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Endoneurial localisation of microvascular damage in human diabetic neuropathy

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Summary. Twenty diabetic patients with neuropathy underwent clinical and neurophysiological evaluation together with a detailed morphometric assessment of capillary pathology in endoneurial and epineurial microvascular beds of the sural nerve. Morphological data were compared with ten non-diabetic control subjects. There were no significant differences in control subjects between basement membrane area, endothelial cell area, endothelial cell profile number or luminal area of endoneurial when compared with epineurial capillaries. In contrast, when compared with epineurial capillaries, endoneurial capillaries from diabetic patients demonstrated a significant increase in basement membrane ($p < 0.001$) and endothelial cell ($p < 0.001$) area and a significant reduction in luminal area ($p < 0.001$). There was no significant difference in endothelial cell profile number between endoneurial and epineurial capillaries amongst diabetic patients. Previous studies have demonstrated a good correlation between the degree of microangiopathy and measures of neuropathic severity. In the present study increased endoneurial capillary basement membrane area was significantly related to reduced peroneal nerve conduction velocity ($p < 0.001$), myelinated fibre density ($p < 0.001$) and elevated vibration ($p < 0.05$) and thermal ($p < 0.001$) perception. Increased endothelial cell area and reduced luminal size were related to a reduced peroneal nerve conduction ($p < 0.05$, $p < 0.01$, respectively), reduced myelinated fibre density ($p < 0.05$, $p < 0.01$) and elevated thermal perception ($p < 0.05$, $p < 0.001$). Epineurial capillary basement membrane, endothelial cell and luminal area failed to relate to measures of neuropathic severity. This study has demonstrated more advanced microangiopathy and a more significant relationship to neuropathic severity in endoneurial compared with epineurial capillaries, thus providing further support for the role of microangiopathy in the pathogenesis of human diabetic neuropathy.

Key words: Diabetes mellitus, microangiopathy, peripheral neuropathy.

Evidence for a role of microvascular disease in the pathogenesis of human diabetic neuropathy is now considerable [1, 2]. Both earlier qualitative and more recent quantitative studies have demonstrated a wide range of abnormalities which include basement membrane thickening, endothelial cell hypertrophy and hyperplasia with vessel closure [3–10]. Such changes, when accompanied by haemorheological abnormalities [11, 12], may well result in the endoneurial hypoxia which has been demonstrated in patients with severe human diabetic neuropathy [13]. Hypoxia in turn may result in the development and progression of neuropathy [14, 15].

Diabetic microangiopathy has also been demonstrated in other tissues affected by long-term complications, namely, retina [16] and kidney [17]. Moreover, a number of studies have shown microangiopathy in muscle [18, 19] and skin [20] capillaries suggesting that this may reflect the extent of microangiopathy in the complicated tissues. However, our recent study demonstrated more advanced microvascular pathology in the sural nerve when compared with muscle and skin capillaries from the same diabetic patients [2]. Furthermore renal tubular basement membrane thickness has been shown to be significantly greater than muscle capillary basement membrane thickness from the same diabetic patient [21]. Therefore the most severe microvascular abnormalities are to be found in those tissues affected by the specific complications of diabetes. Three vascular beds supply the peripheral nerve namely, epineurial, perineurial and endoneurial capillaries [22]. However, nerve fibre damage occurs in the endoneurium [23, 24]. It was therefore considered important to assess the extent of microvessel abnormalities of endoneurial capillaries which lie in close association with nerve fibres when compared with epineurial capillaries which lie outside the endoneurium separated by the perineurium. These studies may provide further insight into the inti-

