Evaluation of conjunctival epithelium of filtering blebs by impression cytology

M.J. Muniesa a, b, *, S. González b, c, P. Buetas b, c, S. López b, c, C. Sánchez a, b, X. Matias-Guiu b, c

a Departamento de Oftalmología, Hospital Universitari Arnau de Vilanova, Lérida, Spain
b Institut de Recerca Biomèdica de Lleida (IRB Lleida), Lérida, Spain
c Departamento de Patología y Genética Molecular, Hospital Universitari Arnau de Vilanova, Lérida, Spain

ABSTRACT

Purpose: To study the ocular surface in filtering blebs using impression cytology, comparing the bleb side and areas outside the bleb edges.

Methods: Twelve filtering blebs of 8 patients were included: 4 cases of trabeculectomy without mitomycin C (MMC), 6 cases of trabeculectomy with MMC, and 2 cases of non-penetrating glaucoma surgery. Impression cytology specimens were taken from filtering blebs as well as outside the bleb area. A classification scale from 0 to 3 was used to describe the distribution of epithelial cells and the density of goblet cells. Grade 0 indicated cohesive epithelial cells and abundant goblet cells; and the grade 3 indicated loss of epithelial cohesion and absence of goblet cells.

Results: The mean grade of cytology in filtering blebs was 2.4 ± 0.9, and in the outside bleb area of 0.8 ± 0.3 (p < .001). These differences were independent of the use of MMC (p = .48). The large majority (83%) of filtering blebs showed a decrease in epithelial cohesion and absence of goblet cells. Outside the bleb area, 100% of the cases had cohesive epithelial cells with different grades of goblet cells.

Conclusion: The conjunctival epithelium overlying the filtering blebs showed significant changes that consisted of increased intercellular spaces and loss of goblet cells. These increased intercellular spaces could explain the trans-epithelial pathway of aqueous humor. The least amount of mucin due to loss of goblet cells could contribute to increase the risk of infection in filtering blebs.

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Estudio del epitelio conjuntival de las ampollas filtrantes mediante citología de impresión

RESUMEN

Objetivo: Estudiar la superficie conjuntival de ampollas filtrantes mediante citología de impresión y comparar los resultados con el área de conjuntiva circundante.

Método: Se incluyeron 12 ampollas de 8 pacientes; 4 tras trabeculectomía sin mitomicina C (MMC), 6 tras trabeculectomía con MMC y 2 tras esclerectomía profunda no perforante sin MMC. Se realizaron citologías de impresión conjuntival de las ampollas filtrantes y del área circundante. Se usó una clasificación de 0 a 3 para describir el grado de cohesión de las células epiteliales y la densidad de células caliciformes, siendo el grado 0 indicador de células epiteliales en placas y abundantes células caliciformes y el grado 3, de pérdida de cohesión del epitelio y ausencia de células caliciformes.

Resultados: El grado medio de la citología de impresión en las ampollas de filtración fue de 2,4 ± 0,9 y en el área circundante de 0,8 ± 0,3 (p < 0,001). Estas diferencias fueron independientes del uso de MMC (p = 0,48). El 83% de las ampollas presentaban células epiteliales cilíndricas dehiscentes y ausencia de células caliciformes. En la conjuntiva circundante, el 100% de los casos presentaban células epiteliales en placas con distintos grados de células caliciformes.

Conclusión: El epitelio de las ampollas filtrantes presentó cambios significativos que consistían en aumento de los espacios intercelulares y pérdida de células caliciformes. Estos espacios intercelulares aumentados podrían explicar un paso transepitelial del humor acuoso. La pérdida de mucina secundaria a la pérdida de células caliciformes contribuiría al riesgo de infección de las ampollas filtrantes.

