



Corneal Epithelial Basement Membrane After Experimental Anterior Stromal Puncture in Guinea Pigs: Immunohistochemical Study

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Abstract: We investigated alterations in the immunolocalization of the components of epithelial basement membrane (BM), type IV collagen, and laminin in guinea pigs subjected to anterior stromal puncture (ASP) of the cornea performed with a standardized needle. Localization of BM components beneath the corneal epithelium was indicated by laminin immunoreactivity. The BM was interrupted by needle punctures immediately after ASP. During healing, type IV collagen immunoreactivity was detected transiently in the BM of some of the ASP-treated corneas, but no reactivity was observed in normal epithelial BM. Development of type IV collagen immunoreactivity was probably caused by an alteration of the α -chains or by an unmasking of the antigenicity of this collagen type, which may be related to an increase in the adhesiveness of the epithelium following ASP. *Jpn J Ophthalmol* 1997;41:376-380 © 1997 Japanese Ophthalmological Society

Key Words: Anterior stromal puncture, collagen type IV, cornea, immunohistochemistry, laminin.

Introduction

Anterior stromal puncture (ASP) is used in treating primary recurrent corneal erosion.¹⁻³ Although ASP appears effective in stimulating the attachment of the epithelium to the underlying connective tissue, its mechanism remains unclear.⁴⁻⁶ Basement membrane (BM), which is destroyed during ASP, is composed of type IV collagen, laminin, and other glycoproteins; anchoring structures of type VII collagen and hemidesmosomal components are also distributed in the BM region. Damage and reconstruction of connective tissue containing extracellular matrix, such as collagen types, laminin, and fibronectin, may contribute to an enhanced healing of epithelial wounds.^{7,8}

ASP may influence the composition of the extracellular matrix in the corneal epithelial BM.

It is difficult to create an experimental model of a standardized persistent epithelial defect in animals. In the present study, we evaluated the immunolocalization of the extracellular matrix components of epithelial basement membrane, collagen type IV, and laminin in guinea pig corneas with a simple epithelial defect after ASP, recognizing that a persistent epithelial defect and a simple epithelial abrasion follow different clinical courses.

Materials and Methods

Anterior Stromal Puncture

The experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee, Wakayama Medical College. Guinea pigs of both sexes ($N = 40$) were used. During general anesthesia produced by the inhalation of ether, we abraded the central corneal epithelium with a small trephine and a scalpel, leaving the BM intact. Multiple superficial nonpenetrating wounds into the ante-

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rior stroma were created with a 26-gauge disposable hypodermic needle. The animals were sacrificed at specific intervals (up to 30 days) by intraperitoneal injection of an overdose of ketamine hydrochloride or pentobarbital sodium, following the inhalation of ether. Each eye was then enucleated.

Immunohistochemistry

The anterior part of each eye was embedded in OCT compound (Miles Inc., Elkhart, IN, USA). Cryosections (5 μm) were fixed in cold acetone. After the sections had been rinsed in 0.1M phosphate-buffered saline (PBS; pH 7.4), primary antibodies were allowed to react with each specimen for 45 minutes in a moist chamber at room temperature. The two primary antibodies used were mouse monoclonal antitype IV antibody ($\times 100$ in PBS, donated by the Department of Pathology, Wakayama Medical College) and rabbit polyclonal antilaminin antibody ($\times 2,000$ in PBS; LSL, Tokyo). After a second rinsing of the sections in PBS, secondary antibodies were allowed to react with the specimens for 30 minutes at room temperature. The two secondary antibodies used were peroxidase-conjugated sheep antimouse IgG antibody and goat antirabbit antibody ($\times 100$ in PBS; Cappel, Organon Technika, West Chester, PA, USA). After another rinse of the sections in PBS, the color of the antibody complex was developed with 0.05% 3,3'-diaminobenzidine (DAB) in tris-HCl buffer containing 0.06% hydrogen peroxide. The specimens were then counterstained with methyl green, dehydrated through a graded ethanol series, and mounted on Canada balsam. Each cryosection was also stained with hematoxylin and eosin and observed by light microscopy.

Results

Histologic Changes

Immediately after the epithelial abrasion and ASP had been produced, a round epithelial defect and some nonpenetrating wounds were visible in each cornea (Figure 1). A sheet of epithelium gradually migrated onto the abraded area from the peripheral normal area. Twelve hours after ASP, two of three corneas were completely covered with new epithelium; all corneas were re-epithelialized after 24 hours. On days 7 and 30, the epithelium of all corneas seemed to be restored to a normal configuration (Figure 2).

