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LABORATORY INVESTIGATIONS

The Expression of Laminin-5 and Ultrastructure of the Interface Between Basal Cells and Underlying Stroma in the Keratoconus Cornea

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Purpose: We investigated the expression of laminin-5 and integrins, and the ultrastructure of the interface between basal cells and the basement membrane in the keratoconus cornea. These findings were compared to those in normal central cornea and limbus.

Methods: Frozen sections of the normal cornea (center and limbus) and the keratoconus cornea were immunostained with monoclonal antibodies against three chains of laminin-5 and integrins. To investigate the ultrastructure of the interface between basal cells and the underlying stroma, we used transmission electron microscopy.

Results: As compared to those in the normal central cornea, immunostaining patterns of the three chains of laminin-5 were thick and irregular in the keratoconus cornea and the normal limbus. Using electron microscopy analysis, the same characteristic structure of the interface between basal cells and the underlying stroma was recognized in the keratoconus cornea and the normal limbus. The expression of integrin $\alpha_6\beta_4$ was restricted to the basal aspect of basal cells in the normal cornea. In the keratoconus cornea, however, integrin $\alpha_6\beta_4$ was expressed in all aspects in basal and suprabasal cells.

Conclusion: The expression patterns of laminin-5 and the ultrastructure of the interface between basal cells and the basement membrane in the keratoconus cornea were similar to those in the normal limbus. **Jpn J Ophthalmol 2001;45:209–215** © 2001 Japanese Ophthalmological Society

Key Words: Integrin, keratoconus, laminin-5, limbus.

Introduction

Keratoconus is a progressive disease characterized by thinning and scarring of the central portion of the cornea. This disease most commonly starts in early adolescence, and gradually causes the cornea to become cone-shaped. In the advanced form, high myopia with irregular astigmatism and corneal opacity decrease visual acuity and the patient has to undergo penetrating keratoplasty. Many studies, including biochemical and molecular biological approaches, to account for the pathogenesis and development of keratoconus, have been advanced.[1–7] However, its etiology remains unclear.

The earliest histopathological changes in the keratoconus cornea were considered to be fragmentation of epithelial basement membrane and discontinuities in Bowman's layer. The epithelium tends to be thin and exhibits loss of normal architecture in the area of the cone as the disease progresses. However, little is known about the mechanism of the loss of normal architecture. The basement membrane not only separates the epithelium from the underlying stroma, but also maintains the structure of the epithelial layer. Recent studies revealed that the basement membrane could play a positive role in determining the differential stage in corneal basal cells.[8,9] Basal

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cells in the corneal epithelium adhere to the underlying basement membrane via hemidesmosomes, and undergo division in such a manner that one daughter cell remains a basal cell, while the other enters the terminal differentiation pathway. The interaction between basal cells and the basement membrane via adhesion proteins is considered to be important for basal cell differentiation and for the maintenance of the epithelial architecture.[10,11] Therefore, abnormalities in the epithelial architecture in the keratoconus cornea may depend on the interaction between basal cells and the basement membrane.

The basement membrane is composed of a mixture of matrix components including collagens, laminins, and heparan sulfate proteoglycans. Among these extracellular matrixes, laminin is a major component of the basement membrane. Of 11 laminin isoforms, laminin-1 and laminin-5 are major components in the corneal basement membrane. Laminin-5 is a new laminin variant composed of $\alpha 3 \cdot \beta 3$ and $\gamma 2$ chains.[12] It is well known that laminin-5 has a strong adhesive ability in vitro and plays an essential role in the formation of hemidesmosomes.[13–15] Hemidesmosomes are composed of several molecules including BP180, BP250, and integrin $\alpha_6\beta_4$.[16,17] Integrin $\alpha_6\beta_4$ is a receptor of laminin-5. Integrin$\alpha_3\beta_1$, another receptor of laminin-5, has been considered to contribute to basal cell adhesion via focal contact.[18,19] Anchoring filaments in the lamina lucida contain laminin-5 and bridge hemidesmosomes with lamina densa. Therefore, laminin-5 and integrins $\alpha_6\beta_4$ and $\alpha_3\beta_1$ are key molecules in the adhesion between basal cells and the basement membrane.

