CORNEAL EPITHELIUM SQUAMOUS METAPLASIA DETERMINATION AS DIAGNOSTIC FACTOR IN LIMBAL DEFICIENCY

DETERMINACIÓN DEL GRADO DE METAPLASIA ESCAMOSA DEL EPITELIO CORNEAL COMO FACTOR DIAGNÓSTICO DE INSUFICIENCIA LIMBAL

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ABSTRACT

Purpose: To determine the correlation between the squamous metaplasia grade of the corneal surface and the clinical severity of the limbal deficiency in patients with this disorder.

Methods: We studied 98 eyes of patients with limbal deficiency by impression cytology. These patients were divided into four groups in relation to their clinical severity: 14 eyes had no symptoms, 34 eyes showed a mild grade of limbal deficiency, 28 eyes had a moderate grade and 22 eyes had severe limbal deficiency. Corneal cytology was performed in each patient. Cellular size, nuclear size, nuclear changes and the nuclear-cytoplasmic (N:C) ratio were defined in corneal epithelial cells, in addition to evaluation for the presence of goblet cells in the corneal epithelium.

Results: In patients with limbal deficiency without symptoms, we found that the cellular size was 477 (SD140) µm² and the N:C ratio was 1:5.25 (SD1.5). In patients with mild limbal deficiency, the cellular size was 764 (SD122.6) µm² and the N:C ratio was 1:8.2 (SD1.4). These patients did not show corneal...
INTRODUCTION

The limbus is very important in the epithelial regeneration of the cornea, because at this level the stem cells which produce the corneal epithelium are found. Said cells, typically located in Vogt’s girdle (1) exhibit a number of features which are shared with other stem cells located at other levels such as multiple potentiality, self-renewal capacity, long life and prolonged cellular cycle (2,3). To date there are no specific markers of these limbal stem cells, which can be proved only through indirect evidence (4).

Limbal deficiency is a clinical entity characterized by vascularization of the corneal epithelium, chronic inflammation, persistent and recurring epithelial defects, photophobia, red eye and loss of vision (5). It is caused by a dysfunction of corneal epithelium progenitor cells, which may be partial or complete depending on the degree of involvement of these stem cells. This limbal dysfunction may arise as the result of a destruction of the limbus due to external aggressions, such as in the case of eye burns, contact lenses, multiple surgery over the limbal area, application of cryotherapy or after severe microbial infections. It can also appear in association with skin pathologies such as cicatricial ocular pemphigoid, Stevens-Johnson syndrome, Lyell syndrome, rosacea, etc. Less frequently, it is associated to congenital diseases such as aniridia, or it exhibits an idiopathic nature (6-8).

Impression cytology allows us to study the most superficial cellular layers of the corneal and conjunctival epithelium, facilitating the diagnosis of a considerable number of eye surface disorders (9-11). Even though the most common application of impression cytology in ophthalmological clinical practice is for assessing the degree of conjunctival squamous metaplasia in patients with dry eye (12), it has also been utilized for assessing the degree of corneal surface recovery in patients treated with amniotic membrane transplant or combined surgery of the latter with limbus transplant (13-15).

In this article we aim to relate the clinical severity of limbal insufficiency with the severity of corneal epithelium squamous metaplasia assessed by impression cytology, in order to determine the diagnostic value thereof in patients with eye surface disorders which are secondary to limbal deficiency.
SUBJECTS, MATERIAL AND METHODS

National and international standards for clinical research were observed in the selection of patients, techniques to be applied and manipulation of samples. All patients who volunteered to participate in this study were asked to accept their participation in writing.

We studies 98 eyes of 57 patients (35 men and 22 women) with limbal deficiency caused by different etiologies: eye burns (24 eyes), aniridia (22 eyes), post-terygium surgery or other processes with conjunctival involvement (12), cicatricial ocular pemphigoid (10), contact lens users (10), Stevens-Johnson syndrome (6), Lyell syndrome (4), rosacea acne (4), others of unknown cause (6). By means of anamnesis and exploration, four clinical groups were established based on patients not exhibiting clinical disorders related to limbal deficiency (group 0) or exhibiting slight disorders (group 1), moderate (group 2) or severe (group 3). The distribution of patients in groups according to etiology is illustrated in Table I.