Collagen Type IV

An antitype IV collagen antibody was found bound to the BM zone under the conjunctival and limbal

epithelium of the normal guinea pig ocular surface, but no DAB reaction was detected in the corneal epithelial BM. On day 3, however, epithelial BM of the central cornea showed a collagen type IV immunoreactivity (Figure 3). Between days 3 and 7, we detected immunoreactivity among the collagen lamellae. We detected no type IV collagen immuno-reactivity of the epithelial BM of the cornea 30 days after ASP.

Laminin

Laminin, a major component of the BM, was detected in the BM beneath the conjunctival, limbal, and corneal epithelium and in the limbal vascular endothelial BM (Figure 4). Immediately after ASP, DAB reaction was seen on the BM. One day later, laminin was detected under the new corneal epithelium. On days 2 and 3, laminin immunoreactivity was restored to the normal linear configuration. Duplication of laminin immunoreactivity was also observed.

Controls

No specific DAB reaction was detected in control stainings, even though endogenous peroxidase had not been inactivated. A nonspecific reaction was sometimes seen in the epithelium or on the epithelial surface (not shown).

Discussion

Anterior stromal puncture is reportedly effective in promoting epithelial wound healing in the presence of a persistent epithelial defect, which is characterized by delayed epithelial covering due to either a compromised adhesiveness of epithelial cells or an abnormal connective tissue scaffold. The exact cause of the persistent epithelial defect and the mechanism of the accelerated epithelial wound healing after ASP are still unknown.

It is reported that corneal incisions are covered by epithelial plug by day 1.⁹ Activated keratocytes are found adjacent to the basement membrane defect by day 7. The BM appears to be healed at 2 and 4 weeks. Proliferation of epithelial cells into the stromal incisions, with an underlying mature BM, are detectable 5 months after surgery.

Treatment by ASP was followed by fibronectin and laminin immunoreactivity, whereas no reactivities were detected in untreated corneal epithelial BM.³ We believe that this development of extracellular matrix components may contribute to enhanced epithelial wound healing. In the present study, ASP was performed in guinea pig corneas with simple epithelial defect, which differs from persistent epithe-

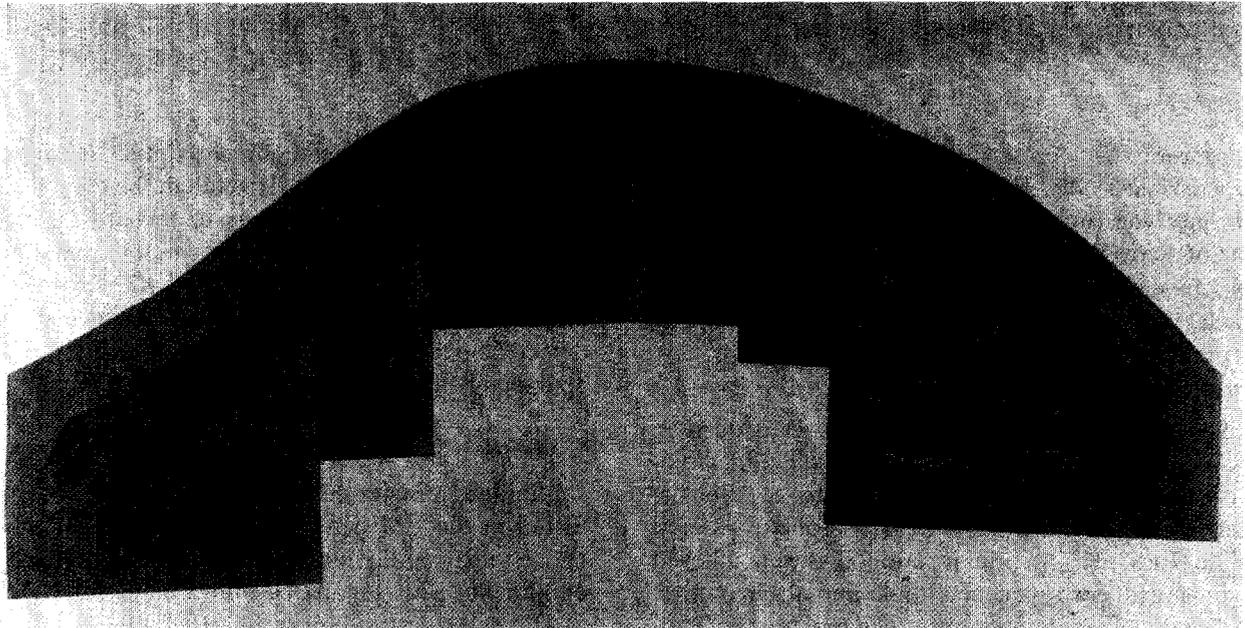


Figure 1. Low-magnification photomicrograph of guinea pig cornea immediately after anterior stromal puncture. Round epithelial defect and some nonpenetrating wounds are seen in the central cornea (between the arrows). Arrowheads show basement membrane defects. H&E staining. Original magnification $\times 22$.

