Pterygium epithelium abnormal differentiation related to activation of extracellular signal–regulated kinase signaling pathway in vitro

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Abstract

• AIM: To investigate whether the abnormal differentiation of the pterygium epithelium is related to the extracellular signal–regulated kinase (ERK) signaling pathway in vitro.

• METHODS: The expression levels of phosphorylated ERK (P–ERK), keratin family members including K19 and K10 and the ocular master control gene Pax–6 were measured in 16 surgically excised pterygium tissues and 12 eye bank conjunctiva. In colony–forming cell assays, the differences in clone morphology and in K10, K19, P–ERK and Pax–6 expression between the head and body were investigated. When cocultured with the ERK signaling pathway inhibitor PD98059, the changes in clone morphology, colony–forming efficiency, differentiated marker K10, K19 and Pax–6 expression and P–ERK protein expression level were examined by immunoreactivity and Western blot analysis.

• RESULTS: The expression of K19 and Pax–6 decreased in the pterygium, especially in the head. No staining of K10 was found in the normal conjunctiva tissue, but it was found to be expressed in the superficial cells in the head of the pterygium. Characteristic upregulation of P–ERK was observed by immunohistochemistry. The clone from the head with more differentiated cells in the center expressed more K10, and the clone from the body expressed more K19. The P–ERK protein level increased in the pterygium epithelium compared with conjunctiva and decreased when cocultured with PD98059. The same medium with the ERK inhibitor PD98059 was more effective in promoting clonal growth than conventional medium with 3T3 murine feeder layers. It was observed that the epithelium clone co–cultured with the inhibitor had decreased K10 expression and increased K19 and Pax–6 expression.

• CONCLUSION: We suggest ERK signaling pathway activation might play a role in the pterygium epithelium abnormal differentiation.

• KEYWORDS: abnormal differentiation; epithelial cells; pterygium; extracellular signal–regulated kinase signaling pathway; in vitro

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INTRODUCTION

Pterygia are the most obvious ophthalmology disease with unknown pathogenesis. This disorder is characterized by the encroachment of altered conjunctival tissue over the cornea [1]. Several hypotheses have been suggested to explain the pathogenesis of pterygia, including the imbalance between proliferation and apoptosis in pterygium epithelial cells (PECs) [2]. It is well known that ultraviolet (UV) irradiation is the major factor [3,4] that induces the hyperproliferation and keratinization of PECs. Abnormal changes in the extracellular matrices and inflammatory cytokines are also attributed to the development of pterygia [5]. The mechanism underlying the abnormal differentiation of PECs remains obscure. Keratins (Ks) and the intermediate filament proteins of epithelia are coordinately expressed as pairs in a cell-lineage and differentiation manner, and they are prominent in simple epithelium and periderm at the late stage [6]. The K5/K14 pair is present in the keratinocytes of the basal layer of the stratified epithelia, and the terminally differentiated keratinocytes contain the K1/K10 pair [7]. As differentiation markers, K19 and MUC5AC are expressed in the human conjunctival epithelium, and CX43 and K12 are found in the corneal epithelium cells [8]. These markers are abundant in the pterygium, especially the conjunctival markers [9]. The pterygium has two parts: one is the head near the invading edges, with multilayer epithelial cells that are
partly atrophied and keratinized; and the other is the body, with decreased epithelial layers with Bowman's membrane breakdown. Abnormal expression of K10 was found in PECs, especially in the head \[9,10\]. We postulate that the differentiation between the head and body is distinct, and this difference might cause phenotypic alterations in different parts.

The extracellular signal-regulated kinase (ERK)1/2 is activated by UV light \[11\]. PD98059 is a noncompetitive inhibitor of ERK1/2. The activation of ERK1/2 could promote cell survival and proliferation in response to mitogenic stimuli. The activation of ERK1/2 also acts as an intermediary in the UVB-mediated induction of MMP-1 in cultured PECs \[12\]. ERK1/2 is required for the terminal differentiation of various types of cells such as marrow mesenchymal stem cells and non-small-cell lung cancer cells \[13\]. Pax-6 protein plays a key role in eye development, proliferation, apoptosis, formation of the limbal stem cells and participation in ocular surface wound healing \[14\]. Previous studies have shown that decreased Pax-6 expression was associated with abnormal differentiation of corneal epithelial cells in severe ocular surface diseases such as Stevens-Johnson syndrome, chemical burns, aniridia and pterygium \[10\]. The role of ERK1/2 or Pax-6 in pterygium remains largely unknown.

