Reduced VEGF expression and intra-epidermal nerve fiber loss in human diabetic neuropathy

C Quattrini\textsuperscript{1} MD, M Jeziorska\textsuperscript{2} PhD, AJM Boulton\textsuperscript{1} MD, RA Malik\textsuperscript{1} MD, PhD.

Division of Cardiovascular Medicine, University of Manchester and Manchester Diabetes Centre, Manchester Royal Infirmary, Manchester, UK\textsuperscript{1}
Division of Regenerative Medicine, University of Manchester, UK\textsuperscript{2}

Running title: VEGF and Intra-epidermal nerve fiber reduction

Address of correspondence and reprint requests to
Dr. R.A.Malik,
Division of Cardiovascular Medicine,
Core Technology Facility,
University of Manchester,
Manchester, M13 9NT, UK.
E Mail- rayaz.a.malik@man.ac.uk

Received for publication 7 August 2007 and accepted in revised form 4 October 2007.
ABSTRACT

OBJECTIVE: To assess the relevance of VEGF in the maintenance of peripheral nerve integrity in diabetic neuropathy we have assessed the expression of VEGF and intraepidermal nerve fibre density (IENFD) in skin biopsies from diabetic patients.

RESEARCH DESIGN AND METHODS: 53 diabetic patients and 12 non-diabetic control subjects underwent neurological evaluation, electrophysiology, quantitative sensory and autonomic function testing. Dermal blood flow responses were evaluated with laser Doppler flowmetry. Skin biopsies were performed on the dorsum of the foot and IENFD was quantified and compared to the expression of VEGF-A, its receptor VEGFR-2, HIF-1α and microvessel density.

RESULTS: IENFD decreased progressively with increasing severity of diabetic neuropathy (P<0.001). Dermal blood flow response to ACh was reduced in diabetic patients with mild and moderate neuropathy (P<0.01) and the intensity of staining for epidermal VEGF-A was significantly reduced in diabetic patients compared to controls (P<0.01). Epidermal HIF-1α and VEGFR-2 expression did not differ between groups.

CONCLUSIONS: Progressive endothelial dysfunction, reduction in VEGF expression and loss of intraepidermal nerve fibers occurs in the foot skin of diabetic patients with increasing neuropathic severity.
The complex causative pathways for the development and progression of nerve damage are as yet not clearly established for human diabetic neuropathy (1). Vascular endothelial growth factor (VEGF) has been shown to have neurotrophic properties (2). Recently herpes simplex virus vector-mediated VEGF prevented a reduction in sensory nerve amplitude and loss of intraepidermal nerve fibres (3). Intramuscular administration of an engineered zinc finger transcription factor, which activates transcription of all major VEGF-A isoforms, improved sensory and motor nerve conduction velocities (4) and yielded a positive indication in a phase 1 clinical trial (5) for establishing a larger phase 2 trial with additional end points including IENF assessment. Of relevance, increased expression of HIF-1α and its target genes, VEGF and erythropoietin has been demonstrated 4-6 weeks after the induction of diabetes with a decline at 8 weeks (6).

IENF density is significantly reduced in diabetic patients with minimal neuropathy (7), is related to painful symptoms and neuropathic deficits (8), and may improve following life style intervention (9). In the present study we utilized skin biopsies to quantify intraepidermal nerve fiber loss in relation to the expression of HIF-1α and its principle target gene, VEGF and its receptor, in diabetic patients with increasing severity of neuropathy.

RESEARCH DESIGN AND METHODS

This study was approved by the Local Research Ethics committee and all patients gave informed consent to participate. Patients and non-diabetic volunteers were invited unless they had absent pedal pulses, to exclude peripheral vascular disease that may impact both on neuropathy and VEGF expression but also wound healing after the biopsy. All subjects were screened for other causes for neuropathy including vitamin B₁₂, thyroid-stimulating hormone, antinuclear antibody and serum protein electrophoresis and those with a family history of neuropathy or a disease known to cause neuropathy were excluded.

Neuropathy evaluation. Neuropathic symptoms were assessed using the diabetic neuropathy symptom score (DNS) (10) and the Visual Analogue Score (VAS) for pain (11). Neurological deficits were assessed using the neuropathy disability score (NDS) (12) which ranges between 0 and 10; neuropathy was diagnosed if NDS was ≥3/10. We also performed quantitative sensory tests including cooling detection threshold (CDT), minimal (HP-VAS 0.5) intermediate (HP-VAS 5.0) and differential (HP-VAS 0.5-5.0) heat as pain thresholds (13,14) and deep breathing heart rate variability (DB-HRV) (15) with CASE IV (WR Medical Electronics, Stillwater, MN). Patients also underwent electrophysiological assessment with a Dantec “Keypoint” system (Dantec Dynamics Ltd, Bristol, UK) to evaluate amplitudes (µV), conduction velocities (m/sec), and latency (msec) of responses.

