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Evaluation of dermal myelinated nerve fibers in diabetes mellitus

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Abstract

Skin biopsies have primarily been used to study the non-myelinated nerve fibers of the epidermis in a variety of neuropathies. In the present study, we have expanded the skin biopsy technique to glabrous, non-hairy skin to evaluate myelinated nerve fibers in the most highly prevalent peripheral nerve disease, diabetic polyneuropathy (DPN). Twenty patients with DPN (Type I, n=9; Type II, n=11) and sixteen age-matched healthy controls (ages 29–73) underwent skin biopsy of the index finger, nerve conduction studies, and composite neuropathy scoring. In patients with DPN, we found a statistically significant reduction of both mechanoreceptive Meissner corpuscles (MC) and their afferent myelinated nerve fibers ($p=0.01$). This myelinated nerve fiber loss was correlated with the decreased amplitudes of sensory/motor responses in nerve conduction studies. This study supports the utilization of skin biopsy to quantitatively evaluate axonal loss of myelinated nerve fibers in patients with DPN.

Keywords

Skin biopsy; diabetic polyneuropathy; glabrous skin; myelinated fibers; Meissner corpuscles

Introduction

Diabetic polyneuropathy (DPN) is known to affect myelinated nerve fibers. This is evident in the decreased nerve conduction velocities of electrophysiological studies as well as segmental demyelination in sural nerve biopsy (Dyck et al., 1980; Sima et al., 1988; Thomas and Tomlinson, 1997). Skin punch biopsies have revealed unmyelinated fiber loss prior to the development of electrophysiological abnormalities in DPN (Herrmann et al., 2004; Polydefkis et al., 2004). To date, parallel histologic examination of both unmyelinated and myelinated fibers in patients with DPN has been limited. Instead, nerve conduction studies (NCS) have been the primary method of analyzing large fibers, although this measure may not be sufficiently sensitive (Malik et al., 2005).

Skin punch biopsies are typically performed on the proximal or distal leg where there are ample unmyelinated nerve fibers, yet a relative paucity of myelinated fibers (Doppler et al., 2012; Provitera et al., 2007). In contrast, glabrous skin contains a high density of mechanoreceptors that significantly enrich non-hairy skin with myelinated fibers similar in

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structure to proximal nerves (Li et al., 2005; Myers et al., 2013). We therefore sought to quantify these dermal myelinated fibers in glabrous skin biopsies of patients with diabetic neuropathy. Because axonal loss has been best correlated with neurological disabilities in a variety of disorders (Krajewski et al., 2000; Trapp et al., 1998), we made this the primary focus of our study.

Materials and Methods

Participants

Thirty-one patients with diabetic polyneuropathy (DPN) were recruited in addition to healthy, age-matched controls (29 to 73 years of age). Of these, 11 DPN patients were screened out for one or more of the following reasons: other potential causes for neuropathy, Michigan Diabetic Neuropathy Score < 7, Hemoglobin A1c >10%, or risks for non-healing (e.g. edema). These criteria ensured the enrollment of twenty patients (Type I, n=9; Type II, n=11) with mild to moderate severity of DPN but with minimal risks for complications. Healthy controls had no history of diabetes, HgA1c < 6.5% (*American Diabetes Association, 2011*), normal neurological exam, and normal nerve conduction studies (NCS). Normal epidermal nerve fiber density was not an inclusion criterion for control patients due to our wide age range and the known reduction of small fibers in ageing (Lauria et al., 2010a).

Approval was obtained from the Vanderbilt University Internal Review Board and all patients gave informed consent according to the principles of the Declaration of Helsinki.

Procedures

All participants received a detailed exam examination with scoring by the Michigan Diabetes Neuropathy Score (MDNS), Michigan Neuropathy Screening Instrument (MNSI) (Feldman et al., 1994), and visual pain analog scale (Carlsson, 1983). Nerve conduction studies (NCS) were performed as previously described (Peltier et al., 2012).

