VEGF and VEGF Receptor Expression in Human Chronic Critical Limb Ischaemia

S. Choksy, A.G. Pockley, Y.E. Wajeh and P. Chan

Division of Clinical Sciences (North), University of Sheffield, Clinical Sciences Centre, Northern General Hospital, Herries Road, Sheffield S5 7AU, UK

Objective. This study quantified endogenous VEGF and VEGF receptor expression in limbs of patients with chronic critical limb ischaemia (CLI).

Methods. Skin and muscle biopsies were obtained from the legs of 25 patients undergoing limb amputation for CLI. Samples were obtained at the amputation level (thigh or calf) and, distally, from the foot and in the vicinity of ischaemic ulcers and gangrene. Control biopsies were obtained from patients undergoing amputation for non-arterial reasons or knee arthroplasty (n = 7). VEGF protein levels in tissue lysates were measured by ELISA, and VEGF and KDR mRNA levels were determined using quantitative PCR.

Results. At the amputation level, VEGF protein and VEGF and KDR mRNA levels in CLI limbs were similar to those in controls. In the foot VEGF mRNA in skin (P < 0.005) and VEGF protein levels in muscle (P < 0.02) were elevated compared to levels in a proximal biopsy from the same limb. VEGF and KDR mRNA levels in the vicinity of gangrene/ulcers (VEGF P < 0.01, KDR P < 0.03) also were elevated.

Conclusions. VEGF expression is not deficient in CLI. Indeed, it is elevated at distal sites in the ischaemic limb. These findings question the rationale for VEGF supplementation in CLI.

Keywords: Therapeutic angiogenesis; Peripheral arterial disease; Collaterals; Angiogenesis; Critical limb ischaemia; Vascular endothelial growth factor; VEGF; KDR; VEGF R2.

Introduction

Vascular endothelial growth factor (VEGF) is a key regulator of angiogenesis which is up regulated by hypoxia. VEGF exists in various isoforms ranging from 121 to 206 amino acids resulting from alternative exon splicing. VEGF increases vascular permeability and induces the migration and proliferation of endothelial cells. VEGF binds to Flt1 (fms like tyrosine kinase receptor) and KDR (kinase insert domain receptor) receptors present on endothelial cells. The role of the Flt1 receptor in angiogenesis is not clear, although it also is present on the surface of monocytes, where it may play a role in monocyte chemotaxis. KDR plays a much more definable role in angiogenesis by mediating the full spectrum of biological responses to VEGF including mitogenesis, migration and survival of endothelial cells. Neuropilin is a co-receptor for the VEGF165 isoform enhancing its binding to KDR.

Although the role of VEGF in arteriogenesis (collateral vessel formation) remains disputed, exogenous VEGF has been shown to be capable of stimulating collateral development in ischaemic limbs. Interest in the potential clinical efficacy of increasing endogenous levels of VEGF in patients with peripheral vascular disease developed from encouraging results in pre-clinical studies and several early clinical reports have indicated that the administration of VEGF by direct intra-muscular gene transfer to patients with critical limb ischaemia (CLI) might result in symptomatic improvement. However, the rationale for VEGF replacement therapy remains to be established since little is currently known about pre-existing levels of VEGF and VEGF receptor expression in human CLI. Furthermore, insight into endogenous levels of VEGF and VEGF receptor expression in different parts of the...
same ischaemic limb is important, since VEGF supplementation is most likely to be therapeutically beneficial if it is delivered to areas in which endogenous expression is reduced.

Evidence from animal studies in which the femoral artery was excised has indicated that VEGF is up-regulated in limb ischaemia. However, the duration of ischaemia in such experimental models, is limited and the histological features more closely resemble those of acute limb ischaemia in humans. Currently there is little data on tissue levels of VEGF in different parts of the ischaemic limb in human disease.

The aim of the current study was to systematically quantify tissue (skin and muscle) VEGF and VEGF receptor gene and VEGF protein expression in critically ischaemic human limbs.

