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Original Paper

Expression of nitric oxide synthase isoforms and arginase in normal human skin and chronic venous leg ulcers

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Abstract

Chronic venous ulcers, an example of abnormal wound healing, show chronic inflammation with defective matrix deposition which together with the underlying vascular pathology, result in delayed healing. L-arginine is known to be metabolized by one of two pathways: nitric oxide synthase (NOS), producing nitric oxide (NO), or arginase, producing ornithine. NO is involved in many pathological conditions including vascular and inflammatory disorders. This study therefore investigated the distribution, level and activity of NOS and arginase in chronic venous ulcers in comparison with normal skin, using immunocytochemistry, western blotting, and enzyme assays. The results demonstrated an increased distribution of both NOS and arginase in chronic venous ulcer tissue compared with normal skin, with inflammatory cells and vascular endothelial cells as the main sources. These data were confirmed by western blot analysis, which showed increased levels of both enzymes in chronic venous ulcers. Moreover, there was significantly increased activity of both total NOS ($p < 0.04$) and inducible NOS ($p < 0.05$) in chronic venous ulcer tissue compared with normal skin, and significantly increased activity of arginase ($p < 0.01$) in chronic venous ulcer tissue in comparison with normal skin. NO is known to combine with hydroxyl free radicals forming peroxynitrite, a potent free radical which causes tissue destruction. NO overexpression in chronic venous ulcers may be involved directly or indirectly (through production of peroxynitrite) in the pathogenesis and delayed healing of chronic venous ulcers, through its effects on vasculature, inflammation, and collagen deposition. Arginase is known to enhance matrix deposition. Thus, increased levels of arginase in chronic venous ulcers could contribute to the pathogenesis of lipodermatosclerosis associated with chronic venous insufficiency, predisposing to the formation of chronic venous ulcers and also to matrix cuff formation around blood vessels.

Keywords: nitric oxide; nitric oxide synthase; inducible nitric oxide synthase; endothelial nitric oxide synthase; arginase; chronic venous ulcer

Introduction

There are many theories regarding the pathogenesis of chronic venous ulcers. These include pressure damage of capillaries, with leakage of fibrinogen leading to the formation of perivascular cuffs, preventing normal oxygen exchange between capillary and tissue [1]; the release of toxic metabolites, proteolytic enzymes, and toxic free radicals by the accumulated leukocytes [2]; and cytokine-mediated matrix cuff formation [3]. Recently, emphasis has focused on the role of the endothelium in the regulation of vascular tone in the micro- and macro-vasculature [4,5]. The endothelium secretes both vasodilators, such as nitric oxide (NO), prostacyclin, and endothelium-derived hyperpolarizing factor, and vasoconstrictors, such as prostaglandins and endothelin [6].

Inflammatory cell metabolism of L-arginine can occur by two pathways. One pathway, catalysed by nitric oxide synthase (NOS), produces NO and citrulline; the other pathway, catalysed by arginase, produces ornithine and urea. NOS is known to be present in at least two isoforms. The first, a calcium-dependent isoform, is normally expressed constitutively in the tissues. It includes endothelial NOS (ecNOS) in the vascular endothelial cells and neuronal NOS (nNOS) in peripheral nerves [7]. The second isoform is the calcium-independent, inducible isoform (iNOS), which is produced mainly in pathological conditions [8]. Constitutive NOS rapidly synthesizes small amounts of NO in response to increases in intracellular calcium, while iNOS is under transcriptional control and its synthetic activity is stimulated by cytokines or endotoxin. NO produced by endothelial cells is important in the regulation of local blood flow [9]. NO produced by iNOS is a major mediator of macrophage cytotoxicity [10]. NO overproduction has been linked to diverse pathophysiological conditions associated with primary vascular, inflammatory, and neurodegenerative diseases [11].

NO is known to be synthesized in the wound [12], but its role in the healing process is only beginning to be defined. The cellular sources of NO during the healing process are probably multiple and are not fully

established, although inflammatory cells such as macrophages and polymorphonuclear neutrophils (PMNs) have been shown to synthesize large amounts of NO [13,14].

Ornithine, produced by arginase, can be converted into proline, required for collagen synthesis, and into putrescine, spermidine, and spermine, which are essential for cell growth and differentiation [15,16]. Both are important for wound healing [17].

