Corneal nerve fiber pathology in Japanese type 1 diabetic patients and its correlation with antecedent glycemic control and blood pressure

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ABSTRACT

Aims/Introduction: Morphological changes to corneal C-fibers in Japanese type 1 diabetic patients were visualized by corneal confocal microscopy (CCM). The effects of prior glycemic control and blood pressure on morphological parameters were clarified.

Materials and Methods: Corneal nerve fibers were visualized by CCM in 38 Japanese type 1 diabetic patients (14 with and 24 without neuropathy) and 38 controls. Morphological parameters were compared and related to annual mean HbA1c, blood pressure, and serum lipid levels of previous years prior to CCM examination.

Results: Compared with controls, diabetic patients had reduced corneal nerve fiber length (CNFL; 9.80 ± 0.38 vs 13.65 ± 0.88 mm/mm^2; $P < 0.001$), reduced density (CNFD; 25.32 ± 1.04 vs 36.62 ± 2.37/mm^2; $P < 0.0005$), lower frequency of beading (22.38 ± 0.73 vs 30.44 ± 1.03/0.1 mm; $P < 0.0001$), and increased tortuosity (3.13 ± 0.09 vs 1.74 ± 0.06; $P < 0.0001$). These changes were found in patients without neuropathy. There was no difference in nerve branches between controls and diabetic patients. The mean annual HbA1c level for the 7–10 years prior to CCM examination was an independent predictor of reduced CNFL and CNFD; HbA1c levels obtained 1–3 months and 1 year prior to CCM, as well as blood pressure 3, 5, and 6 years prior to CCM, were independent predictors of reduced beading frequency.

Conclusions: Corneal confocal microscopy is a novel, noninvasive technique to evaluate morphological changes of corneal C-fibers in type 1 diabetes. Antecedent hyperglycemia and blood pressure have different time-dependent effects on CNFL and CNFD and the frequency of beading. (J Diabetes Invest, doi: 10.1111/j.2040-1124.2011.00157.x, 2012)

KEY WORDS: Corneal confocal microscopy, Diabetic neuropathy, Type 1 diabetes mellitus

INTRODUCTION

The earliest fibers to undergo damage and subsequent repair are small unmyelinated (C) fibers[1], as reported in patients with impaired glucose tolerance and type 2 diabetes mellitus[2]; however, this has not been confirmed in type 1 diabetes. Recently, noninvasive corneal confocal microscopy (CCM) was used to detect morphological changes of the corneal nerve, which is composed mainly of C-fibers[3]. It has been shown that CCM can detect early small fiber changes in patients with type 1 diabetes without neuropathy[4] and accurately quantify the severity of diabetic neuropathy (DN)[4,5]. Corneal nerve pathology reflects the C-fiber damage observed in the somatic nerves of a skin biopsy specimen[4]. Therefore, damage visualized by CCM seems to represent C-fiber changes in somatic DN[4,5]. In patients with type 1 diabetes, long-standing hyperglycemia is an important causative factor of neuropathy[6,7], whereas vascular risk factors, including body mass index (BMI), dyslipidemia, and hypertension[8,9], are involved in the development of neuropathy independent of hyperglycemia[10].

Although a few CCM studies[11,12] have reported a reduction in the number and length of nerve fibers and increased tortuosity in patients with type 1 diabetes compared with healthy subjects, there have been no analyses of nerve beading, which reveals the accumulation of mitochondria in type 1 diabetic patients.

An apparent time discrepancy exists between hyperglycemia and the onset or progression of diabetic complications, including neuropathy[13,14]. Typical clinical symptoms of type 1 diabetes mellitus usually develop rapidly, allowing early intervention and normalization of hyperglycemia. Thus, it may be easier to analyze the time-dependent effect of glycemia and other pathogenetic factors on the development of diabetic complications, including neuropathy. However, unlike retinopathy and nephropathy, the time period necessary for neuropathy, as defined by morphological changes, to develop is as yet unknown; furthermore, little is known about the time-dependent effects of antecedent glycemia and other vascular risk factors on the various morphological findings detected by CCM,

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except that 6 months of euglycemia induced by pancreas transplantation promotes small fiber regeneration[12].

In the present study, we used CCM to quantify pathological changes in C-fibers and assessed the possible time-dependent effects of antecedent glycemia and vascular risk factors on various morphological parameters of corneal C-fibers in Japanese patients with type 1 diabetes, with or without DN.

