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LABORATORY INVESTIGATIONS

TGF-β2, Tenascin, and Integrin β1 Expression in Superior Limbic Keratoconjunctivitis

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Purpose: To determine the pathophysiological etiology of superior limbic keratoconjunctivitis (SLK), we compared the superior limbic conjunctivae of SLK patients and normal controls.

Methods: Frozen sections of conjunctival specimens from five SLK patients and two controls were examined by immunohistochemical methods. Transforming growth factor (TGF)-β2, integrin β1, and tenascin (TN) were chosen for analysis because their expression is known to be affected by mechanical stress or injury. The staining pattern was observed by confocal laser scanning microscopy.

Results: Prominent positive TGF-β2 staining on the surface region and heterogeneous staining in the suprabasal region were observed in the SLK specimens. TN expression was markedly up-regulated in the subepithelial stroma. In addition, suprabasal expression of integrin β1 was observed.

Conclusions: Up-regulation of TGF-β2 and TN suggested that an increased amount of mechanical stress existed in the conjunctivae of the SLK patients. In addition, deposition of TN and suprabasal expression of integrin β1 suggested that chronic minor injury contributed to the pathogenesis of SLK. **Jpn J Ophthalmol 1999;43:251–256** © 1999 Japanese Ophthalmological Society

Key Words: Superior limbic keratoconjunctivitis, transforming growth factor-β, tenascin, integrin β1, mechanical stress.

Introduction

Superior limbic keratoconjunctivitis (SLK) is an ocular surface disorder of unknown etiology originally reported by Theodore.[1] Clinically, it occurs most commonly between 20 and 60 years of age; and at least 75% of the cases occur in women, without racial predilection.[2] In a previous study, we reported that the limbal conjunctivae of SLK patients had alterations of cytokeratin expression and showed signs of hyperproliferation.[3]

Although the pathogenesis of SLK remains unknown, the mechanical stress between the upper tarsal and bulbar conjunctiva is believed to be involved in the pathophysiology of the disease.[4] This hypothesis is supported by the finding that snake-like chromatins, which are commonly observed in SLK,[3] are an indication of mechanical stress on the ocular surface.[5] On the other hand, the clinical observation that wearing a hard contact lens can induce SLK[6] suggested that chronic minor injury of the superior limbal epithelium might have a possible role in the pathogenesis of SLK.

Mechanical stress can induce the expression of various cytokines as well as the extracellular matrix, which can then alter the differentiation status of epithelial cells. Transforming growth factor (TGF)-β is a multipotential cytokine that is known to be induced by mechanical stress[7] and is expressed in the ocular surface epithelium.[8] Mechanical stress can also induce extracellular matrix tenascin (TN) whose expression is controlled spatiotemporally.[9]

It was also reported that both TN and integrin β1 are expressed during corneal wound healing.[10,11] To

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Table 1. Panel of Primary Antibodies

Antibodies	Type/Class	Antigen	Source
TGF-β2	Rabbit polyclonal/affinity-purified IgG	Synthetic peptide* corresponding to amino acid 352–377 of human TGF-β2	Santa Cruz Biotechnology (Santa Cruz, CA, USA)
Tenascin	Rat monoclonal/affinity-purified IgG	Purified human tenascin	Gift from Dr. M. Kusakabe [20]
Integrin β1	Mouse monoclonal/purified IgG1	Integrin β1 from human fibroblasts	Biohit

*Sequence of TGF-β2 peptide: YLWSSDTQHSRVLSLYNTINPEASAS

determine the possible involvement of mechanical stress and the wound healing process in the pathogenesis of SLK, we examined the expression of TGF-β, TN, and integrin β1 in the conjunctivae of SLK patients.