In the acute wound, iNOS predominates in the early stage where the wound environment is cytotoxic. Expression of iNOS at this time is consistent with the known effects of NO, such as vasodilatation [18], antimicrobial activity [19], and anti-platelet aggregation activity [20], but in the late stage of healing, arginase predominates and iNOS returns to the normal level [21]; this promotes healing by enhancing matrix deposition [18]. Thus, the course of wound healing is normally controlled by both iNOS and arginase and abnormality in the expression of either of them may be involved in the pathogenesis of chronic venous ulceration. The aim of this work was to investigate the expression of both iNOS and arginase in chronic venous ulcer biopsies, compared with normal human skin.

Materials and methods

Specimens

This study was approved by the Local Ethics Committee and patients gave written consent. For this study, 16 normal skin and 18 chronic venous ulcer biopsies were used from patients with a mean age of 55 years. Patients with evidence of diabetes, diabetic ulcers, and clinical evidence of infection were excluded. No patients were taking non-steroidal anti-inflammatory drugs or antibiotics. Skin biopsies were taken to include part of the surrounding skin, the ulcer edge, and the ulcer base. Half the biopsies were placed immediately in liquid N_2 for biochemical analysis and half were snap-frozen in iso-propanol in a bath of liquid N_2 for histological and immunohistological observations.

Immunohistochemical staining

Snap-frozen biopsies were embedded in OCT; 6 μm cryostat sections were cut and thaw-mounted on poly-L-lysine-coated slides, air-dried, and stored at $-20°C$ until used. Prior to immunolabelling, sections were fixed in acetone for 10 min and endogenous peroxidases were quenched by treatment with 0.5% H_2O_2 in methanol with subsequent washing in Tris-buffered saline (TBS). Non-specific binding of IgG was blocked using normal serum of the host species of the secondary antibody, diluted 1 : 50 in 0.1% bovine serum albumin in TBS. The sections were incubated with a 1 : 1000 diluted primary antibody to the enzyme of interest at 4°C overnight, washed, and incubated for a further 30 min with a biotinylated goat anti-rabbit secondary antibody (Vector Laboratories) diluted 1 : 1000. Following a further 30 min incubation with Vectastain ABC kits, the substrate, diaminobenzidine tetrahydrochloride in distilled water (Sigma, Poole, UK), was added for the appropriate period (5–10 min). Positive cells were labelled brown. For the negative control, primary antiserum was replaced with normal serum of the host species of the secondary antibody. The antibodies used were polyclonal rabbit anti-human ecNOS (Affiniti, UK), polyclonal rabbit anti-human iNOS (Affiniti, UK), and polyclonal rabbit anti-bovine arginase (Biogenesis, Poole, UK).

Homogenization

Tissues were homogenized in a mixture of protease inhibitors (10 μg/ml leupeptin, 10 μg/ml peptastatin A, and 100 μg/ml phenylmethylsulphonyl fluoride) in 50 mM Tris–HCl, pH 7.5. After spinning for 15 min at 2000 g, the supernatants were used for western blot analysis and measurement of enzyme activities. The protein concentration in the supernatant was measured by the Bradford assay [22], using bovine serum albumin as a standard.

Western blot analysis

The protein concentrations in all the samples were equalized. Tissue homogenates were mixed with gel loading buffer (50 mM Tris–HCl, 10% SDS, 10% glycerol, 10% 2-mercaptoethanol, 2 mg/ml bromophenol blue) in a ratio of 1 : 1 and then boiled for 8 min; 30 μl of each sample was loaded in each well on a 10% SDS-polyacrylamide mini-gel (Hoefer, Staffordshire, UK) using the Laemmli buffer system. The proteins were transferred to 0.45 μm nitrocellulose membranes using a transblotting apparatus (Bio-Rad). The transfer was performed overnight using transfer buffer (25% methanol, 1.5% glycine, and 0.3% Tris-base). Non-specific IgG binding was blocked by incubation with 5% dried milk protein for 30 min. Then the membrane was incubated with primary antibody (diluted 1 : 1000) overnight at 4°C. The bands were detected using an amplified alkaline phosphatase kit (Sigma, Poole, UK) and developed with 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium (Sigma, Poole, UK) as a chromogen. Broad range prestained rainbow markers (Bio-Rad Laboratories, Hemel Hempstead, UK) were used for molecular weight determinations.

