Flow Cytometry Measurements of DNA Content in Primary and Recurrent Pterygia

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PURPOSE. To evaluate DNA content and cellular proliferation rates in primary and recurrent pterygia.

METHODS. Matched pterygium and superior conjunctiva tissue were obtained in 36 eyes of 36 patients undergoing pterygium excision with conjunctival autografting (24 primary pterygia, 12 recurrent pterygia). Epithelial and fibrovascular layers were separated for analysis. Matched superior conjunctiva obtained at the time of surgery were used as controls. Samples were prepared according to Thompson’s method, and flow cytometry was performed with a Becton-Dickinson FACScan. Analysis of histograms and calculations of cell percentages in cell cycle phases were carried out using CellFit software (version 2.0). Mean proliferation indices (MPIs) were compared using the Wilcoxon matched-pair signed-rank test.

RESULTS. The MPI of pterygium fibrovascular tissue (13.4) was significantly higher than the MPI of pterygium epithelium (3.1; P = 0.0001). The MPI of pterygium fibrovascular tissue was also significantly higher than that of superior conjunctival fibrovascular tissue (6.0; P = 0.0001). There was no difference in MPI values between pterygium epithelium and superior conjunctival epithelium (3.55; P = 0.12). The MPI of fibrovascular tissue from recurrent pterygium (73.75) was significantly higher than the MPI of fibrovascular tissue from primary pterygium (7.3; P = 0.003).

CONCLUSIONS. The finding of high levels of cellular proliferation in the subepithelial fibrovascular layer of pterygium confirms that pterygium is a disorder of excessive cellular proliferation and that the fibrovascular layer is the site of cellular proliferation. Markedly raised levels of cellular proliferation in recurrent pterygium tissue suggest a clinical correlation between fibrovascular tissue upregulation and pterygium recurrence after surgery. (Invest Ophthalmol Vis Sci. 2000;41:1684–1686)

Pterygium is a disorder of uncertain etiology, with features indicative of both degenerative processes and disordered growth. Evidence of the degenerative nature of pterygium stems originally from light microscopy findings of elastoid degeneration, which has been linked to actinic degenerative changes from chronic ultraviolet light exposure, and this is supported by the geographical predisposition of pterygium to periequatorial regions, which have high levels of ambient UV radiation.1,2 At the same time, there are features of the behavior of pterygium that suggest excessive or disordered growth (i.e., tumor-like properties). Pterygium recurs aggressively after surgical excision, and treatment modalities mimic radiotherapy, and antimitotic chemotherapy.3 Primary pterygium can also be locally invasive, and the pterygium epithelium has been shown to exhibit various degrees of abnormality ranging from mild dysplasia to carcinoma in situ.4 The relationship of UV radiation exposure and pterygium formation has also been compared with the etiologic role of UV radiation exposure in Bowen’s disease and skin malignancies.2

Pterygium tissue consists of a superficial conjunctival epithelial layer and an underlying fibrovascular component. We previously determined that abnormal p53 overexpression may be present in pterygium epithelium.5 The abnormal expression of p53, a tumor suppressor gene modulating expression of growth controlling genes, which has been shown to be abnormally expressed in a wide variety of human cancers as well as actinic skin lesions,6 suggests that the epithelial layer of pterygium may be involved in the pathophysiological process of pterygium development. However, the aggressive nature of pterygium has long been noted to be related to the subepithelial fibrovascular component. Recurrent pterygium is almost always thick and fleshy, and we have recently shown that the morphology of pterygium, as determined by the degree of fibrovascular tissue present, correlates well with recurrence rates after simple bare sclera excision.7 We have also shown that the surgical technique of conjunctival rotational autografting, in which just removal of the fibrovascular component of pterygium is performed, with replacement of the original conjunctival epithelium overlaying the pterygium, is a successful procedure with a low rate of recurrence of 4%.8

To further evaluate the proliferative growth process in pterygium, flow cytometry was performed in this study to compare cellular proliferation rates in the epithelium and fibrovascular layers of primary and recurrent pterygia, in comparison with matched epithelial and subepithelial layers of superior conjunctiva from these eyes.
METHODS

Tissue specimens from 36 eyes with pterygium were obtained from 36 patients undergoing pterygium excision with conjunctival autografting at the Pterygium Clinic of the Singapore National Eye Center (SNEC). Of these, 24 were primary pterygia and 12 were recurrent pterygia. The mean age of patients was 56 years (range, 39–81 years), with 25 men and 11 women. The majority of patients were Chinese (29 patients, 80.6%), with 7 Malays (19.4%). The study protocol conformed to the Declaration of Helsinki, and study protocol and informed consent forms were approved by the SNEC Ethics Committee and the SNEC Executive Research Committee. Written informed consent was obtained from all patients in the study.

