Delayed Wound Closure and Phenotypic Changes in Corneal Epithelium of the Spontaneously Diabetic Goto-Kakizaki Rat

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PURPOSE. To characterize wound closure and phenotypic changes in the corneal epithelium of the Goto-Kakizaki (GK) rat, a spontaneous model of type 2 diabetes.

METHODS. Corneal wound healing was monitored by fluorescein staining after epithelial debridement. Tear secretion was measured with an esthesiometer in 13- to 15-week-old GK and Wistar (control) rats. The distributions of cytokeratin 12 (K12), K14, and connexin43 in the corneal epithelium were examined by immunohistofluorescence analysis. The proliferation capacity of epithelial cells in the intact cornea and during wound healing was evaluated by immunostaining for Ki-67.

RESULTS. Tear secretion, corneal sensation, and corneal epithelial wound closure rate were all decreased in GK rats compared with those in Wistar rats. Whereas connexin43, K14, and Ki-67 were all restricted to the single layer of basal cells in the corneal epithelium of Wistar rats, they were detected in the two layers of cells closest to the basement membrane in that of GK rats. The frequency of Ki-67–positive cells in the intact corneal epithelium was greater in GK rats than in Wistar rats, and it was increased to a greater extent in the peripheral cornea of GK rats than in that of Wistar rats during wound healing.

CONCLUSIONS. Spontaneously diabetic GK rats manifest characteristics similar to those of diabetic keratopathy in humans, including delayed wound closure, and they exhibit phenotypic changes in corneal epithelial cells. (Invest Ophthalmol Vis Sci. 2007;48:590–596) DOI:10.1167/iovs.05-1168

Complications of diabetes mellitus that affect the eye represent an increasing threat to sight because of the increasing prevalence of this condition worldwide. In addition to diabetic retinopathy, various types of corneal epithelial disorder are relatively common in persons with diabetes. Although the cornea is transparent and appears normal in patients with diabetic keratopathy in the absence of corneal injury, damage to the corneal epithelium reveals that epithelial wound healing and the re-formation of the normal stratified structure of the epithelium are delayed. Abnormalities of the basement membrane of the corneal epithelium, including thickening, degeneration, increased nonenzymatic glycation of protein components, and deposition of advanced glycation end products, have been detected in diabetic humans and animal models of diabetes. Moreover, the functions of corneal epithelial cells are also altered in the diabetic state. The underlying pathology of diabetic keratopathy is thus characterized by abnormalities of the epithelial basement membrane and epithelial cells.

The normal corneal epithelium is a nonkeratinized, stratified, squamous epithelium that consists of basal cells, wing cells, and superficial cells. A single layer of basal cells sits on the basement membrane. These cells mature consecutively into wing cells and superficial cells. The normal structure and functions of the corneal epithelium are maintained by a constant turnover of cells that is the result of an appropriate balance among the proliferation, differentiation, and maturation of epithelial cells. In the central cornea, only the basal cells are able to proliferate, and this ability is lost in association with further maturation. Corneal epithelial cells express cytokeratin 12 (K12). Cytokeratin 14 (K14), a marker of basal cells in stratified squamous epithelia, is expressed only in the basal cell layer of the corneal epithelium. Connexin43 (Cx43), the major protein component of gap junctions, is also expressed only in the basal cell layer of the corneal epithelium. The expression pattern of Cx43 changes during corneal epithelial wound healing but is restored as healing is completed. Components of the basement membrane, including laminin, fibronectin, tenascin, and type IV collagen, also play important roles in maintaining the corneal epithelium and in wound healing.

1, 2 Diabetes that were produced as a result of selective breeding over many generations of nondiabetic Wistar rats with glucose intolerance. GK rats develop mild hyperglycemia in the absence of obesity and hyperlipidemia. Pathologic changes in various organs and tissues, including the kidneys and nerves, of GK rats resemble those apparent in patients with diabetes. The deposition of extracellular matrix proteins such as type IV collagen has thus been shown to be increased in the kidneys of GK rats. Furthermore, GK rats manifest various abnormalities of the eye, including decreased retinal circulation, increased retinal production of nitric oxide, upregulation of vascular endothelial growth factor, and increased abundance of O-GlcNAc–modified proteins in the cornea.