In order to establish the clinical groups, we assessed the severity of different signs and symptoms produced by limbal deficiency. A patient was considered to exhibit slight limbal deficiency disorders (group 1) when he/she referred a maximum of two occurrences of recurring ulcers or relapsing erosions in the last 6 months, and slight photophobia or epiphora. Upon slit lamp exploration, these patients did not exhibit significant alterations, except when it matched a period of activity. When present, the pannus was slight and did not exceed 1 mm from the limbic arch and exhibited slight disorders in the absorption of fluorescein. This group comprised 34 eyes. A patient was considered to have moderate limbal deficiency (group 2) when the number of regressing erosion or recurring ulcer episodes equaled or exceeded 3 in the last 6 months and the slit lamp exploration identified permanent instability of the lacrimal layer and a vascular pannus accompanied or not by sub-epithelial fibrous tissue involving under half of the cornea periphery (fig. 1). Photophobia, epiphora and red eye were constant. This group comprised 28 eyes. A patient was considered to have severe limbal deficiency (group 3) when the slit lamp exploration identified corneal vascularization involving the center of the cornea as well as corneal erosion and (figs. 2 and 3). Photophobia, epiphora and red eye were the norm, as well as loss of vision due to involvement of the visual axis. This group comprised 22 eyes. In group «0» we included 14 eyes of patients with etiological processes susceptible to limbal deficiency but without exhibiting associated symptoms (fig. 4).

The corneal surface study by means of impression cytology was made obtaining samples of the 4 quadrants collected on Millipore HAWP304 5x5 mm paper, fixed with 96% ethanol during 10 minutes and dyed with PAS-hematoxiline according to the Locquin and Langeron protocol as modified by Rivas et al. (16), and subsequent assessment with optical microscope.

The morphometric study of these cells was performed with 40 increases over 10x15 cm photos.

Table I. Distribution of eyes according to etiology and clinical severity

<table>
<thead>
<tr>
<th>Group</th>
<th>B</th>
<th>aniridia</th>
<th>PS</th>
<th>OCP</th>
<th>CLU</th>
<th>SJS</th>
<th>Lyell</th>
<th>A.R</th>
<th>Others</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 0</td>
<td>0</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Group 1</td>
<td>5</td>
<td>10</td>
<td>8</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>4</td>
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<td>Group 2</td>
<td>12</td>
<td>4</td>
<td>2</td>
<td>4</td>
<td>0</td>
<td>4</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Group 3</td>
<td>7</td>
<td>4</td>
<td>0</td>
<td>6</td>
<td>0</td>
<td>2</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

B: Eye burns; PS: Post-surgery; OCP: Cicatricial eye pemphigoid; CLU: Contact lens user; SJS: Stevens-Johnson syndrome; A.R: acne rosacea.
An image analysis equipment was utilized, comprising a Nikon Ophtiphot-2 microscope with photo equipment Microflex-DX and a color video-camera EVI-10011P coupled to a Sony Black Trinitron KT-14 CPI monitor. The system is based on the PP-Plus by Image Pro/Media Cibermetics and the CAD system.

In order to assess the degree of cellular alteration we utilized the squamous metaplasia classification proposed by Murube and Rivas (17). Said classification differentiates 6 degrees of metaplasia, wherein 0 represents normality based on criteria such as shape and size of the cells, the separation between them, the size of their nucleus, the cytoplasm color, nuclear alterations, the nucleus-cytoplasm ratio (N:C) and the conjunctivalization of the corneal epithelium.

The statistical analysis was made with SPSS version 9.0 for Windows. The measures were mean and standard deviation. The results between different groups were compared utilizing the single-factor ANOVA test with subsequent correction by means of Tukey’s HSD multiple comparison test for a statistical significance of p<0.05. The correlation ratio was determined by Spearman’s Rho test.

