

Advanced Glycation End Products in Diabetic Corneas

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PURPOSE. Corneal complications are often associated with diabetes mellitus and can be vision threatening. Corneas in diabetic patients are exposed to increased glucose concentration despite cornea's avascular property, and this condition may contribute to the accumulation of advanced glycation end products (AGEs). The focus of this study was to examine the role of AGEs in the pathogenesis of diabetic keratopathy.

METHODS. An anti-AGE monoclonal antibody (6D12), which recognizes a N^ε-carboxymethyl lysine (CML)-protein adduct as an epitope, was prepared. Immunohistochemical localization of CML was examined in human age-matched diabetic and nondiabetic corneas (8 of each). In vitro, type I collagen-, type IV collagen-, or laminin-coated 96-well plates were glycated by glucose-6-phosphate. In some experiments, aminoguanidine was present in the incubation mixture. The amounts of CML-protein adducts in the extracellular matrix (ECM) were determined by enzyme-linked immunosorbent assay using 6D12. SV40-immortalized human corneal epithelial cells were seeded onto modified or unmodified ECM in 96-well plates and allowed to attach for 3 hours. Attached cells were fixed, and the areas of attached cells in each condition were measured. Attached cells without fixation were removed, and cell number was counted.

RESULTS. In all of the 8 diabetic corneas, CML immunoreactivity was observed in the epithelial basement membrane, whereas CML immunoreactivity was not found in the corresponding area in 7 of 8 nondiabetic corneas. In vitro, nonenzymatic glycation of laminin on the culture dish attenuated adhesion and spreading of corneal epithelial cells. The presence of aminoguanidine in the incubation mixture during glycation inhibited CML formation and promoted the adhesion and spreading of corneal epithelial cells in a dose-dependent manner.

CONCLUSIONS. The accumulation of AGEs on the basement membrane, particularly on laminin, may play a causative role in the corneal epithelial disorders of diabetic patients. (*Invest Ophthalmol Vis Sci.* 2000;41:362-368)

Corneal abnormalities in patients with diabetes mellitus have been demonstrated and have been termed "diabetic keratopathy."¹ Histologically, thickening of the corneal epithelial basement membrane²⁻⁴ and morphologic changes of the corneal epithelium and endothelium⁵⁻⁷ have been reported. Clinically, the damage to the corneal epithelium during vitreous surgery and retinal photocoagulation sometimes induces vision-threatening corneal complications such as persistent epithelial defect, recurrent erosion, and stromal edema in diabetic patients.⁸⁻¹¹ Some reports have remarked that the activation of sorbitol pathway might be involved in the pathogenesis of diabetic keratopathy.^{12,13} However, the pathogenic mechanisms underlying those abnormalities are still unclear.

The accumulation of advanced glycation end products (AGEs) has been suggested as a pathogenic mechanism underlying diabetic complications such as nephropathy,¹⁴ retinopathy,¹⁵ and cataract.^{16,17} Chronic hyperglycemia leads, through early-stage products of nonenzymatic glycation such as Schiff bases and Amadori products, to the formation of AGEs on proteins. Among several structures reported so far, N^ε-(carboxymethyl) lysine (CML) has been identified as a dominant AGE antigen in tissue proteins.^{18,19}

Previous reports^{10,20} indicated that corneas in diabetic patients are exposed to increased glucose concentration despite the cornea's avascular property. This condition may contribute to the accumulation of AGEs in diabetic corneas. However, the existence and the distribution of AGEs in diabetic corneas have not been examined. We prepared an anti-AGE monoclonal antibody and examined the role of AGEs in the pathogenesis of diabetic keratopathy.

