Corneal Subbasal Nerves Changes in Patients with Diabetic Retinopathy: An In Vivo Confocal Study

Stefano De Cilla, Stefano Ranno, Elisa Carini, Paolo Fogagnolo, Gaia Ceresara, Nicola Orzalesi, and Luca M. Rossetti

PURPOSE. To study the subbasal corneal plexus (SCP) in patients with diabetic retinopathy (DR) treated or nontreated with panretinal Argon laser photocoagulation (ALP).

METHOD. Fifty consecutive patients with DR and 50 age- and sex-matched normal control subjects were examined with retinal tomography by a masked evaluator. The following subbasal plexus nerves parameters were considered: number per frame, tortuosity, and reflectivity. Diabetic patients were divided into two groups, according to the presence of proliferative versus nonproliferative retinopathy, according to the Early Treatment of Diabetic Retinopathy Study (ETDRS) classification.

RESULTS. The number of fibers per frame and reflectivity were significantly lower in diabetic patients compared with control subjects (2.4 ± 1 vs. 2.9 ± 0.8, P = 0.01 and 2.3 ± 0.9 vs. 2.6 ± 0.9, P = 0.04, respectively). Tortuosity was significantly higher in diabetic patients (2.5 ± 0.9 vs. 2.0 ± 0.8, P = 0.002). Number per frame and reflectivity were significantly lower in diabetic patients with proliferative diabetic retinopathy (PDR; respectively, 2.0 ± 0.9 vs. 2.9 ± 0.9, P = 0.001, and 2.0 ± 0.8 vs. 2.6 ± 0.7, P = 0.003). Tortuosity was significantly higher in the PDR group (2.2 ± 0.8 vs. 2.8 ± 0.9, P = 0.008). The PDR group treated with ALP had significantly lower subbasal nerves number compared with the nontreated group (P = 0.01).

CONCLUSIONS. DR may induce substantial changes in the SCP. There is a difference between proliferative and nonproliferative retinopathy and in the former group between ALP treated and nontreated patients. (Invest Ophthalmol Vis Sci. 2009;50: 5155–5158) DOI:10.1167/iovs.09-3384

Diabetes is one of the leading causes of vision loss in industrialized countries. Ultrastructural studies have shown that corneal changes may occur in up to 70% of diabetic patients as a result of tissue overglycation,1,2 and polyols deposition in the epithelial basement membrane.3,4 These changes, which include thickening of the basement membrane,5,6 reduction in the number of hemidesmosomes,7 abnormalities of epithelial and endothelial cells,8,9 and changes in innervation,10,11 are commonly associated with superficial punctate epitheliopathy, persistent epithelial erosions, corneal edema, and hypoeesthesia.12 Confocal microscopy (CM) is a novel diagnostic technique that provides noninvasive optical sectioning of the tissues of the anterior segment of the eye and allows high-magnification imaging of the corneal epithelium, Bowman’s membrane, stromal keratocytes, and nerves, as well as the corneal endothelium. In a recent study, it was suggested that changes in the subbasal corneal plexus, studied in detail with CM, may correlate with the clinical involvement of the cornea and with diabetic neuropathy. In particular, patients with proliferative diabetic retinopathy (PDR) show more pronounced nerve changes than do patients without diabetic retinopathy (DR).11 The purpose of this study was to investigate with CM the subbasal corneal plexus involvement in different stages of DR, including patients treated with panretinal laser photocoagulation.

MATERIALS AND METHODS

Patients

This study was conducted at the Eye Clinic of the University of Milan, San Paolo Hospital. It was approved by the local Ethics Committee and adhered to the tenets of the Declaration of Helsinki.

All noninsulin-dependent diabetic patients referred to the Retina Service from May 2006 and July 2007 were considered for the study. Inclusion criteria were represented by noninsulin dependent diabetes, diagnosis of nonproliferative diabetic retinopathy (NPDR) or proliferative diabetic retinopathy (PDR) according to the ETDRS (Early Treatment of Diabetic Retinopathy Study), willingness to participate in the study, and a signed informed consent. Exclusion criteria were other retinal disorders, corneal changes of any kind, media opacities, and history of ocular surgery other than uncomplicated cataract.

