Vascular Endothelial Growth Factor, diagnostic and therapeutic aspects

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Chapter

SKIN CAPILLARY PERMEABILITY IN THE DIABETIC FOOT WITH CRITICAL LIMB ISCHEMIA: THE EFFECTS OF A pHVEGF-GENE CONTAINING PLASMID

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**ABSTRACT**

Local edema is commonly found in the diabetic foot with critical limb ischemia (CLI), and has been linked to increased capillary permeability and to local inflammation. In CLI VEGF administration has been proposed as a new therapeutic avenue, but may also result in edema formation. The aim of our study was to address capillary permeability, as assessed by skin capillary sodium fluoresceïn leakage in patients with diabetes mellitus with CLI, and to compare the effects of VEGF with those of placebo.

Sodium fluoresceïn (NaF) leakage was assessed using a 'large window' capillary microscope technique of the medial ipsilateral ankle in 17 patients with diabetes mellitus with CLI (DM-CLI), in 24 DM patients without clinical signs of macrovascular disease or neuropathy DM-C), in 21 DM patients with clinical and objective signs of neuropathy (DM-NP), and in 22 healthy controls (C). Nerve conduction studies of the peroneal and sural nerve were also performed in all groups. The 17 DM-CLI patients were randomized to ipsilateral intramuscular administration in the calf of a phVEGF<sub>165</sub> gene product (n=11), or of placebo (n=6). Changes over time were assessed after 28 days.

DM-CLI patients had a longer dye arrival time (DAT), but a NaF leakage curve similar to that of C, while capillary permeability was increased in DM-C and DM-NP compared to C. The number of capillaries in the field of observation after arrival of NaF was lower in DM-CLI than in DM-NP. Twenty eight days after VEGF or placebo administration in the DM-CLI group, a significant difference was found in the change in leakage curve and in Iav(7) (the average relative fluorescence light intensity over the first 7 minutes of the videodensitometry) between the patients receiving phVEGF<sub>165</sub> and those receiving placebo with a mean increase of Iav(7) of 6.7 in the VEGF group and a fall of 1.5 in the placebo group. The DAT fell with 9.2 sec in the phVEGF<sub>165</sub> group and rose with 5 sec in the placebo group, this difference between the groups was marginally significant.

In conclusion, in DM-CLI no increase in capillary leakage was found as assessed by NaF leakage, probably because an increase in capillary filtration coefficient is counterbalanced by the marked fall in perfusion pressures. Therefore, edema formation in DM-CLI is probably more due to other processes, including local inflammation. A fall in DAT after VEGF administration suggests an increase in perfusion, and a concurrent increase in capillary NaF leakage may form an explanation for the edema formation after VEGF.
INTRODUCTION

ANKLE and foot edema is commonly seen in diabetes mellitus (DM), and even more so in one of its most feared complications, the diabetic foot with critical limb ischemia (CLI).\(^1\) Several factors have been proposed to contribute to this edema formation. Local factors, including loss of arteriolar precapillary vasoconstriction and absent veno-arteriolar reflexes, have been described for both conditions.\(^2\) In DM, this may be due to autonomic neuropathy, while in CLI this may be secondary to local ischemia or a compensatory reaction to the fall in perfusion pressure distal to occlusions in the larger arteries. In CLI, local changes in endothelial and arteriolar structure are also found, such as larger endothelial gaps and arteriolar wall atrophy, which affect endothelial function and permeability, as well as myogenic and mechanical characteristics of arterioles.\(^3,4\) On the other hand, systemic factors, including endothelial dysfunction, may also result in changes in the capillary filtration coefficient.\(^5\) Increased systemic concentrations of endothelial function markers such as von Willebrand factor and endothelin in CLI support the contention that endothelial dysfunction is not limited to the relatively small zone with ischemia, but may be more widespread.\(^6-8\) Of course, atherosclerosis and other vascular diseases which result in CLI, are well known to be associated with systemic endothelial dysfunction. In type 1 DM, the evidence for endothelial dysfunction is more equivocal than in type 2 DM.\(^9\)