Laser Doppler flowmetry. A subset of the patients undergoing biopsy comprising of 11 controls and 46 diabetic patients underwent skin blood flow Laser Doppler flowmetry with a large area laser scanner (Moor Instruments, Devon, UK) on the dorsum of the foot at the site of the biopsy. A MIC iontophoresis controller (Moor Instruments, Devon, UK) delivered 20µA for 5.5 minutes to the skin through MIC-ION plastic chambers (Moor Instruments, Devon, UK) containing 1% Acetylcholine and 1% Sodium Nitroprusside. The results were measured in arbitrary units of flux (AUF) for baseline, maximal responses and differential increase in blood flow (Delta).

Skin biopsy. Two 3-mm punch skin biopsies were taken from the dorsum of the foot, approximately 2 cm above the 2nd
metatarsal head, under 1% plain lidocaine local anesthesia.

**Intraepidermal nerve fiber density.** The first specimen was immediately fixed in 4% paraformaldehyde and after 18-24 hours, rinsed in Tris-buffered saline and soaked in 33% sucrose (2-4 hrs) before cryoprotection in OCT and rapid freezing in liquid nitrogen. 50 µm sections were cut using a cryostat (model OTF, Bright Instruments Ltd, Huntington, England) and floating sections were transferred onto a 96-well plate. Four sections per case underwent melanin bleaching (0.25% KMnO$_4$ for 15 min, then 5% oxalate for 3 min), 4 hour protein block with a Tris-buffered saline solution of 5% normal swine serum, 0.5% powdered milk, 1% Triton X-100; and incubated overnight with 1:1200 Biogenesys polyclonal rabbit anti-human PGP9.5 antibody (Serotec Ltd, Oxford, England). Swine anti-rabbit secondary antibody 1:300 (1 hour) was then applied; sections were quenched with 1% H$_2$O$_2$ in 30% MeOH-PBS (30 minutes) prior to a 1 hour incubation with 1:500 HRP-Streptavidin (Vector laboratories, Peterborough, England). Nerve fibers were demonstrated using 3,3′-Diaminobenzidine (DAB) chromogen (Sigma-Aldrich Ltd., Manchester, UK). Sections were mildly counterstained with eosin to better localize the basement membrane. Negative controls comprised sections that underwent the same run except the primary antibody was omitted. Developing time was exactly the same for all sections in each separate run and in each run the sections were processed synchronously.

**Vascular factor immunostaining.** The second biopsy was fixed in 4% paraformaldehyde for 18-24 hours, embedded in paraffin wax and cut into 5-micron sections. Thin sections were mounted on positively charged slides (3 per slide), de-waxed in xylene and gradually rehydrated through decreasing ethanol dilutions. Trypsinization was used to disclose the antigen for sections used to immunostain for blood vessels. Optimal visualization was obtained by microwaving for VEGF-A and VEGFR-2; and by adding a tyramide amplification reagent (CSA I, DAKO) for HIF-1α. In all cases epidermal melanin was bleached with 0.25% KMnO$_4$ and 5% oxalate prior to serum protein block. Anti-human primary antibodies were applied overnight at 5°C: mouse monoclonal to CD31 and vWF [DAKO, diluted 1:100], and VEGFR-2 [Santa Cruz, 1:50]; and rabbit polyclonal to VEGF-A [Santa Cruz, 1:300] and HIF-1α [Abcam, 1:300]. Negative controls comprised sections that underwent the same runs except the primary antibody was omitted. Developing time was exactly the same for all sections in each separate run and in each run the sections were processed synchronously.

**Image analysis.** Patterns of immunostaining were examined by light microscopy (Leitz DM RB microscope). Digital images were captured at 400 time magnification with a Nikon digital camera and analyzed with Leica QWin Standard V2.4 (Leica Microsystems Imaging Ltd, Cambridge, UK) set to detect color intensities in a fixed and constant range (16).

IENF density was defined as the number of fibres per millimeter of basement membrane length and expressed as no./mm (nerve density per length). If two nerve endings appeared closer than 5 fibre diameters they were counted as one single nerve (17).