Given the important role of these proteins in ocular diseases, this study aims to examine the differences in the differentiation between the head and body of the pterygium. We investigated the roles of Pax-6 and ERK1/2 in the abnormal differentiation of the pterygium.

SUBJECTS AND METHODS

**Antibodies** Anti-cytokeratin 10 (K10), anti-cytokeratin 19 (K19), anti-Ki67 (Ki67) and anti-cytokeratin 15 (K15) antibodies were obtained from Dako Cytomation (Carpinteria, CA, USA); anti-Pax-6 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, Dallas, Texas, USA); and anti-ERK1/2 and anti-phosphorylated ERK (P-ERK) antibodies were obtained from Cell Signalling Technology (Danvers, Massachusetts, USA).

**Surgical Tissue Specimens** Resected pterygium (n=16, 9 males and 7 females) and normal conjunctiva (n=12, 7 males and 5 females) were obtained from the Second Affiliated Hospital of Guangzhou Medical University, Guangzhou, Guangdong Province, China. Excised tissue was divided laterally into two identical and symmetric pieces. One piece was OCT-embedded and snap frozen in liquid nitrogen immediately after surgery. Another piece was used for pterygium clonal culture with head and body parts (Figure 1).

The present study was reviewed and approved by the Institutional Review Board of the Second Affiliated Hospital of Guangzhou Medical University on Feb. 28, 2013. All of the research protocols were performed in accordance with the tenets of the World Medical Association Declaration of Helsinki.

**Pterygium Clonal Culture** To examine the difference between the head and body epithelial cells and whether PD98059 influences the clonal growth of human PECs, a clonal assay was performed using a previously reported method \[16\]. In brief, NIH 3T3 fibroblasts were grown in DMEM containing 10% newborn calf serum at 80% subconfluence and were treated with 2.5 μg/mL of mitomycin C for 2h and trypsinized and plated at a density of 2×10^4 cells/cm^2. Human pterygium and conjunctiva epithelial sheets were sterilized in Betadine solution (Purdue). The head and body of the pterygium were separated on the limbal line, as shown in Figure 1, and isolated directly from the connective tissue based on our modified digestion method, using 2.5 μg/mL Dispase II in SHEM at 4℃ for 14-16h \[17\]. The isolated epithelial layers of the sheets were carefully removed with a detacher, rendered into single cells by digestion with 0.05% trypsin/0.53 mmol/L EDTA at 37℃ for 15min, and seeded in triplicate at a density of 300 cells/cm^2 (8481 cells/plate) in 60-mm culture dishes containing SHEM for the head and body. For the detection of the PD98059 effect, the PECs were seeded in 60-mm culture dishes containing SHEM for the control group and 10 μmol/L PD98059 for the experimental group. The cultures were incubated at 37℃ under 5% CO2 and 95% humidity, and the medium was changed every two to three days. The colonies were counted at 6, 9, 11d. The colony-forming efficiency (CFE) was calculated as the number of colonies per dish divided by 8481 (i.e. the total seeded cells per dish). On 9, 12d, three plates of pterygium and conjunctiva cultures from each group were terminated for immunostaining.

