Abnormal Epithelial Differentiation and Tear Film Alteration in Pinguecula

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PURPOSE. To investigate the differentiation of conjunctival epithelium and tear film function in pingueculae.

METHODS. Twelve patients (12 eyes) who underwent simple excision for pingueculae were enrolled in the study. Immunostaining for K14, K19, K10, MUC5AC, Pax6, P63, Ki67, and K16 was performed on the pinguecula epithelium and normal conjunctival epithelium. Schirmer I test results and tear film break-up time (TFBUT) were evaluated just before and 1 month after surgery.

RESULTS. Abnormal epidermal differentiation of pinguecula tissue, as evidenced by a decline in or absence of Pax6 expression, was accompanied by a decline in or absence of K19 keratin and MUC5AC, and an increase in K10 and K14 keratin. Furthermore, the pinguecula epithelium was actively proliferating, as evidenced by positive expression of Ki67, P63, and K16 keratin. The Schirmer I test results did not indicate any significant differences before and after surgery. However, the TFBUT was significantly prolonged 1 month after surgery compared with before surgery.

CONCLUSIONS. Pinguecula is a condition of abnormal epithelial differentiation. It is characterized by squamous proliferation and metaplasia, resulting in instability of tear film with normal basic tear secretion. (Invest Ophthalmol Vis Sci. 2009;50:2710–2715) DOI:10.1167/iovs.08-2905

Pinguecula is a common ocular surface disorder, with reported prevalence rates of 22.5% to 90%.1–5 Pingueculae are benign, yellowish, slightly raised, interpalpebral lipid-like deposits in the nasal and temporal limbal conjunctiva.1 Although pinguecula is a relatively benign condition, it can manifest as ocular irritation (e.g., foreign-body sensation, pain, and tearing)6 and represents a significant eye health problem in many communities.

Although the etiogenesis of pingueculae is still not fully understood, prolonged exposure to ultraviolet-B radiation is thought to promote its development.5–7 Other factors (e.g., p53 mutation,8 increased cholesterol metabolism,9 development of abnormal elastic materials,10 age,11,12 and inadequate tear film stability13) may also play roles in pinguecula formation. However, to the best of our knowledge, there are no published studies comparing proliferation and differentiation of pinguecula epithelium to those of normal conjunctival epithelium.

To better understand the alterations of the conjunctival epithelium in this condition, we compared pinguecula samples to normal conjunctiva via immunostaining with monoclonal antibodies to K14, K19, K10, MUC5AC, Pax6, Ki67, P63, and K16 proteins, and examined the extent and distribution of their expressions. Moreover, we evaluated the effect of pinguecula vis-à-vis tear function, based on tear film break-up time (TFBUT) and the results of pre- and postoperative Schirmer I tests.

MATERIALS AND METHODS

The following materials were used: hydrogen peroxide, DAPI, Hoechst-33342 dye, acetonitrile, Triton X-100, bovine serum albumin (BSA), and FITC-conjugated anti-mouse IgGs (Sigma-Aldrich, St. Louis, MO); mouse anti-cytokeratin 10 (K10), K19, -P63, and -Ki67 antibodies (Dako Technology, Glostrup, Denmark); mouse anti-MUC5AC monoclonal antibody (Abcam Biotechnology, Cambridge, UK); mouse anti-K14, K16, and Pax6 monoclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA); diaminobenzidine (DAB; Dako Cytomation, Carpinteria, CA); and the ABC kit for mouse (Vectastain Elite; Vector Laboratories, Burlingame, CA).

Patients and Specimens

We studied pinguecula specimens from 12 patients who had had elective outpatient pinguecula surgery. Clinical diagnosis of pinguecula was based on previously reported criteria.14 All investigators were conducted in accordance with the tenets of the Declaration of Helsinki and were approved by the Ethics Committee of Xiamen Eye Center. Informed consent was obtained from each patient before inclusion in the study. All study patients were examined at the Cornea and Ocular Surface Clinic of Xiamen Eye Center (Xiamen, Fujian, China), between December 2007 and February 2008. All pingueculae were surgically removed by one surgeon (HW). Subjects with previous ocular surgery, contact lens wear, pemphigoid, Sjögren’s syndrome, or any other type of conjunctivitis or keratitis were excluded from the study. The patients were observed for at least 1 month after surgery. Normal conjunctiva obtained from human donors at Xiamen Eye Center and Eye Institute served as the control.

