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Eur J Vasc Endovasc Surg **31**, 516–522 (2006)
doi:10.1016/j.ejvs.2005.12.002, available online at http://www.sciencedirect.com on SCIENCE DIRECT®

Expression of Growth Factors and Growth Factor Receptor in Non-healing and Healing Ischaemic Ulceration

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Objectives. *To characterise the histological and cytokinetic characteristics of purely ischaemic ulcers and the processes that underpin healing following successful revascularisation.*
Design. *Prospective observational study.*
Materials and methods. *Biopsies were taken immediately pre- and 6 weeks following successful revascularisation of solely ischaemic ulceration. They were evaluated for morphological differences using H&E staining for the platelet derived growth factor receptor (PDGFR), epidermal growth factor receptor (EGFR), TGFβreceptorIII (TGFβRIII), transforming growth factor beta 1 and 3 (TGFβ1 and TGFβ3) and von Willebrand factor (vWF) expression using immunohistochemistry. Localisation and quantification of these growth factors and receptors was assessed systematically by three independent investigators who were blinded to the timing of biopsy.*
Results. *Pre-operatively, small vessel vasculitis, necrosis and infection with a profuse neutrophil and macrophage infiltrate was observed in all samples. Post-operative biopsies revealed a proliferation of new capillaries in and around the ulcer edge and base. vWF staining confirmed an endothelial layer within these new vessels. Following successful revascularisation there was less infection and inflammation with minimal vasculitis. These newly formed capillaries had increased staining for TGFβ3, PDGFR and TGFβRIII with staining for PDGFR also localised to dermal fibroblasts which were larger and more numerous. Accelerated epithelial cell proliferation was observed with detachment from the underlying dermis.*
Conclusions. *Healing of purely ischaemic ulcers is characterised by vasculogenesis associated with increased presence of the proangiogenic cytokines PDGF and TGFβ3. These findings show promise for the use of growth factor manipulation to aid healing in ischaemic ulcers.*

Keywords: Ischaemic ulceration; Angiogenesis; Healing; Revascularisation.

Introduction

In the pre-antibiotic era, numerous techniques were developed to aid healing because of the potential life threatening nature of open wounds and the problems attendant to infection. Metchnikoff in his studies of injured tissue, and the immune response evoked, described the migration of microphages (neutrophils) and macrophages to the site of injury.[1] More than 100 years later we now believe these events to be crucial to the regulation of the healing response, particularly in the setting of limb ulceration.

The primary function of skin is to prevent the entry of bacteria and viruses and to maintain internal homeostasis in the face of injury. This barrier function is provided by the external layer of keratinocytes which form the epidermis, but is also reliant on the tensile strength and elasticity provided by the underlying dermis with its abundant matrix of collagen and elastin fibrils. However, this barrier function can be disrupted through either localised damage in isolation or combined with poor tissue perfusion seen in peripheral vascular disease.

Peripheral vascular disease (PVD) contributes to 20% of lower limb ulcers and is present in isolation in about 10% of cases.[2] Recent epidemiological studies have shown that approximately 5% of males over 50 years suffer with intermittent claudication (IC) with up to 25% of this group developing critical limb ischaemia (CLI).[3] Treatment of ischaemic ulceration

This paper was presented in the ESVS Prize session at the ESVS Meeting, Helsinki, 2005.
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poses a therapeutic challenge, usually due to local injury in the setting of poor peripheral perfusion combined with consequent failure of the ischaemic epidermis to heal. Treatment for these patients presents a challenge often necessitating revascularisation to achieve healing,[4–7] whether by interventional radiology, arterial surgery or a combination. These treatments are fraught with significant morbidity and mortality and long-term success is not always assured. In patients with CLI there is a 10% early mortality rate with a further 15% requiring major amputation by 1 year.[8] Aortoiliac and femoro-popliteal disease are amenable to revascularisation in the majority of cases with excellent rates of limb salvage and ulcer healing.[8] Small vessel disease, usually as a consequence of diabetes mellitus or chronic renal impairment, is less amenable to intervention and has much lower rates of limb salvage and ulcer healing.[8]

Following epidermal disruption the process of healing is dependant on an appropriate cellular reaction mediated by cytokines and growth factors, which regulate migration, proliferation, differentiation and metabolism of cells. They act in a paracrine manner on adjacent cells, as autocrine factors on the secreting cell and as endocrine factors bound to carrier proteins. Many such factors have been identified as playing a key role in regulating healing.

