Increased Langerhan cell density and corneal nerve damage in diabetic patients: Role of immune mechanisms in human diabetic neuropathy

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Abstract

Aim/hypothesis: Immune mechanisms have been proposed to play a role in the development of diabetic neuropathy. We employed in vivo corneal confocal microscopy (CCM) to quantify the presence and density of Langerhans cells (LCs) in relation to the extent of corneal nerve damage in Bowman’s layer of the cornea in diabetic patients.

Methods: 128 diabetic patients aged 58 ± 1 yrs with a differing severity of neuropathy based on Neuropathy Deficit Score (NDS—4.7 ± 0.28) and 26 control subjects aged 53 ± 3 yrs were examined. Subjects underwent a full neurological evaluation, evaluation of corneal sensation with non-contact corneal aesthesiometry (NCCA) and corneal nerve morphology using corneal confocal microscopy (CCM).

Results: The proportion of individuals with LCs was significantly increased in diabetic patients (73.8%) compared to control subjects (46.1%), \( P < 0.001 \). Furthermore, LC density (no/mm\(^2\)) was significantly increased in diabetic patients (17.73 ± 1.45) compared to control subjects (6.94 ± 1.58), \( P < 0.001 \) and there was a significant correlation with age (\( r = 0.162 \), \( P = 0.047 \)) and severity of neuropathy (\( r = -0.202 \), \( P = 0.02 \)). There was a progressive decrease in corneal sensation with increasing severity of neuropathy assessed using NDS in the diabetic patients (\( r = 0.414 \), \( P < 0.001 \)), branch density (\( P < 0.001 \)) and length (\( P < 0.001 \)) were significantly decreased whilst tortuosity (\( P < 0.01 \)) was increased in diabetic patients with increasing severity of diabetic neuropathy.

Conclusion: Utilising in vivo corneal confocal microscopy we have demonstrated increased LCs in diabetic patients particularly in the earlier phases of corneal nerve damage suggestive of an immune mediated contribution to corneal nerve damage in diabetes.

1. Introduction

The dominant antigen presenting cells in the cornea and ocular surface are Langerhans cells (LCs) and Dendritic cells (DCs) which are derived from the bone marrow and can stimulate both primary and secondary T and B-cell responses [1]. The function and migration of DCs and LCs have been defined in numerous studies [2]. Dendritic cells were first described in the cornea by Engelmann in 1867, and have been thought to be present only in the periphery of the cornea in conditions associated with immune dysfunction such as herpetic keratitis [3,4], pseudomonas keratitis [5] and corneal allograft rejection [6]. However, recent ex vivo studies have confirmed the presence of LCs in the epithelial layer of the cornea and DCs in the anterior stroma of the central cornea [7], and revised the belief that the cornea is immune privileged and indeed is able to respond to foreign antigens and auto antigens.

Corneal confocal microscopy provides a non-invasive means to readily demonstrate LCs in healthy subjects [8–10] and dendritic cells in patients with herpes keratitis [11] and mixed bacterial keratitis [12,13]. Additionally, a range of stimuli causing corneal irritation including hypoxia, mechanical irritation, and inflammation have also been shown to lead to maturation and migration of LCs. Thus contact lens wear increases LCs and this has been demonstrated both ex vivo [14] and using corneal confocal microscopy in vivo [15]. Another recent study using corneal confocal microscopy in subjects after removal of metal foreign bodies has also shown an increase in the number of Langerhans cells in relation to acute corneal injury [16].

Over the past several years we have employed corneal confocal microscopy (CCM) to demonstrate that corneal nerve damage is directly related to the severity of somatic neuropathy in dia-
of variation for NCCA is 5.6%.

2. Method

2.1. Neuropathy evaluation

This study was approved by Central Manchester Ethics Committee and written informed consent was obtained according to the Declaration of Helsinki. All patients underwent a detailed clinical history and examination. If the patients had neuropathy for any other reason apart from diabetes or had history of contact lens wear or corneal surgery they were excluded from the study. The neuropathy disability score (NDS) was used to grade the severity of neuropathy. NDS is based on a clinical scoring system obtained from a neurological examination which defines abnormalities of vibration perception using a tuning fork, pin prick perception and temperature perception as well as the presence or absence of ankle reflexes, producing a score ranging from 0 to 10 [32]. Based on the NDS score, diabetic patients were classified into the following four groups: NDS = 0–2: ‘no neuropathy’; NDS = 3–5: ‘mild neuropathy’; NDS = 6–8: ‘moderate neuropathy’; and NDS = 9–10: ‘severe neuropathy’.

