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LABORATORY INVESTIGATIONS

Abnormal Deposition of Laminin and Type IV Collagen at Corneal Epithelial Basement Membrane During Wound Healing in Diabetic Rats

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Purpose: To understand the pathophysiology of the corneal basement membrane in diabetes, we compared the localization of laminin and type IV collagen in the epithelial basement membrane during corneal epithelial wound healing in diabetic and nondiabetic rats.

Methods: Streptozotocin was used to induce diabetes in half the rats. Two weeks later, the whole corneal epithelium was debrided. Diabetic and healthy rats (3–5 per group) were sacrificed before debridement and 1, 3, and 7 days and 1 month afterwards. The localization of laminin and type IV collagen was observed in cryosections by epifluorescence microscopy.

Results: In unwounded corneas of both diabetic and normal rats, laminin and type IV collagen were localized in the corneal epithelial basement. The intensity of fluorescence, however, was clearly stronger in the diabetic rats. In normal rats, wounding initially removed laminin and type IV collagen, but during healing these two proteins reappeared beneath the resurfacing corneal epithelium. Although similar results were observed in diabetic rats, the expression of laminin and type IV collagen was delayed, and their deposition was fragmented and irregular.

Conclusions: These results suggest that delayed corneal epithelial wound healing in diabetes might involve delayed reappearance and abnormal reformation of epithelial basement membrane proteins. **Jpn J Ophthalmol 1999;43:343–347** © 1999 Japanese Ophthalmological Society

Key Words: Basement membrane, corneal epithelium, diabetes, laminin, type IV collagen.

Introduction

Corneal complications of diabetes mellitus, reported by several researchers, include delayed corneal epithelial wound healing and a decrease in epithelial barrier function, tear production, corneal sensation, and corneal hydration.[1–6] Among these complications, delayed corneal epithelial wound healing can cause serious clinical problems for diabetic patients after intraocular surgery, especially vitrectomy. Although the mechanisms of this delayed wound healing are not yet fully understood, changes in the synthesis and expression of basement membrane proteins may play a role.

The corneal epithelial basement membrane is a thin extracellular matrix that separates epithelial cells from the corneal stroma. Abnormality of basement membranes has been reported as a common underlying factor in various other types of diabetic complications, such as diabetic retinopathy, nephropathy, and neuropathy.[7–11] In studies of diabetic keratopathy, several researchers have reported abnormal thickening and multilayering of the corneal epithelial basement membrane,[1,2,12] and changes in the interaction of epithelial cells and basement membrane.[13–15]

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Two major components of basement membranes, the protein laminin and type IV collagen, play important roles in the attachment of cells to the basement membrane, and also in cell migration, proliferation and differentiation.[7,16–18] Diabetes alters the synthesis and expression of these proteins in various basement membranes.[19–22]

To study the pathophysiology of the epithelial basement membrane in diabetic cornea, we used immunofluorescence techniques to compare chronological changes in the distribution of laminin and type IV collagen at the corneal epithelial basement membrane in diabetic and nondiabetic rats during corneal epithelial wound healing.

Materials and Methods

Four-week-old male Sprague-Dawley rats (Japan SLC, Shizuoka), weighing about 100 g, were used. Diabetes was induced by injection of streptozotocin (Sigma Chemical, St Louis, MO, USA), 70 mg/kg of body weight in 0.01 mol/L citrate buffer through the tail vein after one night of fasting. Control (nondiabetic) rats were injected with 0.01 mol/L citrate buffer solution. The use and treatment of rats in this study conformed to the ARVO Resolution on the Use of Animals in Research.

Two weeks after injection of streptozotocin, the rats were anesthetized with an intraperitoneal injection of pentobarbital and topical oxybuprocaine drops to each eye. The corneal epithelium was scraped off, from limbus to limbus, with a dulled scalpel blade. The blood sugar levels of diabetic and nondiabetic rats were in the ranges of 500–600 mg/dL and 100–200 mg/dL, respectively.

