Corneal Disorders in KKAy Mouse: A Type 2 Diabetes Model

Hiroko Watanabe,* Chikako Katakami,* Satoshi Miyata[†]
and Akira Negi*

*Department of Ophthalmology, Kobe University School of Medicine, Kobe, Japan;
[†]Second Department of Internal Medicine, Kobe University School of Medicine, Kobe, Japan

Purpose: To observe the clinical and histopathological changes occurring in corneas of KKAy mice, a model of type 2 diabetes, and to elucidate the possible mechanisms involved in these changes.

Methods: Corneal epithelial cell proliferation was analyzed in KKAy and age-matched non-diabetic C57BL/6J control mice using ^{3}H-thymidine autoradiography. Clinical examination and histopathological analysis were also conducted on both types of mice.

Results: KKAy mice showed a significant elevation in blood glucose concentration and body weight compared to age-matched control mice. Fragile corneal epithelial cell attachment and subepithelial opacities were observed in the central area of the cornea of 10-week-old KKAy mice. Corneal epithelial cell proliferation decreased significantly in the 16-week-old KKAy mice. Histological study in the older KKAy mice groups revealed the presence of subepithelial deposits, widening of the intracellular spaces between corneal epithelial cells with poor adherence to the basement membrane (BM) and thickening of the BM itself. At the central area of the cornea, remnants of cell components with deposits and lacuna formation were observed, perhaps secondary to the continuous presence of poor adhesion and detachment of epithelial cells in the area. In the 50-week and older KKAy mice, thinning and atrophy of the corneal epithelial cell layer became more prominent at the central cornea with increases in deposition of materials, blood vessel invasion and activation of keratocytes. The deposits were stained black by von Kossa's method, indicating the presence of tissue calcium. Type IV collagen immunoreactivity was observed not only in the corneal and conjunctival BM but also between the stroma, particularly around the central cornea and in the walls of invading vessels. Laminin staining was intense at the BM around the central cornea, and in the walls of invading vessels along the stroma. Pyrraline, which is one of the major components of advanced glycation end products, was also present in the stroma, and around blood vessels. All these corneal changes were not observed with aging in the age-matched C57BL/6J mice.

Conclusions: Our findings provide evidence of the existence of corneal disorders in KKAy mice. These observations may provide useful information for the explanation of the mechanisms involved in corneal disorders in non-insulin-dependent diabetes mellitus patients. **Jpn J Ophthalmol 2002;46:130–139** © 2002 Japanese Ophthalmological Society

Key Words: Corneal epithelial cell proliferation, diabetic keratopathy extracellular matrix, KKAy mouse, pyrraline.

Introduction

Diabetes mellitus is known to be associated with a number of changes in the cornea.[1,2] Clinically, more than 50% of diabetic patients present with primary corneal lesions during their lifetime.[3] However, the pathologic mechanisms responsible for the development of these lesions have not yet been completely investigated. Increased accumulation of glucose and glycogen in the corneal epithelium,[4] basement membrane (BM) thickening,[5] decrease of hemidesmosomes,[6] and endothelial fluid pump dysfunctions

have been observed in human and diabetic animal models.[7] We have previously reported that chemically induced type 1 diabetic rats developed corneal epithelial fragility and decreased epithelial cell proliferation with aging.[8] These factors may complicate the turnover mechanism in the corneal epithelium and affect BM metabolism. The major type of human diabetes is type 2, with the pathological features differing from type 1. We find it important therefore to investigate corneal disorders in animal models of type 2 in order to fully understand the mechanisms for these changes.

The KK mouse strain is characterized by early onset and prolongation of severe levels of hyperinsulinemia and hyperglycemia, with obesity, accompanied by pathological changes such as nephropathy with exaggerated thickness of the glomerular capillary BM, and retinopathy with microaneurysms, similar to human type 2 diabetes.[9,10] Introduction of the dominantly inherited Ay gene produced the rodent polygenic KKAy mouse, which is a model of genetically induced type 2 diabetes.[11] Because the KKAy mouse therefore carries both the yellow obese and diabetes genes, the KKAy mouse becomes obese and develops hyperglycemia earlier than the KK strain. To elucidate the possible mechanisms involved in diabetic corneal epithelial disorders, we investigated the clinical and histopathological changes in the corneas of KKAy mice.

