Reduced Vascular Endothelial Growth Factor Expression and Intra-Epidermal Nerve Fiber Loss in Human Diabetic Neuropathy

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OBJECTIVE — To assess the relevance of vascular endothelial growth factor (VEGF) in the maintenance of peripheral nerve integrity in diabetic neuropathy we have assessed the expression of VEGF and intra-epidermal nerve fiber density (IENFD) in skin biopsy samples from diabetic patients.

RESEARCH DESIGN AND METHODS — Fifty-three diabetic patients and 12 nondiabetic 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 with the expression of vascular endothelial growth factor A (VEGF-A), its receptor vascular endothelial growth factor receptor 2 (VEGFR-2), hypoxia-inducible factor 1α (HIF-1α), and microvessel density.

RESULTS — IENFD decreased progressively with increasing severity of diabetic neuropathy ($P < 0.001$). The dermal blood flow response to acetylcholine 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 with control subjects ($P < 0.01$). Epidermal HIF-1α and VEGFR-2 expression did not differ between groups.

CONCLUSIONS — Progressive endothelial dysfunction, a reduction in VEGF expression, and loss of intra-epidermal nerve fibers occurs in the foot skin of diabetic patients with increasing neuropathic severity.

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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 intra-epidermal nerve fibers (IENFs) (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 hypoxia-inducible factor 1α (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 (IENFD) is significantly reduced in diabetic patients with minimal neuropathy (7), is related to painful symptoms and neuropathic deficits (8), and may improve after lifestyle intervention (9). In the present study we used skin biopsy samples to quantify IENF loss in relation to the expression of HIF-1α and its principle target gene, VEGF and its receptor (vascular endothelial growth factor receptor 2 [VEGFR-2]), 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 nondiabetic volunteers were invited unless they had absent pedal pulses (to exclude peripheral vascular disease that may have an impact on both neuropathy and VEGF expression but also wound healing after the biopsy). All subjects were screened for other causes for neuropathy including vitamin B_{12}, 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 (10) and the visual analog 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, minimal (HP-VAS 0.5) intermediate (HP-VAS

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Abbreviations: HIF-1α, hypoxia-inducible factor 1α; IENF, intra-epidermal nerve fiber; IENFD, intra-epidermal nerve fiber density; NDS, neuropathy disability score; VAS, visual analog score; VEGF, vascular endothelial growth factor; VEGF-A, vascular endothelial growth factor A; VEGFR-2, vascular endothelial growth factor receptor 2.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

Table 1—*Demographic and clinical characterization of study patients compared with age-matched control subjects*

	Control subjects	No neuropathy	Mild neuropathy	Moderate neuropathy	Severe neuropathy
n	12	12	18	12	11
Age (years)	52 ± 6	55 ± 3	58 ± 3	56 ± 3	60 ± 2
Duration (years)	—	15 ± 4	19 ± 3	28 ± 4	22 ± 4
A1C (%)	—	7.6 ± 0.5	7.9 ± 0.3	8.6 ± 0.4	8.0 ± 0.3
DB-HRV**	—	72 ± 9	40 ± 9‡	16 ± 6‡‡	16 ± 10‡‡
HP-VAS 0.5	—	40 ± 8	33 ± 8	46 ± 11	62 ± 15
HP-VAS 5.0	—	54 ± 12	38 ± 8	51 ± 11	44 ± 12
CDT***	—	67 ± 7	74 ± 7	97 ± 1‡‡	98 ± 1‡
SNAP (μV)***	18 ± 3	14 ± 2	6 ± 1†	5 ± 1††	3 ± 1††
PNCV (m/s)***	47 ± 3	43 ± 1	40 ± 1††	36 ± 2†††	33 ± 3†††
TNDL (ms)***	4.6 ± 0.3	5.1 ± 0.3	5.7 ± 0.2	7.1 ± 0.5†††	8.5 ± 0.7†††
AUF (baseline ACh)	60 ± 13	73 ± 24	42 ± 6	32 ± 6	51 ± 6
AUF (baseline SNP)	55 ± 12	60 ± 18	32 ± 5	33 ± 6	37 ± 7
AUF (peak ACh)**	516 ± 84	318 ± 57	246 ± 38†	144 ± 24††	277 ± 29
AUF (peak SNP)	341 ± 80	261 ± 68	148 ± 22	153 ± 42	141 ± 16
ΔAUF ACh**	456 ± 83	246 ± 47	195 ± 33†	113 ± 22††	197 ± 38
ΔAUF SNP	266 ± 77	201 ± 54	116 ± 21	120 ± 38	104 ± 16