MATERIALS AND METHODS

Patients with type 1 diabetes attending Ishibashi Clinic for >5 years and age- and sex-matched non-diabetic volunteers (HbA1c <5.7% and fasting plasma glucose <5.5 mmol or casual postprandial plasma glucose <7.7 mmol) were invited to participate in the study. Subjects were excluded if their alcohol consumption was >20 g/day, they were diagnosed with neuropathy due to another cause, or wore contact lenses. Written informed consent was obtained from all subjects. The protocol of the present research was approved by the ethics committee of Ishibashi Clinic.

Corneal confocal microscopy

Subjects were examined using a Heidelberg Retina Tomograph 3 equipped with a Rostock Cornea Module (Heidelberg Engineering, Heidelberg, Germany). The laser source used in the module was a diode laser with a wavelength of 670 nm. The two-dimensional images acquired had a definition of 384 × 384 pixels over an area of 0.16 mm^2 with a lateral digital resolution of 1 μm/pixel and a depth resolution of 2 μm/pixel. Each eye examined was anesthetized with one drop of 0.4% benoxinate hydrochloride (Santen Pharmaceutical, Osaka, Japan). The objective lens of the cornea module was disinfected with 70% isopropyl alcohol swabs. A drop of Comfort Gel (Dr Mann Pharma, Berlin, Germany) was applied to the tip of the lens and a disposable sterilized Tomocap was mounted on the holder to cover the objective lens. After applying Comfort Gel to the Tomocap, the lens was slowly advanced forward until the gel touched the cornea, allowing optical contact between the objective lens and the corneal epithelium during the examination. Correct alignment and contact with the cornea were monitored by magnified images captured by a camera tangential to the eye. After focusing on the corneal epithelium, the nerve fiber layer (sub-basal layer) was recorded by fine turning the focus. It took 3–5 min to acquire 10–20 satisfactory images of the corneal nerve fibers. Four to five high-quality images of the sub-basal layer were used to analyze morphological parameters of the corneal nerve fibers. The images collected were used to quantify the following parameters to define corneal nerve fiber changes: (i) corneal nerve fiber density (CNFD; /mm^2); (ii) corneal nerve fiber length (CNFL; mm/mm^2); (iii) corneal nerve fiber branch density (CNBD; /mm^2); (iv) length of the corneal nerve branch emanating from the major nerve trunk (CNBL; mm/mm^2); (v) tortuosity; and (vi) frequency of beading (/0.1 mm). All measurements, except for tortuosity, were made using IMAGE J (Texelcraft, Tokyo, Japan); tortuosity was determined according to the grading system proposed by Oliveira-Soto and Efron[15]. The reproducibility of the assessment of morphological parameters by CCM was evaluated in 14 healthy volunteers by repeating the CCM examination with different examiners and calculating the coefficient of variation (CV) for individual parameters.

Assessment of nerve function

Current perception threshold (CPT) measured using a Neurometer (Neurotron, Baltimore, MD, USA) and vibration perception threshold (VPT) measured using a centrally calibrated biothesiometer (Bio-Medical Instrument, Newbury, OH, USA) were used to assess the neurological function of controls and patients with type 1 diabetes. The CPT was used to quantitatively determine the sensitivity of three different types of peripheral sensory nerve fibers using three current frequencies (2000, 250, and 5 Hz). Stimuli were applied on the dorsal medial side of the left first toe. The lowest electrical stimulus that the subject could perceive was defined as the CPT of the individual current frequency. To determine the VPT, eight readings for the left medial malleolus were obtained, averaged, and expressed as the absolute amplitude (μ) at 120 cycles/s.

Assessment of DN

Simplified diagnostic criteria proposed by the Japanese Study Group of Diabetic Neuropathy[16] was used to diagnose DN in patients with type 1 diabetes. Specifically, DN was diagnosed in subjects with two or more of the following three factors: (i) subjective symptoms of bilateral lower limbs or feet; (ii) loss or reduced ankle jerk; and (iii) abnormal VPT assessed using a C128 tuning fork. Of the 38 patients, 14 were found to have DN. No significant difference was observed between patients with and without DN in terms of mean age (48.8 ± 3.3 vs 45.1 ± 1.7 years, respectively), BMI (22.2 ± 0.7 vs 22.4 ± 0.6, respectively), HbA1c levels at the time of CCM examination (7.3 ± 0.2% vs 7.3 ± 0.2%, respectively), or the duration of diabetes (14.6 ± 2.0 vs 15.5 ± 1.6 years, respectively).

Laboratory data

Following the first visit, patients' HbA1c levels (converted to NGSP units by adding 0.4% to the measured values) and blood pressure were monitored monthly, with serum blood urea nitrogen (BUN), creatinine, and lipid levels examined every 3 months. The annual mean levels of these parameters were calculated.