Microalbuminuria is often considered to be a reflection of systemic endothelial dysfunction. It has been shown from our previous data, that patients with diabetes mellitus and microalbuminuria have increased capillary sodium fluorescein (NaF) leakage in the ankle area, compared to those without microalbuminuria, who have increased leakage compared to nondiabetic controls. All these groups had no clinical signs of macrovascular disease.\(^10\) Although, this might suggest that both microalbuminuria and capillary NaF leakage are expressions of systemic endothelial dysfunction, we demonstrated in a later study that a close relation is also present between increased capillary NaF leakage and nerve conduction abnormalities as a sign of neuropathy.\(^11\) This may be an illustration of the close interrelationships between various pathogenetic mechanisms underlying microangiopathy and neuropathy in DM.

Vascular endothelial growth factor (VEGF) is an angiogenic factor which has received attention because of the beneficial role it may have in improving tissue perfusion and improving clinical outcome in patients with CLI.\(^12,13\) A common adverse reaction is edema formation.\(^14\) The mechanism of edema formation after VEGF administration has been incompletely elucidated.

In the present study we address the mechanisms responsible for edema formation and increased local capillary leakage in DM and CLI. We compare capillary NaF leakage between DM patients without and with CLI. In (part of) the DM patients with CLI the effects of intramuscular administration in the ipsilateral calf of a gene product coding for phVEGF\(_{165}\) were compared to the effects of administration of placebo.
patients and methods.

Study approval was obtained from the medical ethical committee of the University Medical Center Groningen. Patients were recruited from the outpatient clinic of the University Medical Center Groningen, the rehabilitation center Beatrixoord or from the phVEGF\textsubscript{165} study, described in more detail elsewhere.\textsuperscript{15} Healthy control subjects were recruited by an advertisement in a local newspaper.

Patients

84 subjects of a comparable age participated in this study and were distributed in four groups (see Table 1):

- 21 healthy subjects served as controls. None had DM, clinical signs of neuropathy or vascular disease. Mean age was 58 ± 10 years and 10 were men. Normal glucose tolerance was assessed by a blood glucose concentration less than 7.8 mmol/l two hours after ingestion of 75 g of glucose. DM and renal failure were excluded by conventional American Diabetes Association (ADA) criteria, and a serum creatinine < 120 umol/l. All controls had an ankle brachial index (ABI) > 1.0.

- 24 subjects with DM (type 1 and type 2), without clinical signs of vascular disease. Mean age was 52 ± 12 years and 13 were men. DM was diagnosed using conventional ADA criteria. All patients had an ABI > 1.0.

- 22 subjects with DM (type 1 and type 2), without clinical signs of vascular disease, but with clinical signs of neuropathy. For the definition of neuropathy see below. Wagner scores were distributed as follows: 1, n = 5; 2, n = 4; 3, n = 8; 4, n = 5. In all patients the ABI was 0.9 or higher, except in one patient who had, however, a toe brachial index of 1.0 (140 mmHg). Mean age was 58 ± 12 years and 12 were men. DM was diagnosed according to conventional ADA criteria.

- 17 subjects with DM (type 1 and type 2), without clinical signs of neuropathy, but with clinical and objective signs of CLI. CLI was defined according to the criteria defined in the TransAtlantic Inter-Society Consensus on Management of Peripheral Arterial Disease (TASC)\textsuperscript{16}: a) persistently recurring ischemic rest pain requiring opiate or comparable analgesia for more than two weeks, and/or ulceration or gangrene of the foot or toes, and b) ankle systolic pressure < 50 mmHg, or toe-brachial index of 0.5 or lower. Furthermore, occlusion or severe stenosis of larger vessels had been documented with Doppler ultrasound or angiographic studies. The mean age was 68 ± 12 years, 12 were men. Within this group, 6 patients received intramuscular (i.m.) injections with placebo in the ipsilateral calf of the most affected ischemic leg. In 11 patients, the gene product coding for phVEGF\textsubscript{165} was administered i.m. in the ipsilateral calf of the most affected ischemic leg. The protocol for this study has been described previously in more detail.\textsuperscript{15}
The presence or absence of clinical neuropathy in groups 1-3 above was defined using the Dutch Diabetic Neuropathy Symptoms (DNS) scale, the Dutch Diabetic Neuropathy Examination (DNE) scale, and quantitative sensory function testing with Semmes Weinstein monofilaments. In the group of diabetes patients without neuropathy DNS and DNE scores (see below) had to be 0 point, and = 3 points, respectively, while the 10 gram Semmes-Weinstein monofilament was felt on all occasions. In the patients with a foot ulcer and definite neuropathy, the DNS and DNE scores were 1 and 4, respectively, while the 10 gram Semmes-Weinstein filament was not felt.