MATERIALS AND METHODS

Human Corneal Specimens

Sixteen human corneas were obtained from 16 autopsy cases within 6 hours postmortem. We examined 8 corneas from 8 patients with the history of diabetes mellitus (6 men and 2

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women) and 8 corneas from 8 persons without the history of diabetes mellitus (5 men and 3 women). The mean ages of the diabetic and nondiabetic subjects were 69.9 ± 6.79 (mean \pm SD; range, 58–80) and 64.1 ± 10.1 (range, 45–78) years, respectively. The averages of age in the two groups were not significantly different (Mann–Whitney U test, $P = 0.21$). The type of diabetes mellitus was non-insulin dependent in 7 patients and unknown in one patient. The average duration of diabetic history in 5 patients was 11.2 ± 3.6 years (range, 6–16). The duration of diabetic history was unknown in the remaining 3 patients. The diabetic retinopathy was present in 4 diabetic patients, and the presence or absence of diabetic retinopathy was unknown in the other 4 diabetic patients.

Antibody

An anti-AGE monoclonal antibody (6D12) was prepared as previously described.²¹ Briefly, mice were immunized with AGE-modified albumin, and the splenic lymphocytes from the immunized mouse were fused to myeloma cells. Culture supernatant of hybrid cells was screened by its reactivity to AGEs. The selected cell lines were injected into Balb/c mice for production of ascites. The antibody was purified by protein G-Sepharose DEAE-cellulose chromatography. Our recent study revealed that this antibody recognizes a CML-protein adduct as an epitope.¹⁹

Immunohistochemical Localization of CML

Formalin-fixed and paraffin-embedded corneas were cut into 3- μ m-thick sections, deparaffinized by xylene, and dehydrated by graded ethanol. The sections were treated with 3% hydrogen peroxide in phosphate-buffered saline (PBS) for 15 minutes and then rinsed in PBS. To increase the immunoreactivity of AGEs, the sections were placed in 500 ml of 0.01 M of citric acid-buffered solution (pH 7.0) and applied to microwave (500 W) for 5 minutes.^{22,23} After thorough washing, the sections were incubated with the normal rabbit serum for 20 minutes at room temperature to avoid nonspecific binding of the antibodies. The sections were then incubated overnight at 4°C with 3 μ g/ml of the anti-AGE monoclonal antibody (6D12) in PBS containing 1% of bovine serum albumin. Immunoreactivity was detected by the streptavidin-biotin-peroxidase method using a Histofine SAB-PO (M) kit (Nichirei, Tokyo, Japan) according to the manufacturer's protocol. The final reaction product was visualized with 3,3'-diaminobenzidine tetrahydrochloride (DAB). We repeated the experiments three times.

Corneal Epithelial Cell Line and Culture Condition

We used immortalized human corneal epithelial cells with a recombinant SV40-adenovirus vector.²⁴ This cell line exhibits a cobblestone-like appearance similar to that of basal cells of normal corneal epithelium in culture, and can differentiate in a multilayered fashion when grown at air-liquid interface on collagen gel. They were grown in supplemented hormone epithelial medium as previously described.²⁴ The culture was maintained in a humidified atmosphere (95% air and 5% CO₂) at 37°C.

In Vitro Glycation of Extracellular Matrix Components

Type I collagen-, type IV collagen-, or laminin-coated 96-well plates were purchased (Falcon, Oxnard, CA). According to the manufacturer's information, the laminin was produced by mouse Engelbreth-Holm-Swarm tumor and mainly had laminin-1. To glycate extracellular matrix (ECM) components of cultured dishes, 200 μ l of 50 mM glucose-6-phosphate (G-6-P; Wako, Osaka, Japan) in 0.2 M NaHPO₄-buffered solution (pH 7.8) was added in each well under sterile conditions, and dishes were maintained at 37°C for 3 weeks. In some experiments, aminoguanidine (2–20 mM) was present in the incubation mixture. The control wells were incubated without G-6-P.