Three to five diabetic and nondiabetic rats were killed by intraperitoneal injection of sodium pentobarbital before debridement and 1, 3, and 7 days and 1 month after debridement. The eyes were enucleated, embedded immediately in OCT compound (Miles, Elkhart, IN, USA), and frozen in acetone/dry ice. Sections, 8-μm thick, were cut from each eye with a microtome cryostat (HM 505 N; Micom, Walldrof, Germany); each was mounted on a gelatin-coated glass slide. The specimens were rinsed with phosphate-buffered saline (PBS), incubated with 1% bovine serum albumin (BSA; Fraction V; Sigma) in PBS for 30 minutes at room temperature to block nonspecific binding, and again washed with PBS. Sections were then incubated for 60 minutes at room temperature in a moist chamber with rabbit anti-mouse laminin (LSL, Tokyo) diluted 1:1000 with 1% BSA in PBS, or rabbit anti-bovine type IV collagen (LSL) diluted 1:300 with 1% BSA in PBS as a primary antibody. For control staining, normal rabbit serum (Cappel Organon Teknika, Durham, NC, USA) at 1:300 with 1% BSA in PBS was used in place of the corresponding primary antibody. The specimens were rinsed with PBS four times, 5 minutes per rinse, and then fluorescein-isothiocyanate-labeled goat anti-rabbit IgG (Cappel Organon Teknika) diluted 1:600 with 1% BSA in PBS was applied as a secondary antibody; the specimens were incubated for 60 minutes at room temperature in a moist chamber. They were again rinsed with PBS four times for 5 minutes each rinse and were mounted in 1:2 glycerin/PBS solution. Sections were observed with an epifluorescence microscope (Leitz DM IL; Leica, Wetzlar, Germany). Photographs were taken with Fujichrome Provia 100 reversal film (ISO 100; Fuji Film, Tokyo). Only faint background fluorescence was observed in specimens that underwent control staining.

Results

Normal Rat Corneas

In unwounded normal corneas, immunoreactivity in laminin and type IV collagen in the corneal epithelial basement membrane was similar, visualized as sharp, well-defined, continuous lines (Figures 1a, 1f). Specks of fluorescence in type IV collagen were observed also within the stromal layer (Figure 1f). One day after debridement, edema of the corneal stroma was observed. Immunoreactivity in laminin and type IV collagen was visible beneath the migrating corneal epithelial cells, but the fluorescence was slightly irregular and not as intense as that seen in the normal, unwounded cornea (Figures 1b, 1g). At 3 days, strong immunoreactivity in laminin and type IV collagen was observed at the interface between the healed epithelium and stroma (Figures 1c, 1h). At 7 days, the fluorescence specific to laminin and type IV collagen was sharp, well-defined, and continuous (Figures 1d, 1i). One month after debridement, the localization patterns of laminin and type IV collagen were identical to those observed in the normal unwounded cornea (Figures 1e, 1j). These results were in agreement with those reported previously.[23]

Diabetic Rat Corneas

In unwounded corneas of diabetic rats, laminin and type IV collagen were again localized in the basement membrane of the corneal epithelium (Figures 2a, 2f), but the immunofluorescence, especially in laminin, created thicker bands than observed in nondiabetic corneas. Furthermore, the specks of flu-