In diabetic patients, data were collected from chart review regarding items such as known duration of DM, mean HbA1c of the previous year and blood pressure. Clinical details are presented in Table 1. Both type 1 (age at diagnosis of diabetes < 30 year and insulin-dependent from onset) and type 2 diabetic patients participated in all 3 groups. Patients with a history of congestive heart failure or other clinically apparent cardiac disease were excluded. Other exclusion criteria were alcohol consumption of more than four units per day, hepatic insufficiency, folic acid and hydroxycobalamin deficiencies. Furthermore, drugs with vasoactive effects were not allowed in the 24 hours before the measurement. The use of angiotensin converting enzyme inhibitors or angiotensin II receptor antagonists and diuretics was allowed.

Methods

All experiments were performed in a quiet, semi-dark room with a room temperature of 24°C. Patients rested in a semi-supine position for at least 20 minutes before the experiments. Care was taken to keep the skin temperature of the investigated foot above 30°C.

Capillary sodium fluorescein leakage

Capillary NaF leakage was determined according to the method described by Oomen et al previously in more detail. In short, the subjects were studied while lying on their right side. Room temperature was approximately 24°C. The local skin temperature of the right medial ankle was monitored using a thermocouple (Ellab du 3s) and kept between 28°C and 32°C. If necessary, skin temperature was raised by covering the subject with a blanket and by applying a hot fan before the microscopy started. Cooling was not necessary. The average skin temperature during the microscopy was calculated from the skin temperature immediately before and after microscopy. No significant changes in skin temperature occurred during the procedure. Brachial blood pressure was measured simultaneously using an automatic device (Dinamap).

The presently employed system consists of an epillumination microscope (Olympus BHMJ) to which a 75 W Xenon lamp (Osram XBO) is mounted. Emitted light is filtered using a fluorescence filter set (Olympus BH2-UDMB, excitation 380-490 nm, barrier 515 nm). Images were recorded by a video camera (Grundig FA-85) and a S-VHS video recorder. The Xe-lamp was switched on at least 20 minutes before starting the measurements to reach a constant operating temperature. The lower leg and foot were fixed in a vacuum pillow and
placed under the microscope. Immersion oil (Leitz, din 58884) was applied to the skin to increase transparency. A section of 2 x 3 mm containing no markedly enlarged or tortuous capillaries was visualized for measurement (magnification x100). Blood volume was estimated by using a normogram with body weight and length.\textsuperscript{18} After installation of the fluorescence filters, 0.3 ml per l estimated blood volume of a 15\% sodium fluoresceïn solution was rapidly injected through an intravenous catheter positioned just below the left elbow. Images were continuously recorded for 20 minutes. Using this sodium fluoresceïn dosage, fluorescence fading due to constant illumination is reported to amount to no more than 2-3\% after 20 minutes.\textsuperscript{22}

Images were digitized from tape recording (Data Translation 2862 framegrabber with Iris software) every second after first appearance of the dye for 20 minutes. The light intensity of each image was computed and expressed in arbitrary units. One baseline image was digitized to obtain background fluorescence intensity, which was subtracted from subsequent intensities. Individually determined maximal intensity (Imax) was set at 100\%. All other intensities were expressed as a percentage of Imax. In general, Imax was reached between 5 and 10 minutes after appearance of the dye.