RESULTS

In patients without clinical symptoms of limbal deficiency (group 0), the impression cytology revealed epithelial cells with a mean cell size of 477 SD 140 µm², a mean nuclear size of 92 SD 3.13 µm² and a N:C ratio of 1:5 SD 1.5. The cytological samples exhibited abundant cellularity with very homogeneous cell size and small inter-cellular spaces. In all cases the cytoplasm tincture was eosinophile and the presence of nuclear alterations was under 10% of cases, the most frequently observed nuclear alteration being the presence of spiral-shaped nuclei. We did not find cup-shaped cells in any patient of this group. With the exception of 4 cases who exhibited metaplasia grade 2, the rest of eyes exhibited metaplasia grade 1.

The group of patients with slight limbal deficiency (group 1) exhibited squamous metaplasia grade 2. The corneal epithelium cells had a mean cell size of 764 SD 122 µm², a nuclear size of 91 SD 2.7 µm² and a N:C ratio of 1:8 SD 1.4, with few inter-cellular spaces between them. With some exceptions, the cytoplasm tincture was eosinophile. In less than 30% of cases we found nuclear alterations...
tions, the most frequent being the presence of twin-nuclei cells and spiral-shaped nuclei. In none of the patients the corneal cytology revealed cup-shaped cells (fig. 5).

In the group of patients with moderate limbal deficiency (group 2), the corneal epithelium cells exhibited a mean cell size of 1162 SD 340 µm², a nuclear size of 87 SD 2.5 µm² and a N:C ratio of 1:13 SD 3.6. The epithelial cells exhibited a greater dispersion as regards cell size, with the samples having less cellularity and a considerable increase of inter-cellular spaces. The predominant tincture of the cytoplasm was eosinophile but in some cases it appeared meta-chromatic. We found nuclear alterations in 40% of cases, with the most common being the presence of twin nuclei cells, spiral shaped nuclei and cells with picnotic nuclei. Considering all these parameters, these cells exhibited squamous metaplasia grade 2-3. In 25% of this group of patients we found corneal conjunctivalization, defined by the (albeit small) presence of cup-shaped cells with poor mucinic content in the corneal cytology (fig. 6).

In the group of patients with severe limbal deficiency (group 3) we found epithelial cells with a mean size of 2036 SD 382 µm², a nuclear size of 82 SD 2.2 µm² and a N:C ratio of 1:23 SD 4. Said cells exhibited important size differences with large and abundant inter-cellular spaces and even keratinization signs. The cytoplasm tincture was predominantly meta-chromatic, although the presence of cells with basophile cytoplasm was relatively common. The presence of nuclear alterations was the norm, appearing in over 75% of patients. By far, the most frequent nuclear alteration was the presence of picnotic nuclei followed by the presence of enucleated and twin-nuclei cells. The majority of patients exhibited squamous metaplasia grade 4, although we also found in this group patients with squamous metaplasia grades 3 and 5. The presence of a larger or smaller number of cup-shaped cells in the corneal cytology was the norm in all the patients of this group (fig. 7).

Table II summarizes the values of the quantitati-

ve variables determined by impression cytology in the different clinical groups. We found the statistically significant differences in cellular size, N:C ratio and grade of squamous metaplasia among all the clinical groups, whereas in what concerns nuclear size the differences were significant only in the moderate and severe limbal deficiency cases (groups 2 and 3). On the other hand, we found a significant correlation at the bilateral 0.01 level with Spearman's Rho test between the clinical severity of limbal deficiency and the squamous metaplasia grade determined with impression cytology. We found a negative correlation between clinical severity and nuclear size, while this correlation was positive with cellular size and the N:C ratio (table III).

![Fig. 5: Corneal impression cytology of a patient with slight limbal deficiency. The image shows homogeneous cells without important nuclear alterations, with eosinophile cytoplasm and a N:C ratio of 1:8.](image1)

![Fig. 6: Corneal impression cytology of a patient with moderate limbal deficiency. The image shows lower cellularity with frequent nuclear alterations such as twin-nuclei cells and a meta-chromatic cytoplasm tincture, as well as the presence of cup-shaped cells.](image2)
DISCUSSION

Squamous metaplasia is a frequently reversible adaptive response mechanism of some epithelia against external aggressions or pathogenic stimuli of the body. This grade of metaplasia informs about the condition of the eye surface and therefore about the severity of the disease. Even though impression cytology has many applications as a diagnostic test (11), its main application in everyday clinical practice is for studying corneal and mainly conjunctival epithelium metaplasia (17).