Materials and Methods

Patients

The characteristics of the control and CLI patients are summarized in Tables 1 and 2. A total of 25 patients with end-stage peripheral arterial disease were recruited, as characterized by rest pain and, in many cases, tissue loss (Fontaine classification, stage III and IV). There were 14 diabetics (Table 1). All patients had undergone angiography confirming peripheral arterial disease. Eight patients experienced intractable rest pain alone, 10 had gangrene affecting the toe(s) and seven had ischaemic ulcers affecting the calf (n = 3), heel (n = 2), lateral malleolus (n = 1) and arch of the foot (n = 1). Sixteen of the patients with CLI had undergone unsuccessful angioplasty or surgical bypass and nine patients were considered to have un-reconstructable disease (Table 2).

As controls, samples were collected from seven patients without documented arterial disease. These included three patients that had undergone amputations for non-arterial causes, the indications for which were venous disease, congenital tibial non-union and chronic osteomyelitis. The other four patients had undergone knee arthroplasty. During knee arthroplasty, samples were harvested prior to inflation of the tourniquet.

Ethical approval for the study was granted by the North Sheffield Ethics Committee and all patients gave full informed written consent.

Sample collection

Approximately 200 mg of tissue was collected from each site (see below). Samples were obtained immediately after amputation, snap-frozen in liquid nitrogen and stored at −80 °C until analysis.

CLI patients

Proximal biopsies

Biopsies were obtained at amputation level from clinically well-perfused skin and muscle, which healed primarily following amputation. These were the upper calf (gastrocnemius muscle and calf skin) in transtibial (n = 15), and lower thigh (quadriiceps and thigh skin) in transfemoral amputations (n = 10).

Distal biopsies

Distal muscle was obtained from the first layer of muscles in the sole of the foot (flexor digitorum brevis) in selected patients, in whom foot muscle had not been replaced by contracture or fibrotic tissue (n = 12). Distal skin biopsies were obtained from the dorsum of the foot and from edges of the ischaemic ulcer or viable skin immediately bordering gangrenous digit(s) (n = 11). Biopsies were not obtained from areas that exhibited macroscopic features of gross sepsis, inflammation or recent scars. Therefore not all limbs could be biopsied at all sites.

Control patients

A proximal sample (quadriiceps and thigh skin) was collected from the thigh of patients undergoing amputation for non-arterial reasons and in patients undergoing knee arthroplasty (n = 7). Distal control sample were obtained from the foot (dorsal skin and flexor digitorum brevis muscle) only in patients undergoing amputation for non-arterial reasons (n = 3).

Table 1. Patient characteristics

<table>
<thead>
<tr>
<th></th>
<th>CLI</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex, M/F</td>
<td>M = 21, F = 4</td>
<td>M = 5, F = 2</td>
</tr>
<tr>
<td>Median age (IQR), years</td>
<td>71 (15.5)</td>
<td>65 (20)</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>14</td>
<td>1</td>
</tr>
<tr>
<td>Hypertension</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>Ischaemic heart disease</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>Renal failure</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Chronic respiratory disease</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Smoker</td>
<td>10</td>
<td>1</td>
</tr>
</tbody>
</table>

Summary of characteristics of CLI and control patients.
Tissue samples were weighed, an appropriate volume of RNA extraction buffer was added (Trireagent, Sigma, Poole, UK) and tissue was mechanically disrupted using a polytron homogenizer (Kinematica, Lucerne, Switzerland). Total RNA was extracted as described previously22 and quantified by UV spectrophotometry (Cecil Instruments Ltd, Cambridge, UK). RNA integrity was evaluated by the presence of the 18S and 28S ribosomal bands on gel electrophoresis.