Fifty consecutive patients who fulfilled inclusion and exclusion criteria and 50 age- and sex-matched normal subjects were enrolled in the study.

Study Procedures

A medical history was obtained from all the subjects, with particular attention to age at onset of diabetes, serum levels of glucose over the past 2 years (patients were invited to bring to the visit all their previous blood analyses). All the subjects underwent a complete ophthalmic examination including anterior segment biomicroscopy and fundus examination, refraction, and measurement of best corrected visual acuity (BCVA) by means of ETDRS chart and procedure.

Based on fluorescein angiography (Heidelberg Retina Angiograph [HRA]; Heidelberg Engineering, Dossenheim, Germany) diabetic patients were divided into two groups: NPDR (n = 25) and PDR (n = 27) according to the ETDRS classification. Those with PDR were further divided in those who had previously undergone panretinal argon laser photocoagulation (ALP-PDR, n = 14) or not (NALP-PDR, n = 13). The panretinal argon laser photocoagulation was performed in four or five sessions (mean number of spots 1690; range 1256–2125; using 300–500-μm spot size; and 300–500 mW, 0.2 seconds). There was no statistically significant difference in the duration of diabetes and fasting blood glucose among the subgroups of diabetic patients (NPDR versus PDR and NALP-PDR versus ALP-PDR).

In vivo laser scanning confocal microscopy (LSCM) of the cornea was performed (Heidelberg Retina Tomograph 2: Rostock Cornea Module [HRT2-RCM]; Heidelberg Engineering).
Data were collected for both eyes, and one eye of each patient was chosen at random (unless only one eye met the inclusion criteria) for analysis.

In Vivo LSCM of the Cornea

The HRT, which was first designed to evaluate the optic nerve head in glaucoma, in the second version was endowed with a lens system, known as the RCM,\(^1\) allowing an in vivo confocal study of the cornea layers. The laser source used in the RCM is a diode laser with a wavelength of 670 nm. The acquired two-dimensional images have a definition of \(384 \times 384\) pixels over an area of \(400 \mu\text{m}^2\) with lateral digital resolution of 1 \(\mu\text{m/pixel}\) and a depth resolution of 2 \(\mu\text{m/pixel}\).

Data Collection

After administration of 1 drop of 0.4% oxybuprocaine (Novesine; MSD-Chibret, Paris, France) and 1 drop of a lubricant gel, 0.2% carbomer (Lacrigel, carbomer 980 NF; Europhtha, Monaco) the patient was asked to fixate a small, bright-red light as the examination was performed in the contralateral eye. Correct alignment and contact with the cornea were monitored using the images captured by a camera tangential to the eye. The distance from the cornea to the microscope is kept stable using a single-use contact element in sterile packaging (TomoCap; Heidelberg Engineering). The cap is thin with a planar contact surface made from PMMA and is coupled optically to the lens with the aid of a gel.

A scan of the full thickness of the cornea was performed along the optical axis and to prevent biased acquisitions, no tangential movements were allowed. The examination took approximately 3 minutes per eye and approximately 120 images were collected. Only images of the center of the cornea were collected. The best-focused subbasal plexus image was considered for the analysis.

The following parameters of the subbasal plexus nerves were considered: number per frame (defined as the sum of the nerve branches observed in a frame, which may be considered highly correlated to the density of nerve fibers\(^1\)), tortuosity, fiber reflectivity, and background reflectivity. Tortuosity and fiber reflectivity were evaluated by comparison with the reference images, according to the method proposed by Oliveira-Soto\(^1\) and graded from 0 to 4 as in previous published studies.\(^1\)\(^-\)\(^1\)\(^8\)

A grading ranging (low, 1; moderate, 2; high, 3) was used to evaluate also the background reflectivity of the Bowman membrane that was found to be more reflective in diabetic subjects,\(^1\)\(^9\) to evaluate if it could affect the evaluation of the plexus reflectivity (Fig. 1).