2.2. Corneal sensitivity

Corneal sensitivity was quantified using a non-contact corneal aesthesiometer (NCCA) (Glasgow, Caledonian University, UK) which uses a puff of air on the centre of the cornea, lasting 0.9 s and exerting a force expressed in millibars (mbars) [33]. The coefficient of variation for NCCA is 5.6%.

2.3. Corneal confocal microscopy

Patients underwent examination with a Tomey Confoscan corneal confocal microscope model P4 (Erlangen, Germany). One eye of each subject was selected at random for examination. Several scans of the entire depth of the cornea were recorded by turning the fine focus of the objective lens backwards and forwards for approximately 2 min to acquire satisfactory images of all corneal layers providing en face two dimensional images with a lateral resolution of approximately 1–2 μm and final image size of 768 pixels × 576 pixels. On average, three to five high quality images of Bowman’s layer were used to quantify both nerve fibre morphology and Langerhan cell density in all patients and control subjects and the average results of all these images calculated. This layer is of particular relevance for defining neuropathic changes since it is the location of the main nerve plexus that supplies the overlying corneal epithelium. The investigator who examined the cornea with the corneal confocal microscope and undertook morphometric measurements of the images was masked with respect to the identity of the patient. For morphometric measurements of corneal nerves four parameters were quantified [17–19,34]: corneal nerve fiber density (NFD)—total number of major nerves/mm²; nerve fiber length (NFL)—total length of all nerve fibers and branches (mm/mm²); nerve branch density (NBD)—number of branches emanating from major nerve trunks/mm² and nerve fibre tortuosity (NFT) was computed using a novel mathematical approach which was termed ‘tortuosity coefficient’ (TC).

As anatomically Bowman’s layer is an otherwise acellular layer containing c-nerve fibres, it is easy to distinguish other cells in this layer of the cornea. First, the presence or absence of highly reflective cells was assessed and then the number of highly reflective cells ‘Langerhans cells’ (Fig. 1) were counted manually in each image frame from Bowman’s layer of the cornea in diabetic patients and healthy volunteers and the final density was derived as the number of cells in the area of frame assessed in square millimetres (number/mm²) [10].

2.4. Statistical analysis

SPSS 11.05.0 for Windows was used to compute the results. Analysis included descriptive and frequency statistics. One-way analysis of variance (ANOVA) with Scheffe Post hoc tests was used to study differences between means. Pearson’s test was used to analyze correlations between potentially related variables.

3. Results

3.1. Clinical details

128 predominantly Type 2 diabetic patients aged 58 ± 1 yrs with a mean duration of diabetes 15 ± 1 yrs were studied. The patients were stratified in accordance with the severity of somatic neuropathy using the NDS: none (1.29 ± 0.14, n = 42), mild (3.86 ± 0.11, n = 37), moderate (7.17 ± 0.19, n = 24) and severe (9.72 ± 0.09, n = 25) and compared to 26 age-matched control subjects (53 ± 3 yrs). The age, type of diabetes and HbA1c were well matched between the different groups with differing severity of neuropathy. Diabetes duration and as expected the NDS increased with increasing neuropathic severity (Table 1).

3.2. Langerhans cells

The proportion of individuals with LCs was significantly increased in diabetic patients (73.8%) compared to control subjects (46.1%), P = 0.001. Furthermore LC density was significantly increased in diabetic patients compared to control subjects...
Fig. 1. Images from Bowman’s layer of the cornea with highly reflective cells ‘presumably Langerhans cells’: (a) control subject, (b) diabetic patient with mild neuropathy and (c) diabetic patient with severe neuropathy.

(17.73 ± 1.45 no/mm² vs 6.94 ± 1.58 no/mm², P = 0.001) (Table 1 and Fig. 2). With regard to the severity of neuropathy, LC density was significantly increased in diabetic patients with no neuropathy (P = 0.04) and mild neuropathy (P = 0.004). However with progression of nerve damage, diabetic patients with moderate (P = 0.393) and severe (P = 0.932) neuropathy showed a reduction in the LC density which was not significantly different from control subjects (Table 1 and Fig. 2). There was no significant difference between patients with Type 1 and Type 2 diabetes, with LCs being present in 68% of patients with Type 1 and 72% of patients with Type 2 diabetes. The LC density was significantly increased in Type 1 (P = 0.03) and Type 2 (P = 0.009) diabetic patients compared to control subjects but did not differ between Type 1 (18.93 ± 3.85 no/mm²) and Type 2 (17.48 ± 1.57 no/mm²) diabetic patients.