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Introduction

Trabeculectomy is the most utilized filtering procedure for treating glaucoma. After the formation of a filtering bleb, aqueous humor reabsorption through the venous system as well as transconjunctival exit pathway has been demonstrated.\(^1\) After nonperforating filtering surgeries such as nonperforating deep sclerectomy (NPDS), filtering blebs are flatter and more diffuse than those obtained after trabeculectomy.\(^2\) The exact mechanism whereby aqueous humor can be drained through the trans-conjunctival pathway is not known.\(^3\) The presence of microcysts which can be observed with biomicroscopy and confocal microscope associate to a positive predictive factor for bleb performance.\(^4\) Said microcysts could be exit channels for the aqueous humor.\(^5\) Studying the conjunctiva of filtering blebs helps to understand the exit mechanisms of the aqueous humor, the effects of NT fibrotic agents on these blebs and the susceptibility thereof to exhibit late leaks and infections. A number of studies have described the histopathology of the epithelium of filtering blebs by means of incisional biopsy.\(^6-9\) Observed intercellular epithelial spaces evidence that aqueous humor could follow a transepithelial pathway.\(^3\) In contrast with biopsy, an invasive diagnostic technique, impression cytology is a noninvasive diagnostic technique that enables an anatomopathological study of the outermost epithelial layer of a complete conjunctival area. By means of this technique, goblet cells can be quantified and the degree of conjunctival epithelial differentiation can be assessed, in addition to detecting the presence of atypia and inflammatory cells.\(^10\) However, only 2 published papers have utilized impression cytology samples to study filtering blebs.\(^11\) One of said studies utilized conventional staining with periodic acid-Schiff (PAS).\(^11\)

The objective of this study consists in describing cytological findings on filtering bleb epithelium, focused on the changes observed in non-secretory epithelial cells and goblet cells, and comparing these with the condition of the conjunctiva beyond the limits of the bleb.

Material and methods

The study included 12 eyes of 8 different patients who underwent surgery for chronic open angle glaucoma at the Arnau de Vilanova University Hospital of Lérida (Spain): 4 cases after trabeculectomy without mitomycin C (MMC), 6 after trabeculectomy with MMC and 2 cases after nonperforating deep sclerectomy without MMC. All the eyes included in the study had an IOP of ≤21 mm Hg and no signs of bleb infection. None of the patient histories or ophthalmological examinations showed evidence of ocular surface disease. Data on age, sex, type of filtering surgery, use of MMC, use of topical hypotensor treatment and evolution time since surgery up to sample taking of each patient were recorded.

All the filtering surgeries were performed by the same surgeon (MMJ). For trabeculectomy, a fornix-base conjunctival flap of 6–8 mm was made. After cautery of the sclera, a 4 mm × 4 mm flap of half the scleral thickness was dissected. In the cases in which MMC was used, it was applied impregnated in a surgical sponge at a concentration of 0.2 mg/ml...
Table 1 - Classification of cytological findings per amount of goblet cells and degree of cohesion of distribution of non-secretory epithelium.

<table>
<thead>
<tr>
<th>Amount of goblet cells</th>
<th>Distribution of epithelial cells</th>
<th>Grade 0</th>
<th>Grade 1</th>
<th>Grade 2</th>
<th>Grade 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abundant &gt;500/mm^2</td>
<td>Plates</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Moderate 350–500</td>
<td>Plates &gt; isolated</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low 100–350</td>
<td>Isolated &gt; plates</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent &lt;100</td>
<td>Isolated</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

during 2 min below the conjunctiva and the scleral flap, followed by profuse irrigation with saline solution. The scleral flap was sutured with 4 loose stitches of 10-0 vicryl and the conjunctiva with loose stitches of 8-0 vicryl. For NFDS, an 8 mm fornix-based conjunctival flap was made and the sclera was cauterized. A superficial flap of 4 × 4 covering one third of the scleral thickness and a deep 3 × 3 flap were dissected, leaving 10% of scleral thickness. After obtaining an intact trabeculodescemet membrane, Schlemm’s canal was peeled and the deep flap was excised. The superficial flap was sutured with 2 loose 10-0 nylon stitches and the conjunctiva stitched with loose vicryl 8-0 stitches. Post-surgery medication included tobramycin 3 mg/ml–dexamethasone 1 mg/ml (Tobradex eyedrops in suspension, Alcon Cusi, S.A., Barcelona, Spain), adding 1% atropine for trabeculectomy cases.