Statistical analysis

All statistical analyses were performed using the SPSS medical package (SPSS, Chicago, IL, USA). Data are presented as the mean ± SEM. The Mann–Whitney test was used to compare patients with type 1 diabetes and controls. Analysis of variance (ANOVA) was used to compare controls and type 1 diabetic patients with or without DN. Correlations between morphologic parameters of corneal nerve fibers and concurrent or annual

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mean clinical data were assessed by multiple regression analysis, with the CCM morphological parameters as outcome variables and clinical annual mean values as the predictor variables. Receiver operating characteristic (ROC) analysis established cut-off levels for CNFL, CNFD, tortuosity grade, and beading frequency between non-diabetic controls and type 1 diabetic patients. Sensitivity and specificity were equally weighted. $P < 0.05$ was considered significant.

RESULTS

Table 1 shows the clinical and demographic data of the controls and patients with type 1 diabetes (at the time of study entry and at the time of CCM examination), who had a known diagnosis of 3.9 ± 1.3 years at the time of study entry. The HbA1c levels were significantly higher in patients with type 1 diabetes than in controls. The HbA1c levels of the patients decreased by 2.4%, whereas BMI, systolic blood pressure (SBP), and diastolic blood pressure (DBP) increased significantly, during the follow-up period. There was no significant difference in the age of controls and diabetic patients at the time of CCM examination. When nerve function was assessed in terms of CPT (Figure 1a) and VPT (Figure 1b), CPT at 2000, 250, and 5 Hz was significantly higher in diabetic patients than in controls. In addition, VPT was significantly higher in diabetic patients than in controls.

Table 1 | Clinical characteristics of control subjects and type 1 diabetic patients

	Control subjects	Type 1 diabetic patients	
		At entry	At CCM
n (M/F)	38 (19/19)	38 (19/19)	38 (19/19)
Age (years)	42.5 ± 1.9	34.7 ± 1.6*†	46.7 ± 1.6
BMI (kg/m^2)	22.2 ± 0.45	19.6 ± 0.41**†	22.4 ± 0.41
SBP (mmHg)	130.4 ± 1.9	124.2 ± 1.8†	135.0 ± 1.2
DBP (mmHg)	81.2 ± 1.3	76.8 ± 1.0†	83.6 ± 0.7
No. treated with ARB/ACEI (%)	2 (5.3)	0 (0)	3 (7.9)
HbA1c (%)	5.7 ± 0.05	9.7 ± 0.48**†	7.3 ± 0.11**
Total cholesterol (mmol/L)	4.92 ± 0.15	5.0 ± 0.25	4.98 ± 0.15
No. treated with statins (%)	1 (2.6)	0 (0)	2 (5.3)
HDL-C (mmol/L)	1.87 ± 0.06	1.9 ± 0.28	1.92 ± 0.09
Triglycerides (mmol/L)	0.91 ± 0.11	1.09 ± 0.12	1.23 ± 0.21
ACR (mg/g Cr)	7.1 ± 1.6	56.3 ± 23.6	45.9 ± 23.0
eGFR (mL/min)	89.7 ± 3.0	79.1 ± 4.1	84.8 ± 3.2
Duration of diabetes (years)	NA	3.9 ± 1.3	15.4 ± 1.5
Follow-up period (years)	NA	NA	11.5 ± 1.2

Data are the mean ± SEM in control subjects and patients with type 1 diabetes. *$P < 0.01$, **$P < 0.001$ compared with non-diabetic control subjects; †$P < 0.001$ compared with at the time of corneal confocal microscopy (CCM). Statistical analyses were performed with the Mann-Whitney test. To standardize HbA1c values to NGSP units, 0.4% was added to the measured HbA1c values. NA, not applicable; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; ARB, angiotensin receptor blocker; ACEI, angiotensin-converting enzyme inhibitor; HDL-C, high-density lipoprotein–cholesterol; ACR, urinary albumin/creatinine ratio; Cr, creatinine; eGFR, estimated glomerular filtration rate.

Figure 2 shows representative sub-basal nerve plexuses in a control individual (Figure 2a) and a patient with type 1 diabetes (Figure 2b). Compared with the control, the diabetic patient had reduced CNFD, CNFL, and increased tortuosity. Intra-individual variability was established by repeating the CCM examination on three occasions in 14 healthy volunteers and the average CV were as follows: 10.9% for CNFD, 11.0% for CNFL, 31.8% for CNBD, 33.5% for CNBL, 19.8% for beading frequency, and 18.5% for tortuosity.