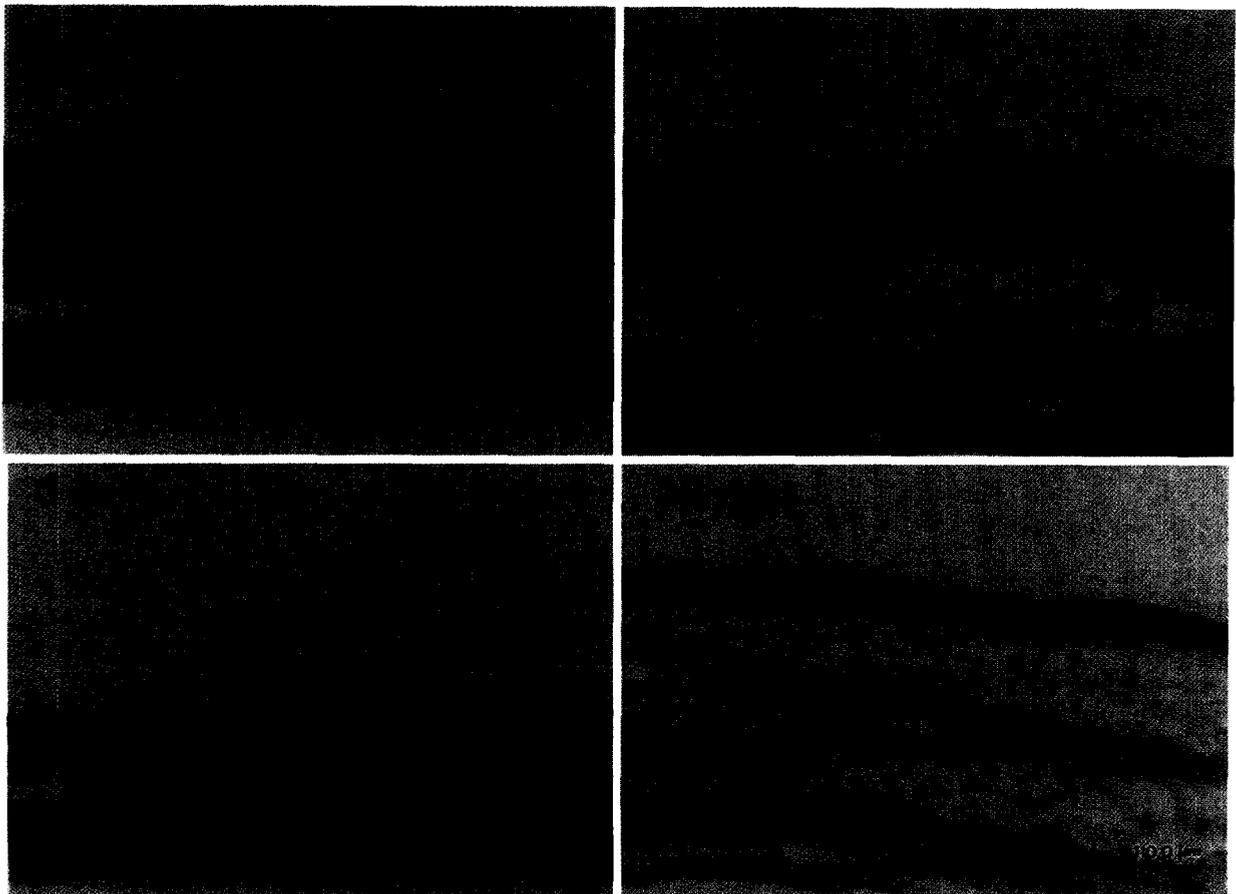


Figure 2. Photomicrographs of guinea pig corneas of normal (C), immediately (0 hr), 48 hours (48 hr), and 30 days after anterior stromal puncture (30 days). Epithelial basement membrane defects (arrowheads) are observed immediately after anterior stromal puncture. Epithelial defect has been re-epithelialized 48 hours and 30 days after anterior stromal puncture. E = epithelium; S = stroma. H&E staining. Original magnification $\times 109$.