Measurement of CML Contents in ECM Components

The amounts of CML-protein adducts in ECM were determined by enzyme-linked immunosorbent assay (ELISA) using 6D12. The glycated and nonglycated wells were washed 3 times with PBS and blocked with 0.5% of gelatin hydrolysate (Sigma, St. Louis, MO) in 0.05 M carbonate-buffered solution (pH 9.6) for 1 hour. The wells were washed 3 times with washing buffer containing 0.05% of Tween-20 in PBS and were incubated for 1 hour with 100 μ l/well of 0.1 mg/ml of the anti-AGE antibody (6D12) in dilution buffer containing 0.3% bovine serum albumin (Sigma) in washing buffer. The wells were then washed with washing buffer 3 times and incubated for 1 hour with 100 μ l/well of horseradish peroxidase-conjugated anti-mouse IgG diluted 1:10,000. After a thorough washing, the wells were developed with 200 μ l/well of substrate solution containing 0.55 mg/ml of ortho-phenylenediamine and 0.002% of hydrogen peroxide in 50 mM of citric acid-buffered solution (pH 5.0). The reaction was stopped by 50 μ l/well of 1 M H₂SO₄, and the absorbance at 490 nm was measured using DigiScan Reader (ASYS Hitech GmbH, Eugendorf, Austria). The experiments were repeated 8 times.

Cell Spreading and Adhesion on ECM Components

Human corneal epithelial cells were seeded at 10,000 cells/0.25 ml onto modified or unmodified ECM in 96-well plates and allowed to attach for 3 hours. The cultured media and unattached cells were aspirated, and the wells were gently washed with PBS. Attached cells were fixed and stained with Diff-Quick staining set (International Reagents, Kobe, Japan), and random pictures of attached cells were taken under light microscope (Nikon, Tokyo, Japan) at the magnification of 200. By analyzing the pictures using NIH image, the areas of 50 attached cells in each condition were measured. Attached cells without fixation were removed by exposing each well to 0.25 ml of trypsin-EDTA (GIBCO, Grand Island, NY) for 20 minutes. Isoton solution (American Scientific Products, McGraw, IL) was added to the cell suspension to a final volume of 10 ml, and cell number was counted with a Coulter counter (type Z1). The experiments were repeated 8 times.

Statistical Analysis

The effect of glycation and aminoguanidine was analyzed by ANOVA. Additionally, the Scheffé multiple comparison test was used to compare the differences between all possible pairs of means within each experiment.

