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Sural nerve pathology in diabetic patients with minimal but progressive neuropathy

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Abstract *Aims/hypothesis:* The early pathological features of human diabetic neuropathy are not clearly defined. Therefore we quantified nerve fibre and microvascular pathology in sural nerve biopsies from diabetic patients with minimal neuropathy. *Methods:* Twelve diabetic patients underwent detailed assessment of neuropathy and fascicular sural nerve biopsy at baseline, with repeat assessment of neuropathy 8.7±0.6 years later. *Results:* At baseline, neuropathic symptoms, neurological deficits, quantitative sensory testing, cardiac autonomic function and peripheral nerve electrophysiology showed minimal abnormality, which deteriorated at follow-up. Myelinated fibre density, fibre and axonal area, and g-ratio were normal but teased fibre studies showed paranodal abnormalities ($p<0.001$), segmental demyelination ($p<0.01$) and remyelination ($p<0.01$) without axonal degeneration. Unassociated Schwann cell profile density ($p<0.04$) and unmyelinated axon density ($p<0.001$) were increased and axon diameter was decreased ($p<0.007$). Endoneurial capillaries demonstrated basement membrane thickening ($p<0.006$), endothelial cell hyperplasia ($p<0.004$) and a reduction in luminal area ($p<0.007$). *Conclusions/interpretation:* The early pathological features of human diabetic neuropathy include an abnormality of the myelinated fibre Schwann cell and unmyelinated fibre degeneration with regeneration. These changes are accompanied by a significant endoneurial microangiopathy.

Keywords Demyelination · Diabetes · Microangiopathy · Nerve fibres · Neuropathy · Schwann cell

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Introduction

Diabetic neuropathy is a cause of significant morbidity as a consequence of foot ulceration [1] and independently predicts increased mortality [2]. Metabolic [3] and vascular [4–7] factors play an important role in its pathogenesis. Detailed neurophysiological studies in diabetic patients have shown that demyelination precedes axonal loss and that the latter may be responsible for the symptoms [8]. Whilst studies in patients with established neuropathy demonstrate a combination of demyelination and axonal degeneration of myelinated fibres and degeneration with regeneration of unmyelinated fibres [9–11] and endoneurial microangiopathy [12–15], the early pathological features of patients with diabetic neuropathy are not clearly defined.

Animal models of diabetic neuropathy demonstrate a reduction in nerve conduction velocity and blood flow [16] yet they do not show structural changes observed in human diabetic neuropathy and there is controversy as to whether the changes they do show are due to maturational delay induced by hyperglycaemia, or due to diabetes itself [17, 18]. Experimental studies demonstrate progressive molecular alterations leading to nodal and paranodal degeneration with axo–glial dysjunction and axonal atrophy [19]. However, these alterations have not been observed in diabetic patients [20, 21]. Increasing attention has focused on the Schwann cell, as it is not only responsible for insulating the axon and maintaining axonal calibre but is also vital for effective nerve regeneration [22]. Also, recent studies have demonstrated demyelination in feline diabetic neuropathy [23] and Schwann cell apoptosis in experimental diabetic neuropathy [24, 25].

We present the results of detailed morphometric studies of the nerve fibres and endoneurial capillaries in diabetic patients with minimal neuropathy. These studies may help to define the major early targets of cell death and repair in human diabetic neuropathy and may further elucidate its pathogenesis.

Subjects and methods

Selection criteria The selection criteria used were as follows: (1) diabetes mellitus diagnosed for more than 6 months; (2) age between 18 and 60; (3) vibration perception threshold at great toe less than 15 V; (4) recordable sural nerve amplitudes; and (5) peroneal motor nerve conduction velocity greater than 38 m/s. Patients with other causes of neuropathy, past or present foot ulceration, nephropathy (creatinine >125 μmol/l), peripheral vascular disease (absent foot pulses and/or history of claudication) or a history of any other serious illness were excluded from the study. All patients had stage N1 (asymptomatic) neuropathy according to published criteria [5].