Materials and Methods

Animals

Male diabetic KKAy mice (n = 38), 8 weeks old, were obtained from Japan CLEA, Tokyo. Age- and sex-matched C57BL/6J mice (n = 38) with a genetic background the same as KKAy mice were designated as the nondiabetic controls.[11]

The plasma levels of insulin, glucose, and cholesterol in both strains of mice were analyzed. All animals were treated in accordance with the Guidelines for Animal Treatment at Kobe University and in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Measurement of Blood Glucose Concentration and Body Weight

Glucose levels were analyzed using glucose oxidase-impregnated test strips and Glu-test sensor (Sanwa Chemical Lab, Nagoya) in tail capillary blood of both strains of mice. Body weights were measured with a weighing scale.

Measurement of Corneal Epithelial Cell Proliferation with ^{3}H-Thymidine Autoradiography

KKAy mice were sacrificed at 8 and 16 weeks of age with an overdose of sodium pentobarbital (20 mg/kg) (Nembutal-R; Abbott Laboratories, North Chicago, IL, USA), and whole eyes were excised.[12] Excised eyes were labeled with 10 μL/mL of ^{3}H-thymidine (100–130 μCi/mMol, 1 mCi/mL) (Amersham, Buckinghamshire, UK) in Dulbecco's modified Eagle medium (1 mL/eye) (Gibco, Grand Island, NY, USA) at 37°C for 6 hours. Corneas were dissected and initially examined by autoradiography. Paraffin sections of the corneas (7 μm) were then prepared after fixation with 10% phosphate formalin for 12 hours at 4°C. Coating with 50% nuclear track emulsion (Konica NR-M2, Konica, Tokyo) was done in a dark room and each section was exposed for 2 weeks at 4°C. Films were developed with Rendol x-ray film developer and Renfix acid-hardening fixer solutions (Fuji Photo Film, Tokyo). Sections were then stained with hematoxylin and eosin and examined under a light microscope (Provis Olympus AX80, Olympus, Tokyo). Photographs were taken with Ektachrome Dyna Ex 200 film (Kodak Japan, Tokyo). An average of three sections was obtained for each cornea. The number of the corneal and limbal cells taking up ^{3}H-thymidine along a 10-mm corrected incision was calculated.

Clinical Observation, Histologic Evaluation and Immunohistochemical Staining

Clinical observation and photography of the anterior segment of the KKAy and C57BL/6J mouse eyes was done under a slit-lamp microscope at 7, 28, 40, and 56 weeks of age.

Histological and immunohistochemical examinations were done in both KKAy and control mice at each corresponding age, as described previously. The excised eyes were immediately embedded in OCT compound (Sakura Finetechnical, Tokyo), frozen at −20°C, sectioned with a cryostat (7 μm) and mounted on PLL-coated micro glass slides (Matsunami Glass, Tokyo). Each section was rinsed in phosphate-buffered saline then stained with hematoxylin and eosin before being examined and photographed under a light microscope.

Rabbit anti-mouse polyclonal antibody against collagen type IV (100 μL, LB-1403) and rabbit anti-mouse polyclonal antibody against laminin (100 μL, LB-1013) (both from LSL, Tokyo) were used for immunohistochemical analysis of the corneal sections. We also investigated the localization of pyrraline,

