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The Role of Apoptotic Pathway in Pterygium Formation: A Flow Cytometry Study with Impression Cytology

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Backgrounds:

Fas is a cysteine-rich transmembrane protein of 335 amino acids — which is also called CD95 — is expressed on cells of the lymphoid system and organs such as the eye and heart. Engagement of this receptor on the cell surface by its natural ligand, FasL, a 278 amino-acid membrane-bound protein, also known as CD95L, results in apoptotic death of the cell. Recent studies have shown that FasL is expressed constitutively in the eye and its expression can limit the ocular inflammation and thus associates with ocular immune privilege. Studies on the expression of FasL by certain tumors also demonstrated that tumors might establish an immune-privileged site to protect themselves from the immune response through this pathway. For example, melanomas and astrocytomas can functionally express FasL and kill tumor-infiltrating lymphocytes. We suspect that pterygium, a common ocular lesion with certain tumor properties such as uncontrolled proliferation, migration, and tissue invasion but with relatively mild signs of ocular inflammation, may acquire the immune tolerance on the ocular surface through FasL-mediated apoptotic pathway. Impression cytology combined with flow cytometry was applied to test the hypothesis.

Patients and methods: Impression cytology specimens were collected with 20 µm polyether sulfone filters (Supor membranes; Gelman Sciences) in 14 patients with primary pterygium and in 14 healthy controls. After 30 minutes of agitation for cell extraction, cells were suspended in PBS with 1% bovine serum albumin and then centrifuged (1600 rpm, 5 minutes). Mouse IgG1 anti-human CD95 (clone DX2, BD PharMingen Technical) and anti-human CD95L (clone NOK-1, BD PharMingen Technical) were used to incubate the cells in a 1:100 working dilution for 30 minutes. Nonimmune mouse IgG1 (Immunotech) was used as a negative isotypic control. After PBS washing, secondary anti-mouse immunoglobulins in a 1:50 dilution was subsequently incubated for 30 minutes. At the end of incubation, they were centrifuged in PBS (1600 rpm, 5 minutes), resuspended in 400 µl PBS, and analyzed on a flow cytometer (FACScan; Becton Dickinson), according to a previously validated method. The number of positive conjunctival cells was then obtained from a histogram representing mean fluorescence intensities on a 4-decade logarithmic amplifier. The superior level of fluorescence intensity obtained for the isotypic control antibody was considered to be the limit of background fluorescence and the threshold of positivity for the tested antibodies. At least 2500 cells were analyzed for each marker. The methods
were summarized in figure 1. Statistical comparisons were performed with the Mann–Whitney test and the Z correlation test, at a 0.05 level of significance (SPSS 10.0).

**Results:** Fas and Fas ligand expression was found in both normal and pathologic eyes (Figure 2). Quantitation of fluorescence intensity of FasL showed a significantly higher expression in pterygia, 12.97% ± 5.06%, than in normal controls, 4.88% ± 2.08% \((P = 0.037)\) (Figure 3). In contrast, Fas expression showed no statistical difference between pterygium eyes, 80.90% ± 11.08%, and control eyes, 87.39% ± 4.16%. (Figure 4) Compared to the normal controls, superior conjunctiva of pterygial eyes showed no difference either in the expression of Fas or FasL.

**Discussion:** Impression cytology combined with flow cytometry is a useful method to evaluate pathological changes on the ocular surface at molecular levels. This technique was first used by Brignole and his associate to demonstrate augmentation of CD40 in dry eyes. With this technique, increased expressions of HLA-DR and vimentin in pterygium have been previously reported by us. In the current study, we demonstrate that FasL is upregulated in pterygium.

Even though pterygium is a very common disease worldwide, it is still a disorder of uncertain etiology. It may arise from excessive cellular proliferation in the subepithelial fibrovascular layer. A recent study showed that p63, a epithelial stem cell marker, is not increased in pterygium, compared to normal conjunctiva. Therefore, the explanation for the hyperplasia of epithelium observed in pterygium is that pterygium may result from a disruption of normal apoptosis in the conjunctival epithelium, instead of excessive cellular proliferation. Indeed, our study provides direct evidence that pterygium is associated with disruption of normal apoptosis regulation. Ultraviolet (UV) irradiation of skin was reported to cause an increased FasL expression in keratinocytes, which leads to the elimination of T cells in psoriasis. As pterygium is known to be associated with long-term UV exposure, we postulate that pterygium can acquire immune tolerance through the same form. Other studies showed that FasL on tumor cells abolished the production of antibodies to the tumor, reduced the number of cytotoxic T cells and suppress necrosis by diminishing immunity against tumor cells. We assume that increased FasL expression may endow pterygium with a local immune privileged site so that the tumor can escape from the normal immunological surveillance and thus favors its growth.

**Conclusion:** Flow cytometry with impression cytology showed an upregulation of FasL expression in pterygium. This finding implies that pterygium development may be in part associated with the model that Fas ligand transfers an immune tolerance to pterygium to escape from ocular immune defensive system against neoplasm.
References:

1. Stuart PM, et al. CD95 ligand (FasL)-induced apoptosis is necessary for corneal allograft survival. *J Clin Invest* 1997;99:396-402
Figure 1. Summary of methods

Methods
1. Sample collection: Impression cytology
2. Sample handling: Agitation & centrifugation for 30 mins in PBS
3. Indirect immunofluorescence staining: Fas & FasL
4. Flow cytometric analysis

Figure 2. Expression of FasL in the control and in pterygium
Figure 3. Positivity of FasL expression

Figure 4. Positivity of Fas expression