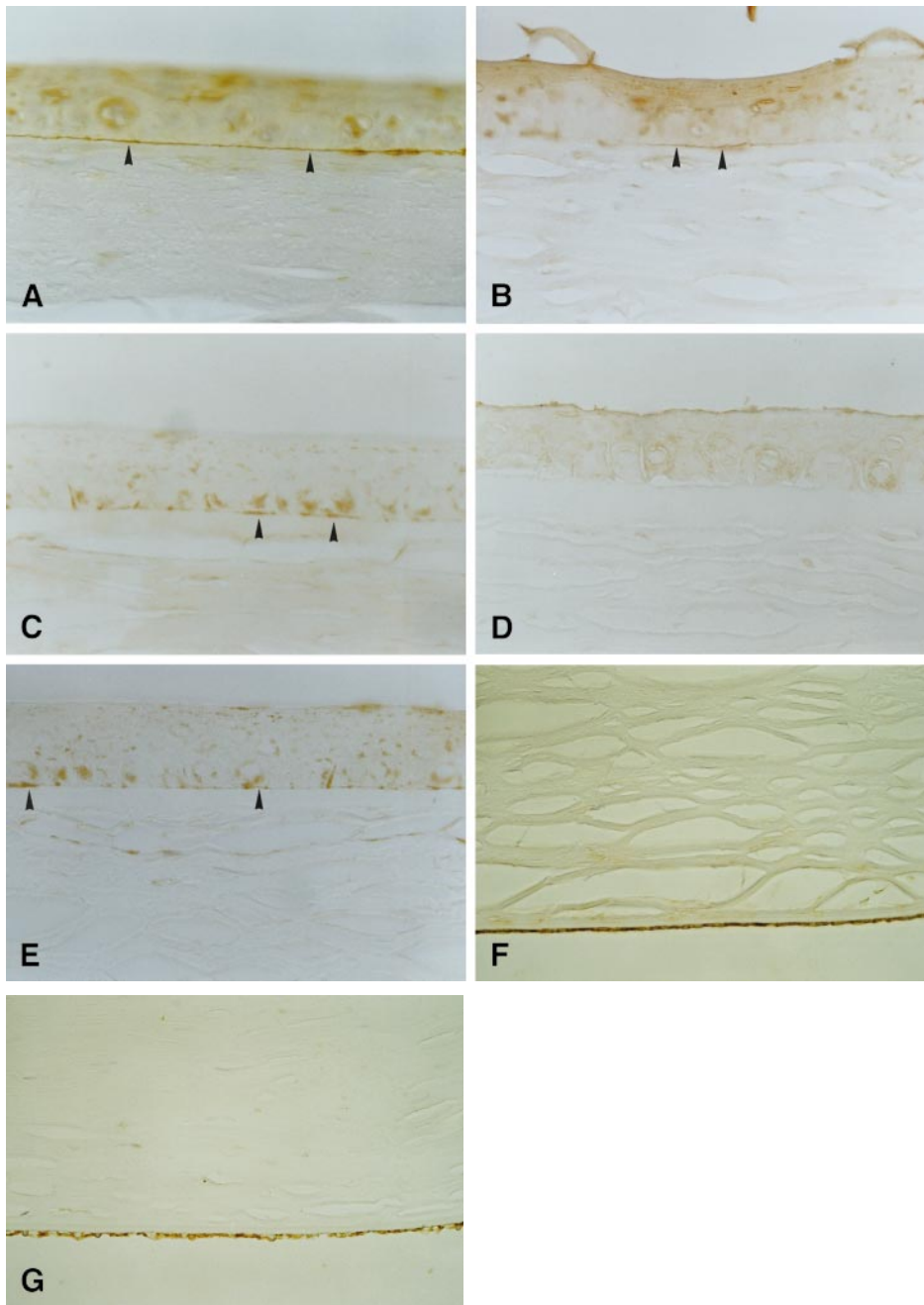


FIGURE 1. Immunohistochemical localization of CML in diabetic (A, B, C, F) and nondiabetic (D, E, G) corneas. In all of the 8 diabetic human corneas, CML immunoreactivity was observed in the epithelial basement membrane area (*arrowheads* in A, B, C), whereas CML immunoreactivity was not found in the corresponding area in 7 of the 8 nondiabetic corneas (D). In one nondiabetic cornea obtained from a 72-year-old man, CML immunoreactivity was observed at the site of epithelial basement membrane (*arrowheads* in E). CML was detected in the endothelial cells of all the examined corneas from both diabetes (F) and nondiabetes (G). Descemet's membrane did not show CML immunoreactivity in the examined corneas from diabetes (F) or nondiabetes (G). Magnification, $\times 200$.

RESULTS

Immunohistochemical Localization of CML in Human Corneas

In all the examined diabetic corneas, CML immunoreactivity was observed at the site of the epithelial basement membrane (Figs. 1A-1C), whereas CML immunoreactivity was not found in the corresponding area in 7 of 8 nondiabetic corneas (Fig. 1D). In one nondiabetic cornea obtained from 72-year-old man, CML immunoreactivity was observed at the site of epithelial basement membrane (Fig. 1E). CML was detected in the endothelial cells of all the examined corneas from both diabetes (Fig. 1F) and nondiabetes (Fig. 1G). Descemet's membrane did not show CML immunoreactivity in the examined corneas from diabetes (Fig. 1F) or nondiabetes (Fig. 1G).

Glycation of ECM

In the culture dishes without ECM, incubation with G-6-P induced little CML. In addition, incubation without G-6-P formed little CML in ECM. After incubation with G-6-P, CML was detected in the culture dishes with type I collagen, type IV collagen, and laminin. The presence of aminoguanidine in the incubation mixture during glycation dose-dependently inhibited CML formation with G-6-P (Fig. 2).