Agreement

All study examinations were performed by a single masked expert operator (SR). Before the study began, the reproducibility of data was tested by using the following procedure. The evaluator collected five scans of the plexus of a volunteer along the corneal axis, during the same day, at intervals of 60 minutes and at the end of each acquisition, he selected the best-focused image of the plexus. Masking was obtained by an independent investigator mixing these 5 images with 10 control images, and the operator was asked to grade all images considering number, tortuosity, and reflectivity of fibers. For each parameter, agreement of the five images was defined in the presence of values within \(\pm 1\) from the mean value, this cutoff value being derived from previous studies on corneal CM.\(^1\)\(^4\) Intraobserver agreement for image scores were calculated using the intraclass correlation coefficient (ICC): ICC = \(s^2/(a^2 + s^2)\), where \(a^2 = QM(e)\), or intraclass variability: \(s^2 = (QM(a) - QM(c))/n\) or (intraclass - intraclass variability)/number of classes.\(^1\)\(^9\)\(^-\)\(^1\)\(^1\) Agreement was defined according to the guidelines proposed by Landis and Koch\(^1\)\(^1\): 0, chance agreement; 0.01–0.19, poor agreement; 0.20–0.39, fair agreement; 0.40–0.59, moderate agreement; 0.60–0.79, substantial agreement; and 0.80–1.00, almost perfect agreement. For our series of data, agreement was 0.70 for tortuosity, 0.75 for reflectivity, and 0.9 for number of fibers.

Statistical Analysis

Intergroup differences were evaluated by means of \(t\)-test for unpaired data. Differences with \(P < 0.05\) were deemed statistically significant (SPSS, ver. 15.0; SPSS Inc., Chicago, IL).

Outcomes of the Study

Primary outcome of the study was the change in the subbasal nerves of the cornea of patients with type 2 diabetes and DR compared with non-diabetic control subjects. Secondary outcomes were subbasal nerves changes in the different subgroups of the study (NPDR, ALP-PDR, and NALP-PDR).

Sample Size Calculation

Formal sample size was calculated to assess the difference in the subbasal plexus between normal and diabetic patients with DR. For this purpose, the number of fibers per frame was chosen, as this measure is more objective than reflectivity and tortuosity. From previous studies, estimated \(\Delta\) was set at 1.5 fibers per frame and \(\sigma\) at 3.4 fibers per frame (the mean value calculated from 4.6 fibers per frame of normal subjects\(^1\)\(^4\) and 2.2 fibers per frame of diabetic patients\(^1\)\(^1\)).

According to the formula

\[
n = \frac{(\Phi^{-1}(\alpha/2) + \Phi^{-1}(\beta))^2 \sigma^2}{\Delta^2}
\]

where \(\alpha = 0.05\) and \(1 - \beta = 0.90\), a sample of 50 patients was needed for each group, to ensure a 95% chance of detecting a difference between the two groups.

Results

The main characteristics of the patients are summarized in Table 1. Mean age and sex were not significantly different in the diabetic and control groups (\(P = 0.23\), \(t\)-test, and \(P = 0.80\), \(\chi^2\), respectively). The same for the duration of diabetes in the different groups: NPDR versus PDR, \(P = 0.9\); ALP-PDR versus NALP-PDR, \(P = 0.8\) (Table 1). The results of the study are summarized in Table 2. The number of fibers (2.4 ± 1 vs. 2.9 ± 0.8, \(P = 0.01\)) and reflectivity of fibers (2.3 ± 0.9 vs. 2.6 ± 0.9, \(P = 0.04\)) of the subbasal plexus were significantly lower in diabetic patients than in control subjects, whereas tortuosity was significantly higher (2.0 ± 0.8 vs. 2.5 ± 0.9, \(P = 0.002\)).