Although glabrous skin on the soles of the feet would be an ideal site for evaluating distal large fibers, complications from infection and prolonged healing were concerns. Instead, skin punches 2 mm in diameter were obtained from the lateral aspect of the non-dominant index finger (Li et al., 2005). As a comparative measure of the axonal integrity of unmyelinated fibers, intraepidermal nerve fiber density (IENFD) was calculated from hairy skin biopsies 10 cm above the lateral malleolus (Beiswenger et al., 2008). All patients tolerated biopsies well with no incidence of infection or wound healing complications.

Immunohistochemistry

Twelve alternating 60 µm thick sections were selected from each glabrous skin biopsy for immunostaining. As described previously (Li et al., 2005), triple-staining was performed with antibodies against protein gene product 9.5 (PGP9.5; AbD serotec; 1:1,000), myelin basic protein (MBP; Chemicon; 1:700) and voltage-gated sodium channels (Pan NaV; Sigma-Aldrich; 1:500). Fluorescent secondary antibodies (Jackson ImmunoResearch, Inc.) allowed for antigen differentiation with epifluorescent (Leica DM 6000B) and confocal (Zeiss LSM510) microscopes.

Hairy skin biopsies were processed similar to glabrous skin with the exception of thinner sectioning (50 µm) and fewer sections randomly selected for quantification, per EFNS guidelines (Lauria et al., 2010b).

Quantification

To quantify axonal loss of myelinated nerve fibers in patients with DPN, we concentrated on the density of intrapapillary myelinated endings (IME) that innervate Meissner Corpuscles (MC). We targeted IME as an index of dermal myelinated fibers for the following reasons: 1. dermal papillae can be readily demarcated, 2. IME are typically not obscured by nerve bundles, and 3. their anatomical proximity to MC already suggests suitability for detecting peripheral nerve disease (Saporta et al., 2009).

Statistical Methods

We report means $\pm$ standard deviation, Wilcoxon rank sum test for continuous variables (IBM SPSS Statistics software), and Pearson chi-square tests for categorical variables (SAS Institute Inc., Cary, NC). Morphological correlations to other neuropathy measures were calculated with partial Spearman correlations. After adjusting for gender, a 1% significance threshold was applied to account for multiple comparisons.

Results

Patient Characteristics

Typical of DPN, patients with diabetes had significantly abnormal pain scores, nerve conduction studies, and IENFD compared to controls (Tables 1 and 2). Despite the longer course of diabetes in Type I v. Type II patients ($p < 0.0001$), there were no significant differences between types in regards to neuropathy score (MDNS), symptom score (MNSI), pain scores, or IENFD (Table 1). Nerve conduction results were similar among Type I and II patients with the exception of significantly lower motor amplitudes of the ulnar and peroneal nerves of Type I patients ($p < 0.05$) (Table 2).

Skin biopsies reveal significant axonal loss in patients with DPN

We examined 240 sections of glabrous skin from diabetic patients and 192 control sections. From all participants, we counted 697 MC and 958 IME (min 3, max 90/patient). These measurements were highly reliable, with an intraclass correlation coefficient of 0.9 for two blinded reviewers.

IME density was significantly reduced in diabetic patients (14.6 ± 14.6 fibers/mm²) compared to controls (21.7 ± 12.3 fibers/mm², $p = 0.01$). This was paralleled by a similar reduction in MC density (10.2 ± 8.4 v. 16.2 ± 9.4 MCs/mm², $p = 0.01$) (Figure 1). A high correlation was seen between IME and MC densities in all patients ($r^2 = 0.86$, $p < 0.0001$).

Separately comparing the Type I and Type II cohorts revealed a more severe loss of myelinated nerve fibers in Type I patients (Table 2). The MC and IME densities of Type II patients did not reach a statistically significant difference from those of controls. This is consistent with the near normal motor amplitudes of the ulnar and median nerves in patients with Type II diabetes.

Correlations between myelinated nerve fiber loss and neuropathy measures

Given the reduced IME and MC densities in diabetic patients, we tested the agreement of these measures with other standard outcome measurements. Median nerve motor and sensory amplitude correlated well to IME density ($r^2 = 0.57$, $p = 0.006$) and MC density ($r^2 = 0.55$, $p = 0.01$), respectively. Ulnar nerve motor amplitude also had similar correlations to IME ($r^2 = 0.57$, $p = 0.006$) and MC densities ($r^2 = 0.43$, $p = 0.05$). Neither the MDNS composite neuropathy score nor the vibration thresholds measured at the toe correlated with IME or

MC. Instead, we found a much stronger correlation between IENFD with MDNS ($r^2 = -0.53$, $p=0.02$) as well as visual analog pain scores ($r^2 = -0.62$, $p=0.002$).