Effect of ECM Glycation on Attachment of Corneal Epithelial Cells

The numbers of attached corneal epithelial cells on glycated type I and type IV collagens did not significantly differ from those of attached cells on non-glycated type I and type IV collagens, respectively (Fig. 3). In contrast, glycation of laminin

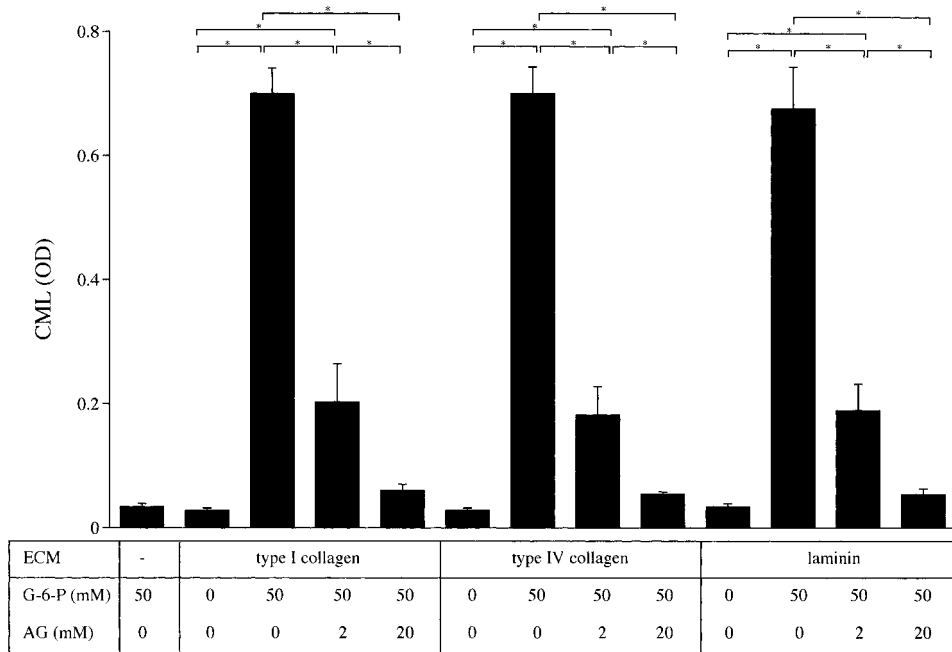


FIGURE 2. Effects of incubation with G-6-P and aminoguanidine (AG) on glycation of ECM components. Incubation with G-6-P for 3 weeks induced glycation of type I collagen, type IV collagen, and laminin. Aminoguanidine dose-dependently inhibited the glycation of each ECM component. **P* < 0.0001.

significantly diminished the number of attached corneal epithelial cells. The presence of aminoguanidine in the incubation mixture during glycation dose-dependently increased the number of adherent cells on glycated laminin.

Effect of ECM Glycation on Spreading of Corneal Epithelial Cells

Glycation of type I and type IV collagens had no significant effect on the surface area of adherent cells (Fig. 4A). The presence of aminoguanidine in the incubation mixture had no effect on the cell area. In contrast, glycation of laminin attenuated the spreading of cells. Most cells on glycated laminin

were spherical (Fig. 4B), whereas most cells on nonglycated laminin were elongated (Fig. 4C). The surface area of adherent cells on glycated laminin was significantly smaller than that of cells on nonglycated laminin (Fig. 4A). The presence of aminoguanidine in the incubation mixture during glycation dose-dependently increased the surface area of adherent cells on glycated laminin (Fig. 4A).

DISCUSSION

Using the anti-AGE antibody (6D12), we previously found a positive correlation between AGE-immunoreactivity and aging