Patients with type 1 diabetes had reduced CNFD and CNFL. When diabetic patients were divided into two groups based on the presence or absence of DN, CNFD and CNFL in patients without DN were lower than in the controls, and the development of DN further reduced these parameters. In contrast, there were no significant differences in CNBD and CNBL between the controls and diabetic patients or between patients with or without DN (Figure 3). An increase in tortuosity and a decrease in beading frequency were observed in diabetic patients compared with controls, whereas no differences were observed between patients with or without DN (Figure 4).

Multiple regression analysis revealed that the annual mean HbA1c level for 7–10 years before the CCM examination was an independent predictor of reduced CNFD (Table 2) and CNFL (Table 3). (Other predictors included age, BMI, duration of diabetes, SBP, DBP, total cholesterol, high-density lipoprotein–cholesterol, triglycerides, and albumin excretion rate.) Beyond 10 years, the correlation between annual mean HbA1c and CNFD and CNFL became insignificant, probably because of a decrease in the number of subjects ($n < 22$). However, no correlation was observed between annual mean SBP ($P = 0.112$–0.767) or DBP ($P = 0.119$–0.917) and CNFL or CNFD at 1–10 years before examination ($P = 0.086$–0.953 and 0.067–0.868 for SBP and DBP, respectively). However, beading frequency decreased relative to concurrent and annual mean HbA1c levels at 1 year before CCM examination (Table 4). Therefore, we analyzed the relationship between beading frequency and monthly HbA1c levels during the first year prior to examination. There was a significant inverse correlation between HbA1c levels 1–3 months prior to CCM examination and beading frequency. Beading frequency was also inversely correlated with mean SBP and DBP at 3, 5, and 6 years before CCM examination, as determined by multiple regression analysis (Table 5).

The sensitivity and specificity of the parameters obtained by CCM examination were assessed using an ROC curve (Figure 5). For the morphological change of C-fibers by CCM examination, the sensitivity, specificity, and tentative cut-off level between controls and type 1 diabetes were: 87, 76%, and

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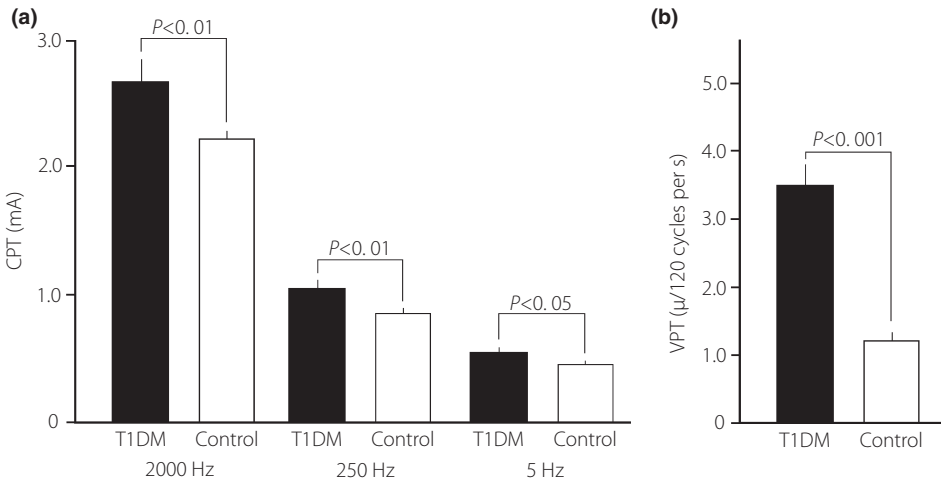

Figure 1 | (a) Comparison of current perception threshold (CPT) at three different frequencies (2000, 250, and 5 Hz) and (b) vibration perception threshold (VPT), expressed as absolute amplitude at 120 cycles/s, in controls and type 1 diabetic patients (T1DM). Data are the mean ± SEM.