Structural disruption of MC in patients with diabetes

We also observed a range of anomalies in the architecture of MC. Most commonly, we identified conspicuous disorganization of the lamellar structure of the corpuscle (Fig. 2B,C). An increase in the irregularity of the spiraling axons around the modified Schwann cells included variable degrees of decompaction. Interestingly, in several cases Schwann cells and nerve fibers associated with MC were hypertrophic in appearance (Fig. 2D).

Characterization of myelin aberrations in patients with diabetes

We also investigated the integrity of dermal myelin. Segmental demyelination is a key pathologic feature of DPN that we readily identified in both the papillary dermal layer of glabrous skin biopsies as well as the deeper dermal layer (Fig. 3). Such segments were identified in the internodal region between the sodium channel-immunoreactive nodes of Ranvier. Demyelination was also identified with sodium channel spreading along the axolemma (Fig. 3C) and heminodes suggestive of de-/remyelination (Fig. 3D).

Discussion

Our data demonstrate a significant loss of myelinated nerve fibers in diabetic patients. Even those with severe neuropathy had quantifiable fibers due to the high density of myelinated fibers in glabrous skin (Provitera et al., 2007). This and our extensive sampling, enabled us to identify characteristic pathology of DPN, including axonal loss and segmental demyelination. The similar correlations we found between IME density and motor amplitudes of both the median and ulnar nerves, suggest our findings are due to diabetic polyneuropathy rather than an isolated compressive median mononeuropathy (Stamboulis et al., 2009).

The greater disease duration in Type I patients (37 years) v. Type II (10 years) was reflected in the more severely reduced amplitudes of sensory/motor nerve responses of Type I patients. This also paralleled a greater reduction in IME/MC densities (Fig. 1). Longer disease duration is expected to result in more severe neuropathy (The Diabetes Control and Complications Trial Research Group, 1993), yet there were no significant differences in MDNS score or neuropathy symptoms (MNSI). MDNS scores had higher correlation with IENFD than IME density, which may reflect a bias toward sensory evaluation primarily in the lower extremity (Feldman et al., 1994). As a result, glabrous skin biopsy from the finger may complement these scoring systems to offer a more comprehensive assessment of neuropathy.

In addition to axonal loss, we are able to demonstrate changes of molecular architecture including degradation of myelin basic protein in internodes, sodium channel spreading in elongated nodes, and heminode formation. All of which have previously been observed in human nerve biopsies (Bai et al., 2006; Li et al., 2005; Li et al., 2006) and animal studies (Arroyo et al., 2004; Bai et al., 2010). In the present study, we qualitatively characterized these myelin aberrations to demonstrate the technical feasibility of our approach.

To our knowledge, Meissner corpuscle hypertrophy (Fig. 2D) has not been previously reported in humans, although Schwann cell proliferation has been described elsewhere in DPN (Kalichman et al., 1998). Interestingly, in a primate model of diabetes, a similar proliferation of MC was identified in an early stage of hyperglycemia that later transitioned to corpuscle atrophy (Pare et al., 2007).

In conclusion, the minimally invasive nature of the procedure and its repeatability make glabrous skin biopsy a particularly attractive method of sampling myelinated fibers in humans. Furthermore, the solitary, vertical course of myelinated fibers through the papillary dermal layer allows quantification akin to epidermal nerve fiber density. Further work characterizing the molecular architecture of these dermal myelinated fibers may more comprehensively describe the pathology of DPN and lend itself well to further studies of its pathogenesis.

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Figure 1.

Axonal loss in diabetic patients compared to controls. IME = Intrapapillary myelinated ending; MC = Meissner corpuscle; IENF = Intraepidermal nerve fiber; Density calculations per square millimeter of skin with the exception of IENFD per millimeter of skin.

Figure 2.