Specimen Collection and Processing

Surgical excision of pterygia was carefully carried out to separate epithelium from underlying fibrovascular tissue. In all instances, it was possible to obtain fibrovascular pterygium tissue without epithelium, but pterygium epithelium samples invariably included a small amount of subepithelial tissue, because epithelium is adherent to the fibrovascular layer via the substantia propria. In all cases, matched specimens of superior bulbar conjunctiva, also separated into epithelial and subepithelial layers, were collected at the time of conjunctival autograft donor harvesting. Specimens were immediately snap-frozen in liquid nitrogen and transferred to a −70°C freezer before processing for flow cytometry analysis.

Single cell suspension was prepared using the method described by Thompson et al., with modifications. Specimens were dissociated in RPMI 1640 medium manually in a Petri dish. Undissociated cells were removed by filtration through nylon mesh (40 μl). Cells and small particles passing through the nylon mesh were collected and centrifuged at 3000 rpm for 5 minutes. The supernatant was discarded, and the pellet was immediately used for staining. Three hundred to 500 μl of propidium iodide (PI) mix (containing 50 μg/ml PI, 0.01 M MgCl2, 0.01 M Tris, 0.2% Triton X-100, and 1 mg/ml RNase) was used to stain nuclear DNA in the pellet. After sufficient minutes, the staining procedure was carried out in the dark for 5 minutes before flow cytometry.

Flow Cytometry Analysis

DNA content from both pterygium and superior bulbar conjunctiva was measured by FACScan (Becton–Dickinson BD) equipped with an argon laser with emission wavelength at 488 nm, and the fluorescence of PI was collected using a 585/42 band-pass filter. The FCM was calibrated each time with chicken erythrocyte nuclei (CEN, BD). A maximum event of 30,000 was collected from each sample. Analysis of the cell cycle compartments was carried out using CellFit software (version 2.0).

Statistical Analysis

Mean proliferation index (MPI) was used to evaluate the proliferative status, which was the ratio of \(S + G_2/M\) to \(S + G_2/M + G_0 + G_1\). The significance of MPI between samples was calculated using the Wilcoxon matched-pair signed-rank test.

RESULTS

The MPIs for the fibrovascular components for normal superior conjunctiva, primary pterygium, and recurrent pterygium were all significantly higher than the epithelial components (Table 1) and were highest in the fibrovascular tissue of recurrent pterygium. There was no difference in epithelial MPI rates between superior conjunctiva (MPI = 3.55), primary (MPI = 3.2) or recurrent (MPI = 2.5) pterygium, whereas MPI for fibrovascular tissue differed significantly. The lowest MPI rate occurred in superior conjunctiva (MPI = 6.0), followed by primary pterygium (MPI = 7.3), whereas the highest MPI value was observed with recurrent pterygium tissue (MPI = 73.75). Statistical analysis by Wilcoxon signed-rank testing revealed these differences to be highly significant when superior conjunctival fibrovascular tissue was compared with both primary and recurrent fibrovascular tissue. In addition, when compared individually, MPI of pterygium fibrovascular tissue in primary pterygium patients was significantly higher than superior conjunctival fibrovascular tissue in primary pterygium (MPI = 0.001), and MPI of pterygium fibrovascular

<table>
<thead>
<tr>
<th>Sample</th>
<th>Median MPI</th>
<th>Minimum MPI</th>
<th>Maximum MPI</th>
<th>P Value</th>
</tr>
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<tr>
<td>Superior conjunctival epithelium</td>
<td>3.55</td>
<td>0.5</td>
<td>9.1</td>
<td>0.0008</td>
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<td>6.0</td>
<td>0.4</td>
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<td>0.8</td>
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<tr>
<td>All pterygia fibrovascular</td>
<td>15.4</td>
<td>0.6</td>
<td>99.5</td>
<td></td>
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<tr>
<td>Primary pterygia epithelium</td>
<td>3.2</td>
<td>1.1</td>
<td>7.4</td>
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<td>Primary pterygia fibrovascular</td>
<td>7.5</td>
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<td>99.5</td>
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<tr>
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<td>0.8</td>
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<td>73.75</td>
<td>2.5</td>
<td>90.5</td>
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</tr>
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MPI, mean proliferative indices. * P by Wilcoxon signed ranks test.
tissue of recurrent pterygium patients was also significantly raised compared with superior conjunctival fibrovascular tissue in these eyes with recurrent pterygium \((P = 0.002; \text{Table } 2)\).