The protocol for assessment of VEGF-A, VEGFR-2 and HIF-1α included a fixed light intensity; a condenser set between 2 and 3, and default Nikon color settings. Every image was evaluated using a standardized Leica program to quantify the amount of stained and total areas (Leica QWin Standard V2.4). The epidermis was assessed including the basal, granular and spinous layers but the keratin layer was excluded. The positively stained area was divided by the total considered area to quantify the amount of
staining as a percentage. Blood vessel cross-sections were counted manually and divided by the dermal area to obtain a density (no./mm$^2$). All observations were performed on coded slides to prevent observer bias.

**Statistical analysis.** Statistical analysis was performed using SPSS 14.0 for Windows (SPSS Inc., Chicago, IL, USA). The data are presented as mean ± SEM. Gaussian data underwent parametric ANOVA and non-Gaussian data non-parametric ANOVA (Kruskal-Wallis test). Following Levene’s test for homogeneity of variances, Tukey or Dunnett’s T3 tests were used for multiple comparisons as appropriate. Comparison between groups was made by unpaired T-test for Gaussian data and Mann-Whitney test for non-Gaussian data. Logarithmic transformation was used to normalize the datasets. Correlations were studied with the Spearman coefficient ($r_s$) and a P-value<0.05 was considered statistically significant.

**RESULTS**

Demographics of the 53 diabetic patients (17 with type 1 diabetes) and 12 age-matched control subjects are listed in Table 1. Diabetic patients had no (n=12), mild (n=18), moderate (n=12) and severe (n=11) neuropathy on the basis of the NDS. Electrophysiology, quantitative sensory and autonomic function testing showed a progressive worsening with increasing neuropathic severity (Table 1). Laser Doppler flowmetry (11 controls, 11 patients with no neuropathy, 18 with mild, 9 with moderate and 8 with severe neuropathy) showed no differences for baseline values (Table 1).

**Intraepidermal nerve fiber density (Fig 2a, b).** IENFD showed a significant and progressive reduction with increasing neuropathic severity (ANOVA P<0.001) (Fig.1D). Comparison with control subjects (10.6±0.7) demonstrated a progressive reduction in diabetic patients without (8.3±1.1, p=NS) and with mild (6.6±0.9, P<0.05), moderate (4.8±1.0, P<0.01) and severe (1.8±0.4, P<0.001) neuropathy.

**Epidermal VEGF-A (Table 2, Figure 3a, d).** VEGF-A staining was prominent in the basal and spinous layers of the epidermis and was significantly reduced in diabetic patients (22±3%) compared to control subjects (53±8%) (P=0.001). VEGF-A expression correlated positively with IENFD ($r_s$=0.361, P<0.01).

**Epidermal VEGFR-2 (Fig 3b, e).** Epidermal VEGFR-2 expression did not differ between control subjects (14±6%) and diabetic patients (9±2%) and was independent of neuropathic severity (Fig.1B), however, it correlated significantly with epidermal VEGF-A staining ($r_s$=0.397, P<0.01).

**Epidermal HIF-1α (Fig 3c, f).** Epidermal HIF-1α expression was low and did not differ between groups (Fig.1C). The VAS score correlated with epidermal HIF-1α expression ($r_s$=0.361, p<0.05).

**Immunolocalization of vascular factors in the dermis.** In the upper dermis, VEGF-A, VEGFR-2 and HIF-1α were immunolocalized in fibroblasts, pericytes, endothelium and smooth muscle of the vascular wall. HIF-1α staining was present both in the nucleus and in the cytoplasm of each cell type and the highest intensity was observed in the pericytes. The amount of staining of VEGF-A, VEGFR-2 and HIF-1α in the dermis was lower than in the epidermis (Table 2) and there was no significant difference between groups. VEGF-A staining was significantly reduced in diabetic patients compared to control subjects in the upper dermis (P<0.05) and on blood vessels (P<0.05) and was the lowest in those with severe neuropathy compared to control subjects (P<0.05). VEGFR-2 staining did not differ in the dermis but was significantly reduced on blood vessels in diabetic patients with severe neuropathy compared to control subjects (P<0.05) and diabetic patients without neuropathy compared to control subjects (P<0.01) (Table 2). It correlated inversely to BVD
VEGF and Intra-epidermal nerve fiber reduction

(r_s=-0.412, P<0.01). HIF-1α expression on blood vessels differed between groups (P<0.05) and dermal HIF-1α expression correlated with the VAS score (r_s=0.288, P<0.05). 