Patients and neuropathy assessment All patients underwent a clinical history and examination to rule out any other cause of neuropathy. Control data for measures of neuropathic severity were derived from our age-matched reference range and not from the subjects who underwent nerve biopsy. The neuropathy symptom score was derived from a detailed questionnaire evaluating the following: (1) sensory symptoms, which included burning, numbness, tingling, aching and cramps; (2) their distribution and time of maximal intensity; and (3) their relieving and exacerbating factors. Responses were graded 0 to 9. The neuropathy deficit score is a composite score that defined the loss, presence or need for reinforcement of ankle and knee reflexes in both lower limbs (maximum score of 8) in combination with loss and level of loss of sensation to vibration, cold, pain and touch (no abnormality = 0, base of toes = 1, mid-foot = 2, ankle = 3, mid-shin = 4, knee = 5; maximum score of 20), giving a maximum score of 28 [26]. Vibration perception was expressed as an average of three readings on the first toe of both lower limbs using a Biothesiometer (Bio-Medical Instruments, Newbury, OH, USA). Warm thermal discrimination threshold was assessed over the lateral aspect of the foot (territory of sural nerve) with a thermo-aesthesiometer (VU Hospital, Amsterdam, The Netherlands) using a forced choice method. Autonomic function was tested from the electrocardiographic R–R interval variation during 1 min of six maximal expirations and inspirations (deep breathing test) to calculate the expiration: inspiration difference (the mean maximal R–R interval during expiration subtracted by the mean shortest interval during inspiration, reflecting parasympathetic, vagal nerve function). The lying : standing 30:15 ratio was also determined by assessing the R–R interval and hence the heart rate on the 15th and 30th beat after standing up, reflecting sympathetic nerve function. Electrophysiological assessment was performed with a Dantec Counterpoint EMG system using surface electrodes. The skin temperature was kept above 32°C using a surface heater. Electrophysiological parameters assessed included median motor nerve conduction velocity, peroneal motor nerve conduction velocity, sural sensory nerve conduction velocity, and amplitude, which were repeated on the contralateral side in ten patients (two patients had died).

Tissue biopsy/processing This study was approved by the Sheffield Physicians Advisory Ethical Committee and informed consent to perform nerve biopsies was obtained from all diabetic patients and closest relatives in the case of control subjects who were multiple organ donors. The sural nerve was exposed posterior to the lateral malleolus under local 2% lignocaine anaesthesia and a fascicular nerve biopsy was removed from diabetic patients and a whole nerve biopsy removed from control subjects (12 multiple organ donors, one traumatic amputation and one amputation for osteosarcoma). The tissue was immersed in 2.5% glutaraldehyde in cacodylate buffer, dehydrated in ascending concentrations of ethanol and infiltrated with Epon 812 resin using propylene oxide (1,2-epoxy-propane) as an intermediary. It was then set in resin blocks in an oven at 60°C for 48 h for light and electron microscopy. For teased fibre analysis, biopsies were infiltrated with unpolymerised Epon using toluene as an intermediary.

Teased fibre analysis Material for teased fibre analysis was available from eight diabetic patients and six control subjects. The analysis was undertaken blindly and the investigator was not aware of the source of biopsy. Approximately ten myelinated nerve fibres, each of at least five internodes in length, from at least ten bundles, totalling approximately 100 nerve fibres from each biopsy, were randomly teased apart into single fibres under a dissecting microscope. These fibres were mounted on glass slides and fixed in polymerised Epon for light microscopic analysis.

Each teased fibre was categorised into the following categories: (1) Wallerian axonal degeneration (breakdown of the axon accompanied by fragmentation of the myelinated fibre into myelin ovoids and balls); (2) axonal regeneration (a minimum of four consecutive, equal, short internodes without longer internodes at either end, where the internodal distance was considered inappropriately small for the fibre diameter); (3) segmental demyelination (axonal preservation with fragmentation or lack of myelin within one or more normally myelinated internodal segments); (4) remyelination (a variable internodal length equal to or shorter than 60% of that which would be expected for fibre diameter, flanked by two normally myelinated segments); (5) paranodal abnormalities (nodal gap at least twice the size of the axonal nodal diameter, or paranodal myelin 50% or less in diameter than the rest of the internode); and (6) normal fibres (any fibre not falling into any of the above categories with adequate fixation).

Morphometry Semi-thin (0.5 μm) sections were prepared and stained with thionin and counterstained with acridine orange. Each fascicle was photographed at a total magnifi-

cation of 400 times using a Vickers light microscope and camera; light micrograph montages were prepared of all fascicles from each biopsy. An image analysis cursor was used to trace the fascicular area, and the endoneurial capillaries and myelinated fibres were counted directly, enabling an assessment of mean fascicular area, endoneurial capillary density and myelinated fibre density [12].