Evaluation of Tear Film Function

All examinations were performed in the morning, and all measured variables for each patient were evaluated during a single office visit in the same darkened room 1 day before and 1 month after surgery. TFBUT measurements with fluorescein were taken and the Schirmer I test (without topical anesthesia) was performed. TFBUT was recorded as the average of three successive measurements. The Schirmer I test result was expressed as the wet length of the strip measured after 5 minutes.
Frozen sections of 6-μm thickness were fixed in acetone at −20°C for 10 minutes. For immunofluorescence staining, fixed sections were incubated in 0.2% Triton X-100 for 10 minutes. After three rinses with PBS for 5 minutes each and preincubation with 2% BSA to block nonspecific staining, the sections were incubated overnight at 4°C with various dilutions of primary antibodies: MUC5AC and K16 (1:50); K10, K14, and K19 (1:200). After three washes with PBS for 15 minutes each, sections were incubated with an FITC-conjugated secondary antibody—rabbit anti-mouse IgG (1:50)—for 1 hour. After three additional 5-minute washes in PBS, the sections were observed by microscope (TE-2000U Eclipse epifluorescence microscope; Nikon, Tokyo, Japan). For immunohistochemical staining, fixed sections were placed in 0.6% hydrogen peroxide for 10 minutes to block endogenous peroxidase activity. Nonspecific staining was blocked by 1% normal horse serum for 30 minutes. Sections were then incubated overnight at 4°C with various dilutions of primary antibodies: P63 (1:50), K67 (1:100), and Pax6 (1:200). After three 15-minute washes with PBS, sections were incubated with biotinylated anti-mouse IgG (1:100) for 1 hour and incubated with ABC reagent for 30 minutes. The reaction product was developed with DAB for 2 minutes. The sections were photographed with a camera mounted on a light microscope (both by Nikon).

Image Analysis
For analysis of integrated optical density (IOD) expression of positive immunostaining in the epithelial layer, images from immunostained (K14, K19, K10, MUC5AC, Pax6, K67, P63, and K16 proteins) sections were processed (Image Pro Plus ver. 6.0; Media Cybernetics, Silver Spring, MD). In brief, correction of unequal illumination (shading correction) and the calibration of the measurement system were performed with a reference slide after the images were recorded. For each sample, different areas of 3 to 10 sections were scored. The images of immunostained slides were converted to gray scale, and the IOD was measured as a linear combination between the average gray intensity and the relative area occupied by positive cells.

Statistics
Summary data were reported as the mean ± SD. Group mean data were compared by using the appropriate version of the t-test, where \( P < 0.05 \) was considered statistically significant.

RESULTS
Clinical Evaluation
Pingueculae from 12 eyes (four right, eight left) of 12 patients (2 men, 10 women; average age, 47.25 ± 7.75 years; range, 34–56) were studied (Table 1). Pingueculae were located nasally (\( n = 8 \)) and temporally (\( n = 4 \)), as shown in Figure 1. No ocular surface complication was observed in any eye after surgery.

<table>
<thead>
<tr>
<th>Age</th>
<th>Sex</th>
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<th>Shirmer I Test (mm)</th>
<th>TFBUT (s)</th>
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<td>56</td>
<td>F</td>
<td>OD 10/16</td>
<td>9/11</td>
</tr>
<tr>
<td>2</td>
<td>52</td>
<td>F</td>
<td>OS 13/13</td>
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<td>3/15</td>
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<tr>
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<td>F</td>
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<tr>
<td>5</td>
<td>38</td>
<td>F</td>
<td>OS 16/13</td>
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<td>M</td>
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<td>3/13</td>
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Data are before/after surgery.

Table 1. Clinical Characteristics of the Patients

Tear Film Function
None of the patients in this study had dry eye. The average TFBUT was 5.4 ± 3.0 seconds. This figure increased significantly to 12.6 ± 1.8 seconds by one month after surgery (\( t = -6.689, P < 0.05 \), paired-sample t-test; Fig. 2A).

The Schirmer I test result, which is important for evaluating the aqueous phase of the tear film, was 12.8 ± 5.4 mm before surgery and increased to 14.2 ± 2.4 mm after surgery, a nonsignificant difference (\( t = -0.949, P = 0.363 \), paired-sample t-test; Fig. 2B).

Epithelial Differentiation in Pinguecula
To investigate whether the conjunctival epithelial phenotype is maintained in pingueculae, immunostaining was performed for K14, K19 keratin, and epidermis-specific K10 keratin. K14 keratin, a basal epithelial cell marker that has been reported to be confined to the basal cell compartment in normal conjunctiva,15,16 was demonstrated in all normal conjunctival epithelial cells (Fig. 3C), whereas K19 expression was dramatically decreased in all pinguecula samples and was even negative in some areas of full-thickness pinguecula epithelium (Fig. 3D). As reported,15,16 expression of K10 keratin, an epidermal keratinocyte-specific intermediate filament, was negative in normal conjunctiva (Fig. 3E). Surprisingly, K10-positive cells emerged in the suprabasal to superficial cell layers of all pingueculae tissues (Fig. 3F).