**Dermal microvessel density and vasodilator responses.** Dermal microvessel density (no./mm²) differed significantly between groups (P<0.01). It was increased in patients with severe neuropathy (145 ± 18) reaching significance in those with moderate neuropathy (180 ± 17) compared to control subjects (129 ± 12) and patients without (109 ± 10, P<0.05) and with mild (105 ± 12) neuropathy (P<0.01) (Fig.1E). Baseline blood flow showed a weak negative correlation (r_s=-0.316, P=0.04) with epidermal expression of VEGF. The maximal blood flow response to ACh was significantly lower in patients with neuropathy (P<0.05) (Table 1). There was no difference in blood flow response to SNP. Blood flow data showed no correlation to dermal microvessel density.

**CONCLUSION**

Vascular factors are thought to be central in the development of diabetic neuropathy (18-19) and VEGF has neuroprotective effects (20-21). The present study combined detailed clinical, neurological and immunohistological evaluation in skin biopsies from the dorsum of the foot. The central finding of our study is that of a reduction in epidermal VEGF expression and intraepidermal nerve fibres in diabetic patients with increasing neuropathic severity. Maximal expression of VEGF was observed in the epidermis consistent with previous studies (22), particularly by keratinocytes (23-24). Whilst these results do not directly imply cause and effect they do provide the first clinical data in support of a link demonstrated in experimental diabetic neuropathy (3-4).

Tissue hypoxia has been proposed to up-regulate HIF-1α expression (25) which is considered to be the main stimulator of VEGF-A synthesis. A previous study in skin wounds has shown increased VEGF-A following topical NGF administration but did not assessed HIF-1α expression (26). In the present study, HIF-1α expression was not reduced in intact skin of diabetic patients without peripheral vascular disease, suggesting adequate tissue oxygenation which is supported by the findings of a baseline skin blood flow not significantly different from controls, even in patients with advanced neuropathy. It is possible that hyperglycemia per se induces a reduction of VEGF expression in the skin; clearly, further mechanistic studies are needed to clarify this relationship.

Dermal blood vessel density was increased in diabetic patients with more severe neuropathy, particularly those with moderate neuropathy, despite a reduction in VEGF and VEGFR-2 expression. Although no significant changes were found in baseline blood flow and HIF-1α did not increase in neuropathic patients it may reflect an angiogenic response to the impaired endothelium-dependent response observed in these patients. In nerves of diabetic rats there has been some evidence of transient HIF-1α up-regulation (6) and a blunted post-injury rise in nerve blood vessel numbers (27). Finally, despite the overall low expression of HIF-1α it was inversely related to the intensity of pain assessed using the VAS score. This finding requires confirmation but emphasizes that vascular factors may play an important role in the development of painful symptoms (28) and also provides a rationale for the use of vasodilating drugs in painful diabetic neuropathy (29).

In conclusion, this is the first translational study which suggests that there may be a link between skin VEGF expression, loss of intraepidermal nerve fibres, and the severity of human diabetic neuropathy. These data demand further quantitative studies assessing VEGF protein and/or mRNA levels to establish a more direct causal link between VEGF and diabetic neuropathy to provide a basis for the
beneficial effects reported to date (3-5, 30).

ACKNOWLEDGEMENTS
The study was supported by DUK (RD03/0002624) and NIH (R01 NS46259-01).

We thank Mrs Sally Mosley and Ms Cristina Mantovani in the University of Manchester.
REFERENCES


Table 1. Demographic and clinical characterization of study patients compared to age matched control subjects. Age and duration of diabetes are expressed in years. CASE IV results are expressed as percentile of the general population: DB-HRV, deep breathing-heart rate variability, HP-VAS 0.5=heat as pain-visual analogue score minimal threshold, HP-VAS 5.0= heat as pain-visual analogue score intermediate threshold, CDT=cooling detection threshold. SNAP=sural nerve amplitude potential is expressed in µV, PNCV=peroneal nerve conduction velocity is expressed in m/sec, TNDL=tibial nerve distal latency is expressed in msec. AUF = arbitrary units of flux as obtained by laser Doppler flowmetry at baseline and following iontophoresis of Ach, SNP (maximal responses). Deltas are the differences between peak responses and baseline. The data are expressed as Mean ± SEM. The asterisk (*) indicates ANOVA significance at P<0.05; (**) at P<0.01; (***) at P<0.001. For post-hoc multigroup comparison: the symbol † indicates significant difference from controls at P<0.05 (†), P<0.01 (††) or P<0.001 (†††), the symbol ‡ indicates significant difference from no neuropathy at P<0.05 (‡), P<0.01 (‡‡) or P<0.001.
**Table 2.** Immunohistological results