For ultrastructural analysis of the myelinated and unmyelinated fibres, ultrathin (<0.1 μm) sections were stained with methanolic uranyl acetate and lead citrate and electron micrographs prepared with a Phillips EM201 electron microscope using a systematic random sampling procedure.

Myelinated fibres Electron micrographs were printed at final magnifications of 3,000×. Grid lines equal to the breadth of the largest myelinated fibre were drawn around two edges of each micrograph and only those structures whose edges lay within the grid lines were analysed to avoid any bias associated with the 'edge effect'. Myelinated fibres sectioned at the perinuclear and paranodal regions or at Schmidt–Lanterman incisures were excluded and the myelinated fibre and axon area were evaluated using a manual digitiser by tracing fibre and axon perimeter of at least 200 myelinated fibres per biopsy. Size frequency distributions were prepared for both myelinated fibre and axon area. A g-ratio was also calculated as a square root of the ratio of the axon to fibre area.

Unmyelinated fibres At least 25 electron micrographs per biopsy were printed at a final magnification of 10,000×. The number of unassociated and associated Schwann cell profiles was counted directly from all micrographs. The unmyelinated axons were identified using established criteria to differentiate axons from Schwann cell cytoplasm [27]. These included the presence of neurotubules and neurofilaments defined from electron micrographs at high magnification and the circularity and distribution of the axons enveloped by the Schwann cell. The number of axons was derived by a direct count and the density was derived from the total endoneurial area assessed. The mean axon diameter was digitised using a hand-held digitiser and an axon size frequency distribution was derived. Bands of Büngner and axons associated with this structure were excluded from the analysis.

Endoneurial capillaries Electron micrographs (6,000×) were prepared of at least ten endoneurial capillaries per biopsy. The luminal, endothelial cell and basement membrane areas were derived by tracing the image analysis cursor around each capillary profile. The endothelial cell profile (inter-endothelial cell junctions) and nuclear number and pericyte cell nuclear number per capillary were counted directly from each micrograph. The methodology for quantifying microangiopathy is established and has been described in detail elsewhere [12, 13].

Statistical analysis All data are presented as means±SEM. Statistical analysis was conducted using the Minitab statistical software (Minitab, State College, PA, USA). The Mann–Whitney *U*-test was used for comparisons between groups. Size frequency distributions of the fibre populations were constructed, their skewness and kurtosis derived and differences between distributions analysed with the Kolmogorov–Smirnov two-sample test.

Results

Studied were 12 diabetic patients (nine male), eight with type 1 and four with type 2 diabetes, aged 49.5±1.2 years, with a mean duration of diabetes of 14.6±3.0 years. HbA_1c was 8.4±0.5% (normal <7%). Three patients had background diabetic retinopathy and one patient had had laser treatment for maculopathy. Two of the three patients with background diabetic retinopathy also had microalbuminuria. Two patients smoked cigarettes. Morphometry was compared with 14 age-matched (47.5±2.5 years) control subjects.

Quantitative evaluation of neuropathy The results are presented in Table 1. The neuropathy symptom score was 0 at baseline and at follow-up. Neuropathy deficit score demonstrated minimal abnormality at baseline and did not change significantly at follow-up. Cardiac autonomic function tests were normal at baseline and were not repeated. Quantitative sensory examination was normal at baseline and showed no significant change in vibration perception but there was a significant increase in thermal thresholds ($p<0.0001$) at follow-up. Neurophysiological tests did not differ between control subjects and diabetic patients at baseline and showed a decline in median ($p<0.0001$) and peroneal

Table 1 Measures of neuropathic severity in control subjects and diabetic patients

	Control	Baseline	Follow-up
Age (years)	47.5±2.5	49.5±1.2	58.3±1.6
HbA_1c (%)	<7%	8.4±0.5	8.2±0.5
Duration of diabetes (years)	–	14.6±3.0	23.5±3.2
NSS	0	0	0
NDS	0	4.5±0.7	9.3±1.4
HRVDB	>15	17.8±2.2	–
L:S ratio	>1.04	1.3±0.07	–
VPT (V)	12.9±0.3	12.0±0.5	14.1±1.1
TDT (°C)	0.7±0.1	0.7±0.1	6.3±0.8**
MMNCV (m/s)	55±0.5	50.6±0.8	45.5±0.9**
PMNCV (m/s)	46.9±0.7	42.6±1.0	39.4±1.1*
SNCV (m/s)	50.4±0.6	45.4±1.1	43.5±1.0
SNAP amplitude (μV)	6.8±0.6	5.4±1.1	4.6±1.1