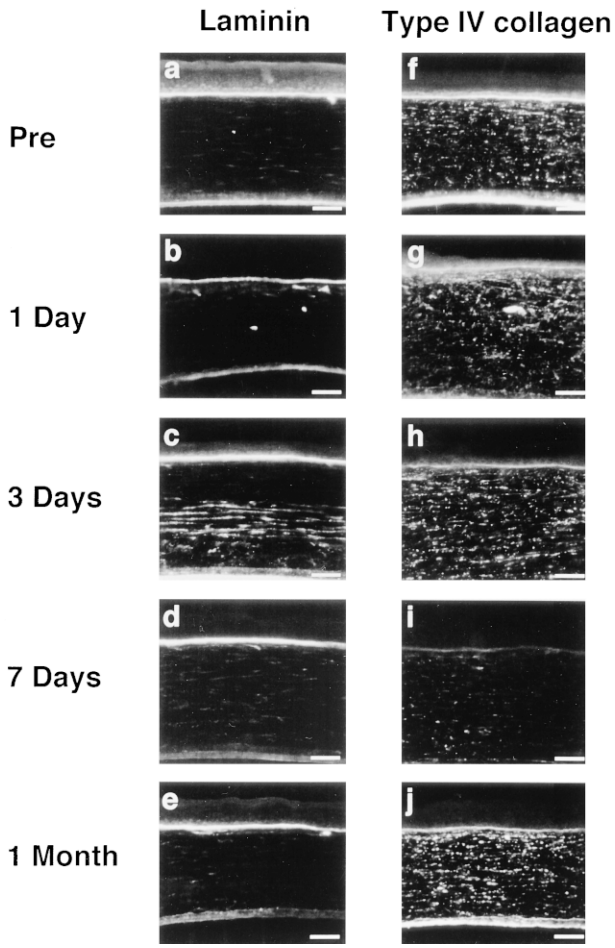

Figure 1. Immunofluorescence micrographs showing laminin (**a–e**) and type IV collagen (**f–j**) before corneal debridement (a, f), at 1 (b, g), 3 (c, h), or 7 days (d, i), and 1 month (e, j) after debridement in nondiabetic rats. Bars = 50 μm.

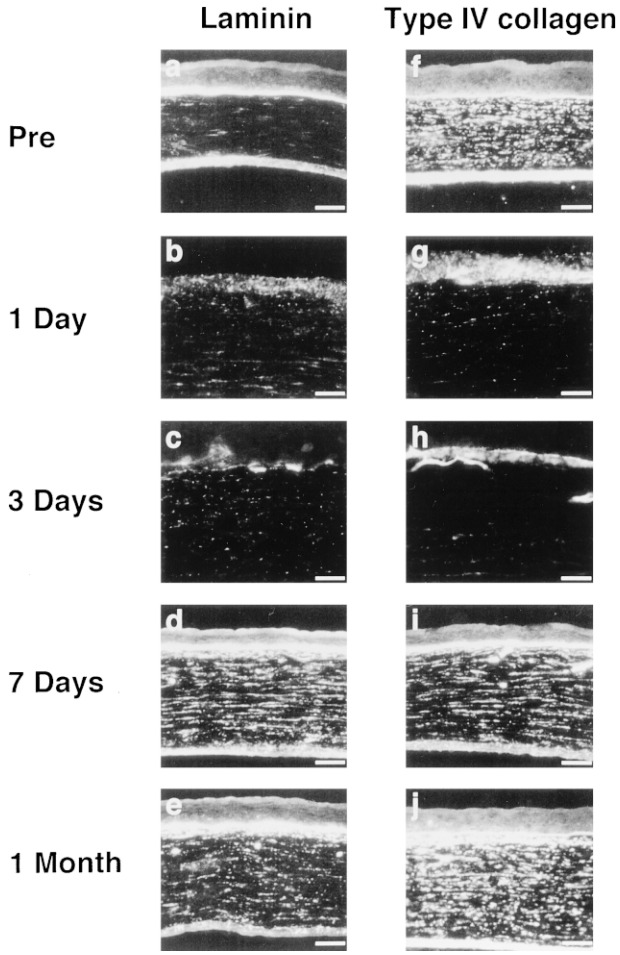

Figure 2. Immunofluorescence micrographs showing laminin (**a–e**) and type IV collagen (**f–j**) before corneal debridement (a, f), at 1 (b, g), 3 (c, h), or 7 days (d, i), and 1 month (e, j) after debridement in diabetic rats. Bars = 50 mm.