Materials and Methods

Patients

The superior limbic conjunctivae of five patients with SLK were resected therapeutically as described previously.[3] Three cases had a severe form of SLK with hyperemia in the upper bulbar conjunctiva showing intense Rose-Bengal and fluorescein staining, and ridge formation of the superior limbus. The remaining two patients had moderate cases of SLK with mild infection and focal Rose-Bengal and fluorescein staining in the superior limbic conjunctiva. All SLK patients were Japanese women aged 49, 54, 58, 62, and 66 years. Two normal superior limbic conjunctivae obtained during malignant orbital tumor surgery were also examined. The patients with orbital tumors were also Japanese women and their ages were 62 and 64 years. All these specimens were collected after obtaining appropriate informed consent.

Immunohistochemistry

Immunohistochemical study was performed essentially as described previously.[3] In brief, the specimens were immediately embedded in OCT compound (Miles, Elkhart, IN, USA), frozen in liquid nitrogen, and preserved at −75°C until used. Frozen sections of 5 μm were mounted on poly-L-lysine–coated slides and air dried for 30 minutes at room temperature. The slides were fixed in 4% paraformaldehyde in 0.1 mmol/L phosphate buffer (pH 7.4). After being washed in 0.01 mmol/L phosphate-buffered saline (PBS) (pH 7.4), slides were incubated in PBS containing 10% normal goat serum and 1% bovine serum albumin for 1 hour at room temperature to block nonspecific staining. The slides were then incubated overnight at 4°C with the primary antibodies listed in Table 1. After washing in 0.05% Tween 20 in PBS (TPBS), they were incubated with fluorescein isothiocyanate (FITC)-conjugated secondary antibodies. All slides were incubated for 1 hour at room temperature followed by three washings in TPBS. The staining was observed and recorded with a confocal laser scanning microscope (CLSM), (MC-1024; Bio-Rad Laboratories, Hercules, CA, USA). For negative control, nonimmune rat, mouse, or rabbit IgG was used instead of primary antibodies at the same concentrations.

Results

The clinical and laboratory findings in the SLK patients are summarized in Table 2. Histopathological analysis of the specimens was the same as published previously.[3] The fixed sections were stained either with hematoxylin-eosin (H-E) (Figure 1) or by indirect immunofluorescence staining with anti–TGF-β2, integrin β1, and TN antibodies. All sections were processed together with the negative controls. The negative control sections did not show any specific staining. The immunohistochemical findings are summarized in Table 3.

Table 2. Clinical Findings in SLK Patients

Patients	Age	Tasal Conjunctiva	Ridge Formation	Schrimer Test	Thyroid Function
A	49	Papillary proliferation		Normal	Hyperthyroidism
B	54	Papillary proliferation	+	Normal	Normal
C	58	Papillary proliferation	+	R 11.5 mm, L 8.5 mm	Normal
D	62	Papillary proliferation		Normal	Normal
E	66	Papillary proliferation	+	R 9 mm, L 14 mm	Normal

Table 3. Summary of Immunohistological Results

	TGF-β2	Tenascin	Integrin β1
Patients	Basal/Suprabasal/Superficial	Epithelium/Subepithelium	Basal/Suprabasal/Superficial
A	++/H/++	−/++	++/++/−
B	++/H/++	−/+++	++/++/+
C	++/H/++	−/+++	+++/++/+
D	++/H/+	−/++	++/++/+−
E	++/H/++	−/+++	+++/++/+
Control 1	++/+/+	−/+	+/+−/−
Control 2	++/+/+	−/+−	++/+−/−

+++: intense staining; ++: moderate staining; +: weak staining; +−: faint staining; H: heterogeneous staining.

Anti–TGF-β2 Staining

Anti–TGF-β2 staining of normal conjunctivae showed staining predominantly in the cytoplasm of the basal cells (Figure 2A). The staining pattern of SLK samples with the anti–TGF-β2 antibody was different from that of the controls. The basal and superficial cells showed strong cytoplasmic staining but the intensity of staining of the suprabasal cells varied from cell to cell. Some of the cells that did not show positive staining were observed as dark spots (Figure 2B, 2C). The section reacted with nonimmune rabbit IgG did not show any specific staining (Figure 2D).

Anti-TN Staining

Only faint linear anti-TN staining was observed at the epithelial–stromal junction and in the perivascular stroma in the normal conjunctiva (Figure 3A). In contrast, there was intense subepithelial staining of the stroma in the SLK sections (Figure 3B).