Assessment of nitric oxide synthase (NOS) activity

NOS activity was measured as the ability of tissue homogenates to convert [^{3}H]L-arginine to [^{3}H]L-citrulline. The enzyme was activated by incubating the samples at 37°C for 30 min in a medium containing a mixture of: enzyme cofactors; 1 mM NADPH, 30 nM calmodulin (Sigma, Poole, UK), 5 μM tetrahydrobiop-

terin (Alexis Corporation, San Diego, USA), and 2 mM $CaCl_2$; 50 mM L-valine (to inhibit arginase activity) and the substrate; and a mixture of unlabelled and 10 μM [^{3}H]L-arginine (Amersham International, Amersham, UK). The contribution of iNOS (calcium-independent) to total NOS activity was assessed by replacing $CaCl_2$ with EGTA (1 mM). In addition, non-specific conversion of L-arginine to L-citrulline was determined by incubation in the absence of NADPH. Reactions were terminated by the addition of 1 ml of HEPES buffer (20 mM, pH 5.5) containing EGTA (1 mM) and EDTA (1 mM). The newly formed L-citrulline was separated from the L-arginine by passing the reaction mixture over 2 ml of Dowex (mesh size 50X8-200, Sigma, Poole, UK) placed in 2 ml chromatography columns and the collected labelled material was measured using a liquid scintillation counter. Results are expressed as pmol [^{3}H]L-citrulline/30 min per mg protein.

Assessment of arginase activity

This was determined according to the method of Corraliza *et al.* [23]. 10 mM $MnCl_2$ in 50 mM Tris–HCl (pH 7.5) was added to each sample and the enzyme activated by heating at 55°C for 10 min. The substrate, 0.5 M L-arginine, was then added and incubated at 37°C for 1 h, after which the reaction was stopped by the addition of an acid mixture: H_2SO_4, H_3PO_4, and H_2O (1 : 3:7). The urea formed was then assessed spectrophotometrically measuring the optical density at 540 nm after the addition of 9% 1-phenyl-1,2-propanedione-2-oxime (in ethanol) and heating at 100°C for 45 min. Results are expressed as μg urea/mg protein.

Data analysis

Results are expressed as the mean ± SEM for nine individual observations. The Mann–Whitney *U*-test was used, with $P < 0.05$ being considered as statistically significant.

Results

Expression of endothelial nitric oxide synthase (ecNOS)

The immunolocalisation profile for ecNOS in both normal skin and chronic venous ulcer sections was investigated. In dermis, few blood vessels were seen in normal skin and they expressed ecNOS (Figure 1a). In chronic venous ulcers, however, numerous vessels were present and all expressed ecNOS immunoreactivity (Figures 1b–1d). The base of the ulcer contained numerous ecNOS-positive blood vessels, the number decreasing towards the edge of the ulcer (Figure 1b). ecNOS was localized in various types of blood vessels: arteries, veins and capillaries (Figure 1c); and was localized only to the vascular endothelial cells (Figure 1d). In both normal skin and chronic venous ulcer tissue, ecNOS was also seen in epidermal cells, with the highest density of staining seen at the ulcer edge, particularly at the junction between the ulcer base and the surrounding skin and in the basal layer of the epidermis (Figures 1a and 1b). This staining was absent from the negative control in which the primary antiserum was omitted (data not shown).

Western blot analysis showed that ecNOS protein occurred at a band of approximately 140 kD (Figure 1e). A positive band was seen in samples from both normal skin and chronic venous ulcer tissue, but in the latter the band was more prominent, correlating with the increased number of blood vessels expressing ecNOS observed in the immunostaining.

Expression of inducible nitric oxide synthase (iNOS)

In normal skin, immunoreactivity was hardly seen in blood vessels (Figure 2a). However, in the ulcer tissue large numbers of blood vessels were present (Figures 2b and 2c), all of which expressed dense iNOS immunoreactivity localized to both the vascular endothelial and the smooth muscle cells. Cells with a morphology like macrophages were also immunopositive for iNOS (Figure 2e). Most iNOS immunoreactivity was present in the ulcer base and edge, with little in the dermis of the surrounding skin (Figure 2b). Some vessels were surrounded by dense cuffs that also showed strong immunoreactivity (Figure 2d). The epidermis showed immunoreactivity to iNOS both in the normal skin (Figure 2a) and in chronic venous ulcers (Figure 2b); the immunoreactivity in the latter was denser and seen in the thick proliferating epidermis, particularly at the ulcer edge. This staining was absent from the negative control in which the primary antibody was omitted (data not shown).

The profile of iNOS protein levels in samples isolated from normal skin and chronic venous ulcer biopsies was also investigated. A protein band of 130 kD, corresponding to the expected position of iNOS, was found in the samples from chronic venous ulcer patients (Figure 2f), while a similar band was hardly detectable in normal skin samples. The increased level of iNOS in chronic venous ulcer samples was confirmatory of the immunostaining data.

Measurement of NOS activity

The radioimmunoassay demonstrated significantly increased levels of total NOS activity in chronic venous ulcer tissue compared with normal skin (Table 1). Most of the NOS activity was attributed to iNOS (Table 2). This was consistent with the results of the immunostaining and western blot analysis.