NSS Neuropathy symptom score, *NDS* neuropathy deficit score, *HRVDB* heart rate variation to deep breathing, *L:S* lying : standing ratio, *VPT* vibration perception threshold, *TDT* thermal discrimination threshold, *MMNCV* median motor nerve conduction velocity, *PMNCV* peroneal motor nerve conduction velocity, *SNCV* sural nerve conduction velocity, *SNAP* sural nerve action potential

At baseline and follow-up expressed as means±SEM with statistical difference between baseline and follow-up. *p=0.04, **p=0.0001

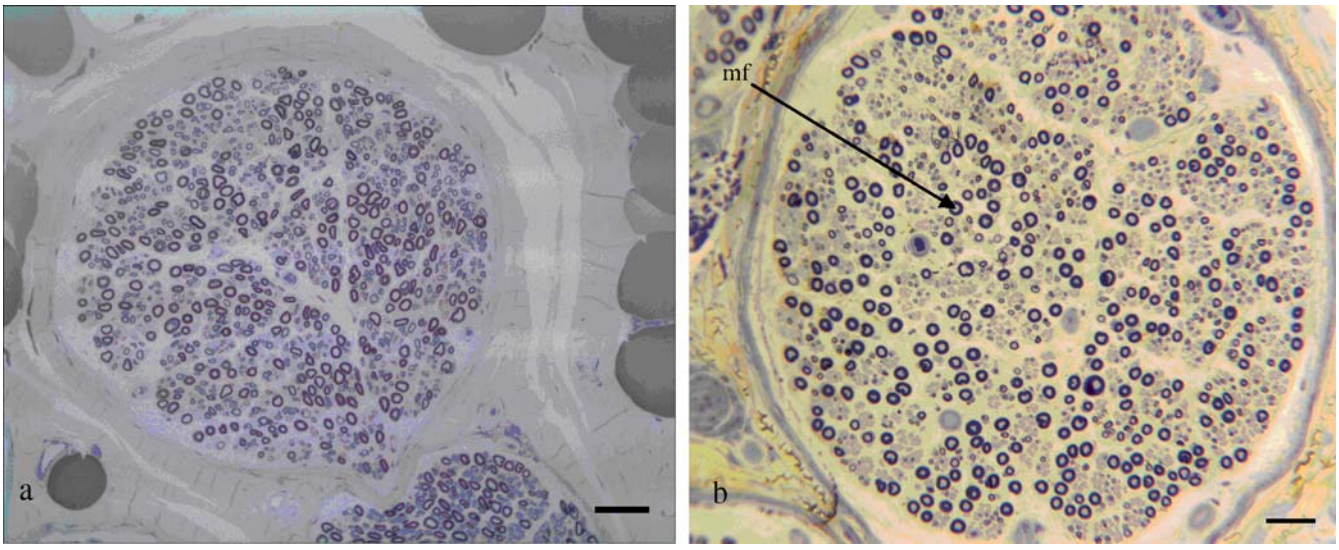

Fig. 1 Transverse semi-thin section from sural nerve biopsy specimen in a patient with minimal neuropathy (**b**) compared with a control subject, and (**a**) showing no loss of myelinated fibres (*mf*). Bar = 50 μm

($p<0.0001$) motor nerve conduction velocity with no significant change in sural sensory nerve conduction velocity or amplitude at follow-up.

Pathology and morphometry At least three fascicles were studied in each nerve biopsy. There was no evidence of vasculitis or an inflammatory infiltrate suggestive of any other cause of neuropathy.

Myelinated fibres There was no evidence of myelinated fibre loss (Fig. 1). Myelinated fibre density, fibre and axon area, and g-ratio did not differ significantly in diabetic patients compared with in control subjects (Table 2). The size frequency distributions of myelinated fibres did not differ but the axon area showed a very small, non-significant shift to the left in diabetic patients compared with in control subjects (Fig. 2). Fixation was inadequate in four biopsies; therefore teased fibre analysis was undertaken in only eight patients. These patients did not differ clinically, neurophysiologically or morphologically from the four who were not studied. An increased incidence of abnormal fibres ($p<0.001$) due to paranodal abnormalities ($p<0.001$), segmental demyelination ($p<0.01$) and remyelination ($p<0.01$), without axonal degeneration or regeneration was observed in diabetic patients (Table 3).