To investigate the underlying mechanisms of corneal epithelial disorders associated with diabetes mellitus, with the use of immunohistochemical techniques we have now characterized the expression of K12, K14, Cx43, and Ki-67 (a marker of cell proliferation capacity) in the intact or wounded cornea of diabetic GK rats in comparison with nondiabetic Wistar (WKY) rats.
METHODS

Animals

Male GK rats and normal male Wistar rats were obtained from Charles River Japan (Yokohama, Japan). The animals were allowed free access to laboratory chow and water and were studied at 13 to 15 weeks of age. The study was performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and was approved by the animal ethics committee of Yamaguchi University Graduate School of Medicine.

Measurement of Tear Secretion and Corneal Sensation

Tear secretion in rats was determined with a modified Schirmer test. The test was performed without topical anesthesia. Schirmer strips (Showa Yakuhin Kako, Tokyo, Japan), cut to a length of 17 mm and a width of 1 mm, were inserted behind the lower eyelid near the medial canthus for 1 minute. The wet length of the strip was then measured. Corneal sensation of rats was measured with a Cochet-Bonnet esthesiometer. Initially, the nylon filament was fully extended to 60 mm. Objective blinking was considered a positive response. If a positive response was not detected, the fiber length was shortened 5 mm at a time, and the procedure was repeated until such a response was obtained. The test was repeated three times, and the average of the three measurements was determined.

Corneal Epithelial Wound Healing

Rats were anesthetized by intraperitoneal injection of sodium pentobarbital (30 mg/kg body weight) and the application of 0.4% oxybuprocan hydrochloride to each eye. The entire epithelium of each cornea was then removed with a blunt blade. The epithelial defect was stained with 1% fluorescein and photographed at 10, 24, 34, 48, 58, 72, 82, and 96 hours after debridement. The area of the epithelial defect was measured on the photographs with a computer-assisted image analyzer. Changes in the defect area were plotted graphically, and the healing rate was calculated by linear regression from the data obtained between 10 and 48 hours after debridement.

Immunohistochemistry

Rats were killed by intraperitoneal injection of an overdose of sodium pentobarbital (Kyoritsu Seiyaku, Tokyo, Japan) before or 36 or 72 hours after total corneal epithelial debridement. Both eyeballs were pentobarbital (Kyoritsu Seiyaku, Tokyo, Japan) before or 36 or 72 hours after total corneal epithelial debridement. Both eyeballs were processed for immunohistochemical staining of Ki-67. The central portion of each cornea was serially sectioned, and four sections were processed for immunohistochemistry.

Antibodies and Other Reagents

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Corneal Epithelial Changes in Diabetic GK Rats

For examination of cell proliferation capacity in the intact or wounded cornea, four eyes of four Wistar rats and four eyes of four GK rats were processed for immunohistochemical staining of Ki-67. The central portion of each cornea was serially sectioned, and four sections of each eye were examined. Frozen sections prepared and fixed as described above and were stained with mouse antibodies to Ki-67.
bien y las células del epitelio que se encuentran en el nivel del borde del limbo fueron medidos para el perfil corneal y para el perfil corneal del limbo en 72 horas después de la depilación, y el número de K67-positivas células por 100 μm bilíaco membrana fue calculado. Dado que los epiteliales heridas no se cerraron en 36 horas después de la depilación, el número de K67-positivas células fue calculado para el un tercero de la longitud total del epitelio que fue medido del limbo.