The average relative intensity over the first 7 minutes after appearance of the dye, Iav(7), represented in the tables, was used as parameter of NaF leakage. Previously, we showed Iav(7) has an estimated intra-individual day-to-day reproducibility (expressed as CV) of 10\%.\textsuperscript{21} Dye arrival time (DAT) was defined as the time from the moment of injection of the dye until its appearance in the skin capillaries. The number of capillaries in the visualized area was counted from tape recording, 60 seconds after appearance of the dye.

**Nerve conduction studies**

Nerve conduction studies were conducted in all groups, in the phVEGF\textsubscript{165} study participants in the CLI group at baseline only. Nerve conduction studies were performed with standard surface stimulation and recording techniques using an electromyograph type Nicolet Viking Iie and IV with standard filter settings. All measurements were performed after warming of forearm and lower leg in hot (38°C) water during at least 15 minutes. Motor nerve conduction velocities and amplitudes were measured in the peroneal nerve (tibialis anterior), and sensory nerve conduction velocities and amplitudes were tested antidromically after stimulation lateral of the Achilles tendon (sural nerve), 10-12 cm proximal from the active electrode, as described elsewhere.\textsuperscript{23} Peak-peak amplitude values were used.

**Statistical analyses**

Differences in capillary NaF leakage between the 4 studied groups were analyzed using ANOVA with post hoc Bonferroni correction. For the analysis of the effects of phVEGF\textsubscript{165} compared to those of placebo the delta NaF leakage was compared between both groups. In case of normal distribution of variables Pearson's correlation was assessed, in case of non-normal distribution or of categorical values Spearman's correlation was assessed. Multivariable stepwise regression analyses were performed for determination of independent effects. Two-
tailed \( P \)-value <0.05 was considered significant. Data are expressed as mean ± standard deviation, when variables were normally distributed. In case of a non-normal distribution data are reported as median (minimum-maximum).

**RESULTS**

**Clinical characteristics and conventional vascular studies**

As shown in Table 1, the diabetes patients with CLI were older than the other 3 groups which were comparable in age. No differences in sex or smoking were found. The distribution between type 1 and type 2 diabetes patients was comparable between the groups. The CLI patients had a lower body mass index (BMI) compared to patients with neuropathy.

ABI and toe pressure were, as expected, lower in the CLI group than in the other 3 groups, which were comparable in ABI and toe pressures.

In 11 of the 17 CLI patients phVEGF\(_{165}\) gene product was administered intramuscularly, while in 6/17 placebo was given. No differences were found in clinical characteristics or in vascular measurements of ABI or toe pressures (Table 2) at baseline. At 28 days no differences were found in the vascular studies between both groups (Table 2).

**Capillary sodium fluorescein leakage**

**Capillary sodium fluorescein leakage in controls and patients with diabetes mellitus**

Results are summarized in Table 1, and NaF versus time leakage curves are shown in Figure 1. The number of capillaries in the field of observation was lower in the group with CLI than in those with neuropathy. The Iav(7) was not significantly different between the 4 groups. The DAT was significantly longer in the CLI group than in those with neuropathy, but was not different from those with diabetes without clinical vasculopathy or neuropathy, nor from controls. When the NaF leakage curves were compared, no difference was found between the CLI patients and non-diabetic controls, a borderline difference was found compared to the diabetes patients without complications \( (P=0.09) \) and the diabetes patients with neuropathy \( (P=0.06) \). The NaF leakage curves were significantly different in the diabetes groups without complications and with neuropathy compared to those of healthy controls, with the largest increase in leakage in patients with neuropathy.
Table 1. Patient characteristics, macrovascular and NaF leakage parameters, and nerve conduction studies in healthy controls (C), diabetes patients without complications (DM-C), with clinical neuropathy (DM-NP), and with critical limb ischemia (DM-CLI).