one of the advanced glycation end products, in the tissue, using mouse monoclonal antibody (pyr-B) to pyrraline.[13] Briefly, frozen sections of eyes were prepared, as described previously. After rinsing thrice in PBS, sections were incubated with 1% bovine serum albumin (Fraction-V; Intergen, Purchase, NY, USA) for 30 minutes at room temperature. After rinsing in PBS three times, sections were incubated with primary antibodies in a moist chamber overnight at 4°C. The primary antibodies against type IV collagen and laminin were diluted in PBS at 1:200. For control staining, sections were incubated with normal rabbit serum (20 mL, Funakoshi, Tokyo) and diluted with PBS at 1:200. The sections were also incubated with pyr-B (1:100 dilution in PBS) in the absence or presence of free hapten (caproyl pyrraline) overnight to examine whether the immunoreactivity was specific to pyrraline. The sections were then rinsed thrice in PBS, and incubated with the secondary antibody, FITC anti-mouse (F0261) and anti-rabbit (F0205) immunoglobulin (DAKO A/S, Glostrup, Denmark) 2 mL at a 1:100 dilution in PBS for 1 hour at room temperature. After rinsing thrice in PBS, each slide was mounted (PermaFluor™ Aqueous; Shadon/Lipshaw, Pittsburgh, PA, USA) and examined under a fluorescence microscope (BH2-RFCA, Olympus).

For detection of tissue calcium, von Kossa's method was used. Each slide was exposed to 5% silver nitrate (Wako Pure Chemical, Osaka) for 1 hour under indirect light, after which 5% sodium thiosulfate (Nacalai Tesque, Kyoto) was applied.

Statistical Analysis

All data represent mean ± SD. The Student *t*-test was used to evaluate the metabolic and morphometric data of each age group of both KKAy and C57BL/6J mice. A *P* value of < .05 was considered statistically significant.

Results

Body Weight and Blood Glucose Level

The changes in body weight and blood glucose levels in both KKAy and C57BL/6J mice are shown
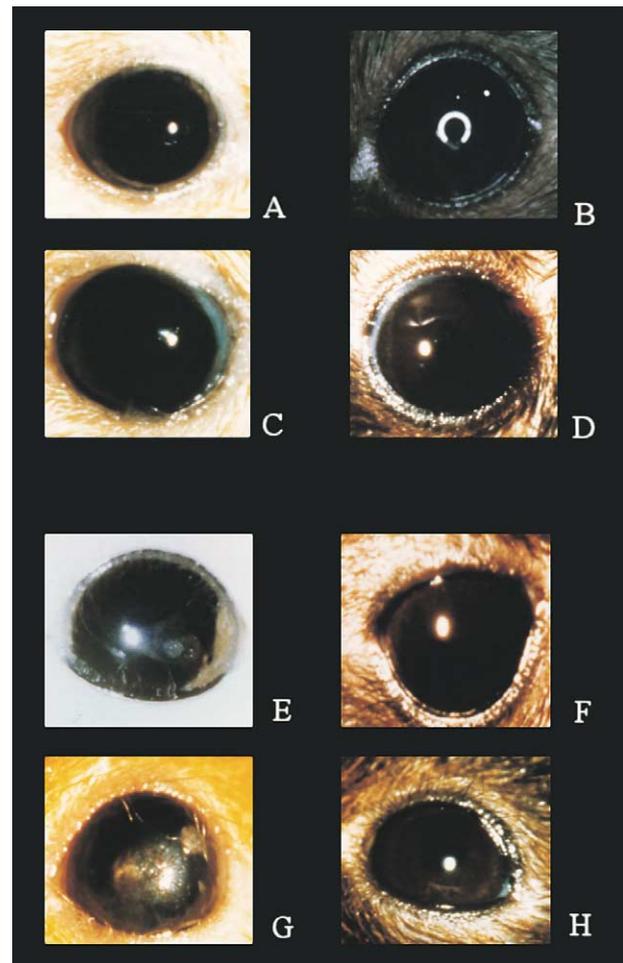

Figure 1. Anterior segment photographs of KKAy and C57BL/6J mice at 7, 28, 40, and 56 weeks of age. Seven-week KKAy (**A**) and C57BL/6J (**B**) mice, 28-week KKAy (**C**) and C57BL/6J (**D**) mice, 40-week KKAy (**E**) and C57BL/6J (**F**) mice, 56-week KKAy (**G**) and C57BL/6J (**H**) mice. Cornea of C57BL/6J mice showed no abnormal changes throughout the experimental period. In KKAy mice, slight opacity of the cornea was observed at 7 weeks of age (**A**). Stromal cloudiness became prominent at 40 weeks of age (**E**). At 56 weeks, the corneal opacity around the center of the cornea became severe and extended to the periphery. Vessel invasion into the stroma was also prominent in some KKAy mice (**G**).