**Transmisión Electrónica Microscopía**

La cornea se desunido del bulbo y se enclavó para 2 horas a 4°C en 0.1 M fosfato buffer (pH 7.4) que contiene 2% formaldehído y 2% glutaldehído. Después de lavado con 0.1 M fosfato buffer, el tejido se expuso a 2 horas a 4°C a 0.1% osmio tetroxido, lavado con agua destilada, incubado durante 3 horas a temperatura con saturación de uracilo acetato, deshebrado en una serie de alcohol soluciones, y embebido en Epon epoxi resina.

**Statistical Analysis**

La data cuantitativa se presentan como promedio ± D y se compararon por el personal Student's *t* test o el Wilcoxon test, como indicado. *P < 0.05* fue considerado estadísticamente significativo.

**RESULTOS**

**Características Clínicas de GK Ratas**

El peso corporal de GK ratas de 13 a 15 semanas de edad fue significativamente más bajo que el de ratas de control Wistar (Tabla 2). Los concentraciones de glucosa sanguínea de GK ratas en el estado alimentado fueron significativamente más altas que en Wistar ratas. El goteo de saliva y la sensación corneal fueron significativamente disminuidos en GK ratas comparado con las de Wistar ratas.

**Epitelio Corneal Herida**

Vimos que el patrón del descubrimiento corneal eran el herida en GK ratas. Inmediatamente después de la depilación del epitelio corneal, no hubo una diferencia estadística significativa en la área del herida en GK ratas (Tabla 2). La juntura en GK ratas y Wistar ratas (25.71 ± 1.1 mm² y 25.41 ± 1.6 mm², respectivamente; *P = 0.61*, Student's *t* test). Cierre del herida corneal después de la depilación corneal es completado en 48 horas en Wistar ratas, pero se mantuvo aún presente a 72 horas en GK ratas. La análisis cuantitativo reveló que la velocidad del herida curación fue 0.611 ± 0.047 mm²/h en Wistar ratas y 0.509 ± 0.043 mm²/h en GK ratas (*P < 0.01; Student's *t* test; Fig. 1B). Herida corneal epitelial herida en GK ratas con el tejido en Wistar ratas.

**Immunoluminalización de K12, K14, y Cx43 en el Epitelio Corneal**

Hicimos el análisis inmunofluorescente El experimento para examinar la localización de K12, K14, o Cx43 junto con que la laminina en el epitelio corneal de GK ratas. En GK y Wistar ratas, K12 se encontró en todas las epiteliales células (Figs. 2A, 2B). En Wistar ratas, K14 (Fig. 2C) y Cx43 (Fig. 2E) se expresaron en las capas de epiteliales basales en GK ratas (Figs. 2B, 2D), mientras que solo la capa de epiteliales basales associated con la membrana base, como revelado por el goteo de laminina. Además, en GK ratas, K14 (Fig. 2D) y Cx43 (Fig. 2F) se observaron en las dos capas de epiteliales celulares adyacentes a la base de la membrana.

Para confirmar la localización de K14 y Cx43 to this double layer of cells in the corneal epitheli de GK rats, we stained for K14 and Cx43 in the same sections (Fig. 3). Again, the two layers of cells at the base of the corneal epithelium were positive for K14 and Cx43 in GK rats (Figs. 3B, 3D), whereas the single layer of basal cells associated with the basement membrane was positive for these two proteins in Wistar rats (Figs. 3A, 3C).

**Electron Microscopía de Transmisión**

La microscopía electrónica de transmisión reveló la corneal epitelium de Wistar rats que se encontraba una estratificación, estrato epitelial queratinizado compuesto de basal, wing, y superficial Celulas. El capa basal celular consistía de columnar celulas, y las wing cells located superior to the basal cell layer exhibited a cell morphology that was spinous and had thin cytoplasmic processes. In contrast, in the corneal epithelium of GK rats, basal celulas were cuboidal and were shorter than those in Wistar ratas. Las basal cells se asociaron directamente con la base de la membrana en Wistar y GK ratas, y hemidesmosomas fueron presentes en el basal surfaces of the cells in contact with the basement membrane. La lamina basale de GK ratas, pero, más grueso que el de Wistar ratas. Las basal cells y wing cells contactaron con cada otras a través de interdigitaciones y desmosomas en ambos tipos de ratas, pero las wing cells de GK ratas manifestaron interdigitaciones menos que las de Wistar ratas. Las tension junctions were observed between superficial cells in Wistar and GK ratas (data not shown).