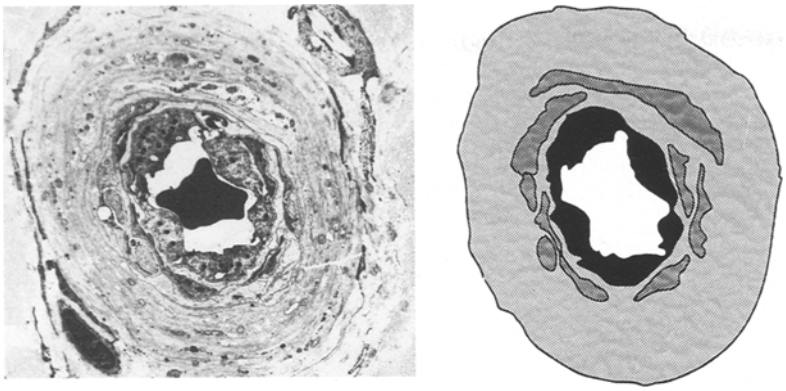

Fig. 1. Electron micrograph (× 3000) and outline of endoneurial capillary displaying morphometric parameters assessed. Basement membrane area ▒. Pericyte profile ▓; luminal area □; endothelial cell area ■

mate role of microangiopathy in the pathogenesis of human diabetic neuropathy.

Subjects and methods

Clinical assessment and electrophysiology

The studies were performed with the approval of the Manchester Central Hospitals and Royal Hallamshire hospital NHS trusts ethical committees. Informed consent was obtained from each patient prior to the study.

Twenty diabetic patients aged 53 [45–65] (median [interquartile range]) years with neuropathy took part in the study. Inclusion criteria were:

(1) Type 1 (insulin-dependent) or Type 2 (non-insulin-dependent) diabetes for more than 6 months.
(2) Peroneal nerve motor conduction velocity (MCV) below the 5th centile for age-related reference values for healthy subjects but greater than 30 ms^{-1}.
(3) Vibration perception threshold over the medial malleolus greater than the 95th centile for age-related healthy subjects, but less than 45 volts [24].

Patients with peripheral vascular disease, renal disease (serum creatinine > 130 μmol/l), or any other conditions known to cause neuropathy were excluded. Control data for measures of neuropathic severity was obtained from 46 age-matched (58 [34.5–74.6] years) subjects who were either healthy volunteers or hospital patients.

All patients underwent a full history and neurological examination. Peroneal nerve motor conduction velocity (MCV) was assessed using surface electrodes and a Medelec MD92A electrophysiological system (Medelec Ltd., Old Woking, Surrey, UK) in a room at 25 °C. The peroneal nerve MCV was considered to be a sensitive indicator of sural nerve pathology as a number of previous studies have demonstrated a significant correlation between this measure and sural nerve myelinated fibre density in diabetic patients with both mild [25] and severe [2] neuropathy. Vibration perception threshold was assessed over the great toe using a biothesiometer (Bio-Medical Instrument Company, Newbury, Ohio, USA). Thermal perception was assessed over the dorsum of the foot using a thermoaesthesiometer (Free University Hospital, Amsterdam, The Netherlands).

Nerve Biopsy

Diabetic patients underwent biopsy of the sural nerve behind the lateral malleolus under 2% lignocaine local anaesthesia. Non-diabetic control sural nerve was obtained from brain dead multiple organ transplant donors and traumatic amputees, aged 45 [21.8–63.8] years, in whom neurophysiological investigations were not feasible at the time of study. The tissue was fixed primarily in glutaraldehyde in cacodylate buffer and secondarily in osmium tetroxide. After dehydration in a graded series of ethanol, the tissue was embedded in epon resin with propylene oxide as an intermediary. Ultrathin sections were prepared and stained with methanolic uranyl acetate and lead citrate for detailed electron microscopy.

Morphometry

Electron micrographs (× 10000) of endoneurial and epineurial capillaries were prepared according to the morphological criteria for capillaries [26].

Exclusion criteria were based on:

(a) More than 8 endothelial cell profiles per capillary.
(b) Microvessel major to minor axis ratio greater than 3:1.
(c) Vessel wall characteristics: 1) presence of elastin; 2) presence of smooth muscle cells as opposed to pericytes found in capillaries. Smooth muscle was characterised by: 1) wider profiles than pericytes; 2) the presence of numerous dense bodies and myofilaments which were less developed in pericytes; 3) clustering of mitochondria and ribosomes which were more dispersed in pericytes; 4) more numerous pinocytic vesicles on the cell surface of smooth muscle; 5) continuity of the cell layer around the endothelium which was characteristically discontinuous for pericytes.

