行政院國家科學委員會專題研究計畫　成果報告

眼翼表面之流式細胞儀檢測暨捺壓式細胞學檢查之研究

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Purpose: To investigate the apoptotic marker expression of pterygial epithelial cells by flow cytometry combined with impression cytology (IC). Methods: IC specimens were collected from 58 primary pterygia and 58 superior bulbar conjunctivas of the lesion eyes in 58 patients and 58 nasal conjunctivas of healthy eyes in 43 normal subjects. Using antibodies directed to Fas, Fas ligand (FasL) and Annexin V, flow cytometry was applied to analyze the percentage of positive cells as well as levels of fluorescence expression quantified as antibody binding capacity (ABC). Results: A significant increase of FasL expression, both in positive rate and ABC intensity, was found in pterygial epithelial cells, compared to sex-age-matched normal subjects. FasL expression was also augmented in superior bulbar conjunctiva of pterygial eyes, compared to normal subjects. In contrast, there was no difference of Fas expression among pterygial epithelial cells, superior conjunctival cells of patients, and normal controls of healthy conjunctiva. Annexin V tended to an equal expression among all groups, in terms of positive rates and intensity levels. Conclusions: Apoptosis is not increased in pterygial epithelial cells. Over-expression of FasL may endow pterygium with a local immune privileged site so that the tumor can escape from the normal immunological surveillance of ocular surface and thus favors its growth.

Key Words: Impression cytology– Immunocytochemistry– Flow cytometry– Fas– FasL– Annexin– Pterygium

Originally believed to be a degenerative disease, another school of thought regarding the pterygium pathogenesis found on the role of inflammation. Our previous studies supported by NSC grants revealed an increased level in expression of Langerhans’ cell and HLA DR on pterygial epithelium, which suggested a cell-mediated Type IV hypersensitivity may play a role in the pathophysiology of pterygial formation. However, our recent study (Cornea 2005;24:583-6) on p53 expression in 127 pterygial specimen also showed an equivocal result on the role of apoptosis on pterygial formation. Therefore, this 3rd-year study aims to further study more apoptotic markers, Fas, FasL, and Annexin, in the pathogenesis of pterygium. The method that we employed to quantify these apoptotic markers on ocular surface was a novel combined technique of impression cytology (IC) and flow cytometry (FCM).
PATIENTS AND METHODS

Patients Selection
Between January 2003 and December 2005, 58 eyes of 58 patients with primary pterygium and 58 eyes from 58 age-, sex-matched normal controls were enrolled in this study, after informed consent and NCKUH ethical committee approval in accordance with the tenets of the Declaration of Helsinki. At the time of presentation, patients were not taking any immunosuppressive agent for any systemic disease. Patients were randomly sent to three groups: Group A (n=21) tested for Fas expression, Group B (n=22) tested for FasL expression, and Group C (n=15) tested for Annexin V expression.

Impression cytology sample handling
Two impression cytology (IC) specimens were taken from each pterygial patient, one from pterygium and the other from superior bulbar conjunctiva of the lesion eye. A IC sample was taken from each control eye at the nasal conjunctiva. Specimen collection was using 0.22 μm polyether sulfone filters (Supor membranes; Gelman Sciences) with a gentle compression under topical anesthesia (0.04% oxybuprocaine) to harvest 1-2 layers of epithelial cells from the ocular surface. Specimens were collected at least 15 minutes after instillation of the last staining eye drop (i.e., fluorescein and lissamine green), to avoid any interference with immunofluorescence (IF) analyses. After collection, membranes were immediately dipped into tubes containing 1.5 ml of cold phosphate-buffered saline (PBS) with fixative (0.05% paraformaldehyde) and kept at or below 4°C until flow cytometry. Cells were extracted by gentle agitation for 30 minutes and centrifuged (1600 rpm, 5 minutes). The cells were then counted in a Malassez cell before processing for flow cytometry, according to previously validated methods. Three different monoclonal antibodies and corresponding negative controls were used in this study. Fluorescein isothiocyanate (FITC)–conjugated mouse IgG1 anti-human Fas/CD95 (clone UB2, 1 mg/ml, Immunotech) was used in Group A. FITC–conjugated mouse IgG1 anti-Fas ligand (clone 3E4, 1 mg/ml, Immunotech, Marseille, France) was used in Group B, and FITC–conjugated mouse IgG1 anti-Annexin V (clone AN5, 1 mg/ml, Immunotech, USA), as an apoptosis marker, were used in a direct IF technique. After incubation, cells were centrifuged in PBS (1600 rpm, 5 minutes), resuspended in 100 ml of PBS, and analyzed on a flow cytometer (FACScan; Becton Dickinson) according to previously validated methods.