**Microscopía Electrónica de Transmisión**

La microscopía electrónica de transmisión de Cx43 expresión confirmada Cx43 inmunoreactividad sólo en la capa de columnar basal cellas in the corneal epithelium of Wistar rats. En GK ratas, Cx43 inmunoreactividad fue observado no sólo entre las celulas asociadas con el base de la membrana but also between the cuboidal cells located superior to them. Higher magnification imágenes revelaron que Cx43 inmunoreactividad was asociado with gap junctions in Wistar and GK ratas (data not shown).

**Proliferación Celular Epitelial**

Vimos que el análisis de la capacidad de proliferación celular corneal epitelial de GK y Wistar ratas por goteo para Ki-67. En Wistar ratas, Ki-67-positivas celulas fueron detectadas en la capa single layer...
of basal cells associated with the basement membrane (Figs. 4A, 4C). In contrast, Ki-67–positive cells were apparent in the basal cell layer directly associated with the basement membrane and in the immediately superior cell layer of GK rats (Figs. 4B, 4D). Quantitative analysis revealed that the number of Ki-67–positive cells per 100-μm length of basement membrane was significantly higher in GK rats (1.40 ± 0.11 cells/100 μm) than in Wistar rats (1.00 ± 0.11 cells/100 μm; Fig. 4E).

**Epithelial Cell Proliferation during Corneal Epithelial Wound Healing**

Finally, we examined the proliferation capacity of epithelial cells in central and peripheral regions of the cornea during epithelial wound healing. The number of Ki-67–positive cells (Figs. 4B, 4D). Quantitative analysis revealed that the number of Ki-67–positive cells per 100-μm length of basement membrane was significantly higher in GK rats (1.40 ± 0.11 cells/100 μm) than in Wistar rats (1.00 ± 0.11 cells/100 μm; Fig. 4E).
in the central region of the intact cornea was greater for GK rats than for Wistar rats, and these cells were present in a double layer in GK rats (Figs. 5A, 5G). At 36 hours after epithelial debridement, Ki-67–positive cells were sparsely distributed in the region of the wound margin in both types of rat (Figs. 5B, 5H). At 72 hours after debridement, when the wound had closed in Wistar rats and had almost closed in GK rats and the multilayered structure of the epithelium had been restored, the number of Ki-67–positive cells was still reduced in both types of rat compared with that apparent in the corresponding intact cornea (Figs. 5C, 5I). However, the Ki-67–positive cells in GK rats were again present in a double layer.

The number of Ki-67–positive cells in the peripheral region of the intact cornea was also greater for GK rats than for Wistar rats, and again these cells were present in a double layer in GK rats (Figs. 5D, 5J). The number of Ki-67–positive cells in the peripheral region of the cornea of Wistar rats was increased 36 hours after epithelial debridement (Fig. 5E), with these cells forming a double layer in places, but it returned to normal by 72 hours (Fig. 5F). The number of Ki-67–positive cells in the peripheral region of the cornea of GK rats was also increased 36 hours after debridement (Fig. 5K), and it remained so at 72 hours (Fig. 5L). Immunostaining for Cx43 and K14 at 36 hours after epithelial debridement also revealed that the immunofluorescence-positive epithelial cells in the peripheral region of the cornea formed a continuous double layer in GK rats but an almost uniform single layer in Wistar rats (Figs. 5M, 5N).