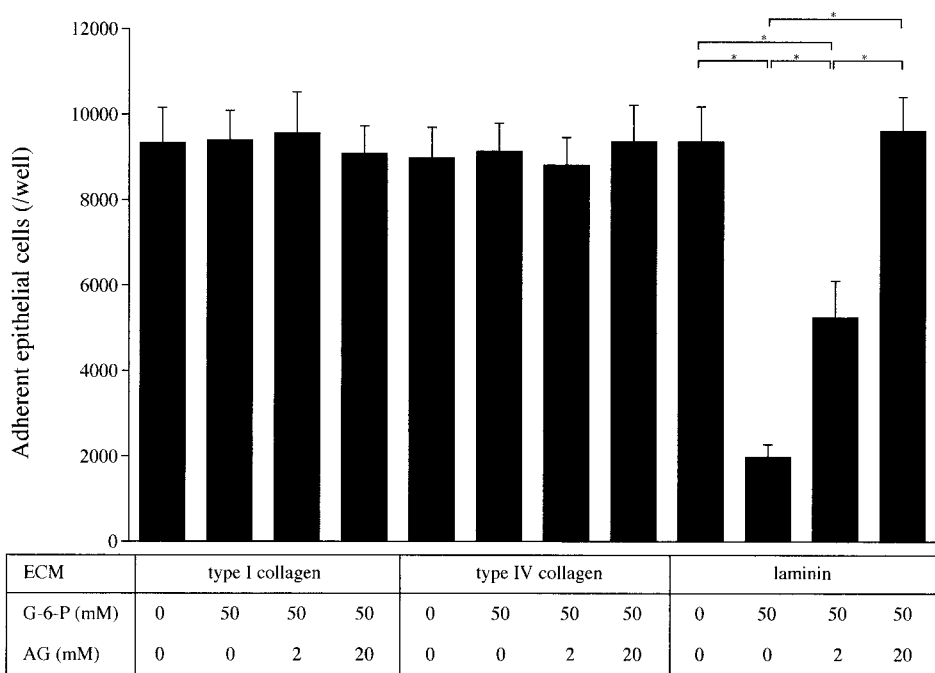


FIGURE 3. Numbers of adherent corneal epithelial cells on glycated or nonglycated ECM components. Human corneal epithelial cells were seeded onto glycated or nonglycated ECM in 96-well plates and allowed to attach for 3 hours. Attached cells were removed, and cell number was counted. The numbers of attached corneal epithelial cells on glycated type I and type IV collagens did not significantly differ from those of attached cells on nonglycated type I and type IV collagens, respectively. In contrast, glycation of laminin significantly diminished the number of attached corneal epithelial cells. The presence of aminoguanidine (AG) during glycation dose-dependently increased the number of adherent cells on glycated laminin.