Anti-Integrin β1 Staining

The sections from normal conjunctivae showed positive peripheral cytoplasmic staining in the basal cells with the anti-integrin β1 antibody (Figure 4A). In contrast, the conjunctivae from SLK patients showed positive integrin β1 staining in almost the entire epithelium (Figure 4B). Integrin β1 was also present in the connective tissue as described previously.[12]

Discussion

We have shown an up-regulation of TGF-β2 expression in the surface region, and heterogeneous TGF-β2 expression as well as ectopic integrin β1 expression in the suprabasal region of the specimens from SLK patients. In addition, the expression of TN on the subepithelial stroma was prominently up-regulated in the limbal conjunctivae of these patients.

The dominant subtype of TGF-β expressed in the limbal epithelium was reported to be TGF-β2.[8] Our results are consistent with this in that TGF-β2 was the predominant subtype found on the limbal epithelium. We have also analyzed TGF-β1 and β3 expression in a preliminary study, and no definitive staining was observed (data not shown). Although the TGF-β2 antibody used was raised against synthetic poly-

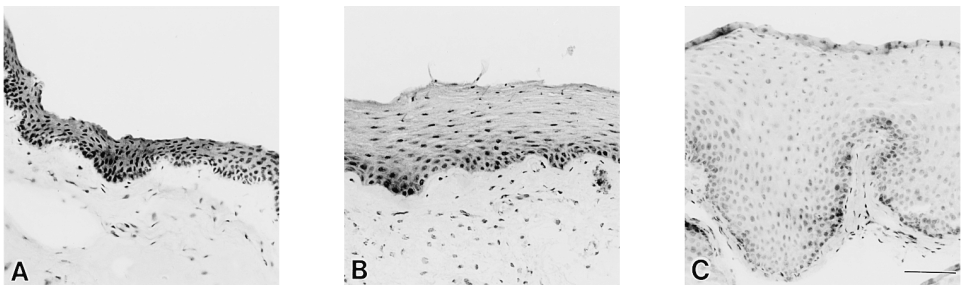

Figure 1. H-E staining of conjunctivae from control and SLK patients. (**A**) Normal superior limbic conjunctiva. Conjunctivae from patients with moderate forms (**B**) and severe forms (**C**) of SLK. Bar = 100 μm.

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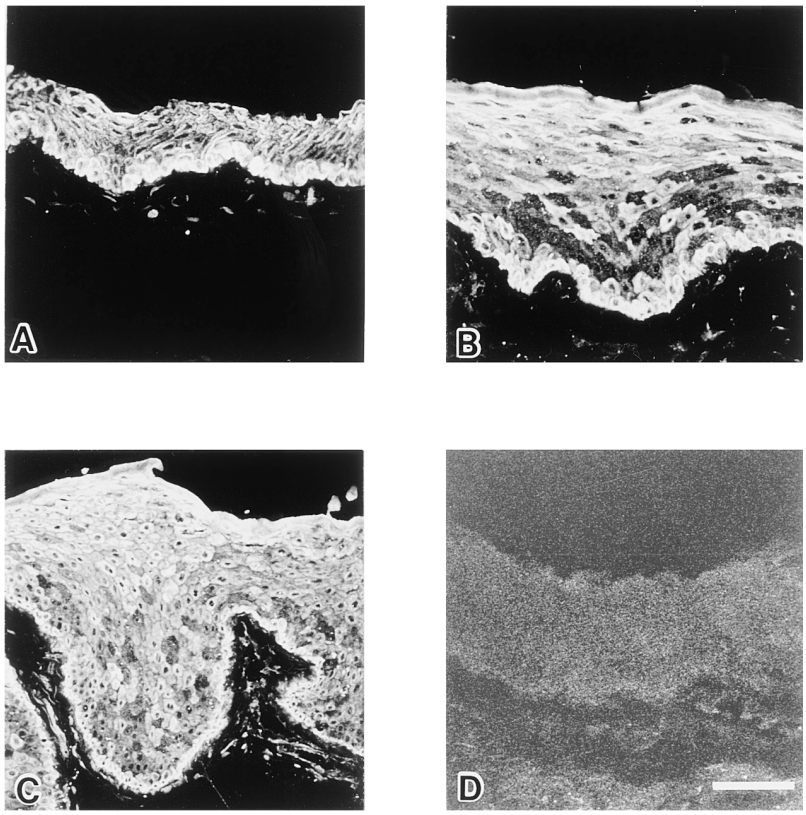