Flow Cytometry Processing
The linear plot giving granulometry versus cell size consistently revealed a single cell population. Analytic gates were set around this population to exclude cellular debris and aggregates. The number of positive conjunctival cells was then obtained from logarithmic cytograms of mean fluorescence intensities. The superior level of fluorescence intensity obtained for the isotypic control antibody was considered as the limit of background fluorescence and the threshold of positivity for the tested antibodies. In each sample, at least 1000 cells were analyzed. All specimens were analyzed in a masked manner, in that the examiner did not know the clinical history of patients. Data were further expressed as a quantimetric assessment of fluorescence intensities by using calibrated fluorospheres to translate the mean fluorescence of each sample into standardized antigen binding capacity (ABC). A calibration curve was established during each flow cytometric procedure by using four different beads (Immunobrite; Coulter, Hyaleh, FL) with standardized fluorescence intensities. This technique allowed quantification and objective comparisons between days and therefore controlled the reliability and quality of measurements.
The real number of ABC was obtained by subtraction of the isotypic negative control from the total ABC calculated for each marker.

**RESULTS**

The patients consisted of 27 women and 31 men ranging from 47 to 81 years old. Fas, FasL, and Annexin V were found to express in both normal and pathologic eyes at various degrees. There was no statistical difference in numbers (shown as percentage) or intensity (shown as ABC) of cells expressing Fas among pterygia, superior bulbar conjunctiva, and controls (Figure 1A & 1B).

![Graphs showing flow cytometry results of Fas expression](image)

Figure 1. Flow cytometry results of Fas expression in primary pterygium (21 patients), bulbar conjunctiva of pterygial eyes (21 patients) and normal control (21 patients), given in mean with 95% confidence interval of Fas-positive cells (Left), and antibody binding capacity (+SE) of Fas (Right). No significant difference was noted among groups. P = pterygium; BC = bulbar conjunctiva of pterygial eye; C = control; ABC = antibody binding capacity.

However, the number of FasL-labelled epithelial cells was increased in pterygial (11.21 ± 8.64%) and superior bulbar conjunctiva of lesion eye (9.08 ± 5.44%), compared to normal controls (5.73 ± 4.17%) (P = 0.015 and 0.019, respectively) (Figure 2A). Quantitation of fluorescence intensity of FasL was also found to be higher in pterygial (238.25 ± 234.16 ABC), and superior bulbar conjunctiva of lesion eye (165.55 ± 115.45 ABC), compared to normal controls (90.30 ± 66.88%) (P = 0.027 and 0.015, respectively) (Figure 2B). In contrast, Annexin V expression showed no statistical difference between pterygium eyes, superior bulbar conjunctiva and control eyes, in terms of cell number (Figure 3A). Interestingly, Annexin V was found to be slightly higher in pterygium (19362.73 ± 14762.13 ABC), compared to control (4432.22 ± 2445.92) (P = 0.007) (Figure 3B). No difference was noted between pterygium and controls. Details of all data were summarized in Table 1.

![Graphs showing quantitation of fluorescence intensity of FasL](image)

![Graphs showing Annexin V expression](image)
Figure 2. Flow cytometry results of Fas ligand expression in primary pterygium (22 patients), bulbar conjunctiva of pterygial eyes (22 patients) and normal control (22 patients), given in mean with 95% confidence interval of Fas ligand-positive cells (Left), and antibody binding capacity (+SE) of Fas (Right). * $P < 0.05$ (Mann-Whitney U test).

CONCLUSION

The results that expression of Fas and Annexin V remained unchanged, but FasL expression was up-regulated indicated that apoptosis is not increased at the surface layer of pterygium but FasL-mediated immuno-regulation maybe endow pterygium an immune privilege to escape from constitutive immune surveillances against tumor.
Table 1. Summary of Fas, Fas ligand and Annexin V expression in all groups

<table>
<thead>
<tr>
<th>Markers</th>
<th>Positive Cells (%)</th>
<th>ABC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pterygium conjunctiva</td>
<td>Bulbar conjunctiva</td>
</tr>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>Range</td>
</tr>
<tr>
<td>Fas</td>
<td>86.01%± 6.18%</td>
<td>72.90%-94.8%</td>
</tr>
<tr>
<td>Fas ligand</td>
<td>11.21%± 8.68%*</td>
<td>0.30%- 1.88%-</td>
</tr>
<tr>
<td>Annexin V</td>
<td>70.67%± 21.23%</td>
<td>28.2%-94.69%</td>
</tr>
<tr>
<td></td>
<td>31.62% 19.59%</td>
<td>% 0%</td>
</tr>
</tbody>
</table>

ABC = antibody binding capacity; * Versus control, P < 0.05 (Mann-Whitney U test)