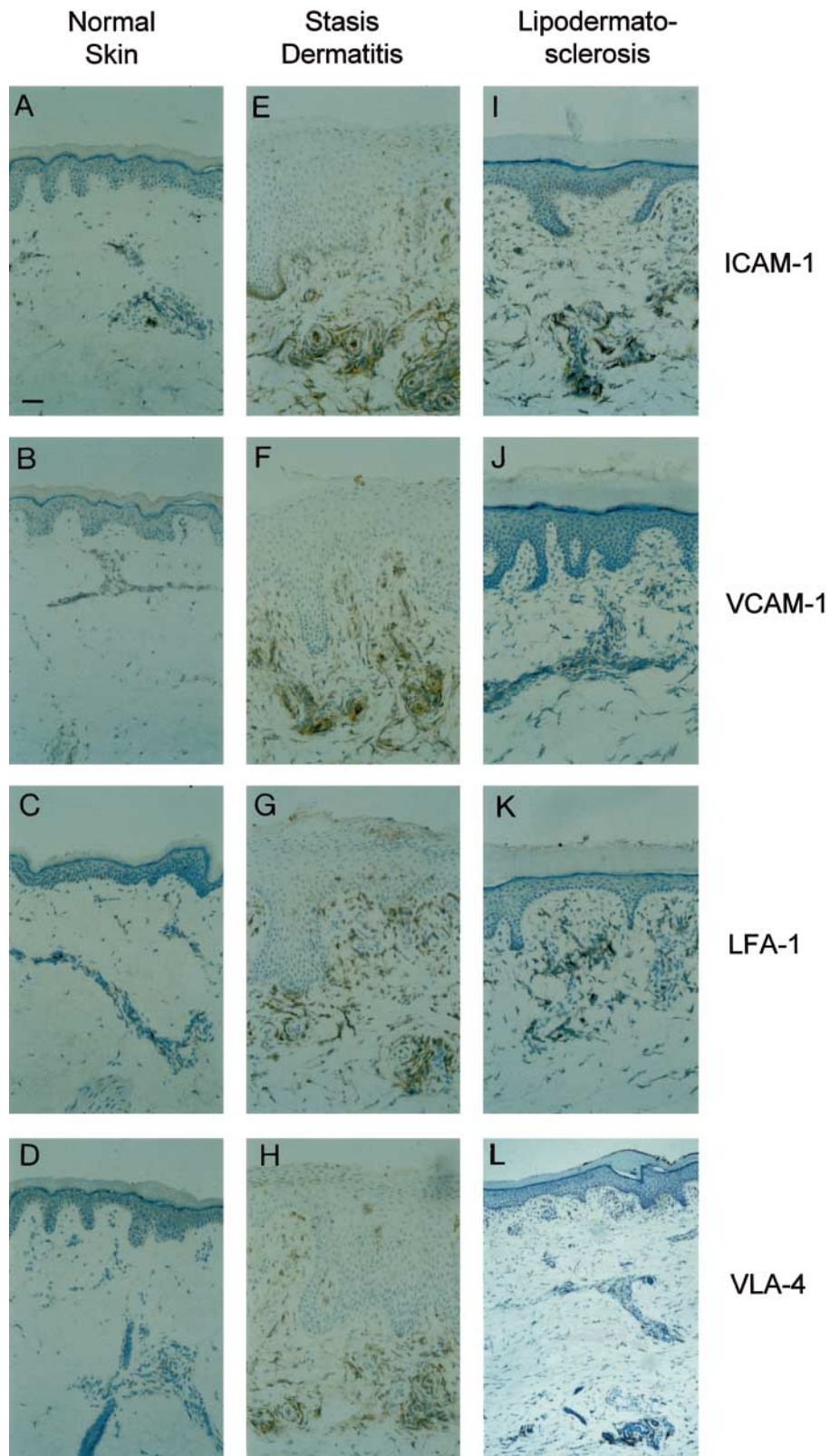


Fig. 1. ICAM-1, VCAM-1, LFA-1 and VLA-4 are markedly expressed in stasis dermatitis and remain upregulated in lipodermatosclerosis. Cryosections of skin samples from healthy volunteers (A–D), patients with stasis dermatitis (E–H) and lipodermatosclerosis (I–L) were immunohistochemically stained with mAbs against ICAM-1, VCAM-1, LFA-1 and VLA-4 as described in PATIENTS AND METHODS (50 × magnification). Scale bar: (A) 150 µm.