Table 1. Changes in Body Weight and Blood Glucose Levels

| Age	Type of Mice	No.	Blood Glucose (mg/dL)	Body Weight (g)
8 Weeks	KKAy	6	534.3 ± 80.1*	41.4 ± 2.3*
	C57BL/6J	7	169.7 ± 23.4	20.9 ± 4.0
12 Weeks	KKAy	5	558.4 ± 54.9*	46.2 ± 2.0*
	C57BL/6J	5	168.0 ± 18.6	27.3 ± 0.7
16 Weeks	KKAy	10	537.5 ± 74.4*	48.5 ± 4.4*
	C57BL/6J	11	151.3 ± 28.6	29.3 ± 2.8
28 Weeks	KKAy	8	430.3 ± 100.6*	49.7 ± 4.8*
	C57BL/6J	7	142.0 ± 11.9	31.4 ± 2.9
40 Weeks	KKAy	5	451.2 ± 81.6*	49.8 ± 4.7*
	C57BL/6J	5	130.4 ± 11.5	34.2 ± 3.5
56 Weeks	KKAy	3	455.2 ± 54.5	50.2 ± 9.8
	c57BL/6J	3	154.3 ± 24.5	33.2 ± 0.2

*$P < .0005$.
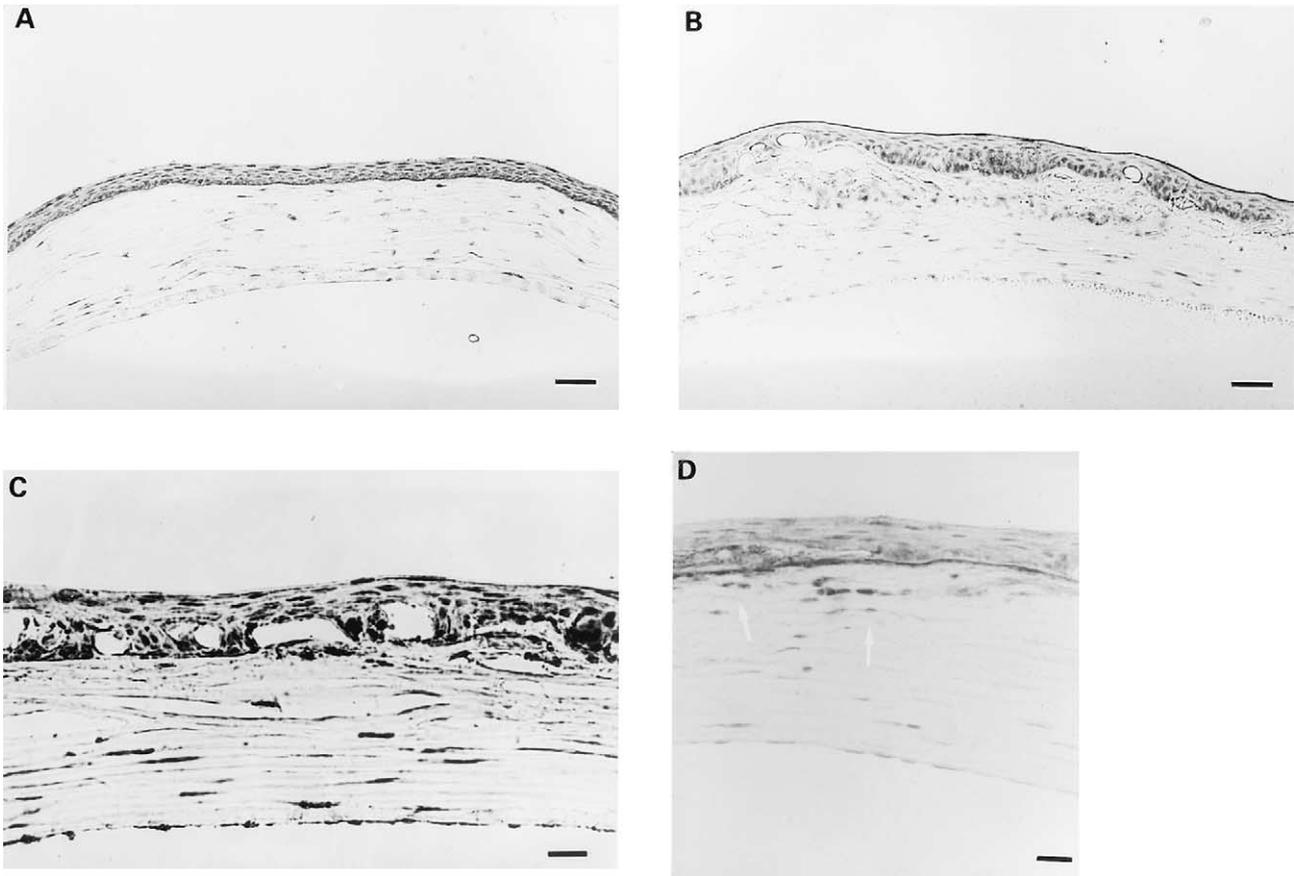
Figure 2. Light microscopic photographs of corneal sections of 7-week (**A**), 28-week (**B,C,D**), and 40-week (**E**) KKAy mice after hematoxylin and eosin (HE) staining. Microphotograph of a 56-week KKAy mouse cornea after HE and Von Kossa's staining (**F**). HE staining of a 7-week (**G**) and a 56-week (**H**) C57BL/6J mouse. In KKAy mice, no remarkable changes were seen in the cornea at 7 weeks of age (**A**). At 28 weeks, however, irregular alignment and wide intracellular spacing was observed between neighboring corneal epithelial cells. Activated keratocytes were found in the stroma under the fragile epithelial cells. Substantial accumulation of materials was also observed (**B,C,D** arrows show