Conjunctival impression cytology was carried out for the filtering blebs and the surrounding bulbar conjunctival area of all the blebs. Millipore HAWP 304 (Millipore Ibérica S.A.U, Madrid, Spain) cellulose acetate paper was used to collect samples of the outermost ocular surface layer. Impression cytology was carried out with topical anesthesia consisting in one drop of 0.4% oxibuprocaine–0.1% tetracaine (Colircusi Anestésico Doble, Alcon Cusi S.A., Barcelona, Spain), instilled in ocular fundus. The filter paper was placed over the conjunctiva to be studied exerting pressure during 5 s. The samples were fixed in 95% ethanol, subsequently staining with PAS and hematoxylin. Cellular morphology was reviewed with an optical microscope at 40× by a specialized technician who was not informed about the origin of the samples. The images were taken with a digital camera.

Cytology results were rated taking into account the amount of goblet cells and the degree of epithelial cell cohesion in values from 0 to 3 (Table 1) (Figs. 1 and 2). For classifying goblet cell density, Nelson classification parameters were used. In addition, the presence of metaplasia was assessed according to the criteria utilized in the Nelson classification for assessing cell differentiation changes (Table 2).

Table 2 - Nelson classification system.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Grade 0</th>
<th>Grade 1</th>
<th>Grade 2</th>
<th>Grade 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell size</td>
<td>Small</td>
<td>Small</td>
<td>Increased</td>
<td>Increased</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>Eosinophile</td>
<td>Eosinophile</td>
<td>Variable</td>
<td>Basophile</td>
</tr>
<tr>
<td>Nuclei</td>
<td>Large</td>
<td>Small</td>
<td>Small</td>
<td>Pyknotic/absent</td>
</tr>
<tr>
<td>Ratio: nucleus/cytoplasm</td>
<td>1:2</td>
<td>1:3</td>
<td>1:4:1:5</td>
<td>1:6</td>
</tr>
<tr>
<td>Goblet cells/mm^2</td>
<td>&gt;500</td>
<td>350–500</td>
<td>100–350</td>
<td>&lt;100</td>
</tr>
<tr>
<td>Goblet cell cytoplasm</td>
<td>PAS+++</td>
<td>PAS+++</td>
<td>PAS++</td>
<td>PAS–</td>
</tr>
</tbody>
</table>

PAS: Schiff-periodic acid stain.
Source: Nelson.

Fig. 1 – Conjunctival impression cytology; PAS-hematoxylin staining, 40×: grade 0 (4×).

Fig. 2 – Conjunctival impression cytology; PAS-hematoxylin staining, 40×: grade 3 (10×).
Statistics

Mean values and standard deviation of age and cytology rating of the blebs and surrounding conjunctival area were calculated. Cytology variables for each patient were compared with the T for student test on the sample. The difference of these variables and the distribution of dichotomic variables such as the use of MMC were compared to establish relationships with this difference. The Levene test was utilized for variance comparison and the T for student test for comparing the mean values. As the influencing variables are dichotomic, this type of analysis was used to determine whether the presence or absence thereof influenced the difference between cytological variables. A statistically significant difference was taken to be p<0.05. The analysis was carried out with SPSS version 16.0 (SPSS Inc., Chicago, IL, USA).

Results

Overall, 12 eyes of 8 patients (4 males and 4 females) intervened for chronic open angle glaucoma were included in the study, 6 after trabeculectomy with MMC, 4 after trabeculectomy without MMC and 2 after nonperforating deep sclerectomy without MMC. The mean age of patients was 77±3 years with a range from 69 to 81. Table 3 shows the variables studied for each patient.