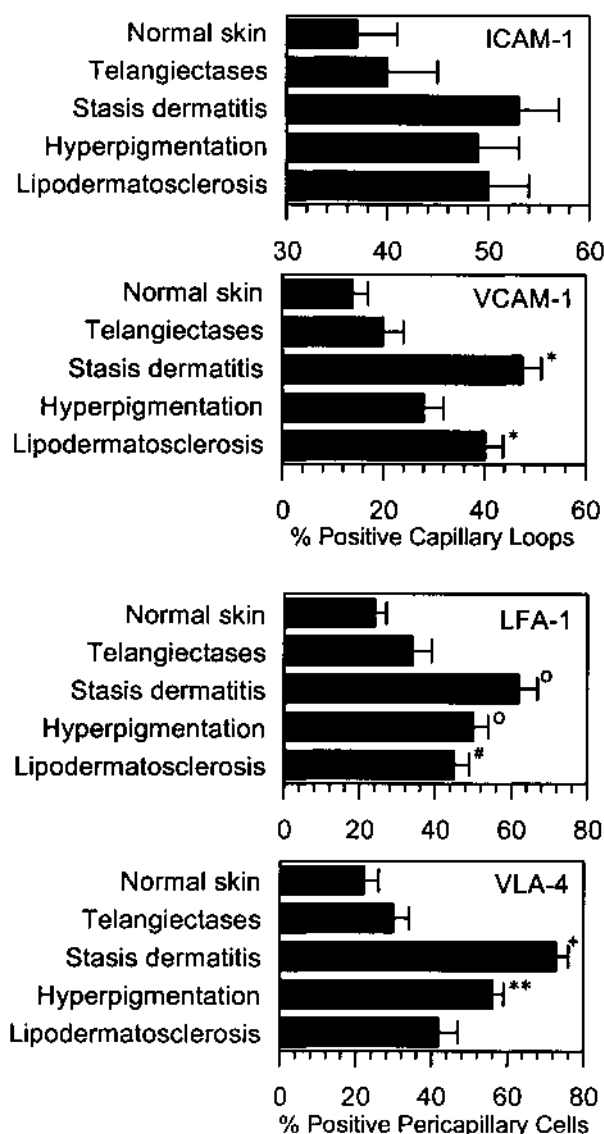


Fig. 2. Modulation of ICAM-1, VCAM-1, LFA-1 and VLA-4 in different stages of CVI: telangiectases ($n=10$), stasis dermatitis ($n=6$), hyperpigmentation ($n=15$) and lipodermatosclerosis ($n=9$). Cryosections of skin samples from healthy volunteers ($n=5$) were stained with the indicated mAbs as described in Fig. 1, and the percentage of mAb positive capillary loops or percentage of mAb-positive pericapillary infiltrating cells were determined as described in PATIENTS AND METHODS. No statistically significant increase was found in ICAM-1 expression on capillary loops. *Statistically significant increase in VCAM-1 expression on capillary loops compared to normal skin. °Statistically significant increase in LFA-1 expressing pericapillary infiltrating cells compared to normal skin and telangiectases. #Statistically significant increase in LFA-1 expressing pericapillary infiltrating cells compared to normal skin. + Statistically significant increase in VLA-4 expressing pericapillary infiltrating cells compared to normal skin, telangiectases and lipodermatosclerosis. **Statistically significant increase in VLA-4 expressing pericapillary infiltrating cells compared to normal skin and telangiectases (mean $\pm$ SD, *°+**statistically significant at $p < 0.05$, Dunn's Method, SigmaStat; Jandel Scientific Software, Corte Madera CA, USA).