Expression of arginase

In normal skin, arginase immunolabelling was localized mainly to blood vessels (Figure 3a). In chronic venous ulcer samples, arginase immunoreactivity was more prominent, with a wider distribution and increased staining density both in the epidermis and

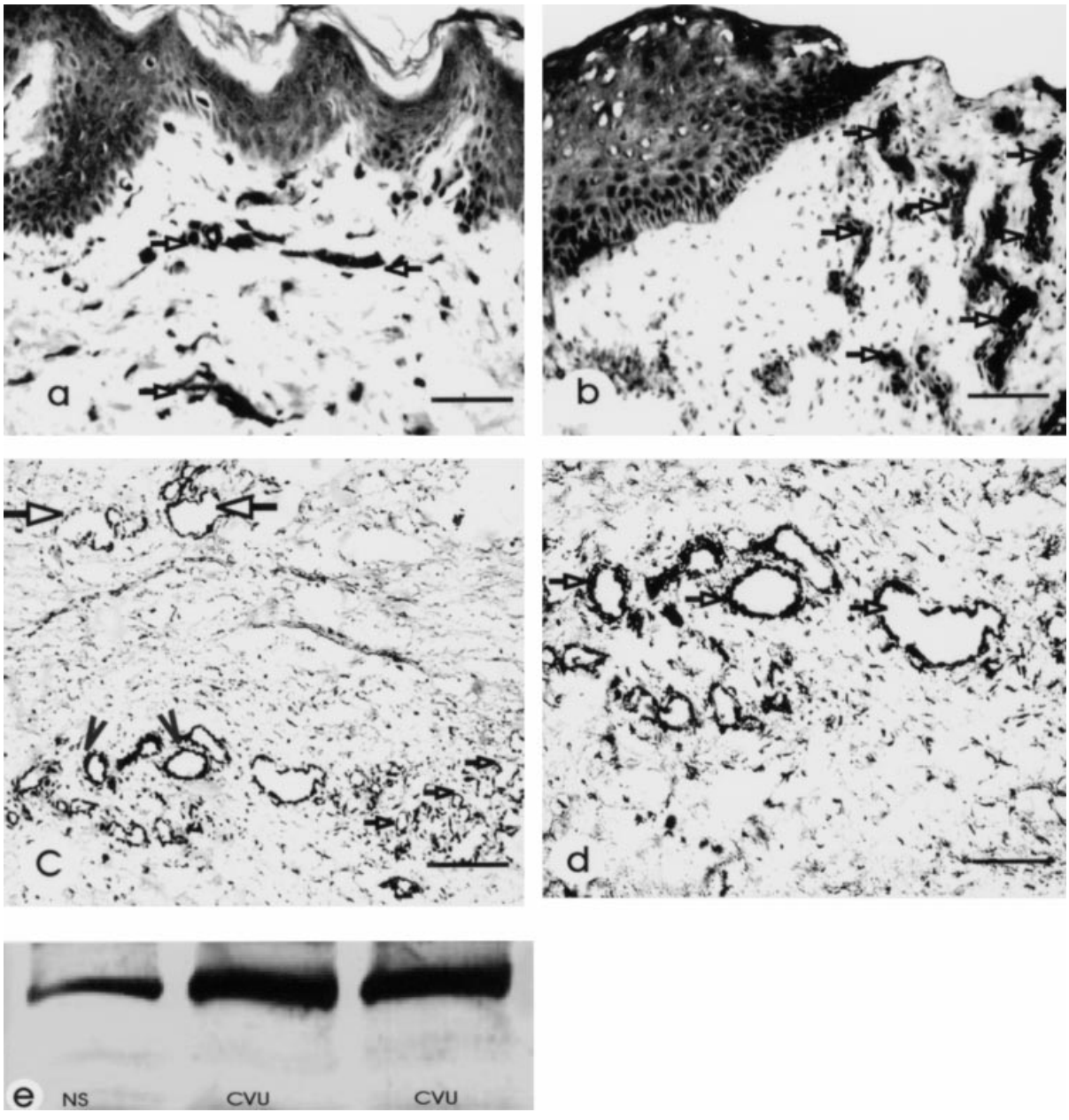

Figure 1. Immunolocalization of ecNOS in (a) normal skin and (b–d) chronic venous ulcer biopsies. (a) Immunoreactivity was localized to blood vessels (arrows). (b) Numerous ecNOS-positive blood vessels (arrows) were seen in the dermis, mainly in the ulcer base with fewer in the dermis of the surrounding skin. (c) A large number of blood vessels of different types and sizes were shown to express ecNOS: veins (large arrows), arteries (arrow-heads) and capillaries (small arrows). (d) Enlargement of some blood vessels in c demonstrating localization of ecNOS in vascular endothelial cells (arrows). Scale bars = 50 μm (a, b); 200 μm (c); 100 μm (d). (e) Western blot analysis of ecNOS, in normal skin (NS) and chronic venous ulcer (CVU) biopsies. ecNOS protein occurred at bands of approximately 140 kD. ecNOS showed denser and thicker bands in chronic venous ulcer samples compared with normal