orescence in type IV collagen within the stromal layer were increased, compared with those seen in nondiabetic corneas. One day after debridement, edema of the corneal stroma was observed. Resurfacing of the corneal epithelium was delayed, and patchy depositions of laminin and type IV collagen were observed in the anterior quarter of the stroma (Figures 2b, 2g). Furthermore, the specks of fluorescence in type IV collagen within the stromal layer had disappeared. After 3 days, the edema of the corneal stroma persisted; areas of specific fluorescence indicating laminin and type IV collagen in the basement membrane were layered and more intense, but fragmented and irregular (Figures 2c, 2h). Specks of fluorescence in type IV collagen within the stromal layer were still absent. At 7 days, the edema of the corneal stroma was no longer observed, and the corneal epithelium was resurfaced. The fluorescence specific to laminin and type IV collagen at the epithelial basement membrane formed continuous lines, but they lacked uniformity, and their borders were unclear (Figures 2d, 2i). Specks of fluorescence in laminin and type IV collagen within the stromal layer were observed. One month after debridement, the corneal stroma and epithelium had returned to their condition before the debridement. Fluorescence specific to laminin and type IV collagen formed continuous lines at the corneal epithelial basement membrane, but they remained irregular and corrugated (Figures 2e, 2j).

Discussion

In the present study, we used immunofluorescence techniques to investigate the chronological changes

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in laminin and type IV collagen, two components of epithelial basement membrane, after corneal epithelial debridement of healthy and diabetic rats. Extracellular laminin and type IV collagen participate in the attachment, migration, proliferation, and differentiation in various types of cells.[7,16–18] We previously reported that laminin and type IV collagen are essential for the maintenance of the epithelial structure during corneal epithelial wound healing.[23] In the present study, diabetes delayed the reappearance of laminin and type IV collagen at the basement membrane after debridement; specific fluorescence revealed the deposition of laminin and type IV collagen to be thick and irregular. Our present results are consistent with previous reports of increased thickness of corneal epithelial basement membrane[2,12] and a delayed rate of re-epithelialization of denuded corneas in diabetes.[24,25] We also have reported delayed epithelial wound healing in the same diabetic model used in the present study.[26,27]

Azar and Gipson[14] reported that the pattern of histochemical localization of bullous pemphigoid antigen (an intracellular hemidesmosome plaque component), laminin, and type VII collagen (the anchoring fibril collagen) were the same following superficial keratectomy of normal rabbits and rabbits with alloxan-induced diabetes. The difference between their results and ours may represent a species difference. Other investigators have found that the rate of corneal epithelial wound healing was the same in diabetic and nondiabetic rabbits,[28,29] but that the rate in diabetic rats was delayed.[24–27] Similarly, Azar and Gipson[14] observed no difference between the morphometric parameters of epithelial adhesion structures in diabetic and nondiabetic rabbits; however, these morphological alterations were observed in diabetic rats.[30] Because delayed corneal epithelial wound healing and morphometric changes of the adhesion structures have been reported in diabetic humans,[2,13,30] diabetic rats may be suitable models for the investigations of diabetic keratopathy.

Our present findings of delayed and abnormal reformation of laminin and type IV collagen may reflect changes in the mechanisms of interaction between epithelial cells and basement membrane, such as reduced numbers of hemidesmosomes and decreased penetration of anchoring fibrils into the stroma.[13,30] Cultured corneal epithelial cells synthesize and deposit basement membrane components, including laminin and type IV collagen.[31] However, diabetes mellitus reportedly causes disorders of the corneal epithelium, such as decreased epithelial barrier function and morphological changes.[4,32] Taken together, these different investigations suggest that delayed corneal epithelial wound healing in diabetes may be related to not only the abnormality of basement membrane, but also the abnormalities of the corneal epithelial cells.

Protein glycosylation, one of the most important problems in diabetes mellitus, might be anticipated to affect the biological functions of extracellular matrix proteins.[33] Indeed, glycosylated laminin and type IV collagen showed reduced ability to participate in cell adhesion and cell spreading.[34] However, glycosylated fibronectin, another extracellular matrix protein, maintained its biological functions in cell adhesion, cell migration, and binding to other proteins.[35] Therefore, fibronectin might be a biologically active, temporary extracellular matrix functioning in place of abnormal basement membrane in denuded diabetic corneas.

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