Data are means ± SEM. CASE IV results are expressed as percentile of the general population. Δ indicates the differences between peak responses and baseline. ANOVA significance at $^{**}P < 0.01$; $^{***}P < 0.001$. For post hoc multigroup comparison: significant difference from control subjects at †$P < 0.05$; ††$P < 0.01$; †††$P < 0.001$. Significant difference from no neuropathy at ‡$P < 0.05$; ‡‡$P < 0.01.001$. ACH, acetylcholine; AUF, arbitrary units of flux as obtained by laser Doppler flowmetry at baseline and after iontophoresis of ACh; CDT, cooling detection threshold; DB-HRV, deep breathing–heart rate variability; HP-VAS 0.5; heat as pain–VAS minimal threshold; HP-VAS 5.0, heat as pain–VAS intermediate threshold; PNCV, peroneal nerve conduction velocity; SNAP, sural nerve amplitude potential; SNP, sodium nitroprusside (maximal responses); TNDL, tibial nerve distal latency.

5.0), and differential (HP-VAS 0.5–5.0) heat as pain thresholds (13,14) and deep breathing heart rate variability (15) with the CASE IV (WR Medical Electronics, Stillwater, MN). Patients also underwent electrophysiological assessment with a Dantec Keypoint system (Dantec Dynamics, Bristol, U.K.) to evaluate amplitudes (microvolts), conduction velocities (meters per second), and latency (milliseconds) of responses.

Laser Doppler flowmetry

A subset of the patients comprising 11 control subjects and 46 diabetic patients who underwent biopsy also had skin blood flow laser Doppler flowmetry with a large area laser scanner (Moor Instruments, Devon, U.K.) on the dorsum of the foot at the site of the biopsy. A MIC iontophoresis controller (Moor Instruments, Devon, U.K.) delivered 20 μA for 5.5 min to the skin through MIC-ION plastic chambers (Moor Instruments) containing 1% acetylcholine and 1% sodium nitroprusside. The results were measured in arbitrary units of flux for baseline, maximal responses, and differential increases in blood flow (Δ).

Skin biopsy

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

IENFD

The first specimen was immediately fixed in 4% paraformaldehyde and after 18–24 h was rinsed in Tris-buffered saline and soaked in 33% sucrose (2–4 h) before cryoprotection in OCT and rapid freezing in liquid nitrogen. Sections (50 μm) were cut using a cryostat (model OTF; Bright Instruments, Huntington, U.K.), and floating sections were transferred onto a 96-well plate. Melanin bleaching (0.25% $KMnO_4$ for 15 min and then 5% oxalate for 3 min) was performed on four sections per case followed by a 4-h protein block with a Tris-buffered saline solution of 5% normal swine serum, 0.5% powdered milk, and 1% Triton X-100; sections then were incubated overnight with 1:1,200 Biogenesis polyclonal rabbit anti-human PGP9.5 antibody (Serotec, Oxford, U.K.). Swine anti-rabbit secondary antibody 1:300 (1 h) was then applied; sections were quenched with 1% H_2O_2 in 30% MeOH-PBS (30 min) before a 1-h incubation with 1:500 horseradish peroxidase–streptavidin (Vector Laboratories, Peterborough, U.K.). Nerve fibers were demonstrated using 3,3′-diaminobenzidine chromogen (Sigma-Aldrich, Manchester, U.K.). Sections were mildly counterstained with eosin to better localize the basement membrane. Negative controls comprised sections that underwent the same run except that the primary antibody was omitted and the developing time was exactly the same for all sections that were processed synchronously.