Amongst the most important growth factors implicated in cutaneous healing in the setting of ischaemia are platelet derived growth factor (PDGF),[9] epidermal growth factor (EGF),[10] and transforming growth factor beta 1 and 3 (TGFβ1 and TGFβ3).[11] The processes underlying healing and failure to heal in chronic venous or neuro-ischaemic ulceration have been extensively studied.[12–16] Indeed numerous published trials, largely limited to chronic venous or neuro-ischaemic ulceration, have sought to improve healing through manipulation of the local environment with local or parenteral administration of growth factors.[17,18] However, knowledge of the complex pathologies involved in chronic limb ischaemia and the healing processes that follow successful revascularisation is limited.

Aims

This study seeks to describe the early changes in the dermal structure, expression of growth factors and associated growth factor receptors (GFR) in chronic lower limb ischaemic ulcers immediately prior to and following successful bypass surgery. We also seek to correlate these changes in dermal and epidermal architecture of healing ulcers with changes in the cytokinetic milieu.

Methods

Committee approval for the study was given by the Ethics Committee of the Central Manchester Healthcare Trust. Non-diabetic patients with purely ischaemic ulceration, defined as lower limb epidermal defect of greater than 1 month duration with an ABPI of <0.8 that had failed to respond to conservative treatment, were recruited to the study. Inclusion criteria included angiographic evidence of femoro-popliteal disease suitable for reconstruction, without significant small vessel disease or calcification. Patients with clinically neuro-ischaemic ulcers, coexistent venous disease identified on duplex scanning, evidence of osteomyelitis and patients without macroscopic evidence of healing at 6 weeks were excluded. Patient demographics are summarised in Table 1.

Eight patients fulfilled the criteria for the study with a mean age of 62.1 years (42–85 years). All patients had evidence of femoro-popliteal disease with at least two patent infra-crural vessels. All patients had punch biopsy to include ulcer base, edge and surrounding skin immediately prior to surgery. Two patients had

Table 1. Patient demographics

Patient no.	Sex	Age	Affected limb	Site of ulcer	Ulcer size (cm)	Hypertension	Smoker	IHD
1	M	42	L	Ankle	3	Y	Y	N
2	F	72	L	Ankle	2	Y	Y	Y
3	F	85	L	Heel/ankle	3	Y	Y	Y
4	M	46	R	Ankle	3	N	Y	Y
5	M	67	R	Ankle	2	N	Y	Y
6	M	49	R	Heel/ankle	6	Y	Y	Y
7	F	62	L	Heel/ankle	4	Y	N	N
8	M	74	R	Ankle	6	Y	Y	Y
Mean		62.1			3.6			

IHD, ischaemic heart disease; L, left; R, right; M, male; F, female.

Table 2. Histology mean scores

	Surrounding skin		Ulcer edge		Ulcer base	
	Pre	Post	Pre	Post	Pre	Post
Infection	3.3	1.3	4.0	1.7	4.7	2.0
Acute inflammation	3.3	1.0	4.0	0.7	3.3	1.3
Chronic inflammation	2.3	2.0	3.3	3.0	3.7	3.0
Necrosis	0.0	0.0	2.3	0.0	3.7	0.3
Fibroblast number	3.3	4.7*	2.7	4.7	2.3	4.0
Angiogenesis	0.0	3.3	0.0	2.7	0.0	4.7
Vasculitis	2.7*	0.7	3.3	1.0*	1.8	1.3

The above table shows the mean scores out of five for morphological features before and after successful revascularisation.
* Scores where there was an inter-observer variation and a consensus opinion reached.

coexistent aorto-iliac disease and were treated with pre-operative iliac angioplasty in both cases. Five men and three women underwent femoro-popliteal or infra-crural bypass with a mean pre-operative ulcer diameter of 3.6 cm (2–6 cm). Patients were reviewed at 6 weeks following successful revascularisation. Following confirmation of macroscopic healing and increased ABPI, the ulcer edge was re-biopsied, as above, under local anaesthetic.