**Immunostaining Analysis** For serial sections (5 μm) of the pterygium, the normal conjunctiva were processed for immunofluorescence and immunohistochemistry staining. The tissue sections and cultured clones were subjected to immunostaining with appropriate dilutions of the primary antibody and their respective secondary antibodies. Briefly, each sample was fixated in cold methanol for 10min at

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Figure 1 Schematic picture shows the different parts of the pterygium A: The head of the pterygia invading the cornea, which is a triangular strap-like tissue; B: The body of the pterygia invade the bottom of the triangle on the conjunctiva.
-20 °C, permeabilized in 0.2% Triton X-100 for 15 min and blocked with 2% bovine serum albumin for 30 min at room temperature. The cells were incubated with the primary antibody for 16 h at 4 °C. After washing three times with phosphate buffer saline (PBS), specific binding was detected by a FITC-conjugated anti-mouse or anti-rabbit secondary antibody incubated for 1 h at room temperature. The sample was mounted in an antifading solution with DAPI (Vector Labs, Burlingame, CA, USA). For P-ERK immunostaining, a DAB kit with an immunoperoxidase protocol (ABC kit Vectastain Elite, Vector Labs) was used. The negative control was performed by substitution of the primary antibody with PBS. The images were photographed with a Nikon Te-2000u Eclipse epifluorescent microscope (Nikon, Tokyo, Japan). Positive immunostaining was counted at 200x magnification in 10 random fields of each sample.

Western Blot Analysis For Western blot analysis, proteins of P-ERK in different epithelium cells cultured or treated with P-ERK inhibitor were extracted with cold RIPA buffer. Equal amounts of proteins extracted from lysates were electrophoretically separated on SDS-PAGE using a 4% stacking and 10% separating gels. Proteins in gels were electro-transferred (100 V, 60 min, 4°C) to polyvinylidene fluoride transfer membranes (Pall, Mexico). After 1 h of blocking solution, which consisted of 5% milk powder in tris buffered saline (TBS) containing 0.05% tween 20 (TBST), then probed with primary antibodies to P-ERK (1:100), and β-actin (1:5000) as a loading control. After three times washes with TBST for 10 min each, the membrane were incubated with HRP conjugated goat anti-rabbit IgG (1:10 000) for 1 h at room temperature. The specific binds were visualized by enhanced chemiluminescence reagents. The protein bands were quantified using ImageJ software.

Statistical Analysis The t-test was used for the statistical analysis. The data was analyzed using SPSS statistical software (SPSS Inc., Chicago, IL, USA). P values less than 0.05 were considered significant.

RESULTS

Different Expression in the Head and Body of the Pterygium Specimens The fragments are composed of epithelium and different types of stroma. The epithelium is stratified and has a variable thickness with irregularities of the underlying stroma. In the head, the epithelium is thick and elevated by proliferation (Figure 2A).

Compared with normal conjunctiva, the staining patterns of keratin were quite different in the pterygium epithelium. As a differentiated skin phenotype, K10 positive expression was typically found (62.50%, n=10) in the head (Figure 2B). As normally expressed in the skin epithelium, K10 was predominantly localized to the superficial head of the pterygium epithelium. In contrast, no epithelial cells expressed K10 in the normal conjunctiva (Figure 2C). The expression of K10 proved the presence of abnormal differentiation in the head of the PECs. Sample epithelial cell expression of K19 primarily and significantly decreased in the PECs (Figure 2D, 2E, 2F), whereas K19 positive expression, as a conjunctiva differentiation marker, was found mostly (68.75%, n=11) in the body of the PECs (Figure 2D, 2E). These findings indicated that the conjunctival marker predominated in the PECs. The abnormal differentiation was typically in the head, and the conjunctiva-like differentiation was significantly decreased. Our studies showed that phospho-ERK1/2 expression was extensive and expressed in all of the pterygium specimens, mostly in the cytoplasm of the pterygium epithelium and the vascular endothelium. The staining was more intense in the head of the PECs compared with the body (Figure 2G, 2H), and positive expression of phospho-ERK1/2 was rarely observed in the normal conjunctiva epithelium (Figure 2I). Pax-6 is the universal master control gene for eye morphogenesis. A significant decrease in Pax-6 expression in the nucleus and cytoplasm was found in all parts of the pterygium; however, the intensity of the positive staining in the head was weaker compared with the staining in the cells in the body (Figure 2J, 2K). The normal conjunctiva epithelial cells expressed Pax-6 (Figure 2L). The distribution pattern of P-ERK and Pax-6 presented the suspicion that these epithelial cells in the head were in a state of abnormal differentiation in accordance with an up-regulated ERK signaling pathway and down-regulated Pax-6.