For the purpose of morphometric assessment the following parameters were measured: perimeter of the lumen (inner border of endothelial cells), perimeter of the outer border of the endothelial cells, perimeter of the outer border of the basement membrane. These were transformed to luminal, endothelial cell and basement membrane area by employing the DIGIT digitising pad interfaced to a BBC microcomputer [27] (Fig. 1). The number of endothelial cell profiles were assessed directly by counting the number of endothelial intercellular junctions in each capillary. Myelinated fibre density was assessed according to previously described techniques [2].

Statistical analysis

All the results were analysed using Minitab Software (Minitab Inc., State College, Pa., USA). All data is presented as the median and interquartile range and compared using the two tailed Mann-Whitney U-test and Spearmans rank correlation.

Results

Clinical details and measures of neuropathic severity are presented in Table 1. There was no significant difference in age between the diabetic patients and control subjects. Nine patients had Type 1 diabetes. Peroneal nerve motor conduction velocity (MCV) was significantly reduced

Table 1. Clinical details and measures of neuropathic severity in diabetic patients ($n = 20$) expressed as median (interquartile range), percentage abnormal and degree of significant difference when compared with control subjects

	Diabetic patients	% Abnormal
Age (years)	53 [45–65]	
Duration diabetes (years)	12.5 [8.3–15.8]	
HbA_{1c} (%)	10.8 [9.7–12.3]	
PNMCV (ms^{-1})	36.1 [29.8–37.7][a]	100
VPT (volts)	27 [20.3–41.5][b]	100
TDT (°C)	5.6 [0.4–21.0][a]	75
MFD (no. mm^{-2})	2558 [1530–5027][b]	100

[a] $p < 0.01$, [b] $p < 0.001$

PNMCV, peroneal nerve motor conduction velocity; VPT, vibration perception threshold; TDT, thermal discrimination threshold; MFD, myelinated fibre density. Normal control values ($n = 46$) (PNMCV: 47.5 (43.2–50.1), VPT: 12.8 (8.5–13.7), TDT: 1.0 (0.5–1.3), MFD ($n = 10$) 6570 (5680–7395) HbA_{1c}: < 8.0 %)

($p < 0.01$) and both vibration ($p < 0.001$) and thermal ($p < 0.01$) perception were elevated in diabetic patients. Myelinated fibre density was significantly reduced in diabetic patients ($p < 0.001$).

Microangiopathy: control vs diabetic patients (Fig. 2)

Results of microvascular pathology are presented in Table 2. Endoneurial basement membrane area ($p < 0.001$), endothelial cell area ($p < 0.01$) and endothelial cell profile number ($p < 0.001$) were significantly increased and luminal area ($p < 0.001$) was significantly reduced in diabetic patients when compared with control subjects. In epineurial capillaries both basement membrane area ($p < 0.02$) and endothelial cell profile number ($p < 0.001$) were significantly increased in diabetic patients when compared with control subjects.

Endoneurial vs epineurial capillaries (Fig. 2, Table 2)

In control subjects no morphological measure of microangiopathy differed significantly between endoneurial and epineurial capillaries. In contrast endoneurial capillaries from diabetic patients demonstrated significantly increased basement membrane area ($p < 0.001$), endothelial cell area ($p < 0.001$) and significantly reduced luminal area ($p < 0.001$) when compared with epineurial capillaries. Endothelial cell profile number did not differ significantly between endoneurial and epineurial capillaries of diabetic patients but was significantly greater in both groups of diabetic capillaries when compared with control subjects ($p < 0.001$). Endoneurial capillary basement membrane area, endothelial cell area and luminal area failed to relate to epineurial capillary basement membrane area, endothelial cell area and luminal area, respectively. However, endothelial cell profile number of endoneurial capillaries was significantly related to endothelial cell profile number of epineurial capillaries ($p < 0.05$).