Figure 2. Anti–TGF-β2 staining of limbal conjunctiva. (**A**) Normal superior limbic conjunctiva. Strong basal staining and relatively homogeneous cytoplasmic staining of suprabasal cells. (**B, C**) Conjunctivae from SLK patients. Intense cytoplasmic staining in basal and superficial cells and heterogeneous cytoplasmic staining in suprabasal cells. (**D**) Section adjacent to figure B was reacted with nonimmune rabbit IgG. This negative control section does not show any specific staining. Bar = 100 μm.

peptides of mature TGF-β2, it is not known whether it is specific for the active form of TGF-β. Thus the data presented here should be accepted with this caution.

A previous study showed that mechanical stress could induce TGF-β expression.[7] Our study demonstrates prominent TGF-β2 expression, especially on the surface region of conjunctiva from SLK patients (Figure 1B, 1C). Because the primary mechanical stress on the SLK conjunctivae was from friction between limbal and tarsal conjunctiva, we believed this expression pattern was reasonable. The prominent papillary proliferation observed in the upper tarsal conjunctivae of all SLK patients supports this hypothesis (Table 2). On the other hand, we cannot fully explain the nature of the heterogeneous expression of TGF-β2 on the suprabasal cells in the conjunctivae of the patients. It should be noted that some of the suprabasal cells were completely devoid of TGF-β2 staining. This region correlated with the proliferating cell's nuclear antigen-positive region found in our previous study.[3] These results suggested the coexistence of a heterogeneous cell population at various differentiation/proliferation stages in the suprabasal cells of SLK patients. In addition, the expression of TGF-β2 in the basal cells did not differ significantly between normal and SLK conjunctivae (Figure 1). This finding suggested that another factor (eg, serum) may control the basal expression of TGF-β2. Factors other than mechanical stress that could affect the expression/activation of TGF-β should also be considered. More specifically, vitamin A (retinol), which is known to affect the activation of TGF-β[13] may also play a role in the pathogenesis of SLK.[14]

Another finding in this study was the prominent deposition of extracellular matrix TN on the subepithelial stroma of the conjunctivae of SLK patients (Figure 2B). To our knowledge, this is the first report showing an abnormality of the subepithelial tissue in SLK. TN is a large hexameric extracellular matrix glycoprotein that is known to be expressed in tumors and during wound healing.[9,10] The precise biological activities of TN remain unknown, but it affects cell adhesion[15] and corneal development.[16] We hypothesized that increased mechanical stress in the conjunctivae of SLK patients can induce prominent TN accumulation on the conjunctival stroma. It is well known that mechanical stress can induce TN expression.[9] As a consequence, increased TN on the

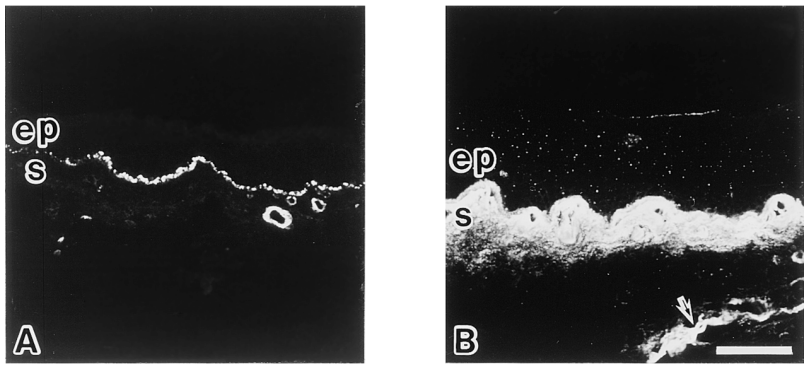

Figure 3. Anti-TN staining of limbal conjunctiva. (**A**) Normal superior limbic conjunctiva. Positive anti-TN staining on subepithelial stroma as well as perivascular stroma. (**B**) Conjunctiva from SLK patient. Intense anti-TN staining on subepithelial stroma. Longitudinal section of perivascular stroma (arrow) also showed positive anti-TN staining. ep: conjunctival epithelium; s: conjunctival stroma. Bar = 100 μm.