In the current study, we separated two distinct regions from the anterior portion in the keratoconus cornea, the nonscarred (presence of Bowman's layer) region and the scarred (defect of Bowman's layer) region. We investigated (a) the expression of laminin-5, integrins $\alpha_6\beta_4$ and $\alpha_3\beta_1$ by immunohistochemical analysis and (b) the structure of the interface between basal cells and the underlying stroma with electron microscopy at the scarred region in the keratoconus cornea. Then, we compared these findings with those in the normal central cornea and limbus.

Patient Profiles

We examined 3 cases of keratoconus.

Patient 1. A 32-year-old man had a past history of rupture in Descemet's membrane. Slit-lamp examination revealed protrusion, thinning, and opacity of the central to the lower part of the cornea. Deeper opacity could be seen at the apex of the cone resulting from the rupture in Descemet's membrane. Visual acuity: left eye 0.01 (0.15 × −7.00 Dcyl −5.00 D Ax85°) (0.20 × hard contact lens).

Patient 2. A 28-year-old man with fine posterior stromal folds known as Vogt's striae found on slit-lamp examination near the apex of the cone. Corneal thinning (one third of normal thickness) was observed in the apex of the protrusion. Visual acuity: right eye 0.03 (0.03 × −15.0 Dcyl −4.00 D Ax140°) (0.08 × hard contact lens). The visual impairment of the eye seemed to be attributable to severe irregular astigmatism.

Patient 3. A 26-year-old man whose slit-lamp examination revealed reticular, subepithelial, and anterior stromal scars within the cone. Visual acuity: left eye 0.2 (0.2 × −8.00 Dcyl −5.00 D Ax90°) (0.2 × hard contact lens).

Materials and Methods

Normal central cornea and limbus were obtained from two retinoblastoma patients (aged 3 and 5 years) and keratoconus corneas were obtained after penetrating keratoplasty from 3 patients (aged 32, 28, and 26 years) after obtaining informed consent. All corneas were embedded in an optimal cutting temperature compound and frozen in liquid nitrogen immediately. Then, 5-μm-thick cryostat sections were prepared and fixed in 100% cold acetone for 15 minutes. Immunohistochemical analysis was carried out using a labeled streptavidin biotin technique (LSAB kit®; DAKO, Tokyo) at room temperature, except for primary antibody incubation, which was performed for 24 hours at 4°C. The primary antibodies used in this study were monoclonal antibodies (mAbs) to the $\alpha 3 \cdot G3 \cdot \gamma 2$ chains of laminin-5 and $\alpha_6\beta_4$, $\alpha_3\beta_1$ integrins. Diaminobenzidine tetra hydrochloride was used as a chromogen, and the sections were counterstained with methyl green. MAbs to the $\alpha 3$ chain (clone P3E4) of laminin-5, integrin β_4 (clone ASC-8) and integrin α_3 (clone ASC-1) were purchased from Chemicon (Temecula, CA, USA). MAbs against the $\beta 3$ and $\gamma 2$ chains of laminin-5 were kindly provided by Dr. Miyazaki (Division of Cell Biology, Kihara Institute for Biological Research). The MAb to the integrin α_6 (clone GoH3) was obtained from Pharmingen (San Diego, CA, USA).

For transmission electron microscopy, these tissues (keratoconus cornea and normal limbus) were fixed in 2.5% glutaraldehyde in Sorensen's buffer, post-fixed in osmium tetroxide and routinely embedded and sectioned.

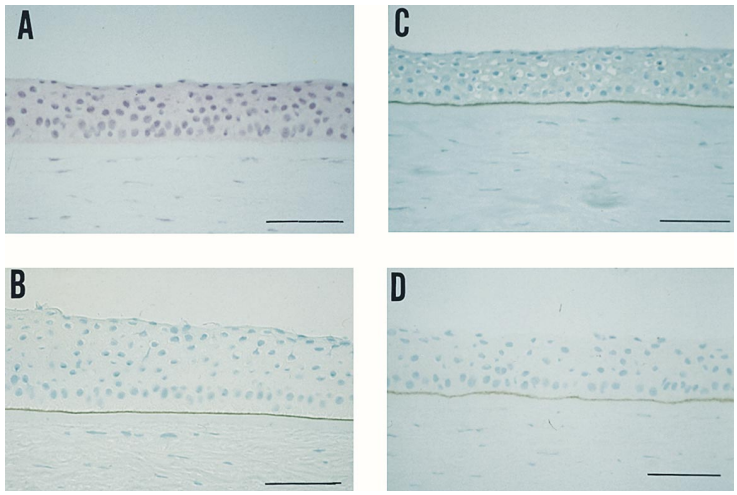

Figure 1. Hematoxylin eosin (HE) stain in normal central cornea (**A**). Expression of laminin-5 in normal central cornea by immunohistochemistry. α3 chain (**B**), β3 chain (**C**), and γ2 chain (**D**) are expressed along basement membrane. These staining patterns are linear.