3.3. Corneal sensation

There was a progressive increase in the NCCA threshold indicative of a decrease in corneal sensation with increasing severity of neuropathy in diabetic patients (Table 1).

3.4. Corneal nerve fibres

Corneal nerve fibre density (P < 0.001), branch density (P < 0.001) and length (P < 0.001) were significantly decreased whilst tortuosity (P < 0.01) was increased in diabetic patients with increasing severity of neuropathy (Table 1).

3.5. Correlations

Despite the reduction in LCs in those with more severe neuropathy there was a significant correlation between the density of LCs and severity of neuropathy assessed by NDS (r = −0.02, P = 0.02). There was also a significant correlation between LC density and age (r = 0.162, P = 0.04). There was no significant correlation between LC density and duration of diabetes (r = −0.082, P = 0.36), or HbA1c (r = 0.145, P = 0.09). Gender had no effect on the number of LCs. In this group of patients, there was also a significant correlation between NDS and NFD (r = −0.341, P = 0.000), NBD (r = −0.311, P = 0.000), NFL (r = −0.430, P = 0.000), NFT (r = 0.217, P = 0.02) and NCCA (r = 0.414, P = 0.000). However, there was no significant correlation between LC density and corneal sensitivity or corneal nerve morphology.

4. Discussion

The key finding is that the density of LCs was significantly increased in diabetic patients, particularly in those with no or mild neuropathy which then decreased in those with moderate and severe neuropathy, though still remained above control values. This suggests that LCs may play a role in the early phase of nerve damage and then this is maintained by other established mechanisms [28]. However, as there was no correlation between glycaemic control (HbA1c) and LC density this suggests that the increase in these cells may be a hyperglycemia independent mech-
animal. Although one might expect for there to be greater immune mediated involvement in patients with Type 1 diabetes, a sub analysis between patients with Type 1 and Type 2 diabetes showed no significant difference in LC density. We also confirm in a larger analysis between patients with Type 1 and Type 2 diabetes showed no significant difference in LC density. We also confirm in a larger

**Table 1**

<table>
<thead>
<tr>
<th></th>
<th>(a)</th>
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<th>(c)</th>
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<td>4/20</td>
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<td>HbA1c (%)</td>
<td>~ 5.8</td>
<td>8.16 ± 0.14</td>
<td>7.8 ± 0.19</td>
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<td>NDS (0–10)</td>
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<td>2.19 ± 0.45</td>
<td>3.86 ± 0.11</td>
<td>7.17 ± 0.19</td>
<td>9.72 ± 0.09</td>
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<td>LC (presence/absence)</td>
<td>488 (12/14)</td>
<td>725 (93/35)</td>
<td>735 (31/11)</td>
<td>836 (31/6)</td>
<td>796 (19/5)</td>
<td>488 (12/13)</td>
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<td>LC density (mm²/m²)</td>
<td>6.94 ± 1.58</td>
<td>17.5 ± 4.15</td>
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<td>NCCA (mbar)</td>
<td>0.72 ± 0.02</td>
<td>1.48 ± 0.10</td>
<td>1.15 ± 0.07</td>
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<td>NFD (mm²/mm²)</td>
<td>40.78 ± 2.64</td>
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<td>NBD (mm²/mm²)</td>
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<td>NFT (TC)</td>
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<td>25.69 ± 1.05</td>
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<td>24.38 ± 1.62</td>
<td>26.86 ± 2.09</td>
<td>31.23 ± 2.34</td>
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Data are expressed as mean ± SEM for diabetic patients and control subjects. Abbreviations: LCs: Langerhans cells; NDS: neuropathy deficit score; NCCA: non-contact corneal aesthesiometer; NFD: nerve fibre density; NBD: nerve branch density; NF: nerve fibre length; NFT: nerve fibre tortuosity; TC: tortuosity coefficient.

1 Statistically significant difference between diabetic patients and controls using ANOVA: P < 0.001.

2 Statistically significant differences between patients and controls using ANOVA: P < 0.01.

3 Post hoc results show a significant difference from control subjects and diabetic patients with different severity of neuropathy: P < 0.05.

4 Post hoc results show a significant difference from control subjects and diabetic patients with different severity of neuropathy: P < 0.01.

5 Post hoc results show a significant difference from control subjects and diabetic patients with different severity of neuropathy: P < 0.001.

6 Post hoc results show a significant difference from control subjects and diabetic patients with different severity of neuropathy: P < 0.001.