Messenger RNA levels of VEGF, KDR and the 18S ribosomal genes were determined by real time RT-PCR using the Taqman™ assay. Total RNA (500 ng) was reverse transcribed into complementary DNA (cDNA) using random hexamers (Promega UK Ltd, Southampton, UK), Maloney Murine Leukemia Virus reverse transcriptase (superscript II, Invitrogen Ltd, Paisley, UK) and deoxyribonucleoside 5'-triphosphates (dNTPs, Promega). RNA was DNase treated to eliminate the possibility of DNA contamination (DNASE 1, Ambion (Europe) Ltd., Huntingdon, UK). cDNA (1 ml) was added to a Taqman™ master mix containing 12.5 ml 2X qPCR mix (Eurogentec, Seraing, Belgium), primers [200 mM] (Sigma Genosys Ltd), fluorogenic labeled probe [50 mM] (Oswell, Southampton, UK) and water in a 25 ml reaction volume. Probe and primer sequences were designed using primer express software (Version 4, Applied Biosystems, Foster City, California, USA).

Each sample was run in triplicate on a 384-well plate on ABI 7900 HT sequence detection system (Applied Biosystems) under universal cycling conditions (50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min). Each plate included known quantities of oligonucleotides

### Table 2. Characteristics of patients undergoing amputation for CLI

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Sex</th>
<th>Diabetes</th>
<th>Indication for amputation</th>
<th>Site of tissue loss</th>
<th>Amputation level</th>
<th>Revasc*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>42</td>
<td>F</td>
<td>N</td>
<td>Gangrene</td>
<td>Toe</td>
<td>Transtibial</td>
<td>N</td>
</tr>
<tr>
<td>2</td>
<td>51</td>
<td>M</td>
<td>N</td>
<td>Rest pain</td>
<td>–</td>
<td>Transemoral</td>
<td>Y</td>
</tr>
<tr>
<td>3</td>
<td>55</td>
<td>M</td>
<td>Y</td>
<td>Ulcer</td>
<td>Arch of foot</td>
<td>Transtibial</td>
<td>N</td>
</tr>
<tr>
<td>4</td>
<td>58</td>
<td>M</td>
<td>Y</td>
<td>Rest pain</td>
<td>–</td>
<td>Transtibial</td>
<td>Y</td>
</tr>
<tr>
<td>5</td>
<td>62</td>
<td>M</td>
<td>N</td>
<td>Rest pain</td>
<td>–</td>
<td>Transemoral</td>
<td>Y</td>
</tr>
<tr>
<td>6</td>
<td>62</td>
<td>M</td>
<td>N</td>
<td>Ulcer</td>
<td>Calf</td>
<td>Transemoral</td>
<td>Y</td>
</tr>
<tr>
<td>7</td>
<td>62</td>
<td>M</td>
<td>Y</td>
<td>Ulcer</td>
<td>Calf</td>
<td>Transemoral</td>
<td>Y</td>
</tr>
<tr>
<td>8</td>
<td>65</td>
<td>M</td>
<td>N</td>
<td>Gangrene</td>
<td>Toes</td>
<td>Transtibial</td>
<td>Y</td>
</tr>
<tr>
<td>9</td>
<td>66</td>
<td>F</td>
<td>N</td>
<td>Rest pain</td>
<td>–</td>
<td>Transemoral</td>
<td>Y</td>
</tr>
<tr>
<td>10</td>
<td>66</td>
<td>M</td>
<td>Y</td>
<td>Ulcer</td>
<td>Calf</td>
<td>Transemoral</td>
<td>N</td>
</tr>
<tr>
<td>11</td>
<td>69</td>
<td>M</td>
<td>N</td>
<td>Gangrene and ulcer</td>
<td>Three toes, heal ulcer</td>
<td>Transemoral</td>
<td>N</td>
</tr>
<tr>
<td>12</td>
<td>71</td>
<td>F</td>
<td>Y</td>
<td>Gangrene</td>
<td>Great toe</td>
<td>Transtibial</td>
<td>N</td>
</tr>
<tr>
<td>13</td>
<td>71</td>
<td>M</td>
<td>Y</td>
<td>Gangrene</td>
<td>Great toe</td>
<td>Transtibial</td>
<td>N</td>
</tr>
<tr>
<td>14</td>
<td>72</td>
<td>M</td>
<td>Y</td>
<td>Gangrene</td>
<td>Great toe amputation site</td>
<td>Transtibial</td>
<td>Y</td>
</tr>
<tr>
<td>15</td>
<td>72</td>
<td>M</td>
<td>Y</td>
<td>Gangrene</td>
<td>Toes</td>
<td>Transtibial</td>
<td>Y</td>
</tr>
<tr>
<td>16</td>
<td>73</td>
<td>M</td>
<td>N</td>
<td>Rest pain</td>
<td>–</td>
<td>Transtibial</td>
<td>Y</td>
</tr>
<tr>
<td>17</td>
<td>74</td>
<td>M</td>
<td>Y</td>
<td>Rest pain</td>
<td>–</td>
<td>Transemoral</td>
<td>Y</td>
</tr>
<tr>
<td>18</td>
<td>75</td>
<td>M</td>
<td>N</td>
<td>Gangrene and ulcer</td>
<td>Heal, toe</td>
<td>Transtibial</td>
<td>Y</td>
</tr>
<tr>
<td>19</td>
<td>77</td>
<td>M</td>
<td>Y</td>
<td>Gangrene</td>
<td>Great toe amputation site</td>
<td>Transtibial</td>
<td>Y</td>
</tr>
<tr>
<td>20</td>
<td>78</td>
<td>M</td>
<td>Y</td>
<td>Gangrene</td>
<td>Amputation site non-healing</td>
<td>Transtibial</td>
<td>N</td>
</tr>
<tr>
<td>21</td>
<td>78</td>
<td>M</td>
<td>Y</td>
<td>Rest pain</td>
<td>–</td>
<td>Transtibial</td>
<td>Y</td>
</tr>
<tr>
<td>22</td>
<td>79</td>
<td>M</td>
<td>N</td>
<td>Rest pain</td>
<td>–</td>
<td>Transemoral</td>
<td>Y</td>
</tr>
<tr>
<td>23</td>
<td>80</td>
<td>M</td>
<td>Y</td>
<td>Ulcer</td>
<td>Heal</td>
<td>Transtibial</td>
<td>Y</td>
</tr>
<tr>
<td>24</td>
<td>82</td>
<td>M</td>
<td>Y</td>
<td>Ulcer</td>
<td>Lateral aspect heal</td>
<td>Transtibial</td>
<td>N</td>
</tr>
<tr>
<td>25</td>
<td>85</td>
<td>F</td>
<td>N</td>
<td>Ulcer</td>
<td>Medial malleolus</td>
<td>Transemoral</td>
<td>N</td>
</tr>
</tbody>
</table>