in the dermis (Figures 3b–3e). Dense staining was seen in the epidermis of the surrounding skin in chronic venous ulcer samples (Figures 3b and 3c). Most of the arginase immunoreactivity in the dermis was present in the surrounding skin (Figure 3c), but little was seen in the ulcer base (Figure 3b). In the dermis, vascular endothelial cells labelled positively for arginase (Figure 3d) and numerous fibroblast- and macrophage-like cells showed intense cytoplasmic staining for arginase (Figure 3e). This staining was absent from the negative control in which the primary antibody was omitted (data not shown).

Western blot analysis using an antibody to arginase detected a discrete band at 70 kD in all samples, but chronic venous ulcer samples showed an increase in the density and thickness of the bands compared with normal skin (Figure 3f).

Measurement of arginase activity

Arginase activity was significantly increased in chronic venous ulcer tissue compared with normal skin (Table 3). This confirmed the results seen with immunostaining and western blot analysis.

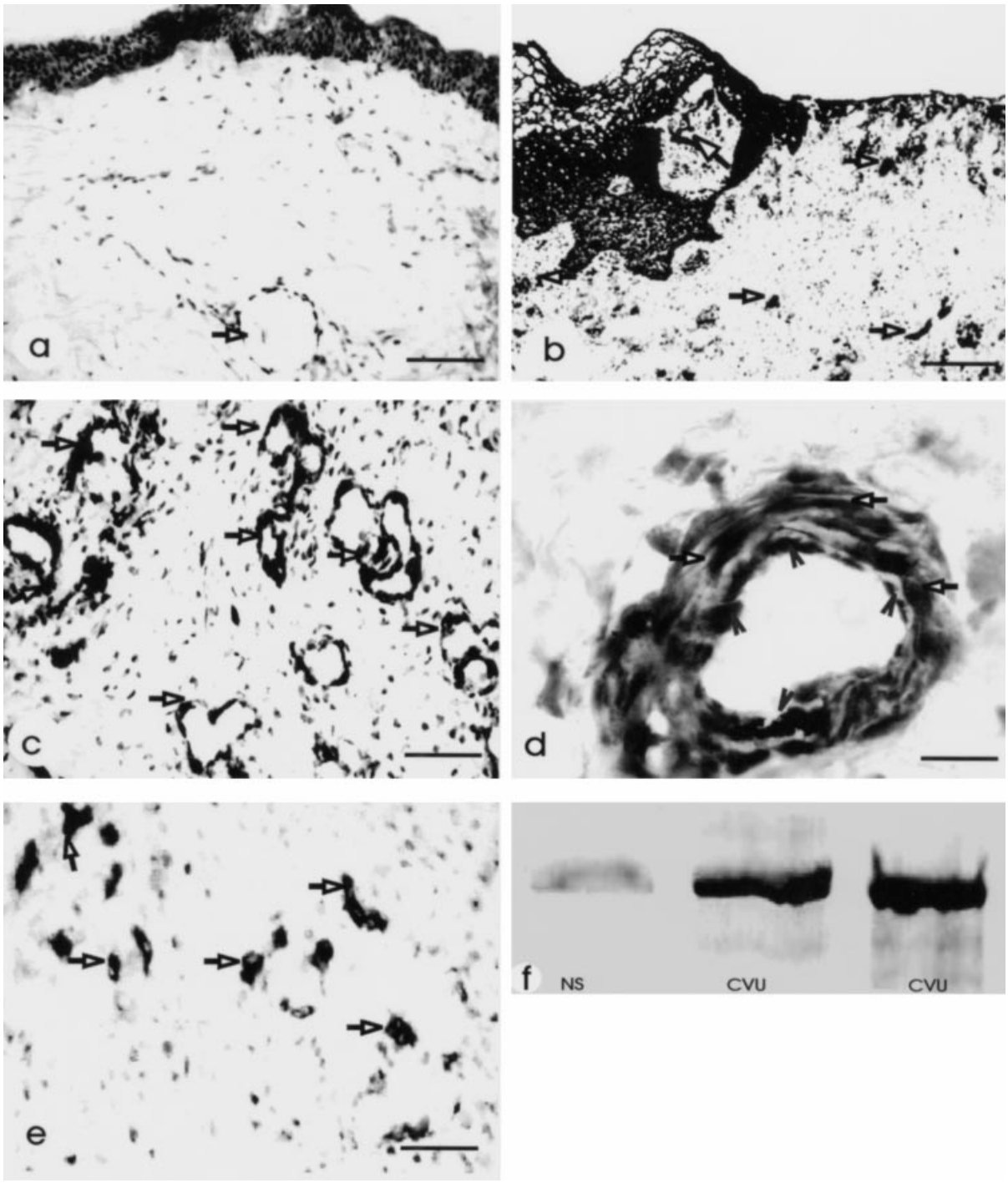

Figure 2. Distribution of immunoreactivity to iNOS in (a) normal skin and (b–e) chronic venous ulcer biopsies. (a) Faint staining for iNOS is seen in the dermis; a large blood vessel is present, with faint staining localized only to the endothelial lining (arrow). (b) Dense immunoreactivity was seen in numerous blood vessels scattered in the base of the ulcer (arrows). (c) Intense immunoreactivity to iNOS in a large number of blood vessels (arrows) was seen, with localization to both the vascular endothelial and the smooth muscle cells. Blood vessels were present in clusters and showed obvious increases in the thickness of their walls. (d) Some vessels showed dense positive staining in all layers of the cuff (arrows) in addition to the endothelial cells (arrow-heads). (e) Macrophage-like cells showed dense cytoplasmic staining for iNOS (arrows). Scale bars = 200 μm (a, b); 100 μm (c); 20 μm (d); 50 μm (e). (f) Western blot analysis of iNOS in normal skin and chronic venous ulcer biopsies. iNOS protein occurred at bands of approximately 130 kD. A dense band was present in chronic venous ulcer samples, but in normal skin the band was weaker