Definition of Pterygium Epithelial Cells Clones To define the PECs clone cells, an immunohistochemistry study was performed. The pterygium epithelium is two or more cells thick with basal cells, polygonal cells and wing cells (Figure 3A). Approximately 93% of the clone cultured adherent cells demonstrated a round epithelioid morphology. The epithelioid cells with extended processes were morphologically distinct from the rounded epithelioid cells. The remaining distinguishable morphologies consistently observed included very large flattened cells in the center of the clones, which suggested differentiated cells (Figure 3E). The majority of the cells detaching from the conjunctival epithelium were labeled with the K19 antibody. The K19 antibody was expressed in the majority of the cells of the PECs, which is the same as in the clone cells (Figure 3B, 3F). A small number of clone cells on the edges of the clone stained positively for K15, which is the stem cell marker for ocular epithelial cells (Figure 3G). The basal cells within the pterygium epithelium stained positively for K15 (Figure 3C). The basal epithelial cells in the pterygium epithelium were labeled by the anti-Ki67 antibody (Figure 3D). Identical positive cells were scattered in the PECs clones, which indicated cells in a state of high proliferation (Figure 3H).
Figure 2 The expression of keratin, phospho–ERK1/2 and Pax–6

A: HE staining of the epithelium; B: Positive staining of K10 in the head superficial cells; C: Negative staining of K10 in conjunctiva; D, E, F: The expression of K19 keratin was predominant in the epithelial cells of the conjunctiva and pterygium, but it decreased in of PECs, especially in the head; G, H, I: Strong p-ERK (brown) in the epithelium in the head and no expression was found in the conjunctiva; J, K, L: Pax-6 expression revealed various degrees that were lower in the PECs, especially in the head, and it is high in conjunctiva.

Figure 3 The definition of pterygium epithelium clone cells

A: The pterygium epithelium is two or more cells thick; B: K19 is expressed in the superficial cells in the PECs; C: The K15 positive cells were in basal cells of the PECs; D: The Ki67 positive cells scattered in the basal cells of PECs; E: The cells in the PEC clones demonstrated a round epithelioid morphology; F: The majority of the clone cells expressed K19; G: The cluster cells of the edges in the clones expressed K15; H: Ki67 expression was found in some cells in the clones.

Comparison of the Differentiation Phenotypes in the Clones of the Head and Body of the Pterygium

The clonal cells, which were formed by pluripotent stem cells in the pterygium epithelium, reflected the differentiation patterns. We asked whether the different expression was confirmed in clonal culture. The epithelium clones were cultured from different parts of the pterygium epithelium. There was no significant difference between the two types of clones in terms of the morphology of the clones, the clone size and the clonal forming efficiency (Figure 4A, 4B, 4C).
Figure 4 Expression of Ks, Pax-6 and P-ERK in clones  

A, B, C: Morphology of the clones of the head, the body and the conjunctiva epithelium; D, E, F: Some strong K10 expressing cells were found in the clones of the head, while no expressing was found in the conjunctiva epithelium; G, H, I: K19 expressing cells were scattered over the clones in the head. Nearly all of the cells in the body and conjunctiva clones were K19 positive cells; J, K: The expression of Pax-6 showed weakly positive cells in the head and body clones; L: The cells in the conjunctiva epithelium clone show strong staining of Pax-6; M, N, O: There were more P-ERK expressing cells in the head clones and no positive cell was found in conjunctiva clone.