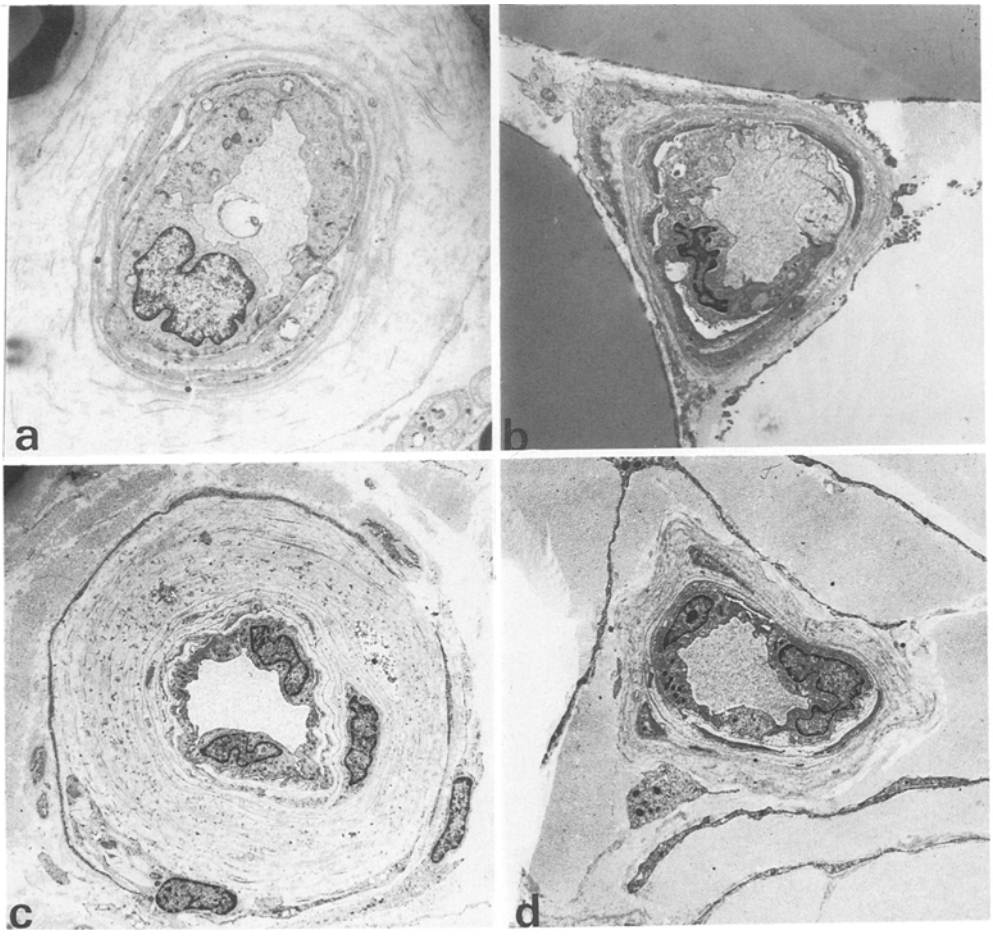

Fig. 2 (a–d). Electron micrographs of endoneurial (**a**) and epineurial (**b**) capillaries (×3000) from a control subject aged 45 and endoneurial (**c**) and epineurial (**d**) capillaries (×2500) from a 56-year-old diabetic patient with chronic sensory motor neuropathy displaying morphological abnormalities between control and diabetic capillaries and between diabetic epineurial and endoneurial capillaries

Table 2. Morphometric data of endoneurial and epineurial capillaries in control subjects and diabetic patients presented as median (interquartile range) and degree of significant difference between groups

	Control endoneurium (A)	Control epineurium (B)	Diabetic endoneurium (C)	Diabetic epineurium (D)	A vs B	C vs D	A vs C	B vs D
Basement membrane area (μm^2)	59.5 (50.5–63.0)	51.0 (49.2–59.3)	85.6 (76.6–96.1)	62.1 (55.9–67.2)	NS	$p < 0.0001$	$p < 0.0001$	$p < 0.02$
Endothelial cell area (μm^2)	35.0 (30.7–41.8)	31.6 (25.6–36.1)	50.5 (40.0–61.1)	29.6 (25.0–39.7)	NS	$p < 0.0001$	$p < 0.01$	NS
Luminal area (μm^2)	34.4 (32.7–39.4)	34.7 (32.1–35.6)	19.1 (12.6–26.3)	31.9 (26.9–35.7)	NS	$p < 0.0001$	$p < 0.0001$	NS
Endothelial cell profile (no.)	3.6 (3.1–4.0)	3.7 (3.4–4.1)	5.4 (4.9–6.2)	5.3 (4.2–5.7)	NS	NS	$p < 0.0001$	$p < 0.001$