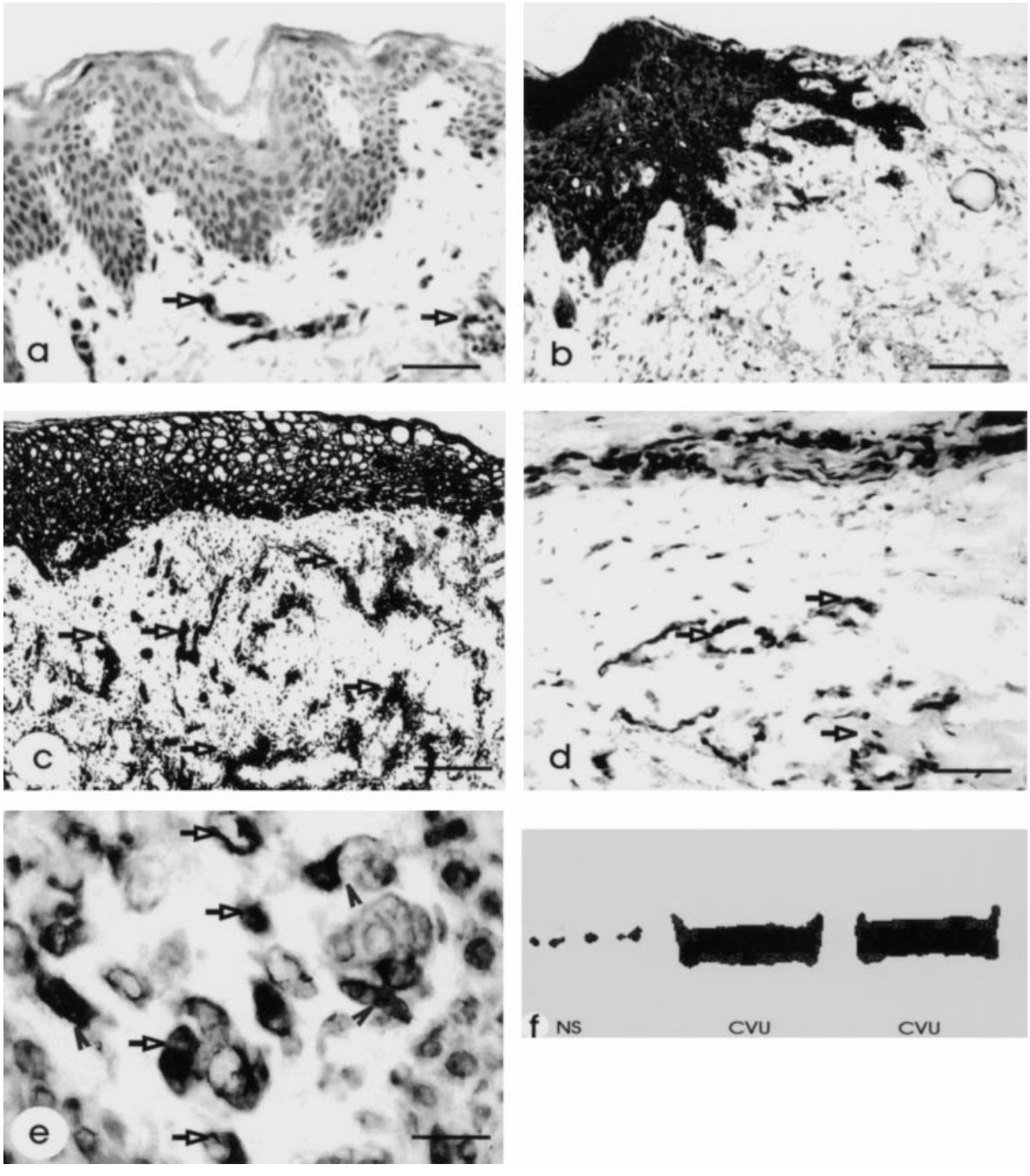

Figure 3. (a) Immunolocalization of arginase in normal skin is localized to blood vessels (arrows). (b) Chronic venous ulcer biopsies showed dense immunoreactivity in the epidermis but little immunoreactivity in the superficial part of the ulcer. (c) The skin surrounding a chronic venous ulcer shows dense staining for arginase both in epidermis and in dermal blood vessels (arrows). (d) Higher power showing arginase in the dermis of the skin surrounding a chronic venous ulcer localized to the vascular endothelial lining (arrows). (e) Higher power of chronic venous ulcer specimens showing cytoplasmic perinuclear localization of arginase in macrophages (arrows) and fibroblasts (arrow-heads). Scale bars = 100 μm (a–c); 50 μm (d); 20 μm (e). (f) Western blot analysis of arginase, a protein band occurred at approximately 70 kD. High levels of arginase were demonstrated in chronic venous ulcer tissue biopsies compared with normal skin, in which only a weak band was visible

Discussion

This work has shown marked up-regulation of both ecNOS and iNOS in chronic venous ulcer tissue compared with normal human skin tissue. This was seen by the wide distribution of both isoforms detected by immunocytochemistry and was confirmed by western blot analysis and enzyme assay, which showed marked increases in the protein level and activity. Several investigations have also demonstrated that iNOS is involved in the pathogenesis of a number of human inflammatory and autoimmune diseases [24,25], and also in dermatological diseases such as cutaneous lupus erythematosus [26] and psoriasis