Further studies on the immunostaining found that more cells in the head epithelium forming clones expressed K10 than the cells in body epithelium forming clones (Figure 4D, 4E). The positive expression was located mostly in the central cells of the clones, which is regarded as the first differentiated cells in the clones, and in some cells on the edges of the clones. Relatively few cells of the body clones expressed K10, with the exception of some clustered cells that showed some fake staining (Figure 4E). No expression of K10 was found in the normal conjunctiva clone cells (Figure 4F). The expression of K19 was completely different. More positive expression cells in the body clones were found than in the head clones, and ratio of positive cells was 95% in the body and 76% in the head clones ($P<0.05$, Figure 4G). In some clones from the body and the normal conjunctiva, almost all of the cells expressed K19 except some cells at the edges. This is reasonable for the undifferentiated cells located in the edges of the clone (Figure 4H, 4I). The decreased expression of Pax-6 had been shown in clones of the head (Figure 4J). The ratio of Pax-6 positive clones was 27%, which was lower than the 65% in the body clones ($P<0.05$, Figure 4K). The ratio was nearly 100% in the normal conjunctiva clones (Figure 4L). The P-ERK staining showed more positive cells in the pterygium head epithelial clones (Figure 4M, 4N) and only some fake staining was observed in conjunctiva clones (Figure 4O).

Changes in the Clones After Inhibition of the Extracellular Signal-regulated Kinase Singling Pathway with PD98059  
In our study, we compared the clonal growth of the pterygium epithelium cocultured with or without 10 µm
ERK inhibitor PD98059. As shown in the Figure 5A, 5B, the clone appearance at day 6 and the clone morphology of the control group without the inhibitor were similar to the group with the inhibitor. Differentiated cells were in the center, and small cells were on the edges with a high nucleus-to-cytoplasm ratio (N/C) (Figure 5A, 5B). The CFE in the inhibitor group was 2.8652±0.1167% at 9d and 3.2897±0.1169% at 11d, which was lower than 3.66113±0.3752% at 9d and 4.5396±0.35835% at 11d in the control group without PD98059. The CFE was statistically lower in the 9, 11d clones with PD98059 (P<0.05, Figure 5C).

The epithelium clones cocultured with or without PD98059 were analyzed by immunostaining to determine the differentiated phenotype changes between the two groups. The central clone cells expressed K10 in the control group as pterygium epithelial clones, which has been shown before (Figure 6A). The clones from the inhibitor group had few K10 positive staining cells (Figure 6B). K19 staining was extensive and localized predominantly to both group clones (Figure 6C, 6D), but fewer cells expressed this marker in the control group. Some cells in the center showed found negative expression of K19 (Figure 6D). The differentiated phenotype was affected by inhibiting specific MARK pathways. The Pax-6 staining supported this; the ratio of Pax-6 positive clones in the control group was 48% (Figure 6E), which was lower than the 76% in clones with PD98059.
WESTERN BLOT

The Western blot indicated the expression of P-ERK increased in the pterygium epithelium clone cells compared with conjunctiva epithelium clone cells and decreased when co-cultured with PD98059 (Figure 6G, 6H).

DISCUSSION

In recent years, histological examinations have identified epithelium proliferation, and the layers decrease from the head to the body with regions of severely damaged Bowman's membrane [18]. The invasion and infiltration in the head has been suggested as the key reason for vision loss. The expression analysis of the human pterygium shows a predominance of conjunctival and limbal markers and genes associated with cell migration [19]. The question regarding the different cell origins and differentiation of the head and the body of the pterygium epithelium remains unanswered.

Abnormal differentiation was detected by the expression of keratin in the pterygium. The keratin gene is expressed during terminal differentiation of the keratinocyte. The changes in keratin composition that take place within each cell during the course of its terminal differentiation play an important role in pathogenesis of epithelial diseases [20]. Abnormal keratin expression was found in the pterygium epithelium in previous studies [20]. In the current investigation, we provide evidence that the epithelial cells of the body expressed more conjunctiva-like markers, and abnormal differentiation was shown in the head of PECs, which expressed more K10 and less K19 in tissue sections and clone staining. In some reports, K19 staining localized to only a few basal cells, and other reports have localized K19 expression to all layers of the corneal and conjunctival epithelium. The majority of these cells likely represent corneal and conjunctival transiently amplifying progenitor cells [21]. In our study, the PECs in all layers labeled with the K19 antibody. The expression decreased in the PECs, especially in the head epithelium compared with the conjunctiva. No expression of K10, as a differentiated marker for skin epithelium, was found in the normal conjunctiva, but it is mostly detected in the superficial cells of the head of the PECs. The abnormal differentiation was seen only in the head. The indication of keratinization in the pterygium epithelium was reasonable because the raised head is always out of tear film protection, which is a necessary factor to keep the ocular surface healthy. Our study showed no staining of MUC5AC (data not shown). MUC5AC, excreted by the epithelial cells of the normal ocular surface, is an important compound of tear film. The highly proliferated epithelial cells might have not enough time for mucin synthesis. We postulate that the keratinized cells in the raised head produce no mucin or nonfunctional mucin, which leads to the deficiency of the tear film; this causes the abnormal differentiation of the epithelial cells.