<table>
<thead>
<tr>
<th>Tissue Area</th>
<th>Controls</th>
<th>No neuropathy</th>
<th>Mild neuropathy</th>
<th>Moderate neuropathy</th>
<th>Severe neuropathy</th>
</tr>
</thead>
<tbody>
<tr>
<td>IENFD (no./mm)</td>
<td>epidermis (***)</td>
<td>10.6±0.7</td>
<td>8.3±1.1</td>
<td>6.6±0.9†</td>
<td>4.8±1.0††</td>
</tr>
<tr>
<td>VEGF-A (%)</td>
<td>epidermis (**)</td>
<td>53±8</td>
<td>28±7</td>
<td>25±6</td>
<td>26±6</td>
</tr>
<tr>
<td></td>
<td>dermis (*)</td>
<td>3±1</td>
<td>1±0.4</td>
<td>1±0.3</td>
<td>2±0.4</td>
</tr>
<tr>
<td></td>
<td>microvessels (*)</td>
<td>16±5</td>
<td>15±5</td>
<td>8±2</td>
<td>14±3</td>
</tr>
<tr>
<td>VEGFR-2 (%)</td>
<td>epidermis (p=NS)</td>
<td>14±6</td>
<td>10±4</td>
<td>13±5</td>
<td>9±3</td>
</tr>
<tr>
<td></td>
<td>dermis (p=NS)</td>
<td>1±0.3</td>
<td>3±3</td>
<td>2±1</td>
<td>1±1</td>
</tr>
<tr>
<td></td>
<td>microvessels (*)</td>
<td>9±3</td>
<td>8±2</td>
<td>6±3</td>
<td>3±2</td>
</tr>
<tr>
<td>HIF-1α (%)</td>
<td>epidermis (p=0.06)</td>
<td>9±2</td>
<td>6±2</td>
<td>5±2</td>
<td>7±1</td>
</tr>
<tr>
<td></td>
<td>dermis (p=0.05)</td>
<td>3±1</td>
<td>2±0.4</td>
<td>2±1</td>
<td>2±0.3</td>
</tr>
<tr>
<td></td>
<td>microvessels (*)</td>
<td>10±2</td>
<td>9±1</td>
<td>6±2</td>
<td>8±2</td>
</tr>
<tr>
<td>BVD (no./mm²)</td>
<td>dermis (**)</td>
<td>129±12</td>
<td>109±10</td>
<td>105±12</td>
<td>180±17†††$$</td>
</tr>
</tbody>
</table>

Table 2. The table shows quantity of staining (mean ± SEM) in control subjects and diabetic patients with increasing neuropathic severity in the epidermis, dermis and microvessels. Intra-epidermal nerve fiber density (IENFD), Vascular endothelial growth factor A (VEGF-A), vascular endothelial growth factor receptor 2 (VEGFR-2), hypoxia inducible factor 1 alpha (HIF-1α), blood vessel density (BVD). The symbol (*) indicates statistical significance at P<0.05; (**) at P<0.01; (***) at P<0.001. For post-hoc multigroup comparison: the symbol † indicates significant difference from controls at P<0.05 (†), P<0.01 (††) or P<0.001 (†††), the symbol ‡ indicates significant difference from no neuropathy at P<0.05 (‡), P<0.01 (‡‡) or P<0.001 (‡‡‡); the symbol $ indicates significant difference from mild neuropathy at P<0.05 ($), P<0.01 ($$) or P<0.001 ($$$). The symbol § indicates significant difference from moderate neuropathy at P<0.05 (§), P<0.01 (§§), P<0.001 (§§§).
Figure 1 A, B, C, D, E. Results of immunohistological assessment of the skin biopsies. The graphs illustrate the mean and SEM.

1A shows the amount of staining (%) for VEGF-A in the epidermis;
1B shows the amount of staining (%) for VEGFR-2 in the epidermis;
1C shows the amount of staining (%) for HIF-1α in the epidermis;
1D shows the intraepidermal nerve fibre density;
1E shows the blood vessel density in the upper dermis.
**Figure 2** a, b. Skin thick sections (50 µm) stained for PGP9.5+ intraepidermal nerve fibers in non-diabetic subjects (a) and patients with established neuropathy (b) (600 times magnification before reduction).
Figure 3 a, b, c, d, e, f. Skin thin sections (5 µm) from control subjects (upper row: a, b, c) and diabetic patients with established neuropathy (lower row: d, e, f) stained for VEGF-A (a, d); VEGFR-2 (b, e); and HIF-1α (c, f) (400 times magnification).