Tissue preparation

Biopsies were immediately frozen in liquid nitrogen and 5 μm sections cut onto poly-L-lysine coated slides (Fisher Scientific, USA). Sections were stained using Haematoxylin and Eosin (Raymond Lamb, UK) for morphological analysis. Additional sections underwent immunohistochemical staining for the growth factors: TGFβ1, TGFβ3 and growth factor receptors: EGF receptor (EGFR), TGFβreceptorIII (TGFβRIII), PDGF receptor (PDGFR), all supplied by R&D systems. In the case of PDGFR and EGFR, the receptor antibodies were chosen due to technical difficulty staining for their respective GF. Finally, sections were stained for von Willebrand factor (vWF) to visualise vascular endothelium.

For GF and GFR visualisation, a three-stage staining procedure was employed. Briefly, endogenous peroxidase activity was quenched with 3% (v/v) hydrogen peroxide and the slides blocked in 10% (w/v) bovine serum albumin (BSA) in phosphate buffer saline (PBS) for 1 h. All primary antibodies were used at 1:200 dilution and applied for 1 h at room temperature (R&D catalogue nos. AF-236, AF-101-NA, AF-243-NA, AF-914, AF-242-PB). The slides were washed for 30 min in PBS and a horse radish peroxidase conjugated secondary antibody applied for a further hour. The slides were developed using an avidin–biotin complex (ABC kit, Vector Laboratories) for 30 min and visualised using a digital camera (Olympus C-8080, Olympus UK Ltd). For vWF staining a similar protocol was followed except fluorescein isothiocyanate (FITC) conjugated secondary antibody was used to visualise the primary antibody (R&D, antibody ref AF-385). Slides were visualised using a confocal microscope (Leica SP2, Leica Microsystems Ltd, UK). In both cases, control slides were made by omitting the primary antibody.

Each sample was examined by three independent investigators who were blinded to the timing of the biopsy. Each section was divided into three zones: surrounding skin, ulcer edge and ulcer base. Each zone was further subdivided into: epithelium (surrounding skin only), papillary dermis and reticular dermis. A standardised scoring system was used for H&E and GF/GFR stained sections. H&E sections were scored on a scale of one to five for the presence of predetermined features in each ulcer (Table 2). A separate proforma was used to detect GF and GFR staining in each ulcer zone. Pooled data was analysed for post-operative alterations GF and GFR staining and architectural changes. The number of microvessels per 10× magnification field in each ulcer zone was counted from vWF stained sections. When there was a sizable inter observer variation, as was the case in only three results, a consensus opinion was reached among the investigators.

Statistical analysis

Data are presented as mean with standard deviation of each score. Student's *t*-test was employed to determine differences between pre- and post-operative data sets with a *P*-value equal or less than 0.05 considered significant.

Results

The pre-operative ABPI of the ulcerated limb was $<$ 0.7 in all patients. Revascularisation was successful in all patients with a median ABPI increase from 0.5

Table 3. Operative details

Patient no.	ABPI		Angiography	Operation
	Pre-op	Post-op		
1	0.7	1.1	AI and SFA to 2	AI plasty and FEM AK POP (RSV)
2	0.1	0.4	SFA and POP to 2	FEM BK POP (RSV)
3	0.0	0.9	SFA and POP to 2	Redo FEM distal (RSV)
4	0.5	1.0	POP OCC to 2	FEM BK POP and toe AMP (RSV)
5	0.7	1.0	SFA to 2	FEM distal+ debride (RSV)
6	0.4	0.9	SFA and POP to 2	FEM BK POP (RSV)
7	0.4	1.3	DIST SFA OCC to 2	FEM AK DIST (PTFE)
8	0.7	0.9	SFA OCC to 2	FEM AK POP (PTFE)
Mean	0.5	1.0		

ABPI, ankle brachial pressure index; AI, aortoiliac; SFA, superficial femoral artery; FEM, femoral; POP, popliteal; AK, above knee; BK, below knee; DIST, distal; OCC, occlusion; 2, 2 vessel run-off; RSV, reversed saphenous vein; AMP, amputation.