stroma may in turn alter the differentiation status of conjunctival epithelial cells and thus contribute to the pathogenesis of SLK. Other factors may contribute to the TN depositions on the subepithelial stroma of SLK patients. Cytokines or growth factors (eg, interleukin-1, basic fibroblast growth factor, and TGF-β) are known to induce TN expression.[17] The contribution of these factors to the pathogenesis of SLK should be examined further.

Suprabasal expression of integrin β1 during corneal wound healing has been reported.[11] When we compared the pathological state of SLK and the typical wound healing process, many similarities were observed. First, infiltration of inflammatory cells is a common feature of both conditions. Second, formation of a temporary extracellular matrix such as TN is commonly observed. Third, the local synthesis of cytokines such as TGF-β, which regulates the formation of granular tissue, is also observed. Considering these findings together with the suprabasal expression of integrin β1 (Figure 3B), we suppose that a chronic wound healing–like process contributes to the pathogenesis of SLK.

In summary, we have shown the expression patterns of TGF-β, TN, and integrin β1 in the conjunctivae of patients with SLK, and the results suggested that mechanical stress contributed to the pathophysiology of SLK. Although it should be noted that other factors (eg, cytokines, vitamin A deficiency) might affect the expression patterns, our findings support previous evidence for the contribution of mechanical stress to the pathophysiology of SLK. Taken together with the results of our previous study that showed the altered cytoskeleton and proliferative status of SLK, we believe SLK to be an excellent pathological model for "tensegrity." Tensegrity hypothesizes that mechanical stress can be transmitted to the cytoskeleton through the cell surface receptor integrin and extracellular matrix, and thus could affect cellular architecture and its response.[18] Further studies are needed to clarify the role of mechanical stress on the pathogenesis of SLK.

Recently, the effectiveness of superior lacrimal duct punctal occlusion as a therapy for SLK was reported.[19] This therapy seems reasonable to improve the wet ocular surface condition and therefore decrease mechanical strain and prevent the chronic injury–like phenomenon in SLK. We believe that additional therapies for SLK focusing on mechanical strain are quite promising.

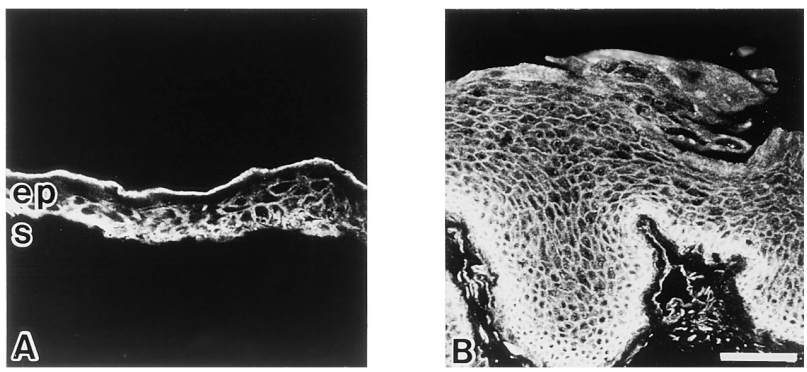

Figure 4. Anti-integrin β1 staining of limbal conjunctiva. (**A**) Normal superior limbic conjunctiva. Positive staining on surface of basal cells. ep: conjunctival epithelium; s: stroma. (**B**) Conjunctiva from SLK patient. Note positive integrin β1 staining in all layers of limbal conjunctiva. Bar = 100 μm.

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