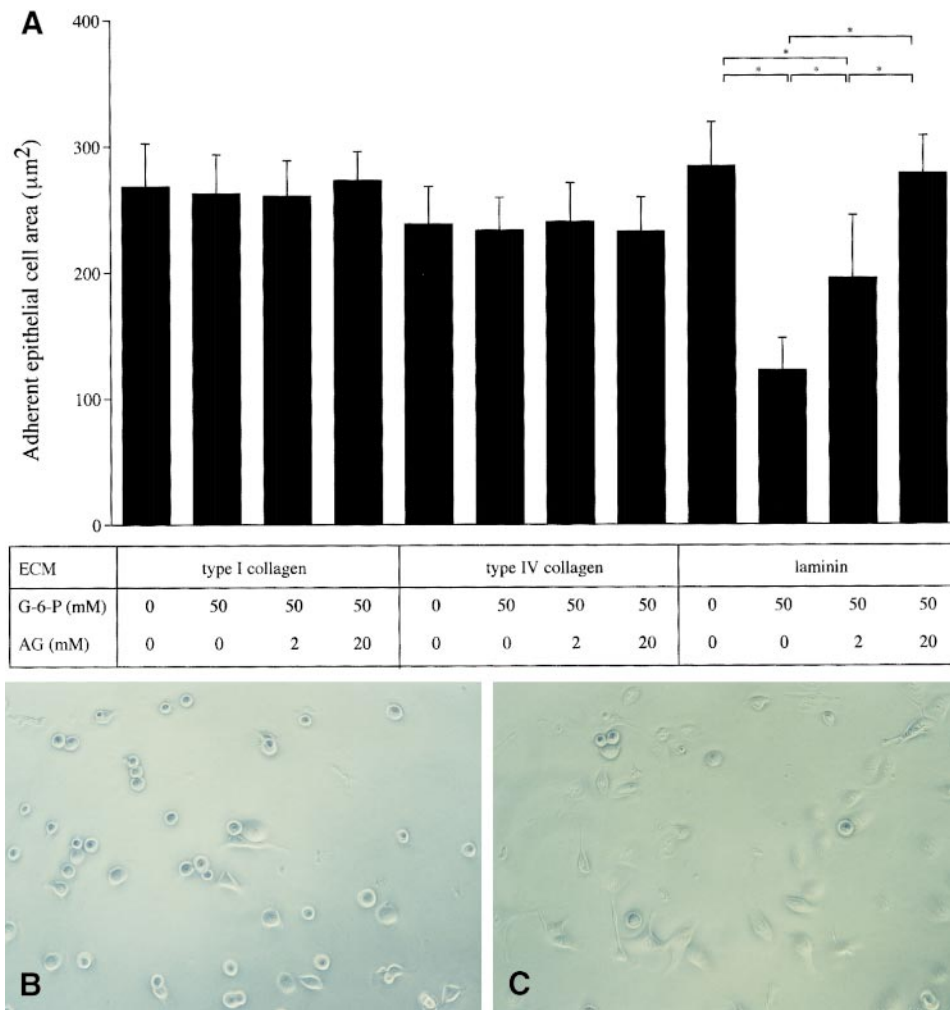


FIGURE 4. Effect of ECM glycation on spreading of corneal epithelial cells. Human corneal epithelial cells were seeded onto glycosylated or nonglycosylated ECM in 96-well plates and allowed to attach for 3 hours. Attached cells were fixed, and the areas of 50 attached cells in each were measured. **(A)** Glycation of type I and type IV collagens had no significant effect on the surface area of adherent cells. The presence of aminoguanidine (AG) in the incubation mixture during glycation of type I and type IV collagens had no effect on the cell area. In contrast, glycation of laminin attenuated the spreading of cells. The surface area of adherent cells on glycosylated laminin was significantly smaller than that of cells on nonglycosylated laminin. The presence of aminoguanidine during glycation dose-dependently increased the surface area of adherent cells on glycosylated laminin. * $P < 0.0001$ (**B** and **C**) Most cells on glycosylated laminin were spherical (**B**), whereas most cells on nonglycosylated laminin were elongated (**C**). Magnification, $\times 100$.

in some tissues.^{17,25} To exclude the aging factor as much as possible and evaluate the effect of diabetic condition on CML accumulation in the cornea, we prepared age-matched diabetic and nondiabetic corneas. In all the examined diabetic corneas, CML immunoreactivity was observed in the epithelial basement membrane area, whereas CML immunoreactivity was not found in the corresponding area in most of the nondiabetic corneas. Considering the previous studies reporting that AGEs accumulate with increasing age in other tissues, it is somewhat puzzling that CML immunoreactivity was absent in most of the nondiabetic elderly subjects and that it was present in only one nondiabetic cornea from a subject (72-year-old) who was not the oldest among the nondiabetic group. One possibility for these points is that, in most of the nondiabetic corneas, the glucose concentration may not reach the level high enough for AGEs to be formed because of the cornea's avascular property. Another possibility is that immunohistochemistry, being a qualitative method, might not be able to detect the amount of AGEs in a linear fashion. More quantitative methods such as high-performance liquid chromatography and ELISA could detect the amount of AGEs accumulated in the corneas of nondiabetic elderly subjects in a linear fashion.

Considering that laminin is a major component in the human corneal basement membrane²⁶⁻²⁸ and that the turnover rate of laminin is as slow as that of other components,^{29,30}

it is likely that laminin in the basement membrane of diabetic cornea contains CML.

The mechanisms by which increased glucose concentration results in altered cell behavior might be multiple. We have examined the effects of one of these mechanisms, glycation of the matrix and its influence on the cell behavior. The presence of aminoguanidine during glycation inhibited the formation of CML on the matrix and regained adhesion and spreading of corneal epithelial cells on the matrix in a dose-dependent manner. These results indicate that glycation of the matrix plays a certain role in altered cell behavior induced by increased glucose concentration.