(0.0–0.7) to 1.0 (0.4–1.3) and macroscopic evidence of ulcer healing at 6 weeks. Operative data are summarised in Table 3. All the biopsy sites within the ulcers healed without complication.

Light microscopy H&E staining

Pre-operatively, a prominent inflammatory infiltrate was present in most wounds, often extending into the surrounding dermis. There was evidence of acute and chronic inflammatory cells in all three zones of all samples with attendant bacterial infection. In addition, the ulcer bases exhibited areas of necrosis with small vessel vasculitis in the surrounding skin edge in most samples (Fig. 1(a)). It was noteworthy that the epidermal edges grew downwards into the dermis rather than across the surface.

Post-operatively, there was a marked reduction in acute inflammatory cells within all zones though a residual chronic inflammatory infiltrate remained ($P=0.003$). This was characterised by moderate numbers of macrophages and small numbers of neutrophils, in the absence of overt infection. There were newly developed small blood vessels within the deep and superficial dermis in all post-operative sections, most notably at the ulcer edge and base (Fig. 1(b)–(d)), confirmed by vWF immunofluorescence (Fig. 1(e)).

In marked contrast to pre-operative biopsies, in the post-operative biopsies the epidermis was proliferating across the surface of the ulcer rather than into the dermis. There was consistent reduction in the degree of necrosis and vasculitis seen in and around the ulcers following revascularisation ($P=0.006$).

Growth factor staining

There was a paucity of staining for TGFβ3 in the pre-operative sections but a marked increase post-operatively in and around the areas of neovascularisation within the dermis of the healing ulcers (Fig. 1(b)). TGFβ3 immunostaining was observed throughout the healing epidermis and fibroblasts in the surrounding skin.

Immunohistochemical staining of surrounding skin revealed the presence of TGFβ1 particularly throughout the epidermis and within dermal fibroblasts. There was little change from pre-operative slides with a small increase in staining localised to the dermal fibroblasts. In contrast to TGFβ3, there was little staining for TGFβ1 around the new blood vessels following revascularisation. At the margins of the ulcers, there was obvious TGFβ1 staining in the epidermis, the epithelium of hair follicles, and fibroblasts. In addition, TGFβ1 expression in the epidermal margin of the ulcers was reduced compared to that seen in normal surrounding epidermis. In the healing ulcers minimal staining for TGFβ1 was observed.

Staining for growth factor receptors

Pre-operative staining for PDGFR was confined to inflammatory cells, vascular smooth muscle cells (VSMC) and fibroblasts. Following revascularisation the PDGFR was again localising to macrophages and dermal fibroblasts but the latter were larger and more numerable ($P=0.012$). In addition, there was avid staining for PDGFR in the areas of new blood vessel formation (Fig. 1(c)).

There also was marked increased staining for TGFβRIII around the areas of post-operative angiogenesis and to a lesser extent dermal fibroblasts (Fig. 1(d)).

There was minimal change for the epidermal growth factor receptor (EGFR) from pre- to post-operative samples and the staining was confined mainly to the basal layer of the epidermis.