Table 2 Myelinated fibre morphometry in sural nerve of diabetic patients compared with in control subjects

	Control	Diabetic
Myelinated fibre density	6,049±342	5,800±600
Fibre area	29.0±1.6	27.8±3.1
Axon area	10.5±0.8	9.7±1.2
g-Ratio	0.60±0.02	0.59±0.02

Data are means±SEM (density, no. [mm^2]/area [μm^2])

Unmyelinated fibres Qualitative assessment of unmyelinated fibres demonstrated increased numbers of unassociated Schwann cell profiles and axonal sprouts suggestive of concomitant degeneration with regeneration (Fig. 3). The density of unassociated Schwann cell profiles was

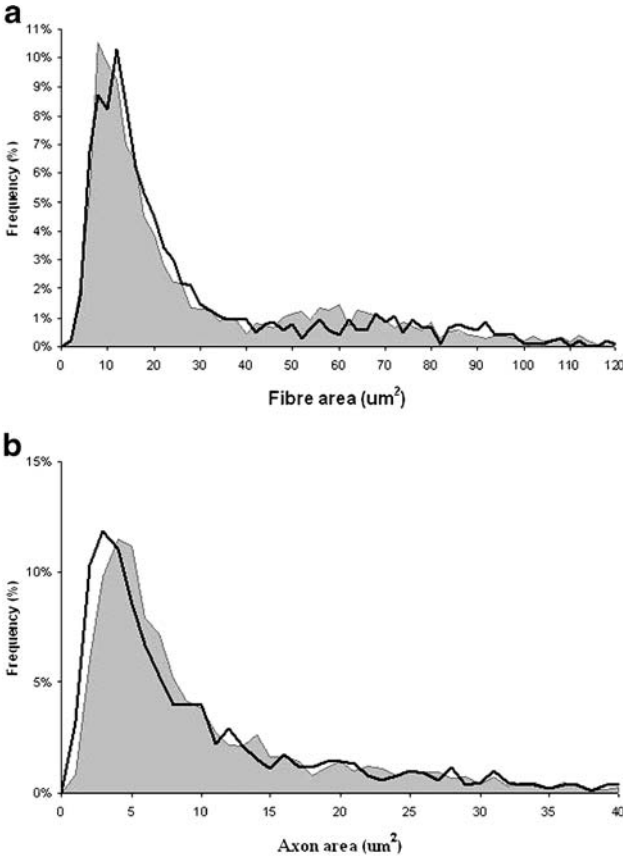

Fig. 2 Size frequency distribution for myelinated fibre and axon areas in control subjects (*grey area*) and diabetic patients (*black line*)

Table 3 Teased fibre abnormalities (%) in six control subjects and eight diabetic patients

	Control	Diabetic
Normal	90.4±1.3	62.7±5.4*
Paranodal abnormalities	3.5±1.0	18.5±2.4*
Segmental demyelination	1.5±0.3	7.9±2.4**
Segmental remyelination	1.8±0.5	5.7±1.4**
Axonal degeneration	1.2±0.6	2.5±0.8
Axonal regeneration	1.6±0.7	2.8±0.6

Data are means±SEM, with significant difference between groups
*p=0.001
**p=0.01

Table 4 Unmyelinated fibre morphometry in sural nerve of diabetic patients compared with in control subjects

	Control	Diabetic
Axon density	64,116±4,889	81,689±9,641*
Axon diameter	0.83±0.02	0.64±0.04**
Unassociated SCP density	8,971+802	14,789+2,264***
Percentage unassociated SCP	19.3±2.0	27.4±3.7
Total SCP density	44,580±2,149	51,715±2,931
Associated SCP	35,608±2,162	36,926±1,984

Data are means±SEM (density, no. [mm^2]/diameter [μm]); with significant difference between groups
SCP Schwann cell profile
*p=0.001
**p=0.007
***p=0.04

significantly increased (p<0.04) in diabetic patients, indicative of degeneration. Unmyelinated axon density was significantly increased (p<0.001) and the mean axon diameter was significantly reduced (p<0.007) in diabetic patients (Table 4). There was a significant increase in the percentage of smaller axons (0.2–0.4 and 0.4–0.6 μm; p<0.04) and a significant shift of the size frequency distribution to the left (skewness 0.89 vs 0.64; p<0.03) suggestive of axonal regeneration (Fig. 4).