* Indicates if patient underwent surgical or radiological intervention e.g. bypass or angioplasty. Indications for amputation were intractable rest pain only (n = 9) or rest pain and tissue loss (n = 16). A thigh skin and muscle biopsy was collected from patients undergoing transfemoral (n = 10) and a calf skin and muscle biopsy (n = 15) in patients undergoing transtibial amputation. A foot skin biopsy was collected in most cases (n = 22), foot muscle biopsies were collected in 12 patients and additional skin biopsies bordering ischaemic ulcers and gangrene were collected in 10 patients.
synthesized commercially (Sigma Genosys Ltd). Fluorescence was detected in real time and analyzed at the completion of the RT-PCR reaction. A standard curve was constructed, from which absolute copy numbers of each gene could be calculated for each sample. Results were normalized to those for 18S ribosomal gene in order to correct for differences in template loading in each sample. The intrasample variation (coefficient of variation, CV) of the assay was 8.6%.

**VEGF protein levels**

Tissue samples were homogenized using a lysis buffer described by Vadiveloo et al.23 (50 mM Tris pH 8, 150 mM NaCl, 0.2% SDS, 0.5% Na Deoxycholate, 0.5% Triton X-100) containing a protease:phosphatase inhibitor cocktail (100 mM NaF, 200 mM NaVO₄, 70 IU aprotinin, 10 mg/ml leupeptin, 1 mM EDTA, 0.1 mM PMSF). The homogenate was centrifuged at 3000 g for 10 min and at 12,000 g for 10 min and the supernatant was stored at −70 °C for a short time (<1 month) prior to protein content determination and analysis by ELISA. The total protein content of the supernatant was determined by a modified Lowry method (DC protein assay, BioRad, Hemel Hempstead, UK).