Table 3. Correlation between abnormalities in endoneurial (Endo) and epineurial (Epi) capillaries and measures of neuropathic severity expressed as the Spearmans rank correlation coefficient (r) and degree of significance

	PMNCV	VPT	TDT	MFD
Basement membrane area				
Endo	$r = -0.67, p < 0.001$	$r = 0.46, p < 0.05$	$r = 0.70, p < 0.001$	$r = -0.91, p < 0.001$
Epi	$r = 0.05$, NS	$r = 0.03$, NS	$r = -0.03$, NS	$r = -0.21$, NS
Endothelial cell area				
Endo	$r = -0.46, p < 0.05$	$r = 0.31$, NS	$r = 0.46, p < 0.05$	$r = -0.47, p < 0.05$
Epi	$r = 0.01$, NS	$r = 0.02$, NS	$r = 0.09$, NS	$r = -0.19$, NS
Luminal area				
Endo	$r = 0.55, p < 0.01$	$r = -0.41$, NS	$r = -0.62, p < 0.001$	$r = 0.61, p < 0.01$
Epi	$r = 0.08$, NS	$r = 0.05$, NS	$r = -0.12$, NS	$r = 0.34$, NS
Endothelial cell profile number				
Endo	$r = -0.32$, NS	$r = 0.18$, NS	$r = 0.34$, NS	$r = -0.27$, NS
Epi	$r = -0.54, p < 0.01$	$r = 0.38$, NS	$r = 0.50, p < 0.02$	$r = -0.37$, NS

PMNCV, Peroneal motor nerve conduction velocity; VPT, vibration perception threshold; TDT, thermal discrimination threshold; MFD, myelinated fibre density

Correlation between clinical details, microangiopathy and neuropathic severity (Table 3)

Only endothelial cell area was related to age ($r = 0.51$, $p < 0.02$) and the level of HbA_{1c} ($r = -0.48, p < 0.05$). All other endoneurial and epineurial capillary abnormalities failed to relate to age, duration of diabetes or HbA_{1c} levels. Increased endoneurial capillary basement membrane area was related to reduced, peroneal nerve MCV ($p < 0.001$), myelinated fibre density ($p < 0.001$) and elevated vibration ($p < 0.05$) and thermal ($p < 0.001$) perception. An increase in endothelial cell area was related to both reduced peroneal nerve MCV ($p < 0.05$), reduced myelinated fibre density ($p < 0.05$) and elevated thermal perception ($p < 0.05$). Conversely a reduction in luminal area was related to reduced peroneal nerve MCV ($p < 0.01$), myelinated fibre density ($p < 0.01$) and elevated thermal perception ($p < 0.001$). Endoneurial capillary endothelial cell profile number failed to relate to any of the measures of neuropathic severity. Epineurial capillary basement membrane area, endothelial cell area and luminal area failed to relate to any of the measures of neuropathic severity. Only epineurial capillary endothelial cell profile number was related to reduced peroneal nerve MCV ($p < 0.01$) and elevated thermal perception ($p < 0.02$).

Discussion

The present study has demonstrated a significant endoneurial microangiopathy in the form of basement membrane thickening, endothelial cell hypertrophy and hyperplasia with luminal narrowing in diabetic patients with neuropathy when compared with normal control subjects, confirming previous findings [2–10]. In addition diabetic patients displayed epineurial capillary abnormalities in the form of basement membrane thickening and endothelial cell hyperplasia.