![Figure 1](image-url)  
**Figure 1.** Grading ranging (low, 1; moderate, 2; high, 3) used to evaluate the background reflectivity of Bowman’s membrane.
**TABLE 1.** Demographics of Study Participants

<table>
<thead>
<tr>
<th>Normal Subjects</th>
<th>Diabetic Patients</th>
<th>B.1 NPDR</th>
<th>B.2 PDR</th>
<th>B.2.1 ALP</th>
<th>B.2.2 NALP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subjects, n</td>
<td>50</td>
<td>50</td>
<td>23</td>
<td>27</td>
<td>14</td>
</tr>
<tr>
<td>Age, y</td>
<td>62.5 ± 8</td>
<td>62.6 ± 6</td>
<td>61.5 ± 6</td>
<td>63.5 ± 6</td>
<td>64.5 ± 6</td>
</tr>
<tr>
<td>Sex (F, M)</td>
<td>27, 23</td>
<td>27, 23</td>
<td>13, 10</td>
<td>14, 13</td>
<td>8, 6</td>
</tr>
<tr>
<td>Refraction, D</td>
<td>1.30 ± 1.4</td>
<td>1.3 ± 1.2</td>
<td>1.10 ± 1.6</td>
<td>1.4 ± 1.1</td>
<td>1.5 ± 1.1</td>
</tr>
<tr>
<td>IOP, mm Hg</td>
<td>18.3 ± 2.4</td>
<td>17.1 ± 2</td>
<td>16.9 ± 2.1</td>
<td>17.3 ± 1.9</td>
<td>16.4 ± 2</td>
</tr>
<tr>
<td>Duration of disease, y</td>
<td>—</td>
<td>15 ± 7</td>
<td>14.8 ± 8</td>
<td>15.1 ± 6</td>
<td>15.4 ± 6</td>
</tr>
<tr>
<td>Fasting blood glucose, mg/100 mL</td>
<td>—</td>
<td>146.4 ± 27</td>
<td>138.6 ± 28</td>
<td>152.5 ± 26</td>
<td>153.6 ± 26</td>
</tr>
</tbody>
</table>

Data are the mean ± SD.

In the subgroups of diabetic patients with or without proliferative retinopathy, relevant differences were found in number (2.0 ± 0.9 vs. 2.9 ± 0.9, P = 0.001, respectively), tortuosity (2.8 ± 0.9 vs. 2.2 ± 0.8, P = 0.008, respectively) and reflectivity (2.0 ± 0.8 vs. 2.6 ± 0.7, P = 0.003, respectively).

The ALP-PDR group showed a lower number of subbasal nerves, higher tortuosity, and lower reflectivity than did the NALP-PDR group (t-test, P = 0.01, 0.03, and 0.04, respectively; Fig. 2). Background reflectivity of Bowman’s membrane was higher in diabetic patients than in control subjects (P = 0.03); furthermore, it was significantly higher in the PDR than in the NPDR (P = 0.03) group, and no statistically significant difference was found between the ALP-PDR and NALP-PDR groups (P = 0.9).

Finally, no correlation was found between number, tortuosity, reflectivity of fibers and background reflectivity and the duration of the disease (R² = 0.00 in all cases) or fasting blood glucose levels (R² = 0.00, 0.01, 0.00, and 0.01, respectively).

**DISCUSSION**

A rich network of nerves known as the subbasal nerve plexus lies between the basal epithelium of the cornea and Bowman’s membrane. This layer appears with the CM as a dense neural plexus characterized by tortuous and thin, beaded nerve fibers, with an homogeneous reflectivity that is distinct from the background. Several qualitative and quantitative studies have been published documenting the appearance of the normal and pathologic subbasal nerve plexus as viewed with the CM using earlier generation instruments, as the TSCM (tandem scanning confocal microscope) and the SSCM (slit scanning CM). The higher image brightness and contrast of the LSCM (scanning confocal microscope) and the SSCM using earlier generation instruments, as the TSCM (tandem scanning confocal microscope) and the SSCM (slit scanning CM). The higher image brightness and contrast of the LSCM offers considerably better images than those obtained with a conventional white-light confocal microscope. Most studies on the involvement of corneal subbasal nerve plexus in diabetes have been performed with TSCM and SSCM, showing a marked reduction of subbasal nerve fibers with increasing severity of DR, and neuropathy. According to Rosenberg et al., this reduction would also explain the loss of corneal sensitivity in these patients. Kallinikos et al. demonstrated that tortuosity of subbasal nerves correlated significantly with the severity of neuropathy. Morishige et al. found a correlation between the corneal light-scattering index and stage of DR, suggesting that tissue reflectivity at the level of the basement membrane increases with increasing severity of DR, but they did not consider nerve morphology. In no study was the effect of panretinal photocoagulation of PDR on the morphology of subbasal nerve plexus analyzed. Using LSCM, we confirmed the results of previous investigations showing significant changes in the subbasal nerve plexus (decreased nerve number per frame, increased tortuosity) in DR compared with normal subjects. Changes were more pronounced in patients with PDR than in those with DR.