Total VEGF protein levels in the supernatants were determined by enzyme immunoassay according to the manufacturer’s protocol (Cytosets™, Biosource, Nivelles, Belgium). Ninety six well Maxisorp microtiter plates (Nunc, Hereford, UK) were coated overnight (18 h) at 4 °C with murine anti-human VEGF monoclonal antibody (clone no. A183C-13G8) at a concentration of 2.5 μg/ml. Non-specific binding was blocked by incubation with phosphate buffered saline (PBS) containing 1% v/v Tween 20 (PBS-T) and 1% w/v bovine serum albumin, for 2 h at room temperature. VEGF standards (0–10,000 pg/ml) and samples (100 μl) were added in duplicate and plates were incubated for 90 min at room temperature, after which they were washed and biotinylated polyclonal rabbit anti-human VEGF detection antibody (100 μl, 0.5 μg/ml) was added. Plates were incubated for 1 h, washed and a streptavidin–horseradish peroxidase conjugate (HRP, 0.5 μg/ml) was added. After 45 min, plates were washed and tetramethylbenzidine (TMB) substrate was added. The enzymatic reaction was stopped after 20 min by addition of 2 M H₂SO₄. The resultant absorbances at 450 nm and 650 nm (reference wavelength) were determined using a Titertek Multiskan MCC/340 microplate spectrophotometer (Thermo Life Sciences, Basingstoke, Hampshire, UK). VEGF levels were calculated from the standard curve using Ascent analysis software (Thermo Life Sciences) and were normalized to the total protein concentration. The CV for the technique was less than 10% within the dynamic range of the assay.

**Statistical analysis**

Non-parametric data are presented as medians (IQR, interquartile range). Gene expression is expressed as mRNA copies per million of 18S and protein expression in picograms (pg) per milligram (mg) of total protein. For statistical analysis the proximal control biopsies from knee arthroplasty and non-arterial amputation were grouped together and were compared with proximal CLI biopsies using the Mann–Whitney test. We also sought to identify regional variation within CLI limbs in order to determine whether there were differences along a proximal to distal axis, corresponding to a presumed gradient of ischaemia. These paired comparisons of data were performed using the Wilcoxon signed rank test.

All statistical analyses were performed using Statview™ (Abacus Concepts, Berkeley, California, USA) and P values less than 0.05 were considered to represent statistically significant differences.

**Results**

**VEGF and VEGF receptor gene and VEGF protein expression in skin**

**CLI vs. control**

There were seven proximal skin and muscle biopsies from controls, and VEGF and VEGF receptor gene and VEGF protein expression in these were compared to that in proximal biopsies from patients with CLI (thigh = 10, calf = 15). VEGF and KDR gene expression and VEGF protein levels in proximal skin samples from control and CLI patients were similar (Fig. 1). In muscle VEGF gene and protein levels were similar in proximal biopsies in CLI and control patients (Fig. 2). However when only thigh muscle samples were compared KDR gene expression was reduced in CLI patients compared to controls (control 3.2 (3.69) vs. CLI 1.65 (1.83), P = 0.016, Mann–Whitney).

**Regional variation in CLI limbs**

There were 22 limbs in which both a proximal (thigh or calf) and foot skin biopsies were available, and 10 limbs from which an additional skin biopsy bordering
ulcer/gangrene was available. Paired foot and proximal muscle biopsies were available in 12 patients.

There was no significant difference in VEGF mRNA levels between proximal skin and muscle biopsies in transfemoral (thigh biopsies) and transtibial amputations (calf biopsies) in CLI patients (Fig. 3). However, VEGF mRNA levels were two-fold higher in foot skin ($P=0.005$, Wilcoxon) and four-fold higher in skin bordering ischaemic ulcers and gangrene ($P=0.01$, Wilcoxon) than those in a proximal biopsy obtained from the same limb (Fig. 3). There was no significant difference in VEGF mRNA levels between proximal and distal muscle biopsies.