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>DM-C</th>
<th>DM-NP</th>
<th>DM-CLI</th>
</tr>
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<tbody>
<tr>
<td>n</td>
<td>21</td>
<td>24</td>
<td>22</td>
<td>17</td>
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<tr>
<td>Male (n)</td>
<td>10</td>
<td>13</td>
<td>12</td>
<td>12</td>
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<tr>
<td>Age (years)</td>
<td>58 ± 10</td>
<td>52 ± 12</td>
<td>58 ± 12</td>
<td>68 ± 12*#^</td>
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<tr>
<td>Smoker (n)</td>
<td>6</td>
<td>7</td>
<td>11</td>
<td>7</td>
</tr>
<tr>
<td>Insulin (n)</td>
<td>0</td>
<td>19</td>
<td>17</td>
<td>10</td>
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<tr>
<td>HbA1c (%)</td>
<td>5.8 ± 0.3</td>
<td>7.5 ± 0.8</td>
<td>8.2 ± 1.0</td>
<td>8.0 ± 1.6*</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>27.22 ± 5.1</td>
<td>28.4 ± 5.0</td>
<td>29.3 ± 4.7</td>
<td>26.2 ± 3.5^</td>
</tr>
</tbody>
</table>

**Macrovascular studies**
- Ankle brachial index: 1.2 ± 0.1, 1.2 ± 0.1, 1.2 ± 0.2, 1.0 ± 0.5#
- Toe pressure (mmHg): 121.8 ± 16.9, 127.0 ± 27.9, 128.1 ± 25.2, 37.2 ± 21.7*#^
- Toe brachial index: 0.9 ± 0.1, 0.9 ± 0.2, 0.9 ± 0.2, 0.3 ± 0.1*#^

**NaF leakage**
- Temperature (°C): 30 ± 0.7, 30.6 ± 1.2, 31.5 ± 0.7, 30.2 ± 1.7
- Dye arrival time (s): 41 (21-76), 38 (18-75), 31 (13-42), 38 (32-51)^
- Iav(7) (%): 53.2 (48-74), 60.1 (47.8-76.2), 64.6 (42.3-94.9), 55.0 (50.6-61.4)
- Capillaries (n): 146 (46-250), 107 (44-232), 155 (16-272), 120 (90-132)^

**Nerve conduction**
- Nervus suralis, sensory
  - Measurable (n): 20/20, 23/23, 12/22, 6/13
  - Velocity (m/s): 46.8 ± 4.3, 44.3 ± 5.1, 36.8 ± 5.1, 35.9 ± 11.4*
  - Amplitude (mV): 8.8 ± 4.6, 8.2 ± 5.3, 1.7 ± 0.9, 3.2 ± 2.8*#
- Nervus peroneus, motor
  - Measurable (n): 20/20, 23/23, 21/22, 10/10
  - Velocity (m/s): 54.7 ± 7.9, 55.1 ± 7.1, 44.2 ± 7.1, 47.0 ± 11.9
  - Amplitude (mV): 9.3 ± 2.1, 10.2 ± 2.6, 6.0 ± 3.0, 5.32 ± 2.6*#

Data are expressed as mean ± standard deviation, or as median (minimum-maximum). * P<0.05 vs. CTL  
# P<0.05 vs. DM-C, ^ P<0.05 vs. DM-NP
Changes in capillary sodium fluorescein leakage in diabetes mellitus with CLI before and 28 days after phVEGF$_{165}$ gene product administration or placebo

The NaF leakage versus time characteristics and curves for the phVEGF$_{165}$ and the placebo groups at baseline and 28 days later are shown in Table 2 and in Figure 2. At baseline, no difference was found in the number of capillaries between both groups, but a significant difference existed in DAT and Iav(7) between the phVEGF$_{165}$ and the placebo group. No differences in ankle or toe pressures existed at day 28 between both groups.