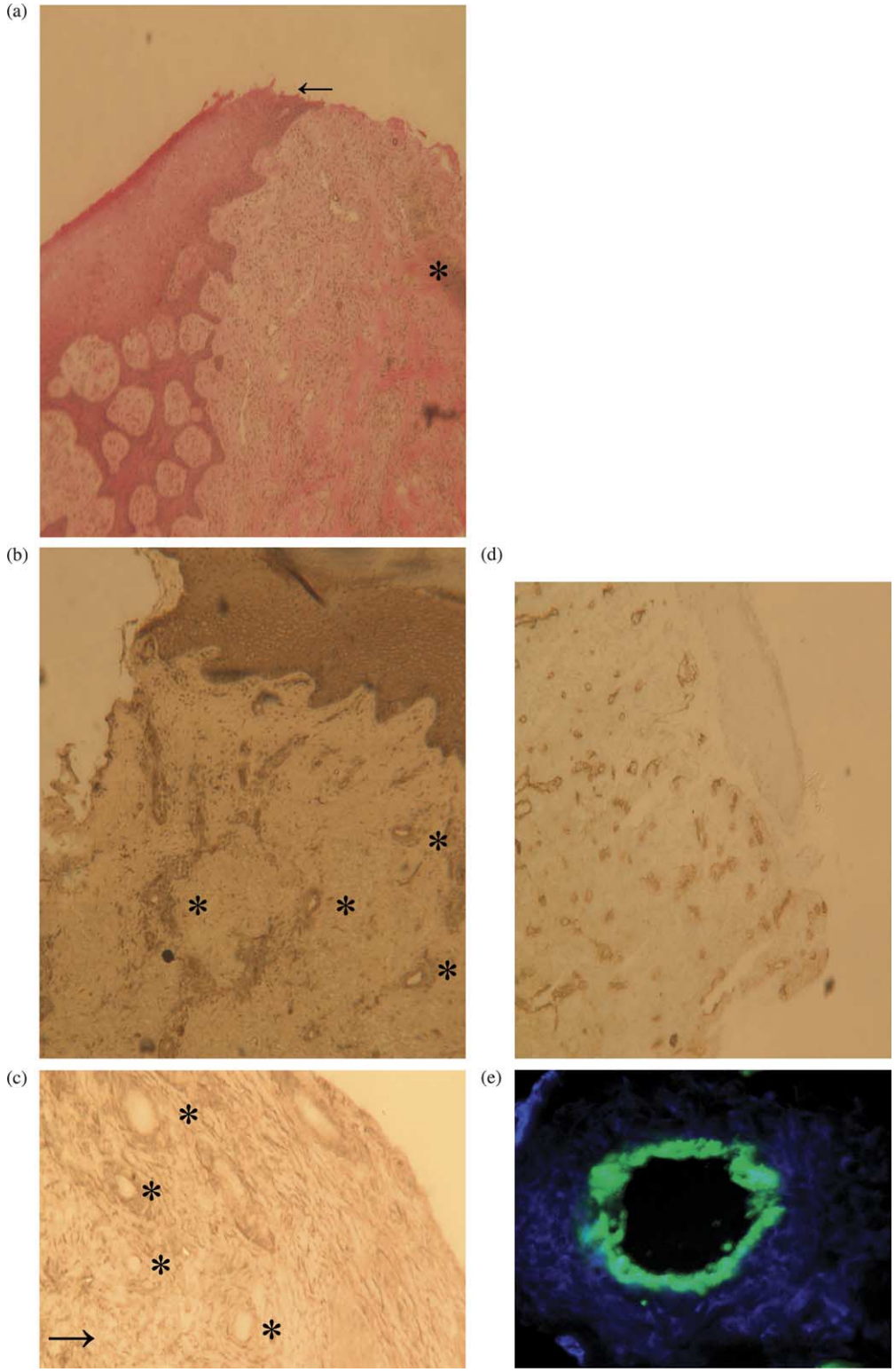

Fig. 1. (a) Pre-operative H&E. Ulcer base necrosis (asterix) with inflammatory cells and infection particularly at the ulcer edge (arrow). (b) Post-operative TGFβ3 immunohistochemistry. Staining of epidermis, fibroblasts and areas of dermal vasculogenesis (asterix). (c) Post-operative PDGFR immunohistochemistry. Staining of new blood vessels (asterix) with larger and more numerable fibroblasts within the deep dermis (arrow). (d) Post-operative TGFβRIII immunohistochemistry. Localised to areas of vasculogenesis. (e) Post-operative vWF immunofluorescence. Demonstrates the endothelial lining of lumen (green) of area of vasculogenesis (blue).

Discussion

Revascularisation has been the gold standard treatment for non-healing ischaemic ulcers for almost 50 years, yet little is known of the mechanisms by which revascularised ischaemic ulcers re-epithelialise. This gap in knowledge is surprising but perhaps relates to the difficulty in getting histological samples in this setting. This is the first study of the processes by which these ulcers heal following revascularisation.