Endoneurial capillaries There was no significant difference in the mean fascicular area or endoneurial capillary density between diabetic patients and control subjects. Qualitatively, the most prominent abnormality was that of basement membrane thickening (Fig. 5). A significant increase in endoneurial capillary basement membrane area (p<0.006) and endothelial cell profile number (p<0.004) with a reduction in luminal area (p<0.007) was observed in diabetic patients compared with in control subjects (Table 5). Endothelial cell area, pericyte nuclear number and endothelial:pericyte cell nuclear ratio did not differ significantly between diabetic patients and control sub-

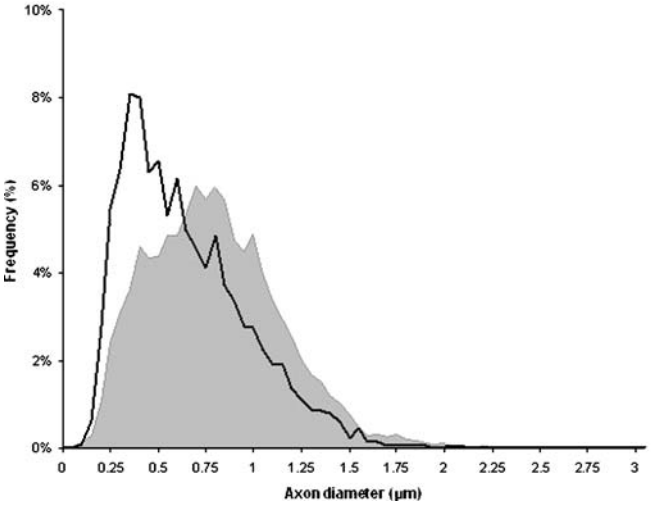

Fig. 4 Size frequency distribution for unmyelinated axon diameters in control subjects (*grey area*) and diabetic patients (*black line*)

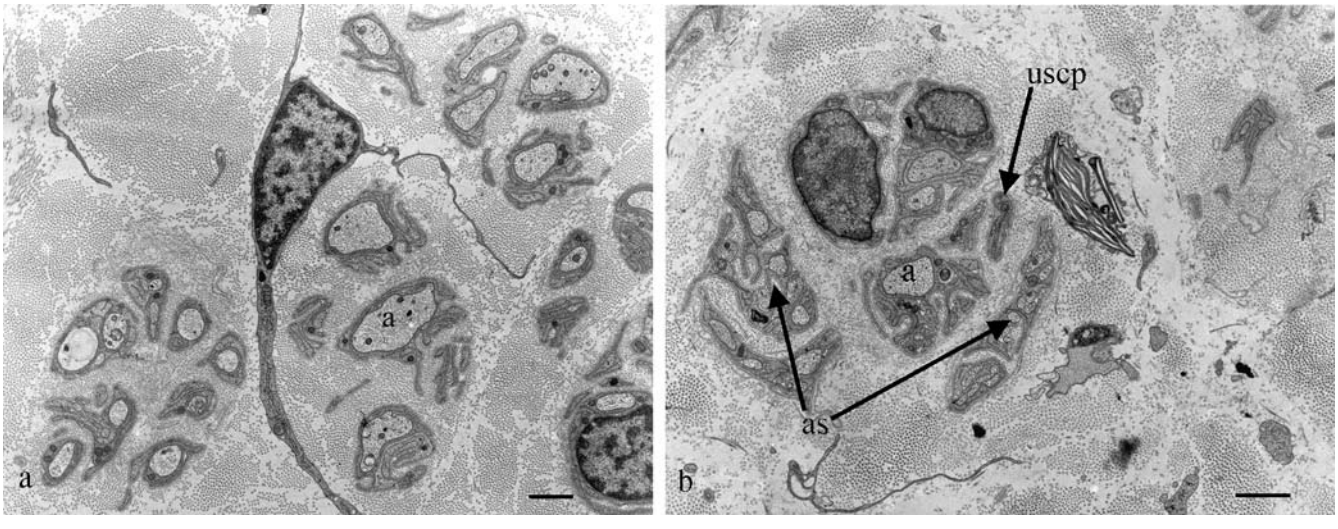

Fig. 3 Electron micrograph of a transverse section of unmyelinated fibres showing normal axons (*a*) in a control subject (**a**), with axonal sprouts (*as*) and unassociated Schwann cell profiles (*uscp*) in a diabetic patient (**b**). Bar = 1 μm