In the present study, however, a further statistically significant difference was found within the PDR group, as the patients who had undergone ALP exhibited more marked changes in comparison to those with nontreated PDR. Of note, the duration of disease and fasting blood glucose levels did not correlate with the alterations of subbasal corneal plexus in any subgroup of study patients. Our findings also confirm that fiber reflectivity may be influenced by background reflectivity (Bowman’s layer reflectivity), which increases in diabetic patients compared with normal subjects and in PDR compared with NPDR, but fiber reflectivity in the ALP-PDR and NALP-PDR groups was not influenced by Bowman’s layer reflectivity (P = 0.9).

It is well known that panretinal ALP may be followed by clinical changes at the level of the cornea, such as corneal hypoesthesia and, in extreme cases, dilated pupil and loss of

**TABLE 2. Features of the Subbasal Plexus Nerves in the Study Population**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Number</th>
<th>Tortuosity</th>
<th>Reflectivity</th>
<th>Background</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Normal subjects</td>
<td>2.9 ± 0.8</td>
<td>2.6 ± 0.9</td>
<td>1.9 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>B. Diabetic patients</td>
<td>2.4 ± 1.1</td>
<td>2.3 ± 0.9</td>
<td>2.3 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>B.1. NPDR</td>
<td>2.9 ± 0.9</td>
<td>2.6 ± 0.7</td>
<td>2.1 ± 0.8</td>
<td></td>
</tr>
<tr>
<td>B.2. PDR</td>
<td>2.0 ± 0.9</td>
<td>2.0 ± 0.8</td>
<td>2.5 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>B.2.i. ALP-PDR</td>
<td>1.6 ± 0.7</td>
<td>1.6 ± 0.7</td>
<td>2.5 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>B.2.ii. NALP-PDR</td>
<td>2.4 ± 0.9</td>
<td>2.3 ± 0.8</td>
<td>2.5 ± 0.5</td>
<td></td>
</tr>
</tbody>
</table>

Probabilities

| A vs. B | 0.01 | 0.002 | 0.04 | 0.03 |
| 1 vs. 2 | 0.001 | 0.008 | 0.003 | 0.03 |
| i vs. ii | 0.01 | 0.03 | 0.04 | 0.9 |
| A vs. i | 0.00005 | 0.00007 | 0.0003 | 0.04 |
| A vs. ii | 0.1 | 0.06 | 0.05 | 0.05 |

Data are the mean ± SD.
accommodation,28,29 which have been associated with damage to the short ciliary nerves. Schiodte29 suggested that damage to the short ciliary nerves by the argon laser occurs as they cross the suprachoroidal space having left the ciliary ganglion on their way to the ciliary body and iris. He also suggested that, as pain arises when the laser burn hits the ciliary nerves, local anesthesia should be avoided to better monitor laser-induced damage during panretinal photocoagulation. In previous studies the alterations in the subbasal nerveplexus found with CM were referred to DR and PDR without taking into consideration that in the latter group significantly more damage could occur in patients with ALP-PDR. As far as we know, this is the first study showing, by means of LSCM, morphologic changes in the subbasal nerveplexus of patients with ALP-PDR, which may justify the above-mentioned clinical changes found in this group of patients. As a clinical consequence of our findings, we justify the above-mentioned clinical changes found in this group of patients. As a clinical consequence of our findings, we justify the above-mentioned clinical changes found in this group of patients.

References