Most studies of lower limb ulceration are population, rather than aetiology based and so are a reflection of an unselected patient group of ischaemic, neuro-ischaemic and mixed ulceration. In this study, we examine the histological changes that underpin healing ischaemic ulceration. It is known that purely ischaemic ulcers, even with only moderately impaired ABPI of 0.9 fail to heal in 70% of cases.[7]

The biopsies showed prominent areas of angiogenesis within the dermis of the ulcer base in all cases. Although angiogenesis is commonly seen within the granulation tissue at the edge of healing wounds, the larger dermal capillaries within the ulcer base seen in this study are an unexpected finding. The formation of true new blood vessels or angiogenesis and the development of collateral vessels from pre-existing blood vessels or atherogenesis are both important processes in the pathophysiology of peripheral vascular disease.[19] Following injury and haemostasis, hypoxia, high lactate levels and acidosis, stimulate angiogenesis. Under the influence factors such as bFGF and VEGF, endothelial cells proliferate and form capillary buds. These cells secrete matrix metalloproteases which allow the vascular buds composed of endothelial cells and SMC to dissect through the wound matrix, while EGF and PDGF recruit pericytes which stabilise the new vessel formation.[20]

Manipulation of these processes has been an attractive therapeutic target for over a decade and the subject of extensive investigation in cardiovascular, oncological and neurological research. Topical and systemic delivery of both vascular endothelial cell growth factor (VEGF) and basic fibroblast growth factor (bFGF) have been used in the setting of end stage coronary ischaemia[21–24] and PVD[25,26] in the hope of alleviating ischaemic symptoms. It was widely held that these factors were deficient in arterial ulcers, hence their use in therapeutic angiogenesis. However, more recent studies have questioned whether there is a true deficiency of bFGF and VEGF in this setting,[27,28] providing a possible explanation for the poor efficacy of these potent angiogenic factors in randomised control trials.[21–24] In addition, concerns abound about the deleterious effects of pro-angiogenic factors in vascular patients with occult malignancies, proliferative retinal disease and unstable atherosclerotic plaques[22] common in an elderly arteriopathic patient group. Perhaps a less potent but more physiological approach is warranted.

Because of histological evidence of angiogenesis we then looked at the expression and localisation of proangiogenic cytokines. VEGF and bFGF have been extensively studied in ischaemia but because of their failure to impact on healing rates in randomised control trials some have questioned their importance. We, therefore, chose to further examine the tissue samples for other pro-angiogenic ligands namely PDGF and the TGFβ superfamily, in particular TGFβ3.

In the healing ischaemic ulcers we found minimal staining for TGFβ1. This contrasts with healing in normal dermal wounds where the addition of exogenous TGFβ1 to existing native TFGβ1 and ligand receptors enhances the repair process through increased cellularity, keratinocyte migration and collagen deposition.[29] Based on our work it is more likely that TGFβ3 would stimulate the angiogenic processes that seems essential to the type of healing we observed in ischaemic tissue. It is noteworthy that this GF has shown efficacy in human skin trials as an anti-scarring agent, reportedly by simulating the healing that occurs *in utero* where higher levels of TGFβ3 occur than in adult healing. It is possible that TGFβ3 may be responsible for regeneration rather than repair and so would seem a promising adjunct to healing in ischaemic ulceration. TGFβRIII is predominantly expressed on endothelial cells and binds TGFβ1 and TGFβ3 but not TGFβ2. TGFβRIII is upregulated in tumour associated vasculogenesis and so has been proposed as a target for anti-angiogenic therapy in tumour patients.[30] However, it is not known if it could serve as an angiogenic target to increase vascularisation. In our cohort, when the ulcers entered the healing phase, expression of TGFβ3 and TGFBRIII were unregulated and at this stage exogenous addition of growth factors may be effective.

We anticipated that EGF and EGFR would be at the forefront of epithelialisation in ischaemic ulcers because of their central role in normal wound healing, however, we found only minimal increases in EGFR post-operatively. The reason for this difference in ligand expression may reflect different functions of growth factors in ischaemic epidermal cells and point to the different wound healing processes following revascularisation.

In summary, this study has revealed that the healing that occurs following revascularisation of ischaemic ulcers is by angiogenesis within the dermis of the ulcer base. There is surprisingly little involvement of EGF in

this process that is chiefly achieved by upregulation of proangiogenic cytokines TGFβ3 and PDGF and little change in TGFβ1. An increased understanding of the processes that underpin successful healing in revascularised arterial ulcers may well provide a therapeutic target for improving ulcer healing rates following bypass or provide alternate therapy for those unsuitable for intervention.

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Accepted 7 December 2005
Available online 20 January 2006