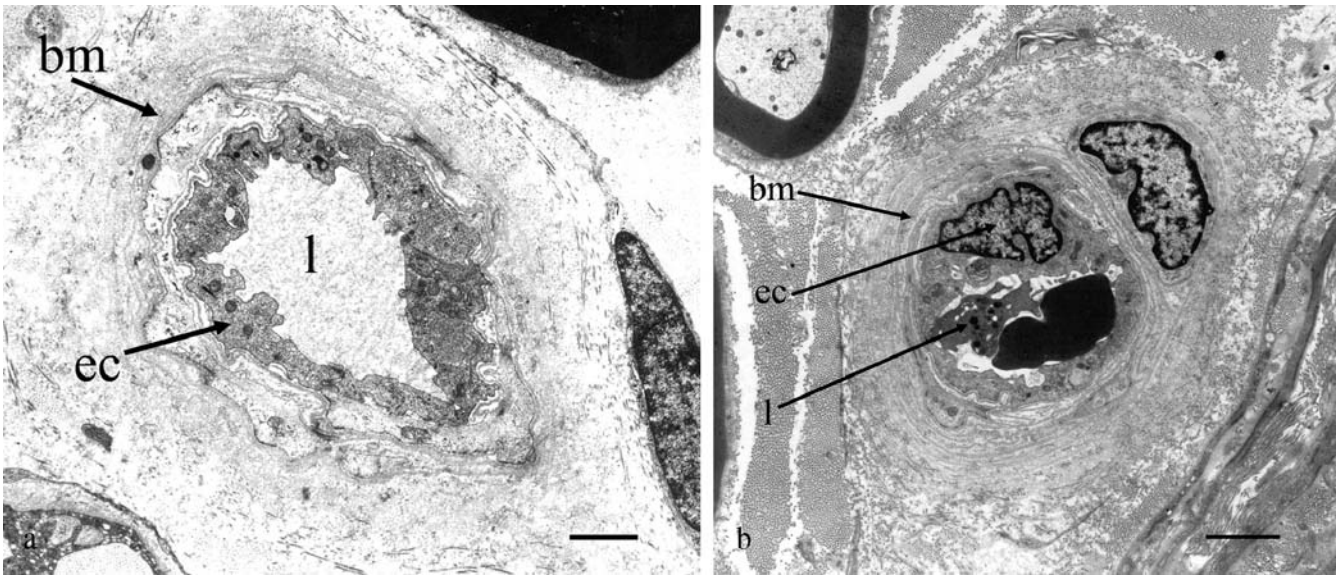

Fig. 5 Electron micrograph of endoneurial capillary from a control subject (**a**), and a diabetic patient (**b**) demonstrating basement membrane (*bm*) thickening, endothelial cell (*ec*) hyperplasia and luminal (*l*) narrowing. Bar = 2 μm

Table 5 Endoneurial capillary morphometry in sural nerve of diabetic patients compared with in control subjects

	Control	Diabetic
Fascicular area (mm^2)	0.09±0.01	0.09±0.01
Capillary density (no./mm^2)	67.9±4.8	57.4±7.5
Basement membrane area (μm^2)	47.3±5.4	135±16.9*
Endothelial cell area (μm^2)	32.5±6.2	43.9±4.8
Endothelial cell profile no.	3.9±0.2	6.0±0.4**
Luminal area (μm^2)	32.6±3.7	12.9±3.7***

Data are means±SEM, with significant difference between groups
*p=0.006
**p=0.004
***p=0.007

jects. All parameters of microangiopathy did not relate to age, duration of diabetes or HbA_1c.