mRNA levels in skin biopsies from healthy skin and the 5 different CVI stages, including leg ulcers, were quantified by image analysis of gels, digitally quantifying mRNA fluores-

cence intensity values at 312 nm UV. Analysis of the mRNA expression levels of the four adhesion molecules in normal and CVI specimens revealed low levels in healthy patients ($n=5$), with mean fluorescence intensity values of 278.3 and 704.0 for ICAM-1 and VCAM-1, respectively, and 64.5 and 443.8 for LFA-1 and VLA-4, respectively (Fig. 3, lane 1). In telangiectases ($n=4$), mean values of mRNA expression showed no significant change in the expression level for the determined adhesion molecules compared to normal skin. mRNA levels of all four CAMs peaked first in stasis dermatitis ($n=4$) (mean fluorescence intensity values: ICAM-1: 498.3, VCAM-1: 1065.8, VLA-4: 989.0, LFA-1: 321.4) (Fig. 3, lane 3). In the CVI stages, hyperpigmentation ($n=4$) and partly in lipodermatosclerosis ($n=4$), mRNA levels decreased but remained elevated compared to healthy skin (Fig. 3, lanes 4, 5). Peak levels of mRNA in all investigated CAMs were found in leg ulcer specimens ($n=4$) (ICAM-1: 893.8, VCAM-1: 1.257.0, VLA-4: 1.306.0, LFA-1: 542.0) (Fig. 3, lane 6).

DISCUSSION

Here we show that during the progression of CVI the adhesion molecules ICAM-1, VCAM-1, LFA-1 and VLA-4, which facilitate the influx of leukocytes into the inflammatory site, are upregulated on microvascular ECs and infiltrating leukocytes. Expression on the protein level correlates with the mRNA levels of these CAMs in skin biopsies of CVI. Following an initial peak expression in stasis dermatitis, increased expression of these CAMs was found throughout the late stages of CVI, peaking again in leg ulcers.

Our findings are in accordance with data from other skin inflammatory diseases that are associated with upregulation of certain CAMs, such as psoriasis, atopic dermatitis, scleroderma and erythema multiforme (18–20). All of these conditions demonstrate differential patterns of increased ICAM-1 and VCAM-1 expression. However, the progression of CVI differs from these diseases in that its maximal form is characterized by tissue destruction cumulating in poorly healing skin ulcers. In this study, we were therefore interested in how microvascular CAMs might contribute to such disease progression in CVI.

We have previously shown that CAMs are highly upregulated in the skin of leg ulcers (12). We now extend these results to demonstrate that in progressing stages of CVI, expression of ICAM-1 and VCAM-1 on ECs and LFA-1 and VLA-4 on infiltrating leukocytes initially peaks in stasis dermatitis. A central pathogenic factor inducing this expression, which is characterized by a strong inflammatory response, might be the ambulatory venous hypertension extending into the skin microcirculation (15, 21, 22).

The advanced CVI stages in hyperpigmentation and lipodermatosclerosis are characterized by a rapid sclerotic process with diminished microvessels (23–25). A central finding of this study is that on remaining ECs, surface expression of ICAM-1 and VCAM-1 is increased. This increase is accompanied by a perivascular leukocyte infiltrate positive for the respective receptors LFA-1 and VLA-4. In both stages, mRNA levels of ICAM-1 and VCAM-1 are upregulated. We therefore speculate that increased mRNA levels in skin biopsies correlate at least partially with upregulated protein expression of ICAM-1 and VCAM-1 on ECs and an increased perivascular leukocyte infiltration positive for LFA-1 and VLA-4 (4).