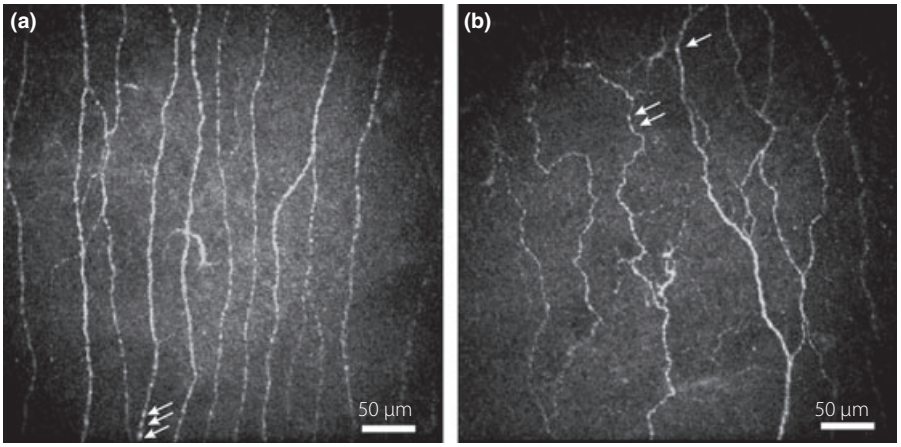

Figure 2 | (a) Confocal microscopic images of the corneal sub-basal nerve plexus in a 36-year-old healthy subject with normal current perception threshold (CPT) and vibration perception threshold (VPT); normal parameters of corneal confocal microscopy (CCM). Arrows indicate beadings. (b) A 38-year-old patient with type 1 diabetes, who did not have diabetic neuropathy, with normal CPT and increased VPT. Significantly reduced corneal nerve fiber density, corneal nerve fiver length, increased tortuosity, and reduced frequency of beading (white arrows) are shown.

28.4/mm^2, respectively, for CNFD; 87, 77%, and 11.0 mm/mm^2, respectively, for CNFL; 92, 94%, and 2.47, respectively, for tortuosity grade; and 81, 79%, and 25.2/0.1 mm, respectively, for beading frequency. The ROC curves for CNBD and CNBL overlapped the reference line and so their cut-off levels could not be determined.

There were no correlation between changes in morphological parameters on CCM examinations and BMI, duration of diabetes, or annual mean serum lipid levels (i.e. total cholesterol, high-density lipoprotein–cholesterol, or triglycerides) in diabetic patients (data not shown).

DISCUSSION

Invasive procedures, such as sural nerve[17] and skin[2] biopsies, have been used to evaluate DN morphologically. A skin biopsy is a reliable and reproducible way in which to quantify pathological changes in small fibers of various etiologies[18], including neuropathy due to diabetes mellitus and impaired glucose tolerance. However, because of the invasiveness of biopsy, this procedure is not applicable to studies comprising a large cohort (i.e. diabetic patients and non-diabetic controls). Corneal confocal microscopy is an alternative noninvasive method to quantify morphological parameters due to pathological changes[19,20] and to stratify the severity of somatic nerve neuropathy in diabetic patients[4]. Corneal confocal microscopy has been used to demonstrate the strict correlation between corneal and intra-epidermal nerve fiber (IENF) degeneration in DN[4]. The cornea is the most innervated tissue in the human body[21,22]. The corneal nerve is composed mainly of C-fibers[3], which are the earliest nerve fibers to undergo damage and

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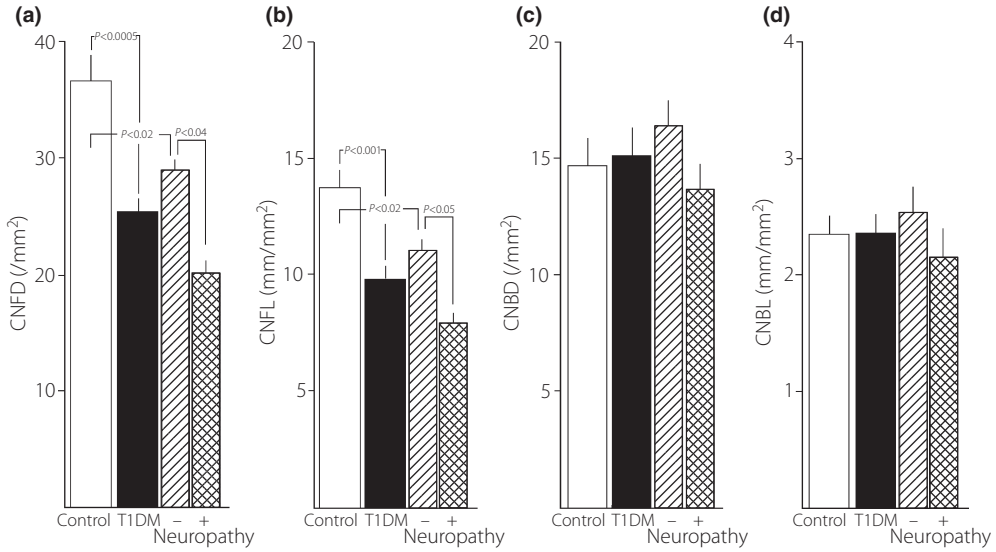

Figure 3 | Comparison of the (a) number of corneal nerve fibers (CNFD), (b) length of corneal nerve fibers (CNFL), (c) number of nerve branches (CNBD) and (d) length of nerve branches (CNBL) in controls and type 1 diabetic patients (T1DM) with (+) or without (–) diabetic neuropathy assessed by simplified diagnostic criteria proposed by the Japanese Study Group of Diabetic Neuropathy[16]. Corneal nerve fibers were visualized by corneal confocal microscopy (CCM) and analyzed using IMAGE J (Texelcraft). Values are the mean ± SEM. The significance of differences between groups was determined by ANOVA.