Discussion

The present study was performed to define the early pathological changes in the sural nerve of diabetic patients with minimal evidence of neuropathy. We have shown that neuropathy progressed electrophysiologically at a rate comparable with other studies in patients with type 1 [28] and type 2 diabetes [29]. Alterations in nerve conduction velocity and amplitude have been considered to reflect underlying structural pathology of the myelinated fibres [30]. Such pathology includes demyelination and a significant loss of myelinated fibres due to axonal degeneration [9, 10]. In addition axonal atrophy and axo–glial dysjunction have also been demonstrated in some [31, 32] but not in other [20, 21, 33] studies. Unmyelinated fibres demonstrate both degeneration and regeneration in a variety of clinical syndromes of diabetic neuropathy [10, 11, 31, 34]. However, the majority of studies have been performed in patients with advanced diabetic neuropathy and thus do not define the early changes associated with the development of neuropathy. Therefore quantification of the early pathological changes will allow the identification of vulnerable cell types and fibre populations involved in the genesis of human diabetic neuropathy.

The present study confirms the excess of paranodal and segmental demyelination with remyelination without significant axonal degeneration observed previously in patients with minimal neuropathy [9, 33]. However, we did not show a reduction in either myelinated fibre or axonal area, nor did we show evidence of axonal atrophy. The occurrence of demyelination in the absence of morphologically apparent axonal damage suggests that the Schwann cell may be a primary target of damage in human diabetic neuropathy [22–25]. The reduction in unmyelinated fibre axon diameter with an increase in the density suggests degeneration with concomitant regeneration of the small fibres. The consequences of this early small fibre damage are borne out in the follow-up studies by demonstrating that thermal thresholds deteriorate, whilst vibration perception and electrophysiology remain preserved. Similarly, thermal discrimination has been shown to be abnormal prior to deficits in large fibre function, in newly diagnosed type 1 diabetic patients [35]. Furthermore epidermal small nerve fibre degeneration was demonstrated in the presence of normal vibration sensation and electrophysiology in a recent study of patients with impaired glucose tolerance [36]. Thus the present study confirms the early occurrence of unmyelinated fibre degeneration, but because of the proximal site of the biopsy, also shows significant regeneration. Similar changes have been seen in the unmyelinated fibres of five diabetic patients with an exceptionally severe early onset neuropathy [34] and in diabetic patients

with severe neuropathy [10, 31]. Small fibres appear to be prone to early damage but retain the ability to repair themselves even in chronic end-stage neuropathy [10, 31]. This suggests that treatments that act on small fibres may be successful even in established neuropathy. Furthermore, the presence of significant myelinated and unmyelinated fibre pathology in patients with apparently minimal neuropathy defined by current tests, suggests the need for further refinement or development of alternative tests which may be able to detect early damage to both myelinated and unmyelinated fibres [37].

Support for the vascular hypothesis is based on structural alterations contributing to reduced nerve perfusion and include a reduction in vascular density and or structural alterations to the endoneurial capillaries themselves, referred to as endoneurial microangiopathy. Thus endoneurial microangiopathy has been demonstrated in diabetic patients with established neuropathy and related to neuropathic severity [12–15]. We have previously demonstrated a significant reduction in endoneurial capillary density and endoneurial microangiopathy in patients with mild diabetic neuropathy [13] and in a limited group of patients with minimal diabetic neuropathy [12]. Endoneurial microangiopathy has been observed in a group of relatively young diabetic patients with an acute onset of neuropathy [34] and in small numbers of diabetic patients without evidence of neuropathy [38, 39]. We have recently demonstrated significant endoneurial microangiopathy in patients with impaired glucose tolerance, specifically in the patients with neuropathy [40]. However, an increase in endoneurial capillary density was observed and associated with current or future diabetes [40]. In the present study we show no alteration in endoneurial capillary density. We believe this simply reflects the situation in patients who have established diabetes and confirms a number of previous studies [12–15]. We do, however, demonstrate endoneurial microangiopathy characterised by basement membrane thickening and endothelial cell hyperplasia in diabetic patients prior to a clinically detectable neuropathy [39]. Additionally, we also demonstrate a reduction in luminal size which has not been demonstrated previously, though these studies were performed in patients with mild and established neuropathy [12–14]. This is important as it provides a morphological basis for an early reduction in nerve blood flow and endoneurial oxygenation [7].

In conclusion, we have characterised and quantified the early pathological changes in a group of diabetic patients with initially minimal but progressive neuropathy. The Schwann cell is an early target for myelinated fibre damage with no effect on the myelinated axon. Unmyelinated fibre degeneration with regeneration is also prominent at an early stage of diabetic neuropathy. Whilst we show no alteration in capillary density, the presence of significant endoneurial microangiopathy provides support for the role of vascular factors in the initiation of nerve damage in human diabetic neuropathy.

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