In 83% of cases (10 eyes) goblet cell number diminished and epithelial cohesion reduced with increase of intercellular space in filtering blebs. No sign of atypia or presence of inflammatory cells was found. In all cases, the non-secretory epithelium at the level of the bleb maintained its cylindrical morphology with absence of metaplasia signs (Fig. 3). In the epithelium of the area around the filtering blebs, 100% of cases exhibited the cylindrical epithelium arranged in plates without intercellular space increase, with different goblet cell density and in 25% of cases with areas exhibiting metaplastic changes.

Taking into account epithelial cell cohesion and the amount of goblet cells, the mean grade of impression cytology in filtering blebs was of 2.4±0.9 and in the surrounding area of 0.8±0.3 (p<0.001). This indicates that the mean of both distributions exhibits statistically significant differences. When MMC was used, the mean test has a significance of p=0.483, which indicates that the difference of cytological variables is the same regardless of MMC use.

Occasional polymorphonuclears were detected in case 11 in the conjunctiva area surrounding the filtering blebs, while

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### Table 3 – Category of patients including cytological findings.

<table>
<thead>
<tr>
<th>Case</th>
<th>Age, sex</th>
<th>Surgery type</th>
<th>Use of MMC</th>
<th>Hypotensor treatment</th>
<th>Appearance of bleb</th>
<th>Evolution time (months)</th>
<th>Bleb cytology grade</th>
<th>Surrounding area cytology grade</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>75, F</td>
<td>Trabeculectomy LE</td>
<td>No</td>
<td>0</td>
<td>Diffuse</td>
<td>6</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>2</td>
<td>79, M</td>
<td>Trabeculectomy RE</td>
<td>Yes</td>
<td>2</td>
<td>Diffuse relatively avascular</td>
<td>36</td>
<td>2.5</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>79, M</td>
<td>Trabeculectomy LE</td>
<td>Yes</td>
<td>3</td>
<td>Diffuse relatively avascular</td>
<td>36</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>69, F</td>
<td>NPDS LE</td>
<td>No</td>
<td>0</td>
<td>Diffuse</td>
<td>8</td>
<td>3</td>
<td>0.5</td>
</tr>
<tr>
<td>5</td>
<td>78, M</td>
<td>Trabeculectomy RE</td>
<td>Yes</td>
<td>0</td>
<td>Cystic</td>
<td>24</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>76, F</td>
<td>Trabeculectomy RE</td>
<td>No</td>
<td>0</td>
<td>Diffuse with microcysts</td>
<td>2</td>
<td>1.75</td>
<td>1</td>
</tr>
<tr>
<td>7</td>
<td>76, F</td>
<td>NPDS LE</td>
<td>No</td>
<td>0</td>
<td>Diffuse with microcysts</td>
<td>9</td>
<td>1.25</td>
<td>1</td>
</tr>
<tr>
<td>8</td>
<td>78, F</td>
<td>Trabeculectomy LE</td>
<td>Yes</td>
<td>3</td>
<td>Flat</td>
<td>60</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>9</td>
<td>78, F</td>
<td>Trabeculectomy RE</td>
<td>Yes</td>
<td>0</td>
<td>Diffuse relatively avascular</td>
<td>48</td>
<td>3</td>
<td>0.5</td>
</tr>
<tr>
<td>10</td>
<td>78, M</td>
<td>Trabeculectomy LE</td>
<td>No</td>
<td>0</td>
<td>Diffuse relatively avascular</td>
<td>12</td>
<td>3</td>
<td>0.5</td>
</tr>
<tr>
<td>11</td>
<td>75, M</td>
<td>Trabeculectomy LE</td>
<td>No</td>
<td>0</td>
<td>Diffuse</td>
<td>2</td>
<td>3</td>
<td>0.5</td>
</tr>
<tr>
<td>12</td>
<td>81, M</td>
<td>Trabeculectomy RE</td>
<td>No</td>
<td>0</td>
<td>Topical corticoids and atropine</td>
<td>2 weeks</td>
<td>3</td>
<td>1</td>
</tr>
</tbody>
</table>

M: male; F: female.
case 12 exhibited a higher number thereof in the patient with the shortest evolution time.