Down-regulation of Pax-6 is associated with abnormal epidermal differentiation in severe ocular surface diseases [15]. Pax-6 is a homeobox transcription factor in the pax gene family, which is made up of nine members in vertebrates. Pax-6 plays a critical role in determining early stage cell differentiation in the eye [22]. The loss of Pax-6 activity inhibits cell differentiation. Pax-6 helps to maintain the normal corneal epithelial phenotype postnatally. In our study, we demonstrated decreased expression of Pax-6 in the pterygium epithelium, which indicated a differentiation disorder of pterygium epithelium. The decreased K19 expression and abnormal K10 differentiation was mostly in the head of the pterygium epithelium according to the deceased Pax-6 expression, which suggested that Pax-6 was related to abnormal differentiation in the pterygium, especially in the head. The pterygium with thickening and a vascularized head proved to be the progressive stage. The head of the PECs was closely related to the pathogenesis or maintenance of pterygium.

The pterygium is a highly proliferative and invasive fibrovascular lesion related to UV-light damage. ERK1/2 is the member of the MAPK family that plays an important role in the regulation of cell growth, differentiation, and the control of cellular responses to cytokines and stress [23]. Previous studies have shown that extracellular signals such as UV light or osmotic stress could activate the intracellular ERK signaling pathway through the phosphorylation of growth factor and the cytokine receptors epidermal growth factor receptor, tumor necrosis factor receptor and interleukin-1 receptor in cultured PECs in vitro [24]. It is generally thought that the activation of ERK1/2 could confer a survival advantage to cells, and short-lived activation of ERK is associated with cell proliferation [20]. In the present study, we showed a significant increase in the ERK1/2 expression in PECs and suggested that PECs are in a high proliferation state and might be a survival reaction for UV light. Strong P-ERK expression in the head was suggested as a risk factor for the recurrence of pterygium. Studies on tumor cells determined that inhibiting the ERK1/2 signaling pathway could suppress the epidermal growth factor mediated cell proliferation [24]. Using the ERK1/2 inhibitor PD98059, we demonstrated that cell proliferation in clonal forming culture was reduced, which inversely suggested that activation of ERK1/2 is associated with high proliferation.

Although the mechanism by which ERK1/2 signaling acts on the pterygium epithelium cells is incompletely understood, it is likely that the ERK1/2 signaling pathway is involved in proliferation and in cell differentiation [26]. We showed that the cell differentiation outcome changes, and abnormal differentiation decreased by specifically inhibiting ERK1/2 because the cells in the epithelial clones with the ERK1/2 inhibitor PD98059 expressed less K10 and more K19. Pax-6...
expression increased with the ERK1/2 inhibitor according to the conjunctiva-like Ks expression and less keratinization. The abnormal differentiation decreased in the cultured PECs with the ERK1/2 inhibitor. Our results suggested that the pterygium epithelium might receive continuous signals, and the abnormal differentiation might be responsible for extensive ERK1/2 expression in vivo. Inhibition of the phosphorylation of ERK1/2 might help pterygium epithelium cells retain normal growth conditions and keratinization.

Our study demonstrated that abnormal differentiation occurred in the head of the epithelial cells in the pterygium via K10 production and K19 Pax-6 down-regulation. The inhibition of the ERK1/2 signaling pathway might modulate the pterygium epithelial cell abnormal differentiation. An improved understanding of the molecular receptors and growth factors activated by the ERK1/2 signaling pathway might provide us with new insight allowing for the prevention of the abnormal differentiation of the pterygium epithelial cells.

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