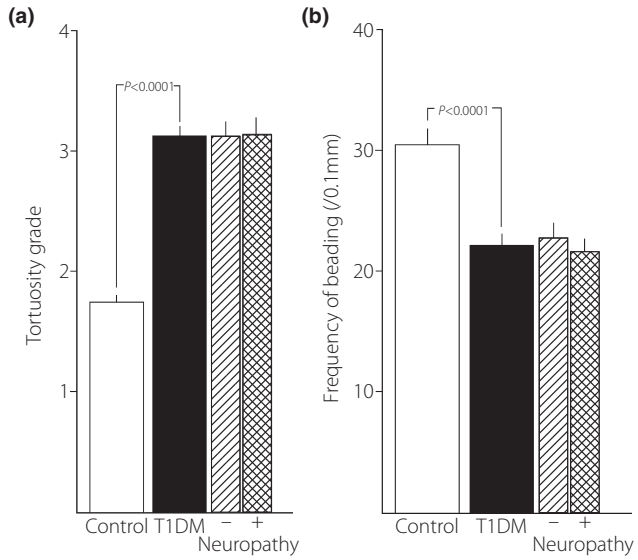

Figure 4 | Comparison of (a) corneal nerve fiber tortuosity and (b) the frequency of beadings in controls and type 1 diabetic patients (T1DM) with (+) or without (–) diabetic neuropathy assessed by simplified diagnostic criteria proposed by the Japanese Study Group of Diabetic Neuropathy[16]. Corneal nerve fibers were visualized by corneal confocal microscopy (CCM) and analyzed using the grading system of Oliveira-Soto and Efron[15] for tortuosity and IMAGE J (Texelcraft) for beading frequency. Values are the mean ± SEM. The significance of differences between groups was determined by ANOVA.

subsequent repair in patients with impaired glucose tolerance and type 2 diabetes[23]; however, this has not been confirmed in type 1 diabetes. In the present study, the intra-individual reproducibility of CCM was rated as 'good' for the assessment of CNFD and CNFL, 'fair' for tortuosity and beading frequency, and 'relatively poor' for CNBD and CNBL. The ROC curve of the morphological parameters determined by CCM revealed excellent separation between healthy controls and diabetic patients for tortuosity, good separation for CNFD, CNFL, and beading frequency, and was comparable with the sensitivity and specificity of IENF density by skin biopsy[24]. The ROC curves for CNBD and CNBL could not be confirmed. Because our patient group had a long duration of type 1 diabetes (>15 years), long enough for considerable morphological changes to occur to the corneal nerve, the excellent separation between the control and diabetic groups may be a reflection of this long period of morbidity.

Because CPT of three different frequencies and VPT in patients with type 1 diabetes increased significantly compared with healthy controls, diabetic patients already had impaired neurological function. When DN was assessed by simplified diagnostic criteria, 14 of 38 patients (36.8%) had probable DN. Corneal confocal microscopy showed reduced sub-basal CNFD and CNFL in diabetic patients compared with non-diabetic controls. Patients without DN already had reduced CNFD and CNFL, and the development of DN further impaired these parameters, which is comparable with previous reports[4,5]. These reports also noted reduced CNBD and CNBL in diabetic patients[4,5]. In contrast, CNBD and CNBL in diabetic patients, regardless of the presence or absence of DN, were similar to those of controls. The relatively poor reproducibility of CNBD and CNBL may make the comparison between healthy subjects and type 1 diabetic patients difficult. Moreover, tortuosity was

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Table 2 | Correlation between the number of corneal nerve fibers and HbA1c at the time of corneal confocal microscopy or previous annual mean values

	HbA1c at time of CCM	Mean annual HbA1c								
		−1 year	−2 years	−3 years	−4 years	−5 years	−6 years	−7 years	−8 years	−9 years	−10 years
β	−0.1	−0.018	−0.173	−0.026	−0.288	−0.133	−0.291	−0.454	−0.627	−0.445	−0.696
P value	0.958	0.937	0.435	0.899	0.184	0.608	0.292	0.018	0.003	0.02	0.011
R^2	0.109	0.119	0.126	0.113	0.145	0.197	0.155	0.315	0.437	0.449	0.456

CCM, corneal confocal microscopy.