Abnormal Differentiation in Pinguecula
To further investigate goblet cell differentiation in pinguecula epithelium, we used immunofluorescence to examine MUC5AC, the most abundant mucin of the ocular surface.19–21 Results showed that MUC5AC expression was prominent in normal conjunctiva (Fig. 3G), but was significantly downregulated in the epithelial cells of pinguecula (Fig. 3H) and was totally absent in five samples.

One distinguishing characteristic of the ocular surface epithelium, as opposed to the epidermal epithelium, is expression of Pax6, a transcription factor that plays a key role in eye morphogenesis.22,23 To determine whether the aforementioned abnormal epidermal differentiation was associated with the loss of differentiation of ocular surface lineage in pinguecula, we performed immunohistochemical staining for Pax6. Pax6-positive nuclei were present throughout the full thickness of the epithelium in normal conjunctiva (Figs. 3I), but was decreased in all samples of pinguecula epithelium, regardless of level. Pax6 expression was lost in some basal epithelial cells, and was even negative in some full-thickness areas of epithelium (Figs. 3J). Our previous study showed that abnormal epithelial differentiation correlated highly with downregulation of Pax6 expression in severe ocular surface diseases.24 Results of the present study indicate that Pax6 downregulation also correlates with abnormal epithelial differentiation in mild ocular surface abnormalities such as pinguecula.

To quantify the expression levels of the above-mentioned cell differentiation markers, the IOD of positive immunostaining in the epithelial layer was analyzed. Results showed that IODs of K10 (3403.90 ± 3204.21) and K14 (5163.89 ± 1687.318)-positive cells were higher in pinguecula than in normal conjunctiva (\( P < 0.05 \), Fig. 3K). However, the IODs of K19 (2089.12 ± 12,222.85) and MUC5AC (517.47 ± 859.61)
were significantly downregulated in pingueculae compared with normal conjunctiva \((P < 0.05)\), respectively (Fig. 3K). These results further confirm that abnormal epidermal differentiation occurs in pinguecula.

**Epithelial Proliferation in Pinguecula**

To further characterize the proliferation status of pinguecula epithelial cells, we performed immunostaining for Ki67, p63, and K16 keratin. All negative control samples without primary antibody showed no staining (data not shown). Nuclear Ki67 staining, indicative of cell proliferation, was detected in the basal and suprabasal layers of normal conjunctival epithelium (Fig. 4A) and pinguecula (Fig. 4B). Expression of Ki67 was significantly increased in pinguecula samples \((767.24 \pm 454.9)\) compared with normal tissue \((161.32 \pm 131.28)\), as shown by the IODs (Fig. 4G). Positive nuclear staining of p63, a marker originally thought to be expressed in basal epithelial progenitor cells of all stratified epithelia, including normal conjunctiva (Fig. 4C), but now recognized as a marker of cells with proliferation potential, was found in almost all basal and some suprabasal epithelial cells in pinguecula (Fig. 4D). However, there was no significant difference \((t = -4.433; P = 0.227)\) in the IOD of p63-positive cells between pinguecula \((2053.46 \pm 1014.79)\) and normal conjunctiva \((1661.61 \pm 400.79)\). K16 keratin is indicative of an alternative pathway of keratinocyte proliferation and is also known for the marked enhancement of its expression in stratified squamous epithelium showing hyperproliferation or abnormal differentiation. K16 keratin was negative in normal conjunctiva (Fig. 4E), but was expressed in suprabasal and superficial epithelial cells in pinguecula (Fig. 4F). Collectively, these results indicate that abnormal epithelial differentiation is indeed associated with hyperproliferation in pinguecula.

**DISCUSSION**

The results of this study show, for the first time, that pinguecula is a condition of abnormal differentiation characterized by squamous metaplasia with proliferation.