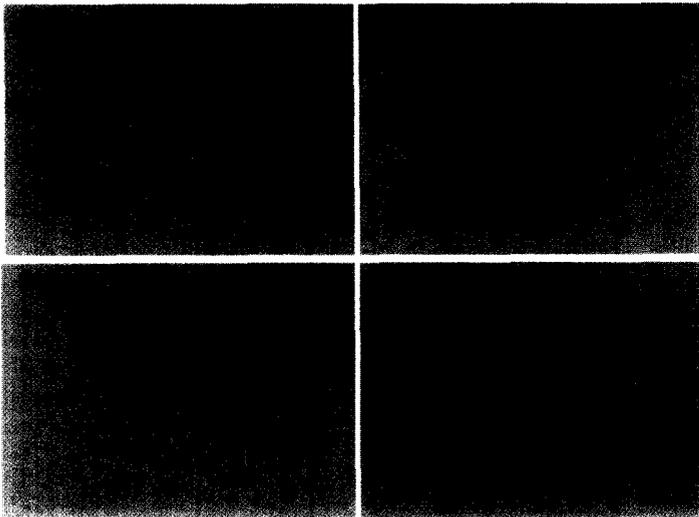


Figure 3. Photomicrographs of immunohistochemical examination using antitype IV collagen monoclonal antibody in guinea pig corneas of normal (C), immediately (0 hr), 48 hours (48 hr), and 30 days after anterior stromal puncture (30 days). In cornea 48 hours after anterior stromal puncture (48 hr), type IV collagen localizes in the basement membrane (arrows), but the corneas of normal, immediately, and 30 days after anterior stromal puncture show no signs of type IV collagen. Arrowheads show basement membrane defects. Original magnification $\times 109$.

lial defect, most likely in the adhesiveness of epithelial cells and in BM scaffold.

Our results indicated that laminin reactivity was interrupted immediately after ASP and that, in the healing phase, type IV collagen immunoreactivity developed transiently in the epithelial BM, but no type IV immunoreactivity occurred in normal corneal epithelial BM. We used a monoclonal antibody that reacts with the $\alpha 1(\text{IV})_2 \alpha 2(\text{IV})$ molecule. The results of efforts to localize type IV collagen in corneal epithelial BM are controversial; some investigators have reported that conjunctival epithelial BM shows type IV collagen immunoreactivity^{10,11} but that BM in the cornea does not.¹²⁻¹⁵ Others have reported that both corneal and conjunctival epithelial BM show immunoreactivity for type IV collagen.¹⁶ Ljubimov et al¹⁷ reported that type IV collagen in

the corneal epithelial BM is composed of $\alpha 3(\text{IV})$, $\alpha 4(\text{IV})$, and $\alpha 5(\text{IV})$, whereas that in the conjunctival epithelial BM is composed of $\alpha 1(\text{IV})$ and $\alpha 2(\text{IV})$. It is also possible that the antigenicity of type IV collagen is masked in corneal epithelial BM. It remains unclear whether the development of type IV collagen immunoreactivity in the corneal epithelial BM exposed to ASP is caused by an alteration of α chain of type IV collagen or by an unmasking of the antigenicity of this type of collagen.

We reported earlier a similar expression of type IV collagen protein in the healing corneal epithelial BM following alkali burns.^{18,19} In both ASP-treated cornea and alkali-burned cornea, not only is the squamous epithelium damaged, so too is the underlying anterior stroma. Some unknown factor(s) secreted by the repopulating keratocytes, which are

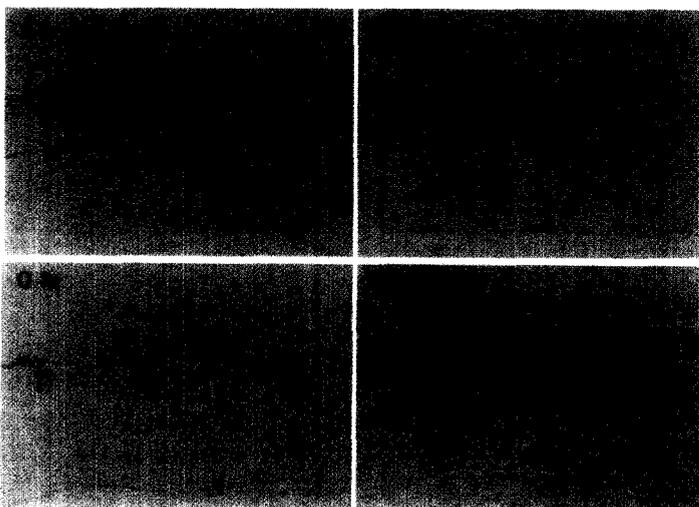


Figure 4. Photomicrographs of immunohistochemical examination using antilaminin monoclonal antibody in guinea pig corneas of normal (C), immediately (0 hr), 48 hours (48 hr), and 30 days after anterior stromal puncture (30 days). Laminin is detected in the basement membrane (arrows) in each cornea. Arrowheads show basement membrane defects. Original magnification $\times 109$.

thought to resemble activated fibroblasts, may influence the development of an alteration of type IV collagen in squamous epithelium.

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