In addition, we performed quantitative analysis of corneal epithelial cell proliferation during epithelial wound healing. The number of Ki-67–positive cells per 100-μm length basement membrane at 36 or 72 hours after debridement was significantly greater in GK rats than in Wistar rats (Fig. 6).

**DISCUSSION**

We have shown that tear secretion, corneal sensation, and corneal epithelial wound closure rate are all decreased in the GK rat, a spontaneous model of type 2 diabetes, compared with those in the nondiabetic Wistar rat. Immunoreactivity for Cx43, K14, and Ki-67 was also detected in the two layers of cells adjacent to the basement membrane in the corneal epithelium of GK rats, whereas only the single basal layer of cells was positive for these proteins in the corneal epithelium of control rats. Furthermore, we found that the proliferation capacity of corneal epithelial cells was increased in GK rats compared with that in Wistar rats. Our results thus demonstrate that the structure of the corneal epithelium and the proliferation capacity of corneal epithelial cells are abnormal in
GK rats, with these abnormalities possibly contributing to the delay in corneal epithelial wound healing also apparent in these animals.

Immunostaining for Ki-67 indicated that the proliferation capacity of basal cells in the corneal epithelium of GK rats is greater than in Wistar rats. This difference was apparent in the intact cornea and during the healing of corneal epithelial wounds; during wound healing, it was more pronounced in the peripheral region of the cornea than at the wound margin in the central region. This increased proliferation capacity of epithelial cells in the cornea of GK rats was associated with a delay in the resurfacing of epithelial defects. Increased cell number and cell migration are required for epithelial wound healing. The proliferation of corneal epithelial cells in the process of migration during wound healing is inhibited until the wound is covered. The apparent discrepancy between the increased proliferation capacity of corneal epithelial cells and the delayed corneal wound healing in GK rats may reflect differential regulation of the proliferation and migration of these cells during wound healing. For example, whereas epidermal growth factor stimulates the proliferation and migration of corneal epithelial cells, fibronectin stimulates only cell migration. However, EGF and fibronectin stimulate the healing of corneal epithelial defects in vivo.

Our immunohistochemical results on the localization of Cx43, K14, and Ki-67 indicate that the corneal epithelium of GK rats has an immature phenotype characterized by a double layer of basal-like cells. This phenotype might reflect the increased proliferation of corneal epithelial basal cells in GK rats. This increased proliferation may thus result in upward migration of the basal cells before they have matured into wing cells. It does not appear that the increased production of basal cells in GK rats results in increased lateral migration of these cells during wound healing.

The proliferation of epithelial cells is thought to be influenced by the local glucose concentrations surrounding the cornea, such as those in tear fluid, aqueous humor, and basement membrane. The proliferation of human corneal epithelial cells has thus been shown to be increased or decreased by exposure to high glucose concentrations in vitro. The proliferation of corneal epithelial cells, as revealed by the uptake of [3H]-thymidine, was found to be increased in rats with streptozotocin-induced diabetes, and the corneal epithelium of these animals appeared immature. The results of this previous study are thus consistent with our present observations in the GK rat. In contrast, the proliferation of corneal epithelial cells was found to be decreased in the KK mouse model of type 2 diabetes. However, KKAY mice develop obesity and hyperlipidemia in addition to hyperglycemia, possibly explaining this difference in findings.

GK rats manifest characteristics similar to those of diabetic complications of the cornea in humans, including delayed epithelial wound healing, lowered corneal sensation, and reduced tear secretion. Many animal models have been studied for investigation into diabetic complications, including genetic models such as the KKAY mouse, the db/db mouse, and the Otsuka Long-Evans Tokushima Fatty (OLETF) rat. However, these models develop obesity or hyperlipidemia and hyperglycemia, which complicates the attribution of pathologic changes specifically to hyperglycemia. In contrast, the GK rat develops hyperglycemia during embryonic development but does not develop obesity or hyperlipidemia. Our present results thus suggest that the GK rat is a suitable animal model for studies into the pathogenesis of diabetic keratopathy.

**References**