Table 3 | Correlation between the length of corneal nerve fibers and HbA1c at the time of corneal confocal microscopy or previous annual mean values

	HbA1c at time of CCM	Mean annual HbA1c								
		−1 year	−2 years	−3 years	−4 years	−5 years	−6 years	−7 years	−8 years	−9 years	−10 years
β	−0.053	−0.102	−0.160	−0.062	−0.251	−0.184	−0.284	−0.391	−0.516	−0.415	−0.635
P value	0.776	0.648	0.468	0.765	0.250	0.406	0.255	0.042	0.019	0.029	0.002
R^2	0.117	0.119	0.134	0.169	0.125	0.192	0.176	0.299	0.358	0.442	0.440

CCM, corneal confocal microscopy.

Table 4 | Correlation between beading frequency and HbA1c at the time of corneal confocal microscopy or previous monthly or annual mean values

	HbA1c at time of CCM	Mean monthly or annual HbA1c									
		−1 month	−2 months	−3 months	−4 months	−5 months	−6 months	−7 months	−1 year	−2 years	−3 years
β	−0.351	−0.442	−0.407	−0.332	−0.185	−0.239	−0.033	−0.044	−0.390	−0.123	−0.013
P value	0.042	0.005	0.013	0.037	0.282	0.212	0.851	0.796	0.023	0.555	0.941
R^2	0.239	0.334	0.339	0.304	0.358	0.301	0.247	0.238	0.247	0.218	0.276

CCM, corneal confocal microscopy.

Table 5 | Correlation between beading frequency and systolic and diastolic blood pressure at the time of corneal confocal microscopy or previous annual mean values

	At time of CCM	Mean annual value									
		−1 year	−2 years	−3 years	−4 years	−5 years	−6 years	−7 years	−8 years	−9 years	−10 years
β											
SBP	−0.08	−0.122	−0.255	−0.481	−0.326	−0.486	−0.794	−0.285	−0.254	−0.253	−0.444
DBP	−0.204	0.008	−0.384	−0.469	−0.312	−0.481	−0.552	−0.153	−0.205	−0.179	−0.183
P value											
SBP	0.636	0.536	0.245	0.018	0.083	0.045	0.001	0.246	0.325	0.307	0.119
DBP	0.243	0.961	0.088	0.02	0.123	0.036	0.022	0.58	0.437	0.386	0.493
R^2											
SBP	0.239	0.256	0.218	0.276	0.262	0.223	0.418	0.151	0.162	0.214	0.232
DBP	0.266	0.247	0.256	0.272	0.311	0.237	0.262	0.114	0.147	0.211	0.146

CCM, corneal confocal microscopy; SBP, systolic blood pressure; DBP, diastolic blood pressure.

greater in diabetic patients than in controls, confirming the results of a previous report[25]. The presence of DN did not influence tortuosity in diabetic patients, which is similar to previous results[26]. Increased tortuosity in these patients may represent degeneration by hyperglycemia and concomitant vascular risk factors and an attempt at fiber repair[27].

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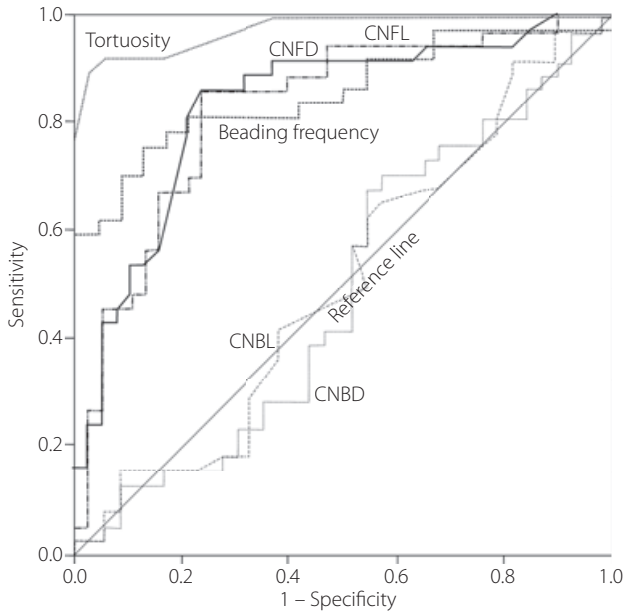

Figure 5 | Receiver operating characteristic (ROC) curves establishing cut-off levels between controls and type 1 diabetic patients for corneal nerve fiber length (CNFL), corneal nerve fiber density (CNFD), corneal nerve fiber branch density (CNBD), corneal nerve fiber branch length (CNBL), beading frequency, and tortuosity grade visualized by corneal confocal microscopy (CCM) and analyzed by IMAGE J (Texelcraft) and the grading system of Oliveira-Soto and Efron[15].