**Discussion**

The results of this study demonstrate significant cytological changes in the conjunctival surface of the blebs. In the majority of filtering bleb samples, absence of goblet cells was detected together with significant cylindrical epithelial cell dehiscence, with intercellular space increases. This was a significant contrast with the conjunctiva studied in the rest of the bulbar conjunctiva, where cylindrical epithelial cells were arranged in plates with various goblet cell densities and, in 25% of cases, areas with a certain degree of metaplasia were detected. Accordingly, filtering blebs involve a regional variation in the distribution of epithelial cells and the amount of goblet cells with regard to the rest of the conjunctiva, which would depend more on the condition of the conjunctiva prior to surgery. In the reviewed literature, the authors have only found one published study which utilized impression cytology with conventional staining (hematoxylin + PAS) to study the epithelium of filtering blebs in 22 eyes of 19 patients. Said study describes the loss of goblet cells in the blebs but did not compare this with the cytological findings beyond the limits of the bleb. In contrast with said study, the present study did not find changes in epithelial differentiation.

The results reported herein are compatible with those found in a more recent study which included 32 blebs of 29 patients and studied both filtering blebs as well as surrounding areas with live confocal microscopy and immunofluorescence on the basis of impression cytology samples. Said study described that, in functional filtering blebs, goblet cells did not maintain their characteristic mucin content and therefore, with the PAS staining in the present study, these empty cells would not be detected even though they could occupy the intercellular spaces between non-secretory epithelial cells. Mucin is a highly hydrophilic glycoprotein and therefore goblet cells would be a preferential pathway for the aqueous humor, diluting the amount of mucin in said cells, which would appear hydrated and with low mucin content. These hydrated, mucin-deprived goblet cells could correspond to the microcysts observed in vivo in confocal microscopy. These microcysts have been described as channels for aqueous humor filtering. In the present study, the apparently empty spaces could correspond to the microcysts observed in vivo confocal microscopy and, at the cellular level, they could be channels for the passage of aqueous humor. On the other hand, mucin acts as a physical and biological barrier on the ocular surface against foreign bodies and microorganisms, so the absence thereof on the surface of blebs entails a dysfunction facilitating bacterial infections.

Nelson et al. developed a rating system for impression cytology samples based on the morphology of the conjunctival epithelium and the density of goblet cells, applying a scale of 0–3. In this study, goblet cell density has been assessed according to this classification. However, as only epithelial cells without changes in morphology were found at the level of the blebs, this classification could not be applied to assess said morphology or differentiation. Instead, the degree of cohesiveness thereof has been rated on a scale from 0 to 3.

A review of the literature has not produced any cytological study of blebs from nonperforating filtering surgery. The present study, with only 2 NPDS cases, has made it necessary to carry out studies with larger sample sizes to determine whether different surgical techniques influence cytological changes in blebs.

Mitomycin C, which is frequently used during surgery to increase the success rate of the surgical procedure, has exhibited increased incidence of cystic blebs having very thin walls with a tendency to hypotony and belated infection. As in the study by Amar et al., the cytology results presented herein were not significantly different when MMC was used even though MMC was utilized in half of these cases. These findings appear to demonstrate that changes in the conjunctival epithelium in blebs are more influenced by the effect of the aqueous humor on said epithelium. However, the study by Amar et al. found that the use of MMC was associated to the greater presence of inflammatory components. The present study did not find inflammatory cells in the blebs. Only 2 cases of patients with the shortest evolution time exhibited polymorphonuclears in the bulbar conjunctiva as indicators of acute inflammation possibly related to the surgical intervention.

The main limitation of this study is the small sample size. Studies with more cases are necessary to confirm these findings and make progress in the study of the aqueous humor transconjunctival pathway.

By way of conclusion, filtering bleb epithelium exhibits significant changes consisting in increase of intercellular space between epithelial cells and the loss of goblet cells. Said increase intercellular space could explain the extracellular passage of aqueous humor. The loss of mucin secondary to the loss of goblet cells would contribute to a greater risk of infection of filtering blebs.

**Conflict of interests**

No conflict of interests has been declared by the authors.

**References**