activated keratocytes). At 40 weeks, remnants of cell components and deposits with lacuna formation were observed in the stroma (**E**). Beyond 50 weeks, activated keratocytes, deposits, and microvessel formation were observed in the corneal stroma. The overlying corneal epithelial cell layer was very thin and atrophic. Calcium in the basement membrane and the stroma of the 56-week KKAy mouse stained black by Von Kossa's method (**F**). No abnormal findings were noted in the corneal epithelial layer of C57BL/6J mouse (**G,H**) throughout the experimental period. Bar = 100 μm (**A,B,E,F,G**), 50 μm (**C,D,H**).

in Table 1. Body weights and blood glucose levels were significantly elevated in the KKAy mice compared with C57BL/6J controls in all age groups, except at the 56-week period.

Morphological Examinations

Clinical examination. At 10 weeks of age, slight opacity of the cornea was observed in KKAy mice (Figure 1A). The corneal opacity became more apparent with increasing age (Figures 1C,E), particularly at the central area of the cornea. The corneal opacities were observed to extend to the corneal limbus in some 56-week-old KKAy mice. Vessel inva-

sion was also observed along the corneal stroma (Figure 1G). In age-matched C57BL/6J mice, no epithelial disturbance was observed and the corneas remained transparent (Figures 1B,D,F, and H).