It was noted that no significant differences in MPI were found between superior conjunctival fibrovascular tissue of primary and recurrent pterygia patients \((P = 0.13)\), suggesting that the main differences in MPI lay in the fibrovascular tissue underlying the pterygia. This was confirmed by a significant difference in MPI between primary and recurrent pterygia fibrovascular tissue \((P = 0.003)\).

**Discussion**

DNA flow cytometry measures the DNA content of individual cells, which provides an accurate indication of cell cycle stage, and has been used to analyze cell cycle kinetics in corneal epithelium after wounding.\(^6\) The use of flow cytometry to evaluate cell cycle kinetics in pterygium was previously reported by Karukonda et al.,\(^{10}\) who studied 93 pterygium specimens and 19 controls from patients in Singapore, Hong Kong, and Little Rock, Arkansas. In that study, flow cytometry provided no evidence for increased proliferation in pterygium tissue, compared with normal conjunctiva, nor was there any difference in tissues between the three sites of various geographical latitudes. In addition, specimens for recurrent lesions were not found to be more proliferative in nature.

The present study, in contrast, showed major differences in subepithelial fibrovascular mitotic activity between pterygium tissue and matched superior conjunctiva, with significantly higher proliferative rates in pterygium subepithelial fibrovascular tissue, and the highest proliferative rates in recurrent pterygium fibrovascular tissue. In contrast, epithelial samples did not vary significantly in mitotic status between pterygium and superior conjunctiva, suggesting that the major proliferative aspect of pterygium lies in the underlying fibrovascular layer. The cellular components of subepithelial pterygium fibrovascular tissue comprise primarily of fibroblasts and capillary blood vessel cells, both of which may be highly proliferative, and this study did not distinguish exact cellular components responsible for proliferative activity.

The use of superior bulbar conjunctiva as matched control tissue for the present study deserves some comment. It should be noted that environmental exposure of pterygium tissue, within the interpalpebral fissure, must be significantly greater than that of superior bulbar conjunctiva, and it is not surprising that prolonged sunlight or UV light exposure and other environmental conditions (such as drying) have been implicated in pterygium etiology.

Clinical evidence that the fibrovascular layer is important in pterygium growth and recurrence is seen in our randomized clinical trial comparing bare sclera excision to conjunctival autograft.\(^7\) In that study, pterygium morphology was graded according to the relative degree of fibrovascular tissue present in the body of pterygium, obscuring underlying episcleral vasculature under slit-lamp biomicroscopic examination. Atrophic pterygium, with translucent tissue at the pterygium body resulting in clear visualization of underlying episcleral vessels (and hence minimal fibrovascular tissue), were graded as T1. Thick, fleshy pterygia were graded as the least translucent (T3), denoting complete obscuration of episcleral vessels by the fibrovascular component, whereas all pterygia in which partial episcleral vessel obscuration was noted were graded as intermediate (T2). The study results showed that in bare sclera excision, pterygium recurrence was linked to the preoperative grade, with T3 pterygia having the highest recurrence rate, and T1 the lowest, providing clinical evidence that the fibrovascular component of pterygium is responsible for aggressive recurrent growth after surgery. The proliferative nature of pterygium fibrovasculature is now borne out in our present study. Additional supportive evidence that the fibrovascular component of the pterygium was removed, with the original pterygium epithelium replaced.\(^8\)

The implication of our findings lies in determining new treatment measures for pterygium removal and its recurrence. Antimitotic measures have long been used in pterygium surgery to prevent recurrence, in the form of the use of mitomycin C, thiopeta, and \(\beta\)-irradiation, which all act to reduce the proliferative capacity of tissues at the site of the pterygium in a nonspecific manner. Complications related to mitomycin C or \(\beta\)-irradiation usually relate to an initial breakdown in surface conjunctiva, with the development of a nonhealing epithelial defect, which ultimately results in scleral necrosis and melting or secondary infective scleritis and endophthalmitis. In the light of our findings, a more focused approach to antiproliferative treatment could be targeted at underlying fibrovascular tissue while sparing overlying epithelial cells.

Finally, our finding of selective upregulation of pterygium fibroblast proliferation further supports the theory that pterygium is a proliferative growth disorder, as opposed to a simple degenerative condition, and future therapies should focus on selective reduction in proliferation in the fibrovascular layer of this lesion.

**Acknowledgment**

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**References**