Vascular factor immunostaining

The second biopsy sample was fixed in 4% paraformaldehyde for 18–24 h, embedded in paraffin wax, and cut into 5-μm sections. Thin sections were mounted on positively charged slides (three per slide), dewaxed 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 before serum protein block. Anti-human primary antibodies were applied overnight at 5°C: mouse monoclonal antibodies to CD31 and von Willebrand factor (diluted 1:100; Dako) and VEGFR-2 (1:50; Santa Cruz); and rabbit polyclonal antibodies to VEGF-A (1:300; Santa Cruz) and HIF-1α (1:300; Abcam). Negative controls comprised sections that underwent the same runs except that 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.

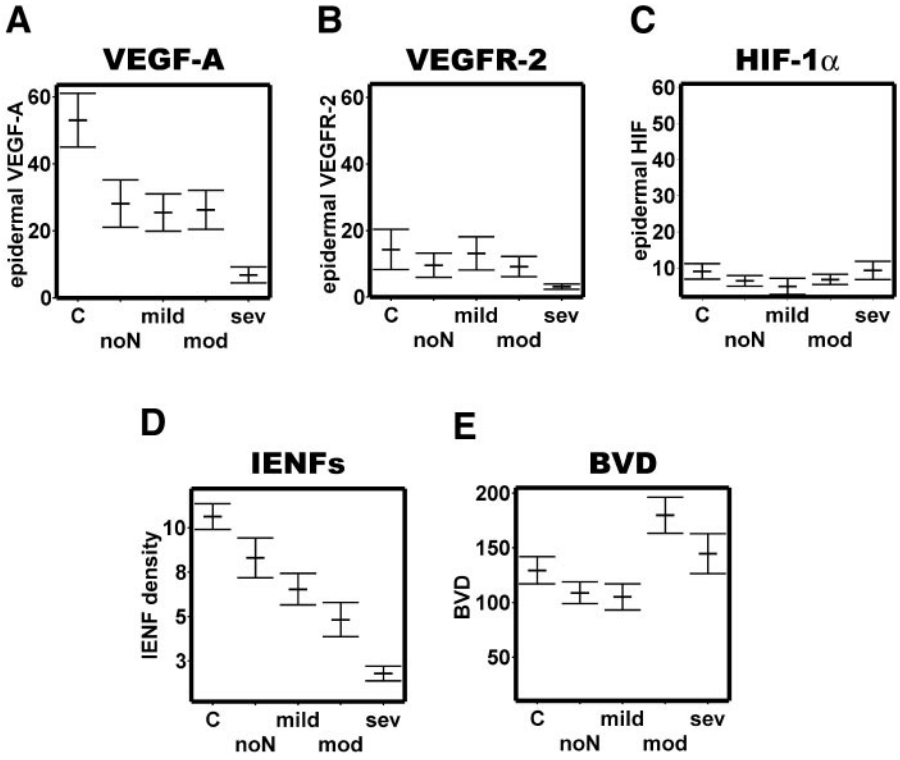

Figure 1— *Results of immunohistological assessment of the skin biopsies. The graphs illustrate means and SEM.* A*: Amount of staining (percentage) for VEGF-A in the epidermis.* B*: Amount of staining (percentage) for VEGFR-2 in the epidermis;* C*: Amount of staining (percentage) for HIF-1α in the epidermis.* D*: IENFD.* E*: Blood vessel density (BVD) in the upper dermis. C, control; NoN, no neuropathy.*

Image analysis

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

IENFD was defined as the number of fibers per millimeter of basement membrane length and expressed as numbers per millimeter (nerve density per length). If two nerve endings appeared closer than five fiber 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 area considered 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 (number per square millimeter). All observations were performed on coded slides to prevent observer bias.