Histological evaluation. The 7-week-old KKAy and C57BL/6J mice showed normal corneal histology. Corneal epithelial basal cells were firmly attached to each other and to the underlying BM (Figures 2A: KKAy mouse, 2G: C57BL6J mouse). In the 12- to 16-week-old KKAy mice, irregular cell alignment and wide intercellular spaces became apparent in the corneal epithelium. Beyond 20 weeks of age, substantial accumulation of amorphous substances
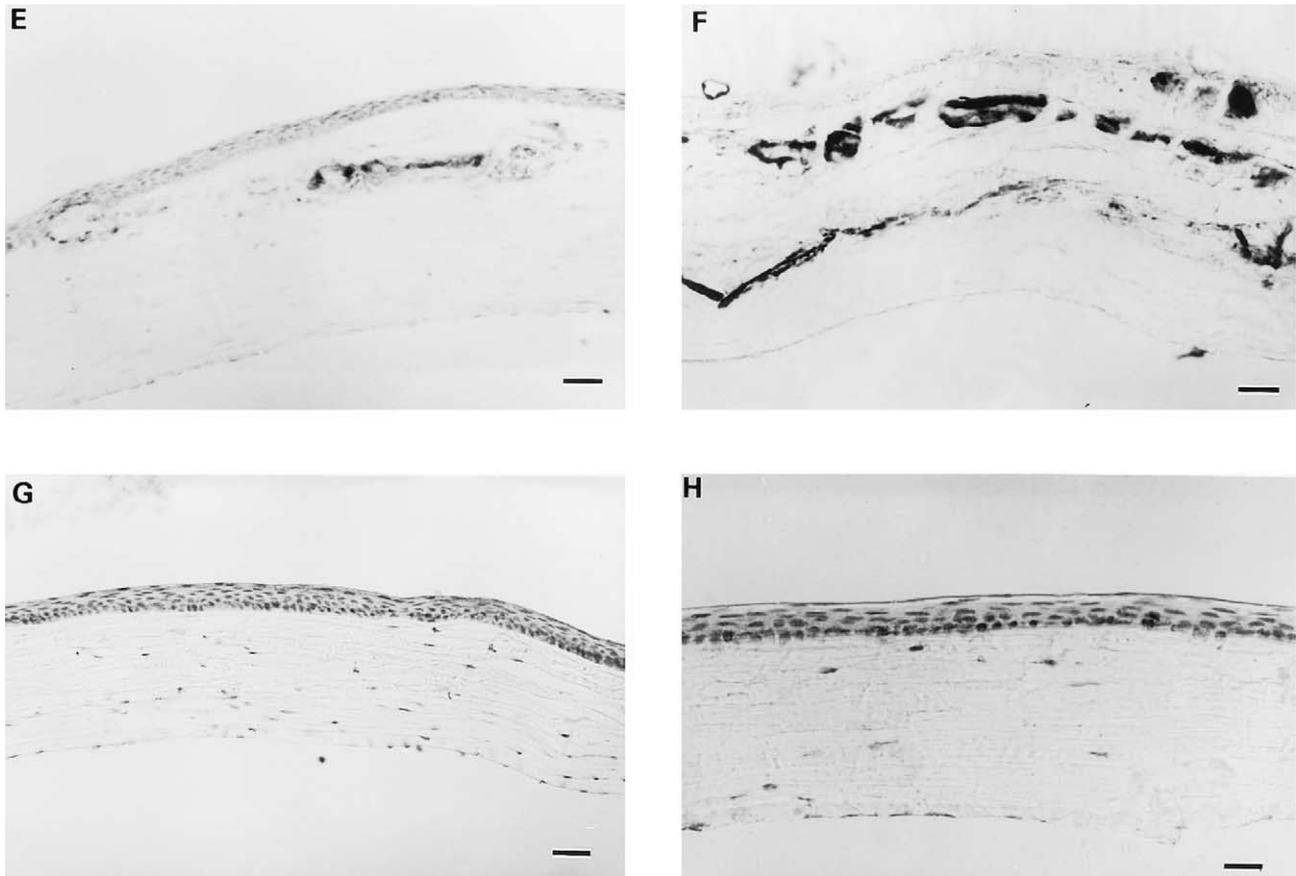
Figure 2. Continued.