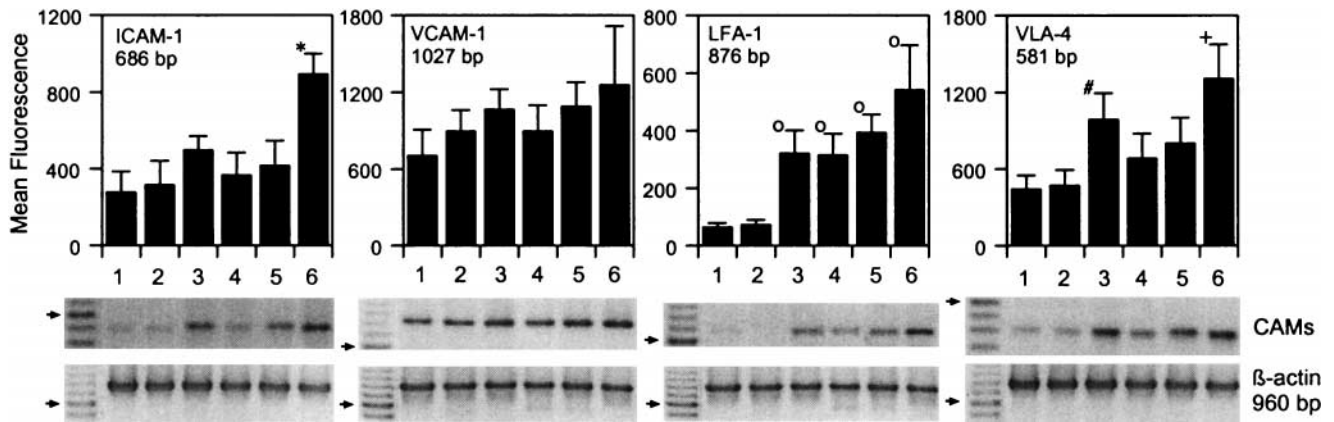


Fig. 3. High levels of ICAM-1, VCAM-1, LFA-1 and VLA-4 mRNA are detected in stasis dermatitis, lipodermatosclerosis and venous leg ulcers. Expression of ICAM-1, VCAM-1, LFA-1 and VLA-4 mRNAs was examined in biopsies by RT-PCR. Healthy skin (lane 1, $n=5$), telangiectases (lane 2, $n=4$), stasis dermatitis (lane 3, $n=4$), hyperpigmentation (lane 4; $n=4$) lipodermatosclerosis (lane 5, $n=4$), and venous leg ulcer (lane 6, $n=4$). Mean fluorescence intensity was determined semiquantitatively, as described in PATIENTS AND METHODS. β -actin was used as control. Arrow indicates 800 bp fragment in the 100 bp DNA ladder. *Statistically significant increase in ICAM-1 expression compared to lanes 1, 2, 3, 4 and 5. No statistically significant increase was found in VCAM-1 expression. ^oStatistically significant increase in LFA-1 expression compared to lanes 1 and 2. [#]Statistically significant increase in VLA-4 expression compared to lanes 1 and 2. ⁺Statistically significant increase in VLA-4 expression compared to lanes 1, 2, 4 and 5. (mean $\pm$ SD, *,^o,[#],⁺ statistically significant at $p < 0.05$, Student-Newman-Keuls Method, SigmaStat; Jandel Scientific Software, Corte Madera CA, USA).

In addition to ECs, we found that keratinocytes express ICAM-1 in stasis dermatitis, however, less prominently compared to the expression on ECs. This finding is in accordance with previous reports showing an increased expression of ICAM-1 on focally grouped keratinocytes in contact with subepidermal inflammatory infiltrates in skin from dermatomyositis or leg ulcers (12, 26). Inflammatory cytokines, e.g. interferon-gamma (IFN-gamma) produced by infiltrating leukocytes, are likely to be involved in expression of ICAM-1 on keratinocytes (27). Since whole skin biopsies were used for mRNA analysis, we cannot exclude the possibility that other dermal cells, such as activated dendritic cells invading inflamed tissues, might contribute to mRNA upregulation of CAMs (28).

We propose that a central mechanism in the progression of CVI to non-healing skin ulceration seems to be the persistent upregulation of endothelial CAMs, following initial stasis dermatitis and continuing throughout skin ulceration. It is uncertain what factors are responsible for the dysregulated expression of these EC CAMs. In addition to high vascular pressure, the reduced oxygen pressure found in late stages of CVI may induce constant expression of ICAM-1 and VCAM-1 on vascular ECs (29–33). Hypoxia in the capillaries and venules has been demonstrated to induce the release of endothelium-derived cytokines, such as IL-1, that subsequently induce EC CAM expression (34–37). By inducing the influx of activated LFA-1- and VLA-4-positive inflammatory cells, high VCAM-1/ICAM-1 expression on ECs may in part be responsible for the perpetuated inflammatory reaction, inducing continuing tissue damage and preventing ulcer closure (5, 36).

In summary, we have shown that the protein and mRNA levels of ICAM-1, LFA-1, VCAM-1 and VLA-4 are upregulated in stasis dermatitis, then downmodulated only slightly in lipodermatosclerosis followed by high expression in leg ulcers. These findings suggest that CAM upregulation on ECs might play an

important role in continuous leukocyte migration into the dermis, finally inducing non-healing venous leg ulceration.

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