The results of the current in vitro and in vivo studies raise the following presumption: nonenzymatic glycation of ECM (particularly laminin) in the corneal epithelial basement membrane decreases adhesion and spreading of the corneal epithelial cells, leading to corneal epithelial disorders in diabetic patients. This presumption is compatible with the clinical observation that in corneal epithelial defects of diabetic patients, corneal epithelial cells proliferate onto the underlying stroma but often easily detach from the stroma.^{31,32} Previous studies have shown that nonenzymatic glycation of ECM weakens adhesion and spreading of mesangial cells, glomerular epithelial cells, and vascular endothelial cells and that it probably causes chronic diabetic complications such as nephropathy.

thy and atherosclerosis.³³⁻³⁶ Our results support the hypothesis that AGEs may be responsible for diabetic corneal epitheliopathy. However, we have not shown that the specific inhibition of AGEs' formation normalizes corneal epithelial behavior in diabetic corneas. Additional experiments are needed to prove that AGEs are responsible for diabetic corneal epitheliopathy.

A recent study reported that the immunoreactivity for entactin/nidogen and for chains of laminin-1 and laminin-10 was very weak or absent in the corneal epithelial basement membrane of diabetics with diabetic retinopathy, whereas corneas from nondiabetic individuals or diabetics without diabetic retinopathy showed such alterations much less frequently.³⁷ As they stated in their report, their results suggest that, as retinal diabetic disease worsens, concomitant alterations of the corneal epithelial basement membrane occur in parallel. They suggested that these alterations might be due to the action of growth factors or proteinases abnormally expressed in the eyes with diabetic retinopathy. In addition to those mechanisms, it is possible that advanced accumulation of AGEs concomitant with a long history of diabetes mellitus altered the immunoreactivity of the ECM components in the corneal epithelial basement membrane. This hypothesis should be confirmed in the future study using a greater number of corneas from diabetes, with or without diabetic retinopathy, and nondiabetes.

CML immunoreactivity was not observed in the Descemet's membrane of diabetic or nondiabetic corneas, which is the basement membrane of the corneal endothelial cells. This result is somewhat puzzling because Descemet's membrane should be exposed to a higher concentration of glucose than the epithelial basement membrane (being closer to the aqueous humor, which is the source of most of the corneal glucose). Why did Descemet's membrane contain no CML immunoreactivity? One possibility is that high concentrations of oxygen may accelerate the formation of CML in the epithelial basement membrane. Considerable evidence shows that oxidative mechanism is involved in the process of AGEs' formation.³⁸⁻⁴⁰ Because oxygen in the cornea is supplied primarily by diffusion from the tear fluid, the epithelial basement membrane should be exposed to a higher concentration of oxygen than Descemet's membrane. Further experiments are necessary to determine the actual mechanisms of the discrepancy of CML immunoreactivity between epithelial basement membrane and Descemet's membrane.

In both diabetic and nondiabetic corneas, CML immunoreactivity was found in endothelial cells. Human corneal endothelial cells do not proliferate in vivo, and the number of endothelial cells gradually decreases with aging.⁴¹ Because the correlation between the accumulation of AGEs and the aging process has been shown in other tissues,^{17,23,42,43} the accumulation of AGEs might cause the decrease of corneal endothelial cells with aging. Further studies are necessary to elucidate these points.

In summary, the present study indicates that CML immunoreactivity is increased in the epithelial basement membrane of diabetic corneas and that nonenzymatic glycation of laminin reduces adhesion and spreading of corneal epithelial cells. Thus, the accumulation of AGEs on the basement membrane, particularly on laminin, may play a causative role in diabetic corneal epitheliopathy. With the relative accessibility of the cornea to the local drug delivery, the potential for the devel-

opment of anti-AGE drugs for diabetic corneal disorders appears promising.

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E R R A T U M

Erratum in: "Constitutive Expression of VEGF, VEGFR-1, and VEGFR-2 in Normal Eyes" by Kim et al. (*Invest Ophthalmol Vis Sci.* 1999;40:2115-2121).

In the Introduction and Discussion sections, the following work should have been referenced:

Gerhardinger et al. Expression of vascular endothelial growth factor in the human retina and in nonproliferative diabetic retinopathy. *Am J Pathol.* 1998;152:1453-1462.