A. MC from a healthy control showing axons (blue), myelin (green) and voltage-gated sodium channels (red). Unmyelinated axons create a spiral structure with each corpuscle also innervated by a myelinated nerve fiber. B. MC of patients with DPN exhibit a range of anomalies including disruption of the lamellar structures as well as moderate demyelination (B, arrows). C. Severe demyelination (C, arrow) may present with (B) or without (C) heminode formation. D. Proliferative changes of Schwann cells were also observed and may represent a unique time point in the disease process.

Figure 3.

A. In a healthy control patient, a normal dermal internode contains MBP-immunoreactive (MBP-ir) compact myelin that insulates PGP9.5-ir axons (blue). Each internode is flanked by two nodes of Ranvier, stained by pan voltage-gated sodium channel antibodies (red). B. An internode from a patient with diabetic neuropathy is shorter than the control (A) as a result of a segment without myelin that reveals the denuded axon beneath. Despite this demyelination, sodium channel localization is preserved (arrow). C. Delocalization of the sodium channel staining (arrow) may also accompany demyelination or two discreet areas of sodium channel localization (D) described in the process of remyelination (Bai et al., 2006).

Table 1

Patient demographics.

	Healthy control patients (n=16)	Patients with diabetes **	
		Type I (n=9)	Type II (n=11)
Length of diabetes (years)	0	36.9 ± 11.4 *	9.6 ± 7.6 *
Age	52.7 ± 11	56.2 ± 8.4	54.5 ± 13.4
Gender	6 Male	4 Male	5 Male
	10 Female	5 Female	6 Female
Body mass index	25.0 ± 3.5	30.8 ± 5.3 *	34.0 ± 7.3 *
HgA1c	5.5 ± 0.3	7.5 ± 1.0 *	7.1 ± 1.5 *
MDNS	0.2 ± 0.5	15.9 ± 6.8 *	12.3 ± 3.4 *
MNSI	0.4 ± 0.6	6.3 ± 4.0 *	6.2 ± 2.8 *
VAS worst pain [mm]	3.3 ± 8.9	46.7 ± 30 *	31.36 ± 28.5 *

MDNS = Michigan Diabetic Neuropathy Score; **MNSI**= Michigan Neuropathy Screening Instrument; **VAS**= visual analogue scale

* p = 0.01 compared to controls

** No significant differences in any measures between Type I and Type II patients with the exception of length of diabetes p<0.0001

Table 2

Nerve conduction study and skin biopsy pathology in Type I and Type II patients.

	Healthy control patients (n=16)	Type I (n=9)	Type II (n=11)	Type I v. Type II Wilcoxon rank sum
Motor amplitude [mV]				
Ulnar	9.5 ± 1.4	5.7 ± 2.5 *	9.7 ± 1.6	p = 0.001
Median	13.2 ± 20.4	6.2 ± 2.5	7.4 ± 2.9	p = 0.30
Peroneal	5.2 ± 1.8	0.38 ± 0.6 *	2.6 ± 2.2 *	p = 0.04
Sensory amplitude [µV]				
Ulnar	30.5 ± 21.8	8.9 ± 11.2 *	14.8 ± 14.3 *	p = 0.2
Median	22.1 ± 8.3	9.4 ± 10.2 *	18.1 ± 12.2	p = 0.1
Sural	13.4 ± 4.8	3.1 ± 6.4 *	5.2 ± 5.6 *	p = 0.3
Motor latency [ms]				
Median	3.5 ± 0.4 (n=15)	4.9 ± 0.8 (n=8) *	4.2 ± 1.0 (n=9)	p = 0.1
Conduction velocity [m/s]				
Peroneal	49.2 ± 3.7 (n=15)	40.6 ± 0.2 (n=2) *	39.9 ± 6.1 (n=8) *	p = 0.8
Morphology				
MC/mm ²	16.2 ± 9.4	6.6 ± 4.9 *	13.1 ± 9.6	p = 0.01
IME/mm ²	21.7 ± 12.3	7.8 ± 6.7 *	20.3 ± 17.2	p = 0.01
IENF/mm	11.3 ± 5.8	2.0 ± 2.6 *	5.0 ± 4.8 *	p = 0.2

MC = Meissner corpuscle, IME= Intrapapillary myelinated ending, IENF = Intraepidermal nerve fiber

* p 0.01 compared to controls; All other values not significantly different from controls