We also found a lower frequency of beading in diabetic patients compared with controls, but no significant differences between the two groups of patients with or without DN. Manually reducing the brightness of the frame to make the nerve beading more prominent could have led to the high beading frequency in the control group (30.44 ± 1.03/0.1 mm) compared with previous reports (18.2–22.2/0.1 mm)[15,28].

The influence of strict glycemic control after pancreas transplantation for nerve fiber repair in type 1 diabetes has been reported[12]. In a preliminary report, Iqbal *et al.*[29] reported that glycemic control for 2 years (HbA1c level reduced from 8.1 to 7.5%) improved CNFD, CNBD, and tortuosity (the type of diabetes was not reported). However, further clarification of the effects of antecedent glycemic control and vascular risk factors on the morphological changes detected by CCM are needed in patients with type 1 diabetes. In the present retrospective study, the mean HbA1c level at the first visit was 9.7%, which decreased to 7.3% at the time of CCM examination. Thus, we assessed the influence of the annual mean HbA1c level during the period before CCM examination on the changes in various morphological parameters of the corneal nerve fibers detected by CCM in patients with type 1 diabetes. We found an inverse correlation between CNFD and CNFL and the mean annual HbA1c levels for 7–10 years prior to the examination, suggesting that the mean HbA1c level during this period was an independent predictor of reduced CNFD and CNFL in type 1 diabetes. However, the significant correlation disappeared abruptly beyond 10 years, probably because of a decrease in the number of subjects. Importantly, corneal nerve fiber damage (reduced CNFD and CNFL, increased tortuosity, and a decrease in beading frequency) was present in patients deemed to have no evidence of DN. When corneal nerve fiber pathology was compared between patients with or without probable DN, CNFD and CNFL in patients with probable DN were further reduced without changes in other parameters. These results suggest the impact of hyperglycemia during a period earlier than 7–10 years before the examination on the development of DN by the induction of pathological changes in tortuosity and beading as a predecessor of changes in CNFD and CNFL. Hypertension and other vascular risk factors are also causative factors for DN in patients with type 1 diabetes[9]. No independent correlation was observed between CNFD and CNFL and SBP or DBP. Therefore, high blood pressure was not considered an influential factor for the changes in CNFD and CNFL in type 1 diabetes.

Electron microscopy can be used to examine thin sections obtained from freshly isolated human cornea at the level of beads, showing mitochondria, vesicles, and a few glycogen particles[21]. Beading can be clearly demonstrated along nerve fibers by CCM[22]. Increasing evidence indicates that mitochondria are intimately associated with high glucose-induced programmed cell death in neurons[30]. The hyperglycemic condition first induces swelling, followed by degeneration of the mitochondria through increased generation of reactive oxygen species, which initiates programmed cell death in neurons[31]. We found beading frequency to be inversely correlated with the concurrent HbA1c level or the level for the preceding 1–3 months and the annual mean HbA1c level for the year prior to CCM examination. Hypertension also reduces beading frequency. Blood pressure 3, 5, and 6 years prior to CCM examination seems to be an independent predictor and to have a separate time-dependent effect from hyperglycemia in reducing the frequency of beading. Because previous investigations that quantified beading frequency in normal subjects used slit-scanning confocal microscopy, and not laser scanning microscopy[32], and because different sampling and quantification processes were applied by the investigators, the comparison of beading frequency between healthy controls and diabetic patients is difficult[32].

In conclusion, CCM demonstrated reductions in CNFD, CNFL, and beading frequency and an increase in tortuosity in type 1 diabetic patients without probable DN compared with non-diabetic controls. Antecedent glycemic control had different time-dependent effects on CNFD and CNFL and beading frequency. The reduction in beading frequency prior to decreases in CNFD and CNFL may represent a causative role of mitochondria in diabetic C-fiber neuropathy. A limitation of the present study was that the data were cross-sectional and correlated with clinical factors retrospectively. A longitudinal study would provide more vigorous data concerning the relationship between the morphological anomalies on CCM and the contributing clinical factors.

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