Table 1. Total NOS activity in normal skin (NS) and chronic venous ulcer (CVU) biopsies

Group	Mean	SD	SEM
NS	146	82	27
CVU	392	137	46

Table 2. iNOS activity in normal skin (NS) and chronic venous ulcer (CVU) biopsies

Group	Mean	SD	SEM
NS	125.8	50.8	16.9
CVU	342.8	152.6	50.9

Table 3. Arginase activity in normal skin (NS) and chronic venous ulcer (CVU) biopsies

Group	Mean	SD	SEM
NS	0.6	0.2	0.1
CVU	2.2	1	0.3

[27,28]. Moreover, several animal models of inflammation have shown iNOS up-regulation, such as the carrageenan-induced rat paw oedema model [29], the murine model of chronic granulomatous inflammation [30], and carrageenan-induced pleurisy [31]. Interestingly, transgenic mice lacking iNOS exhibited less paw swelling in response to carrageenan injection than wild-type controls [32], and NOS inhibitors have been reported to ameliorate a variety of experimental inflammatory diseases in animals [11,33–35]. In these inflammation models, iNOS was the predominant isoform and the main stimuli for its induction were lipopolysaccharide and cytokines. Although inflammation and microbial contamination associated with chronic venous ulcers probably induce iNOS expression, these are unlikely to be the only inducers because significant iNOS expression is seen in the endothelial lining of deep blood vessels (Figure 2c), where there is little inflammatory cell infiltration or bacterial contamination.