beneath the epithelial layer and the BM was observed (Figures 2B,C). Activated keratocytes, which were differentiated by their spindle shape and absence of granules in the cytoplasm, were found in the stroma beneath the damaged corneal epithelium (Figure 2D). At 40 weeks of age, remnants of cell components and deposits with lacuna formation were observed in the anterior central area of the stroma (Figure 2E). At 56 weeks of age, histological examination revealed that the corneal lesions had extended into the peripheral limbus, with activated keratocytes present in the stroma. Collagen fibers were in disarray with subepithelial and stromal deposits seen especially in the central area of the cornea. The overlying corneal epithelial cell layer was noted to be thin and atrophic with goblet cell invasion as well as inflammatory cells, especially in the periphery. Activated keratocytes were also found infiltrating the peripheral cornea. Von Kossa's method revealed calcium deposits in the BM and stroma of the 56-week KKAy mice (Figure 2F). The 56-week C57/BL6J mice showed no remarkable histological change in the cornea (Figure 2H).

Immunohistochemical Examination

The cornea of a 56-week-old C57BL/6J mouse is shown in Figure 3. These findings were consistent with those in a previous report.[14] A section of a KKAy mouse cornea is shown in Figure 4. At 40 weeks of age, type IV collagen stained intensely around the BM and the stroma, and around the walls of newly formed vessels (Figures 4A,B). Laminin stained intensely at the BM around the central area of the cornea and at the walls of blood vessels (Figure 4C). Pyrraline staining was intense in the walls of new vessels and diffuse in the stroma of corneas of 40- and 56-week KKAy mice (Figures 4D,F). The immunoreactivity by pyr-B was absorbed by free pyrraline, indicating its specificity to pyrraline (Figure 4E).