Results

Laminin-5

We used monoclonal antibodies against the α3 · β3 · γ2 chains of laminin-5. These three chains were expressed along the basement membrane in the normal central cornea. These staining patterns were linear (Figure 1). At the scarred (defect of Bowman's layer) region in the keratoconus cornea, the staining patterns of the three chains of laminin-5 were thick and irregular (Figure 2). These staining patterns were similar to those in the normal limbus (Figure 3).

$\alpha_6\beta_4$ Integrin and $\alpha_3\beta_1$ Integrin

The $\alpha_6\beta_4$ integrin is a receptor of laminin-5 and a component of hemidesmosomes. In the normal central cornea, the expression of the β4 subunit was restricted to the basal aspect of basal cells, and the α_6 subunit was strongly expressed in the basal aspect and weakly expressed on the lateral membrane of basal cells. At the scarred region in the keratoconus cornea, the β_4 subunit was expressed in not only the basal aspect, but also in all aspects of basal and suprabasal cells, except for superficial cells. The staining pattern of the basal aspect in basal cells was thick and irregular. The expression of the α_6 subunit was also recognized in all aspects in the basal cells and suprabasal cells, except for superficial cells. The staining pattern of the α_6 subunit of the basal aspect in basal cells was thick and irregular (Figure 4). $\alpha_3\beta_1$ integrin was expressed in all aspects of the basal and suprabasal cells in normal and keratoconus corneas (Figure 5). Frames of Figures 1–5 were taken with a ×100 lens.

Electron Microscopic Analysis

At the scarred region in the keratoconus cornea, basal cells had fine processes directly into the under-

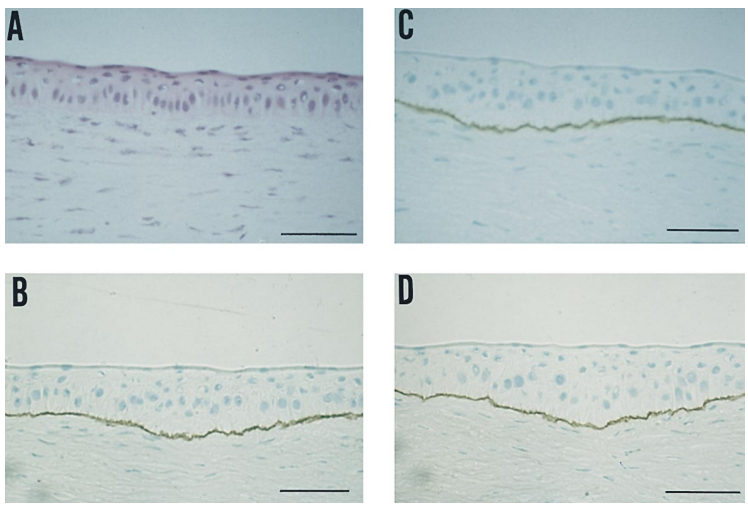

Figure. 2. HE stain in scarred region (defect in Bowman's layer) in keratoconus cornea (**A**). Expression of laminin-5 in keratoconus cornea by immunohistochemistry. α3 chain (**B**), β3 chain (**C**), and γ2 chain (**D**) are expressed in scarred region on keratoconus cornea. These staining patterns are irregular and thick.