Limbus alterations cause important disorders on the surface of the corneal epithelium which can be detected by means of impression cytology (18). In this article we propose a transversal study to determine the cytological characteristics of the corneal epithelium in different etiological entities which have in common the presence of a limbal deficiency. However, this etiological diversity calls for prudence before generalizing the results because the limbal damage is not always caused by the same pathogenic mechanisms (19-21). Equally, the greater or lesser chronicity of the clinical symptoms influences the degree of reactive response by the corneal epithelium and we must bear in mind that this response is not due exclusively to the limbal deficiency. Accordingly and in order to standardize the sample as much as possible, we have classified patients on the basis of clinical parameters derived from the degree of limbal deficiency, without taking into account other characteristic signs or symptoms of each entity.

In this study we have found a correlation between the clinical severity of limbal deficiency and the morphological alterations observed in corneal epithelium cells through impression cytology. We found a higher grade of squamous metaplasia with higher severity of the clinical symptoms. Thus, patients with a severe limbal deficiency (group 3) exhibited an average metaplasia grade 4, whereas patients with slight limbal deficiency (group 2) exhibited metaplasia grade 2. If we analyze separately the different parameters studied by means of impression cytology, we will see they are quantitative parameters such as cell size and N:C ratio in which this correlation is particularly noteworthy. In addition, these two parameters exhibited statistically significant differences between all clinical groups. On the other hand, we have seen how some

Table II. Ratio of mean quantitative parameter values obtained in the different groups (mean and standard deviation)

<table>
<thead>
<tr>
<th>Group</th>
<th>Cell size</th>
<th>Nuclear size</th>
<th>N:C ratio</th>
<th>Metaplasia</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>477SD140</td>
<td>92.5SD3.1</td>
<td>5.25SD1.5</td>
<td>1.25SD0.45</td>
</tr>
<tr>
<td>1</td>
<td>764SD122</td>
<td>91.0SD2.7</td>
<td>8.26SD1.4</td>
<td>1.93SD0.25</td>
</tr>
<tr>
<td>2</td>
<td>1162SD340</td>
<td>87.2SD2.5</td>
<td>13.2SD3.6</td>
<td>2.61SD0.7</td>
</tr>
<tr>
<td>3</td>
<td>2036SD382</td>
<td>82.6SD2.2</td>
<td>23.6SD4.0</td>
<td>4.18SD0.4</td>
</tr>
</tbody>
</table>

(*) Microns².

Table III. Estimated correlation coefficient by means of Spearman's Rho test. In all cases the correlation is significant at the 0.01 level

<table>
<thead>
<tr>
<th></th>
<th>Severity</th>
<th>Cell size</th>
<th>Nucleus size</th>
<th>N:C ratio</th>
<th>Metaplasia</th>
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</thead>
<tbody>
<tr>
<td>Severity</td>
<td>.871</td>
<td>-.779</td>
<td>-.682</td>
<td>.906</td>
<td>.864</td>
</tr>
<tr>
<td>Cell size</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nucleus size</td>
<td>-.779</td>
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<tr>
<td>Metaplasia</td>
<td>.864</td>
<td>.923</td>
<td>-.726</td>
<td>.932</td>
<td></td>
</tr>
</tbody>
</table>
patients who did not exhibit limbal deficiency clinical symptoms (group 0) had a squamous metaplasia grade 2. According to these data, impression cytology would allow us to diagnose limbal deficiency even before it became clinically evident. In this sense, we must say that the limbal deficiency diagnostic is easy in moderate and sever cases, above all with trauma history. However, in slight limbal deficit cases this diagnostic may easily go unnoticed. In these cases, with slight or pre-clinical involvement, impression cytology as well as fluor-photometry can be very useful for diagnostic purposes (22).

According to our results, we can conclude by saying that there is a correlation between the clinical severity of limbal deficiency and the grade of squamous metaplasia determined by corneal impression cytology. Likewise, impression cytology assists in establishing the limbal deficiency diagnostic in slight and sub-clinical cases and allows us to monitor the changes occurring in the corneal surface as a result of the treatment.

REFERENCES