NO has been shown in a number of animal models to be an important regulator of angiogenesis and an inhibitor of agents which promote an angiogenic response [36]. However, the situation may be different in human diseases and NO might have an angiogenic effect in chronic venous ulcers. Angiogenesis is known to occur coincidently with vasodilatation and hyperaemia of pre-existing microvessels [37]; these functions are regulated by NO produced by ecNOS. In addition, NO stimulates endothelial cell proliferation, migration, the release of proteases, and the increase of vascular permeability [38–40], all of which are factors which contribute to angiogenesis. Vascular endothelial growth factor (VEGF), a potent angiogenic factor contributing to the angiogenesis in chronic venous ulcers [41], up-regulates ecNOS and so increases NO production by endothelial cells [42]. Thus, we hypothesize that up-regulation of ecNOS in chronic venous ulcers promotes angiogenesis.

Although this study has demonstrated that both NOS isoforms are up-regulated in the chronic venous ulcer, analysis of the activity demonstrated that iNOS is the predominant isoform. Once iNOS is induced, NO is produced in a high level (nmoles) and for a long time; on the other hand, ecNOS produces a low level of NO (pmoles) [43]. Thus, the main source of NO in chronic venous ulcers is iNOS.

The epidermis of both normal human skin and chronic venous ulcers (CVUs) expressed both ecNOS and iNOS, in agreement with others [44]. In CVUs, the epidermis showed increased staining for iNOS, which could be due to the local presence of inflammatory cytokines [45]. Thus, in CVUs, keratinocytes might have a role in the increased production of NO.

Large amounts of NO produced by the induction of iNOS will interact with oxygen free radicals derived from PMNs and macrophages to form peroxynitrite [15]. Peroxynitrite is a potent oxidant that can attack many types of biological molecules and has strong oxidizing and cytotoxic properties causing tissue damage [46,47]. NO, either alone or when combined with other oxygen or nitrous free radicals, could result in the tissue damage associated with chronic venous ulcers. Moreover, excessive vasodilatation may damage the endothelium of the microvasculature, causing increased permeability and oedema [48].

It has been shown recently that NO can cause apoptosis in several cellular experimental systems [49,50]. In macrophages, activation of inducible NOS generates sufficient amounts of NO to promote apoptotic cell death [50]. The apoptotic effect of NO is due to its damaging effects on DNA and the subsequent expression of the tumour supressor gene, *p53* [51]. Recently, it has been found that peroxynitrite induces apoptosis in a time and concentration-dependent manner [52], leading to cell death either by apoptosis or by necrosis depending on the concentration of peroxynitrite [53]. Thus, induction of apoptosis could be another mechanism through which NO produces its damaging effects in CVU tissues.

Thus, in CVUs, NO, rather than being protective, may actually be deleterious and may be a major contributing factor to the chronicity of CVUs.

It is clear from these results that arginase activity in chronic venous ulcers is up-regulated, compared with normal skin, and that fibroblast- and macrophage-like cells are the major cellular sources. Arginase activity results in the production of ornithine, which is subsequently converted to proline, and as proline is a building block for collagen, it can lead to increased matrix deposition. Although arginase contributes to the normal healing process through promoting matrix deposition, in chronic venous ulcers we have shown up-regulation of arginase and yet in spite of this, there is defective matrix deposition. This could be explained by the up-regulation of other factors. In previous

studies, we showed up-regulation of proteases such as serine protease and neutrophil elastase [54], which are the major proteases involved in the breakdown of fibronectin in chronic venous ulcers. In the present study, we have shown up-regulation of NO, which could counteract the healing effect of arginase. Moreover, immunolocalization of NOS and arginase supported this explanation, as they showed different distributions. While NOS is mainly localized to the ulcer base (Figures 1b and 2b), where there is defective matrix deposition, arginase is seen mainly in the dermis of the surrounding skin (Figure 3c), where lipodermatosclerosis and matrix cuffs are present. The lipodermatosclerosis lesion is characterized by fibrous scar tissue formed from collagen fibres and degraded elastic fibres, in addition to perivascular cuffs surrounding dermal capillaries [3]. It is one of the predisposing factors to chronic venous ulceration in patients with chronic venous insufficiency. In liposclerotic skin adjacent to venous ulcers, enhanced levels of transforming growth factor $\beta 1$ (TGF $\beta 1$) have been reported [55]. This cytokine is known to up-regulate arginase activity [56] but down-regulate NOS activity [18]. Previously we have shown that the matrix cuffs surrounding blood vessels are formed by a mixture of extracellular matrix components [3,57]. Up-regulation of arginase might contribute to matrix deposition in these two locations. Recently we have shown that arginase up-regulation is involved in the pathological fibrosis in the callus formed around diabetic foot ulcers [58].

In conclusion, this study has demonstrated for the first time that iNOS and arginase are up-regulated in chronic venous ulcers. Inhibition of iNOS using selective iNOS inhibitors might overcome the deleterious effect of NO and eventually provide a therapeutic tool to improve healing.

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