Statistical analysis

Statistical analysis was performed using SPSS 14.0 for Windows (SPSS, Chicago, IL). The data are presented as means ± SEM. Gaussian data were subjected to parametric ANOVA and non-Gaussian data nonparametric ANOVA (Kruskal-Wallis). After Levene's test for homogeneity of variances, Tukey's or Dunnett's T3 test was used for multiple comparisons as appropriate. Comparison between groups was made by an unpaired *t* test for Gaussian data and a 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 $P < 0.05$ 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 control subjects, 11 patients with no neuropathy, 18 with mild neuropathy, 9 with moderate neuropathy, and 8 with severe neuropathy) showed no differences for baseline values (Table 1).

IENFD

IENFD showed a significant and progressive reduction with increasing neuropathic severity (ANOVA, $P < 0.001$) (Fig. 1*D*). Comparison with control subjects (10.6 ± 0.7) demonstrated a progressive reduction in diabetic patients without (8.3 ± 1.1, 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 (Fig. 2*A* and *B*).

Epidermal VEGF-A

VEGF-A staining was prominent in the basal and spinous layers of the epidermis and was significantly reduced in diabetic patients (22 ± 3%) compared with control subjects (53 ± 8%) ($P = 0.001$) . VEGF-A expression correlated positively

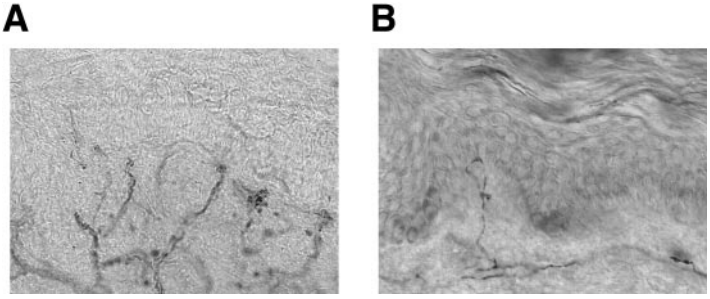

Figure 2—*Skin thick section (50 μm) stained for PGP9.5$^+$ IENFs in a nondiabetic subject* (A) *and in a patient with established neuropathy* (B)*. Original magnification ×600.*

Table 2—*Immunohistological results*

Tissue area	Quantity of staining (%) in different groups				
	Control	No neuropathy	Mild neuropathy	Moderate neuropathy	Severe neuropathy
IENFD (*n*/mm)					
Epidermis***	10.6 ± 0.7	8.3 ± 1.1	6.6 ± 0.9†	4.8 ± 1.0††	1.8 ± 0.4†††
VEGF-A (%)					
Epidermis**	53 ± 8	28 ± 7	25 ± 6	26 ± 6	6 ± 2††
Dermis*	3 ± 1	1 ± 0.4	1 ± 0.3	2 ± 0.4	1 ± 0.2
Microvessels*	16 ± 5	15 ± 5	8 ± 2	14 ± 3	3 ± 2†‡§
VEGFR-2 (%)					
Epidermis (NS)	14 ± 6	10 ± 4	13 ± 5	9 ± 3	3 ± 1
Dermis (NS)	1 ± 0.3	3 ± 3	2 ± 1	1 ± 1	1 ± 0.3
Microvessels*	9 ± 3	8 ± 2	6 ± 3	3 ± 2	2 ± 2†‡‡
HIF-1α (%)					
Epidermis ($P = 0.06$)	9 ± 2	6 ± 2	5 ± 2	7 ± 1	12 ± 3
Dermis ($P = 0.05$)	3 ± 1	2 ± 0.4	2 ± 1	2 ± 0.3	2 ± 0.4
Microvessels*	10 ± 2	9 ± 1	6 ± 2	8 ± 2	8 ± 3
Blood vessel density (*n*/mm^2)					
Dermis**	129 ± 12	109 ± 10	105 ± 12	180 ± 17‡‡$$	145 ± 18

Data are means ± SEM in control subjects and diabetic patients with increasing neuropathic severity in the epidermis, dermis, and microvessels. Statistical significance at *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. For post hoc multigroup comparison: significant difference from control at †$P < 0.05$; ††$P < 0.01$; †††$P < 0.001$. Significant difference from no neuropathy at ‡$P < 0.05$; ‡‡$P < 0.01$. Significant difference from mild neuropathy at $$$P < 0.01$. Significant difference from moderate neuropathy at §$P < 0.05$.

with IENFD ($r_s = 0.361$, $P < 0.01$) (Table 2 , Fig. 3*A* and *D*).