VEGF protein levels in skin and muscle biopsies from the thigh and calf were similar as were VEGF protein levels in foot and proximal skin biopsies (Fig. 4). In contrast, VEGF protein levels in foot muscle were higher than those in the corresponding proximal biopsy ($P=0.02$, Wilcoxon; Fig. 4). Although VEGF protein levels were elevated distally in ischaemic ulcers and gangrene, this difference did not reach statistical significance ($P=0.07$, Wilcoxon).

There was no significant difference in KDR mRNA levels in the skin of the thigh and calf from limbs of patients with CLI, whereas KDR expression in the thigh muscle was lower than that in calf muscle ($P=0.002$, Mann–Whitney, thigh vs. calf; Fig. 5).

Distally, KDR gene expression appeared elevated in foot skin compared to proximal biopsies, although this did not reach statistical significance ($P=0.06$). However, KDR mRNA levels in skin bordering ulcers and

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**Fig. 1.** Box plot of VEGF and KDR mRNA expression and VEGF protein levels in proximal skin samples from critically ischaemic limbs (thigh and calf) and controls. mRNA levels are expressed as copies normalized to 18S and VEGF protein levels are expressed as picograms (pg) per milligram (mg) of protein. Boxes represent interquartile range (IQR), horizontal bars represent group medians and whiskers represent 5th and 95th percentiles. Filled circles represent outliers. There were no statistical differences in VEGF mRNA and protein in CLI vs control in proximal skin biopsies.

**Fig. 2.** Box plot of VEGF and KDR mRNA expression and VEGF protein levels in proximal muscle samples from critically ischaemic limbs (thigh and calf) and controls. mRNA levels are expressed as copies normalized to 18S and VEGF protein levels are expressed as picograms (pg) per milligram (mg) of protein. Boxes represent interquartile range (IQR), horizontal bars represent group medians and whiskers represent 5th and 95th percentiles. Filled circles represent outliers. There were no statistical differences in VEGF mRNA and protein in CLI vs control in proximal muscle biopsies. However, in thigh muscle KDR mRNA levels were significantly less in CLI patients than controls ($P=0.016$, see text).
gangrenous areas was significantly higher than that in proximal biopsies \((P = 0.03; \text{ Fig. 5})\). There was no overall difference in KDR gene expression levels between foot and proximal muscle biopsies.

Control patients

There were three control amputees from whom a proximal and foot biopsy was available. Unlike ischaemic limbs, no difference was detected in VEGF protein or VEGF/VEGF receptor gene expression in either skin or muscle between proximal and distal biopsies (data not shown).

**Discussion**

This study has demonstrated that VEGF gene and protein expression is greater in ischaemic skin and that VEGF protein expression is greater in ischaemic muscle. Importantly, these data clearly indicate that the mechanisms for upregulation VEGF in the ischaemic limb remain intact.

Since tissue loss affecting the skin (ulcers and gangrene) is a common feature of critical limb ischaemia in humans, VEGF expression in skin as well as muscle was examined. VEGF expression in...
proximal control and CLI biopsies was similar; proximal CLI biopsies were taken from relatively well perfused skin and muscle which healed primarily following amputation and can be regarded as ‘internal controls’, against which distal ischaemic biopsies could be compared. This strategy was appropriate as it has previously been demonstrated that proximal muscle samples from ischaemic limbs exhibit relatively normal histology and low VEGF/KDR expression. Another benefit of comparing a distal sample with a corresponding proximal sample from the same limb is that it is likely to reduce variability resulting from patient diversity. This diversity resulted in the considerable variation of VEGF/KDR expression observed. Factors such as age and diabetes, the presence of tissue loss and differences in severity and duration of ischaemia may all contribute to variability.