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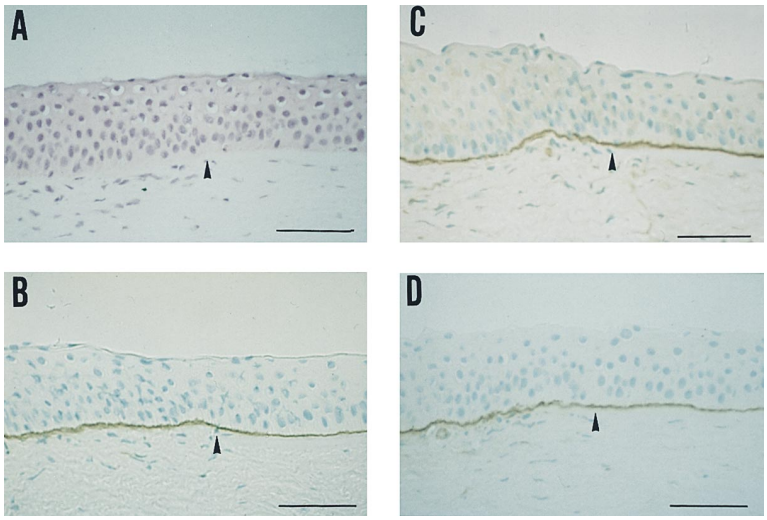

Figure 3. HE stain in limbus (**A**). Expression of laminin-5 in limbus by immunohistochemistry. α3 chain (**B**), β3 chain (**C**), and γ2 chain (**D**) are expressed in limbus. To left of arrow is limbus area. These staining patterns in limbus are irregular and thick.

lying stroma. The surface of these processes displayed many hemidesmosomes (Figure 6). The sections perpendicular to the plane of the normal limbus showed a highly convoluted basal aspect of basal cells with many hemidesmosomes (Figure 7).

Discussion

Laminin-5 is a recently identified laminin variant composed of three nonidentical subunits, α3 · β3 × γ2, and is a major component of the corneal basement membrane.[20,21] It is well known that laminin-5 has a strong adhesive capability in vitro and maintains the normal architecture of the epidermis. Mutations in the gene which encode for the α3 subunit of laminin-5 have been identified in patients with junctional epidermolysis bullosa.[22] This is a severe skin-blistering disease in which the epidermis separates from the basement membrane. Furthermore, recent study showed that laminin-5 could play an essential role in the formation of hemidesmosomes.[13,15] In the normal central cornea, the expression of three chains of laminin-5 was recognized along the basement membrane. Its staining pattern was linear. However, at the scarred region of the keratoconus cornea, the staining pattern was thick and irregular. This staining pattern was similar to that of the normal limbus. This result may indicate that there were similar interface structures between the basal cells and the underlying stroma in the scarred region in the keratoconus cornea and the normal limbus. In our electron microscopic analysis of the scarred region in the keratoconus cornea, basal cells had fine processes extending into the stroma. The surface of these processes displayed many hemidesmosomes. On the other hand, the section perpendicular to the

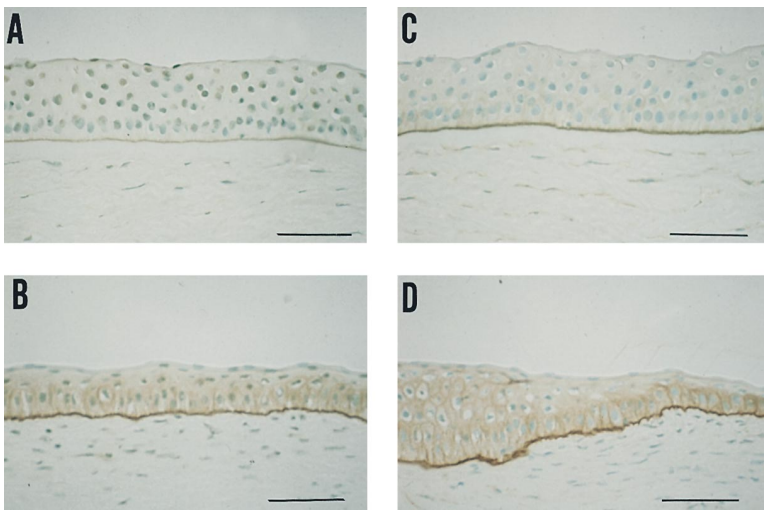

Figure 4. Expression of β_4 integrin is restricted to basal aspect of basal cells in normal cornea (**A**). This staining pattern is linear. In scarred region of keratoconus cornea (**B**) the expression of β_4 integrin is recognized in all aspects of basal and suprabasal cells, except for superficial cells. Staining pattern of basal aspect in basal cells is irregular and thick. On the other hand, expression of α_6 integrin is strongly expressed on basal aspect and weakly expressed on lateral aspect of basal cells in normal cornea (**C**). In scarred region of keratoconus cornea (**D**) expression of α_6 integrin is recognized in all aspects of basal and suprabasal cells, except for superficial cells. The staining pattern of basal aspect in basal cells is irregular and thick.