However, most importantly the present study has shown significantly more pathology in endoneurial capillaries in comparison to epineurial capillaries, particularly in the form of basement membrane thickening, endothelial cell hypertrophy and luminal narrowing. Furthermore

endoneurial capillary abnormalities were significantly related to both neurophysiological and morphological measures of neuropathic severity in the absence of any relationship between epineurial capillary abnormalities and neuropathic severity. We have previously shown both a more advanced microangiopathy and a stronger relationship to neuropathic severity in endoneurial capillaries when compared with muscle and skin capillaries from the same diabetic patients [2]. Renal tubules also displayed more severe basement membrane thickening than muscle capillaries from the same diabetic patients [21]. Moreover, in a recent study of the sural nerve no relationship was observed between epineurial arteriolar abnormalities and neuropathic severity [28], in the presence of a strong relationship between endoneurial capillary abnormalities and neuropathic severity in the same diabetic patients [7, 8]. Therefore, these studies suggest that more advanced microvascular disease occurs in the tissues most affected by the diabetic complications, particularly at the site of the most advanced pathology.

Considering the abnormality of basement membrane thickening, a number of mechanisms are likely to underlie this change [8, 19, 21]. However, the more significant basement membrane thickening of endoneurial compared with epineural capillaries would suggest that only part of these mechanisms are expressed in the epineurium or that the endoneurial environment provides optimal expression of such abnormalities. Basement membrane thickening may compromise, not only microvascular compliance [29, 30] and function [31] but may damage the endothelium via an increased shear stress [32].

One pathological abnormality which is common to both groups of diabetic capillaries is that of endothelial cell hyperplasia which may be caused by a number of factors including ischaemia [33] and physical-chemical stress [32, 34]. This finding suggests that the mechanisms causing endothelial cell hyperplasia may differ from those which cause basement membrane thickening and endothelial cell hypertrophy. A discordant pattern of cellular (endothelial cell) and matrix (basement membrane) abnormalities have been demonstrated previously in the renal vasculature [17]. Furthermore, an improvement of glycaemic control reduces renal basement membrane thickening [35] in the absence of any change in cellular mesangial structure [36]. Pericyte cell loss is the pathological hallmark of diabetic retinopathy [37]. However, previous studies in endoneurial capillaries have demonstrated either no change [9, 10, 38] or conversely an increase in the pericyte profile number [2, 8, 39]. The pathogenetic relevance of pericyte cell abnormalities in endoneurial microangiopathy is therefore debatable and was therefore not assessed in the present study.

The effect of age and duration of diabetes on all these abnormalities has been considered to be important [38], but the present study in agreement with previous studies [2, 7–10] failed to find any relationship with these variables.

Recent studies have shown an increased blood flow in nailfold capillaries [40] and the retina [37] of diabetic patients, with an accompanying increase in vessel luminal area [37]. The presence of an increased capillary luminal size in endoneurial capillaries of patients without [8] or with mild [39] neuropathy would also suggest an increased flow at an early stage of neuropathy. However, as pathology progresses, blood flow is compromised due to endothelial cell hyperplasia and hypertrophy with luminal narrowing and reduced vessel compliance due to basement membrane thickening. These abnormalities may result in a reduced endoneurial capillary oxygen diffusing capacity [2] and endoneurial hypoxia in patients with established diabetic neuropathy [13]. A relationship between nerve function and transcutaneous oxygen tension [41], the inability to improve nerve conduction on exercise [42] and neuropathy in hypoxic patients with chronic obstructive airways disease [15] provide strong evidence for the role of hypoxia in the development of human diabetic neuropathy.

The present study has demonstrated significant correlations between morphometric measures of microangiopathy and neuropathic severity assessed by both functional and pathological means. It is not, of course, possible to directly relate microangiopathy in the pathogenesis of human diabetic neuropathy. However, this study has shown that endoneurial tissue is the most important site for assessing factors in the pathogenesis of neuropathy, and therapies to prevent and perhaps reverse microangiopathy and neuropathy must be aimed at having an optimal effect in the endoneurium. Therefore, the isolation of more advanced microangiopathy to the site of nerve damage, namely the endoneurium, further strengthens the evidence for the role of microangiopathy in the pathogenesis of human diabetic neuropathy.

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