We observed that VEGF gene expression in distal skin and VEGF protein levels in distal (foot) muscle were about two-fold greater than in the corresponding proximal biopsy from the same patient. This may be partly explained by a gradient of ischaemia from proximal to distal thereby exposing distal tissue to the severest hypoxia. The resultant hypoxia is likely to induce increased VEGF gene expression in keratinocytes, myocytes and dermal fibroblasts, which are all known to up regulate VEGF in hypoxic conditions, partly mediated by a transcription factor, hypoxia inducible factor 1α (HIF1α). The levels of HIF1α levels are increased under hypoxic conditions. HIF1α binds to a HRE (hypoxia response element), an enhancer site upstream of the VEGF gene, causing the transcriptional upregulation of VEGF. Rissanen et al. demonstrated that elevated HIF1α was associated with an elevation of VEGF expression in ischaemic muscle obtained from CLI limbs. In regions of tissue loss such as margins of ulcers and skin bordering gangrene, we found that VEGF and KDR expression were about four times greater than corresponding proximal biopsies. At these sites other mechanisms are likely to contribute to VEGF up regulation such as inflammation and wound healing. Elevated VEGF mRNA levels have also been found in homogenates of granulation tissue adjacent to chronic sacral pressure ulcers and in hyper-plastic epithelium from wound margins in venous ulcers. Although VEGF mRNA levels were elevated significantly, there was only a tendency towards elevated levels of VEGF protein in skin adjacent to ulcers and gangrene ($P=0.07$). The converse was apparent in muscle, in that muscle VEGF protein levels were elevated whereas VEGF mRNA levels were unchanged. These inconsistencies might be explained by the sequestration of VEGF in the extra cellular matrix (ECM). Apart from the VEGF$^{121}$ isoform which is freely diffusible, all other isoforms of VEGF bind, to differing degrees, to the ECM. The mechanisms that govern the release/storage of VEGF isoforms from the ECM in different tissues are not fully understood.
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understood and might result in a lack of association between total VEGF protein levels and gene expression.

Our finding that VEGF levels vary in different parts of the ischaemic limbs is similar to that observed in a rat model of severe limb ischaemia in which the onset of ischaemia has been shown to be followed by a two fold increase in VEGF in distal muscle, but no significant increase proximally in thigh muscle. However, in addition to it is conceivable that clinical benefit might result from over-expression. Might supra-physiological levels of VEGF rather than distal supplementation is most likely to be

An unexpected and potentially important finding of our study is the reduction in KDR gene expression in the thigh muscle in CLI patients compared to both control thigh biopsies and CLI calf biopsies. KDR mediates almost all of the angiogenic functions of VEGF. On binding by VEGF, an array of intracellular signal transduction pathways are stimulated, leading to a variety of biological responses including mitogenesis, migration and survival of endothelial cells. Therefore, deficient KDR expression might limit the effectiveness of VEGF gene therapy for critically ischaemic limbs, although it is conceivable that exogenous VEGF could correct reduced KDR expression via its autocrine actions.

Diabetes is a major risk factor for peripheral arterial disease and a greater proportion of diabetic patients with PVD are destined for amputation. This is reflected in the greater proportion of patients with diabetes in the CLI patient group. However, VEGF and KDR gene expression in skin or muscle (proximally, distally) from patients with diabetes and patients without diabetes in the CLI group were similar (data not shown). We, like others, have not shown a decreased ability to up regulate VEGF in diabetes.

In summary, VEGF is upregulated appropriately in the ischaemic limb, which indicates that the mechanism for VEGF up regulation is intact. There is a regional variation of VEGF expression. Distally in foot skin and muscle and in skin bordering tissue loss, VEGF levels are elevated. However, proximally, where collateral development is likely to be more important VEGF levels are no different to control biopsies. There is also a reduction in KDR expression in thigh muscle in CLI patients.

This study has raised important questions regarding VEGF biology e.g. why are high VEGF levels distally not reflected in higher levels proximally? Could this result from a failure of propagation? These data question the rationale of VEGF gene therapy. Should additional VEGF be given to ischaemic limbs, which already have high levels of VEGF distally? Might supra-physiological levels of VEGF result in side effects that could exacerbate the symptoms of CLI such as oedema? Finally, if VEGF gene therapy is to be used therapeutically, proximal rather than distal supplementation is most likely to be...
beneficial, since VEGF levels are not elevated proximally.

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