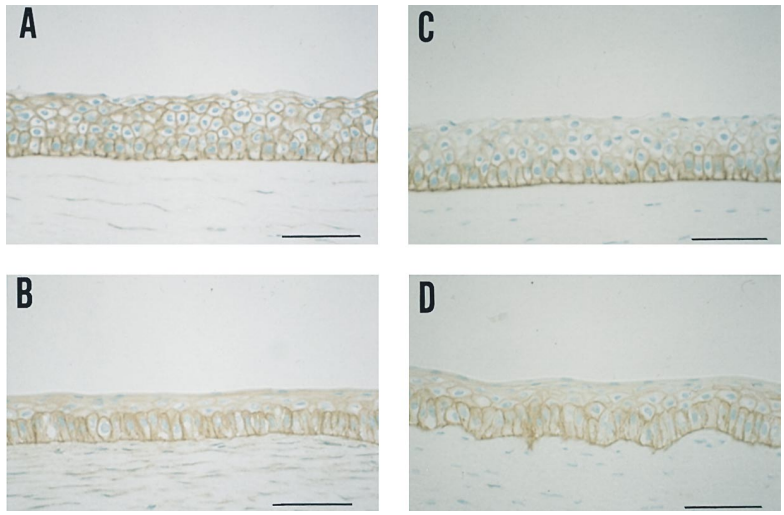

Figure 5. Expression of β_1 and α_3 integrin in normal central cornea and keratoconus cornea. β_1 integrin is expressed in all layers of epithelium in normal (**A**) and keratoconus cornea (**B**). α_3 integrin is also expressed in all layers of epithelium in normal (**C**) and keratoconus cornea (**D**), except for superficial cells. Intensity of staining for α_3 integrin decreases from basal to suprabasal cells.

plane of the normal limbus showed a highly convoluted basal surface of basal cells with many hemidesmosomes. Therefore, thick and irregular staining patterns of laminin-5 in the keratoconus cornea and the normal limbus may depend on a similar structure in the interface between the basal cells and underlying stroma. Especially, we noted that the presence of these structures between the basal cells and underlying stroma gave rise to a large increase in basal cell surface compared to the area covered. Also, increasing the attached area may promote firm adhesion between basal cells and the underlying stroma. Firm adhesion may prevent normal differentiation of basal cells in the keratocunus cornea. Basal cells of the limbus are considered to be stem cells for the corneal epithelium.[9,23,24] Stem cells have high potential for proliferation and a low differentiation capability. Kenney et al demonstrated that tenascin-C, fibrillin-1, and the α_1-α_2 chains of type IV collagen were expressed along the basement membrane at the scarred region in the keratoconus cornea.[25] These three proteins were absent in normal central corneas, but were expressed in the normal limbus. It was speculated that the different composition of the basement membrane in the keratoconus cornea may reflect changes in epithelial basal cell differentiation toward a less differentiated limbal phenotype. Therefore, these characteristic structures in the

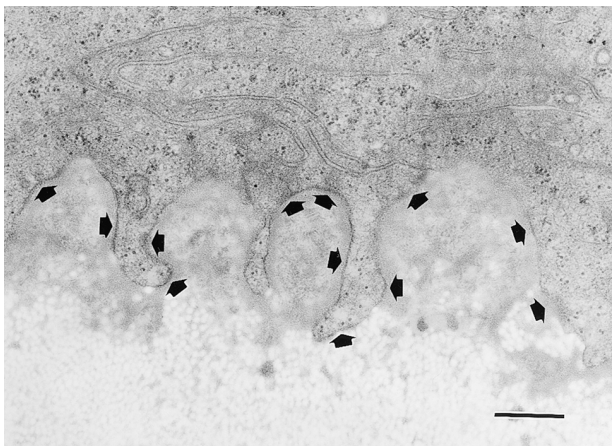

Figure 6. Ultrastructure of interface between basal cells and underlying stroma in keratoconus cornea. At scarred region in keratoconus cornea, basal cells have fine processes into underlying stroma. Surface of these processes displays many hemidesmosomes (large arrows).