Epidermal VEGFR-2

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

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

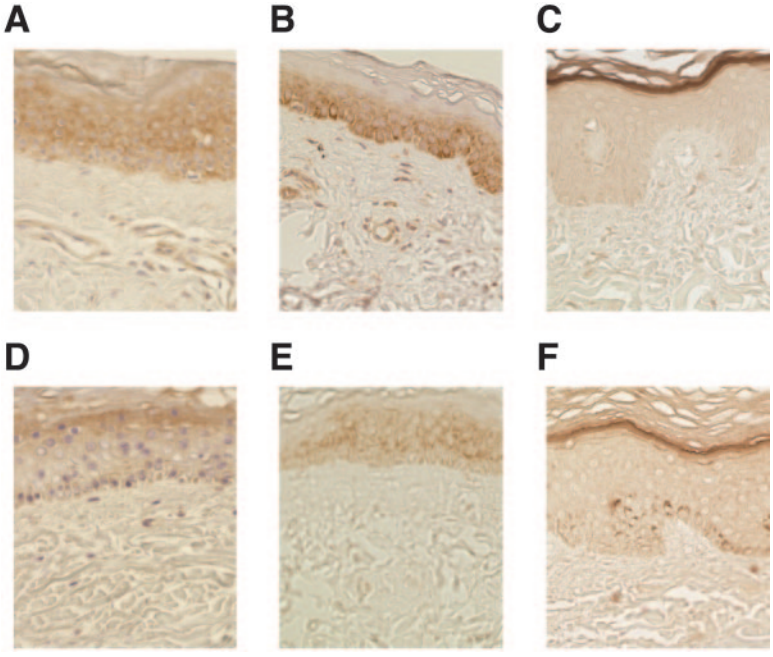

Figure 3—*Skin thin sections (5 μm) from control subjects* (A–C) *and diabetic patients with established neuropathy* (D–F) *stained for VEGF-A* (A *and* D)*; VEGFR-2* (B *and* E)*; and HIF-1α* (C *and* F)*. Original magnification ×400.*

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 that in the epidermis (Table 2), and there was no significant difference between groups. VEGF-A staining was significantly reduced in diabetic patients compared with 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 with 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 with control subjects ($P < 0.05$) and diabetic patients without neuropathy ($P < 0.01$) (Table 2). It correlated inversely to blood vessel density ($r_s = -0.412$, $P < 0.01$). HIF-1α expression on blood vessels differed between groups ($P < 0.05$) and dermal HIF-1α expression

correlated with the VAS ($r_s = 0.288$, $P < 0.05$).

Dermal microvessel density and vasodilator responses

Dermal microvessel density (number per square millimeter) 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 with control subjects (129 ± 12) and in patients without (109 ± 10, $P < 0.05$) and with mild (105 ± 12) neuropathy ($P < 0.01$) (Fig. 1*E*). 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 acetylcholine was significantly lower in patients with neuropathy ($P < 0.05$) (Table 1). There was no difference in blood flow response to sodium nitroprusside. Blood flow data showed no correlation to dermal microvessel density.

CONCLUSIONS — 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 a reduction in epidermal VEGF expression and IENFs 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). Although 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 upregulate 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 after topical nerve growth factor administration but did not assess 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 that in control subjects 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 upregulation (6) and a blunted postinjury 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. 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 summary, this is the first translational study to suggest that there may be a link between skin VEGF expression, loss of IENFs, 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).

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