Squamous metaplasia has been defined as the pathologic transition of a nonkeratinized stratified secretory epithelium to squamous epithelium. The average TFBUT was 5.5 ± 3.0 seconds before surgery and increased to 12.6 ± 1.8 seconds by 1 month after surgery \((P < 0.05, \text{paired-sample} \, t\text{-test})\) (A). The Schirmer I test result was 12.8 ± 3.4 mm before surgery and 14.2 ± 2.4 mm after surgery \((P = 0.363, \text{paired-sample} \, t\text{-test})\) (B).
a keratinized nonsecretory epithelium.\textsuperscript{29,30} It is the hallmark of a variety of severe ocular surface disorders manifesting dry eye caused by the lack of lacrimal gland secretion such as Sjögren syndrome, Stevens-Johnson syndrome, mucous membrane pemphigoid, chemical/thermal burns, and vitamin A deficiency.\textsuperscript{29,31} Chan et al.\textsuperscript{32} used impression cytology to evaluate ocular surface abnormalities in four eyes with pinguecula and pterygium and found that the surface cells in pinguecula exhibited squamous metaplasia. In the present study, we thoroughly analyzed epithelial differentiation of pinguecula by means of immunostaining, which permitted visualization of full-thickness changes rather than only superficial cell profiles from impression cytology. We confirmed that squamous metaplasia, as evidenced by expression of K10, was accompanied by loss of MUC5AC and K19 expression in all pinguecula samples (Figs. 3C–H). Our recent study described squamous metaplasia induced by air exposure of human limbal explant cultures.\textsuperscript{33} The pathogenesis of squamous metaplasia in pinguecula may be also interpreted by using this model. First, pinguecula is a manifestation if lift-up from normal con-
junctiva that mimic airlift culture. Second, TBUT is shorter in pinguecula, which can cause more exposure time of the abnormal tissue than normal ocular surface. In the urothelial model of squamous metaplasia, Liang et al. proposed five models to explain the etiogenesis of squamous metaplasia: transdifferentiation at the terminal differentiated cell level, dedifferentiation, pluripotency or plasticity of stem cells, selected expansion of different stem cells, and expansion and replacement by a different lineage. In our study, we performed immunostaining of Pax6 to determine the differentiation lineage of epithelial cells in pinguecula tissue. Postnatal Pax6 expression is restricted to corneal, conjunctival, lens, and iris epithelia and amacrine cells of the retina. Our previous study clearly showed that Pax6 helps maintain the normal postnatal corneal epithelial phenotype. Downregulation of Pax6 is associated with abnormal epidermal differentiation in severe ocular surface diseases. In the present study, we also found downregulation of Pax6 in conjunctival epithelium of pinguecula. Moreover, Pax6 expression was attenuated and even absent in basal epithelial cells, in which conjunctival progenitor cells were present, suggesting that abnormal differentiation in pingueculae may occur in some progenitor cells. Other than Pax6, which is related to in vivo phenotype maintenance of ocular surface epithelial cells, our previous study showed that p38 signaling pathway is involved in abnormal differentiation of limbal epithelial cells in ex vivo explant culture. Further investigation is warranted to determine whether p38 signaling pathway is also related to the abnormal differentiation in pinguecula epithelium.

Traditionally, pingueculae have been considered to be degenerative lesions, described histologically as having elastic degeneration of its collagen. However, pingueculae also exhibit features associated with p53 mutation and increased cholesterol metabolism in agreement with the hypothesis that a pinguecula comprises a potentially proliferative tissue. In the present study, expressions of Ki67 and K16 were significantly greater in pingueculae than in normal conjunctiva. This observation also supports the notion that there is hyperproliferation in pinguecula epithelium. Since overexpression of p63 is associated with the development of conjunctival neoplasms, including conjunctival squamous cell carcinoma and pterygium, we performed P63 immunostaining to investigate the proliferative capacity of pinguecula. Although the difference was not statistically significant, there were more p63-positive nuclei in pingueculae than in normal conjunctiva, further indicating that pingueculae exhibit characteristics of squamous proliferative diseases.

A previous study has demonstrated shortened TBUT but normal tear secretion in pingueculae. In our study, the Schirmer I test result was in the normal range in 10 patients, whereas TBUT was shorter than normal, consistent with the prior study. Moreover, TBUT was significantly prolonged after a single excision, further indicating that pingueculae are associated with a significant perturbation of tear film stability. There are several interpretations of the shortened TBUT in pinguecula: abnormal eyelid blinking, presence of an irregular formation, and mucus deficiency. Another explanation may be the presence of squamous metaplasia in the surface epithelium of pinguecula, which could compromise the stability of tear film. Although squamous metaplasia is common in ocular surface epithelium of dry eye, further study is needed to reveal whether there is a causative relation between squamous metaplasia and tear function in pingueculae.

In summary, our study confirmed the deleterious effect of pingueculae on tear film stability and ocular surface health status, as evidenced by decreased mucosal defense capability and damage to the ocular surface epithelia—that is, squamous metaplasia, resulting in tear film instability. A correlation may exist between squamous metaplasia and ocular tear dysfunction in the pathogenesis of pingueculae. Furthermore, it strategies of restoring Pax6 expression, as well as use of abnormal differentiation inhibitors and artificial tears, could be beneficial in the prevention and treatment of pingueculae.

Acknowledgments

The authors thank Yueping Zhou and Jianxing Ma for critical review of the project proposal and Zhaosheng Liu and Suzhen Xie for generous support in the clinical study.

References