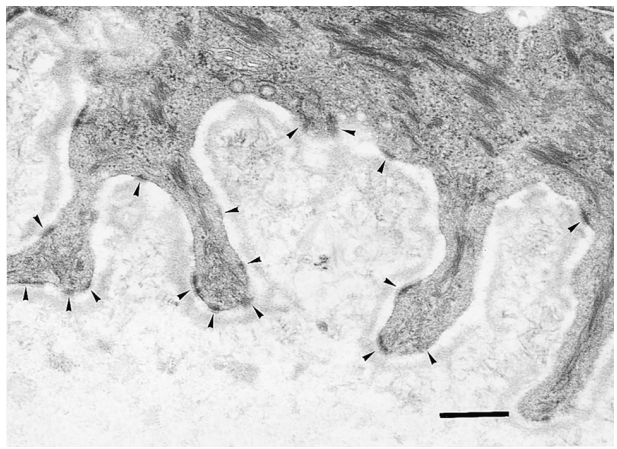

Figure 7. Ultrastructure of interface between basal cells and underlying stroma in normal limbus. Section perpendicular to plane of limbus shows highly convoluted basal aspect of basal cells with many hemidesmosomes (small arrows).

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keratoconus cornea and normal limbus may force these basal cells to change in cell differentiation towards a less differentiated stage.

Integrins are transmembrane glycoproteins, recognizing cell–cell and cell–matrix interactions. The ligand specificity of integrins depends on the α/β subunit combinations. $\alpha_6\beta_4$ integrin is a receptor of laminin-5 and a component of hemidesmosomes.[16,17] Recent studies revealed that $\alpha_6\beta_4$ integrin is an essential molecule for the maintenance of normal epidermis architecture. Also, mutations in the gene which encode for the β_4 submit of integrin have been identified in patients with junctional epidermolysis bullosa.[26,27] In the normal cornea, the expression of the β_4 integrin subunit was recognized in only the basal aspect of basal cells. Its staining pattern was linear. However, at the scarred region in the keratoconus cornea, the β_4 subunit was expressed in not only the basal aspect, but also the lateral and apical aspects of basal and suprabasal cells, except for superficial cells. In addition, the staining pattern of the basal aspect in basal cells was thick and irregular. In the normal cornea, the α_6 subunit was strongly expressed in the basal aspect and weakly expressed in the lateral aspect of basal cells. At the scarred region in the keratoconus cornea, the α_6 subunit was recognized in all aspects in basal cells and suprabasal cells, except for superficial cells. The staining pattern of the basal aspect in basal cells was thick and irregular. This staining pattern of $\alpha_6\beta_4$ integrin in the basal aspect may also depend on the structure of the interface between the basal cells and underlying stroma. In quiescent conditions of the corneal epithelium, $\alpha_6\beta_4$ integrin is strictly located in the basal aspect of basal cells. Recent studies showed changes in the localization of the $\alpha_6\beta_4$ integrin in wound healing. Stepp et al revealed redistribution of the β_4 subunit to nonhemidesmosomal locations at the apical and lateral aspects of basal and suprabasal cells after corneal wound healing in vivo.[28] Latvala T et al showed the same changes in the distribution of the α_6 subunit after corneal injury.[29] Recent reports indicated that integrin polarization on cell surfaces is not established in fetal skin and is lost to virally transformed keratinocytes. These reports indicated that depolarization of the $\alpha_6\beta_4$ integrin showed changes in cell proliferative capability toward a high potential for proliferation. Therefore, the depolarization of the $\alpha_6\beta_4$ integrin in the keratoconus cornea may show changes in the proliferative capability of basal cells toward a high potential for proliferation. The expression of $\alpha_3\beta_1$ integrin was similar to that in the normal cornea.

In conclusion, we demonstrated the possibility that at the scarred region in the keratoconus cornea direct contact between the epithelium and underlying stroma would produce firm adhesion, a change in the capability of differentiation and in the proliferative capability of basal cells, resulting in the loss of normal architecture in the epithelium.

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