At 28 days after VEGF plasmid or placebo administration a mean increase in Iav(7) of 6.7 in patients receiving phVEGF$_{165}$ and a fall of 1.5 in the placebo group was seen which was significantly different ($P=0.009$). The DAT decreased with 9.2 sec in the phVEGF$_{165}$ group and rose with 5 sec in the placebo group. This difference between the groups was marginally significant. No differences were found in baseline and 28 day values, or changes over time between responders and non-responders in DAT or Iav(7).
Table 2. Patient characteristics, macrovascular and NaF leakage parameters, and nerve conduction studies at baseline (visit 1) in diabetes patients with CLI receiving phVEGF$_{165}$ or placebo administration. For NaF leakage the results at 28 days (V3) after VEGF or placebo are also given.

<table>
<thead>
<tr>
<th>VISIT 1</th>
<th>PLACEBO</th>
<th>VEGF</th>
<th>∆V3-V1</th>
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<tbody>
<tr>
<td>n</td>
<td>6</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>Male (n)</td>
<td>4</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>67 ± 13</td>
<td>69.5 ± 11</td>
<td></td>
</tr>
<tr>
<td>Smoking (n)</td>
<td>2</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Insulin (n)</td>
<td>4</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>8.1 ± 1.7</td>
<td>7.9 ± 1.8</td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m$^2$)</td>
<td>26.5 ± 4.6</td>
<td>26 ± 3.1</td>
<td></td>
</tr>
</tbody>
</table>

**Vascular studies**
- Ankle brachial index: 1.2 ± 0.6 vs. 0.51 ± 0.51
- Toe pressure (mmHg): 31.2 ± 13.6 vs. 27.5 ± 25.4
- Toe brachial index: 0.26 ± 0.2 vs. 0.62 ± 0.38

**NaF leakage**
- Temperature (°C): 30.8 ± 1.2 vs. 29.9 ± 1.9
- Dye arrival time (s): 31 (28-34) vs. 48 (34-55)* +5 -9.2
- Iav(7) (%): 60.3 (57.4-65.3) vs. 53.2 (49.1-55.0)* -1.5 +6.7#
- Capillaries (n): 114 (91-174) vs. 120 (88-132)

Data are expressed as mean ± standard deviation, or as median (minimum-maximum).

*: $P<0.001$ compared to placebo.

#: $P<0.05$ compared to placebo.

**Nerve conduction studies**

Nerve conduction studies in controls and patients with diabetes mellitus

Main results are presented in Table 1. Adequate nerve conduction recordings were available for all healthy controls and diabetes patients without complications. However, data were obtained for the sural nerve and peroneal nerve in only 12/22 and 21/22, respectively, of patients with neuropathy, and 6/17, and 10/17 of CLI patients. For the sural nerve, the conduction velocity was lower in the CLI group than for the healthy controls ($P=0.03$), and borderline significantly compared to DM patients without complications ($P=0.08$), but was similar to that of the group with clinical neuropathy. The amplitude of the sural nerve signal was lower in the CLI group compared to controls and to DM patients without vasculopathy or neuropathy (both $P=0.01$), but similar to the neuropathy group.
No differences in the peroneal nerve in conduction velocity existed between the CLI and neuropathy group compared to controls and DM patients without complications. The peroneal nerve amplitude was lower in the CLI group compared to controls and DM patients without complications, but similar to that in the neuropathy group.

**DISCUSSION**

Capillary permeability as measured with NaF leakage was, remarkably, similar in diabetes patients with CLI compared to nondiabetic controls, and diabetes patients without signs of vasculopathy. Therefore, the often occurring edema in diabetic patients with CLI may be more due to other factors such as local inflammation, than to a more widespread vascular leakage. As expected from the severely compromised circulation to the CLI foot, the DAT was longer.
in the CLI group compared to those with a predominantly neuropathic foot. Furthermore, the administration of the phVEGF\textsubscript{165} gene product led, remarkably, over a period of 28 days to a fall in the prolonged DAT, suggesting an improvement in circulation.

In previous studies we have shown that NaF leakage is increased in patients with DM, and even more so in those with microalbuminuria.\textsuperscript{10} ACE inhibitors normalized this increased capillary permeability. Therefore, we proposed it as an alternative method for assessing endothelial (permeability) function. CLI is an end stage of a combination of both macro- and microvascular disease, which are both associated with endothelial dysfunction, and thus increased NaF leakage. In addition, CLI is associated with a reduced local perfusion pressure. The present results suggest that in CLI a balance exists between a low capillary perfusion pressure and increased permeability of the capillary barrier. Although, patients with CLI were older and endothelium dependent vasodilation may depend on age, this would, extrapolated to endothelial permeability, have been expected to further increase capillary NaF leakage and, thus, cannot explain the lack of difference in capillary leakage.\textsuperscript{24,25} In another study using NaF leakage, we found that diabetes patients with neuropathy had more pronounced NaF leakage than those without, and we suggested that sympathetically mediated vasomotion is related to capillary permeability.\textsuperscript{11} The fact that nerve conduction abnormalities were similar in the current study in the CLI group compared to those with clinical neuropathy, in combination with the difference in NaF leakage, suggests that the role of disturbed sympathetically mediated vasomotion may be limited. It also argues against the hypothesis that the diffusion coefficient of the capillary membrane may be altered in both diabetes patients with neuropathy and those with CLI, secondary to neuropathically mediated vasomotion loss.

Alternative methods to assess endothelial function, such as flow-mediated vasodilation, are not feasible in the leg in CLI patients, and will, therefore, not offer an answer. Perhaps microvascular assessment of endothelial function with laser Doppler flow iontophoresis with acetylcholine and/or nitroprusside may offer an alternative method to address endothelial function than the microcirculatory level in CLI.\textsuperscript{26}

The high ankle-brachial index (which was $>1.3$ in 2 patients), and the fact that ankle pressures could not be determined in 5/17 patients in the CLI group due to incompressible arteries confirms that the ankle pressure cannot be considered as a reliable classification criterion in diabetes patients with ischemia, and that toe pressures should be preferred instead, unless toe ulcers prevent this.\textsuperscript{27}

As for the increase in perfusion and NaF leakage in our study with the phVEGF\textsubscript{165} gene compared to the placebo group, VEGF is a well known, potent inducer of blood vessel formation (angiogenesis).\textsuperscript{28} Although the process of angiogenesis is complex and dependent upon a variety of growth factors and other components, the critical importance of VEGF in regulating vessel formation has been well established.\textsuperscript{12,13} Considering the lack of change in ABI and TBI, this fall in DAT cannot be explained by macrovascular changes, and may be more due to improvements on the microcirculatory level and changes in collateral vessels. The increase in capillary permeability, as suggested by the increase in Iav(7) in the phVEGF\textsubscript{165} but not in the placebo group, is compatible with the clinical observation that VEGF administration is associated with an increased prevalence of edema. Because no relation was found between clinical responders and non-responders in either the VEGF or placebo-group,
the above effects can be considered to be secondary to the phVEGF_{165} gene product, and not due to other, clinically relevant changes in perfusion.

It is remarkable that the nerve conduction studies showed similar results for the neuropathy and CLI groups, even when we attempted to separate both groups on the basis of clinical signs of neuropathy. In fact the contrast between both groups was more pronounced in the second differentiation inclusion criterion, the toe pressures, but also in the microvascular parameters than for the nerve conduction studies. This is compatible with clinical observation of a major overlap between neuropathy and vascular compromise in patients with a diabetic foot, but also with experimental and clinical evidence that ischemia affects nerve function.\textsuperscript{29} Unfortunately, no nerve conduction studies were available before and at 28 days after VEGF and placebo administration.

In conclusion, in CLI no increase in capillary leakage was found as assessed by NaF leakage, probably because an increase in capillary filtration coefficient is counterbalanced by the marked fall in perfusion pressures. Therefore, edema formation in CLI is probably more due to other processes, including local inflammation. A fall in DAT after VEGF administration suggests an increase in perfusion, and a concurrent increase in capillary NaF leakage may form an explanation for the edema formation after VEGF.
REFERENCES