Corneal Epithelial Cell Proliferation

Comparison between corneal epithelial cell proliferation both at the cornea and in the limbus in KKAy and C57BL/6J mice is shown in Figure 5 and
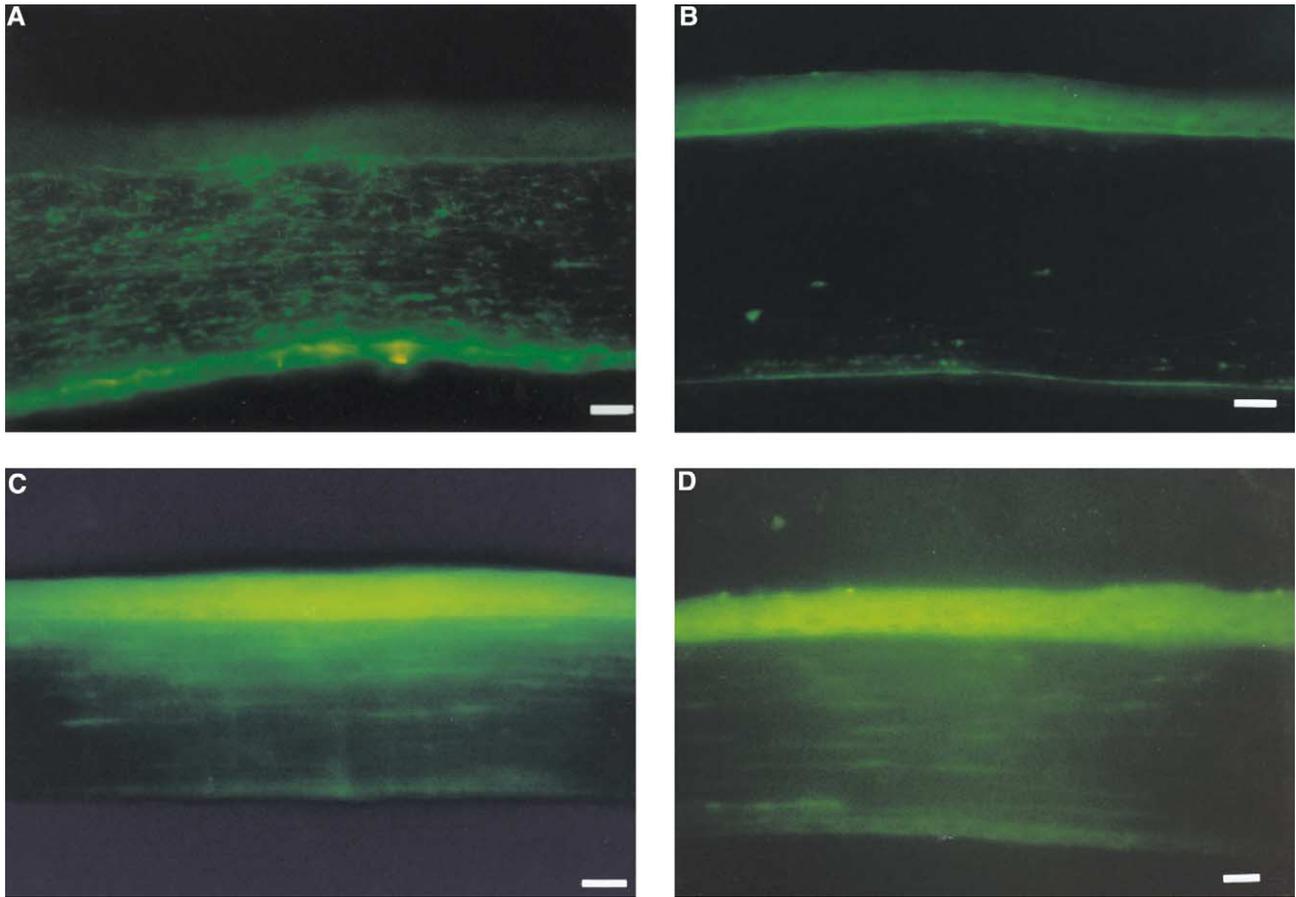
Figure 3. Immunohistochemical staining results with type IV collagen (**A**), laminin (**B**), and pyrraline (**C**) in corneal sections of 56-week C57BL/6J mice. Staining with type IV collagen was observed at the basement membrane (BM) and stroma, while laminin staining was observed only at the BM. No staining with pyrraline (**C**) was observed. The negative control also showed no staining (**D**). Bar = 50 μm (**A,B,C,D**).

summarized in Table 2. The number of cells along a 1-mm corrected length was calculated. At 8 weeks of age, corneal proliferation in KKAy and C57/BL6J mice was active (Figures 5A,B). However, at 16 weeks of age, corneal epithelial cell proliferation decreased significantly in the KKAy mice (Figures 5C,D, Table 2) ($P < .05$).

Discussion

Animal models of diabetes mellitus can be classified into two groups: one consisting of chemically induced hypoinsulinemic models of type 1 diabetes, and the other comprising genetically induced diabetic models, which include both type 1 and type 2 diabetes mellitus. In this study, we investigated corneal disorders in KKAy mouse, a rodent polygenic model of genetically induced non-insulin-dependent diabetes mellitus (NIDDM).

A high incidence of spontaneous NIDDM has been found among the KK strain of mice. The KK mice are characterized to be polygenic, obese, with early onset and prolonged levels of hyperinsulinemia and hyperglycemia.

Histopathologic changes include islet cell hypertrophy, exaggerated thickness of the glomerular capillary BM and microangiopathy of retinal vessels, which correspond to several pathological changes observed in human NIDDM.[9,10] KKAy mice are more obese and more hyperglycemic than the KK strain. Corneal disturbances in KK mice have been previously reported.[15–17] A centrally located oval-shaped opacity in the corneas of KK mice were observed at about the third week of age. Furthermore, the presence of calcium deposits in these lesions which were not metastatic or due to serum hypercalcemia was also noted. It was suggested that these changes might be of both genetic and metabolic
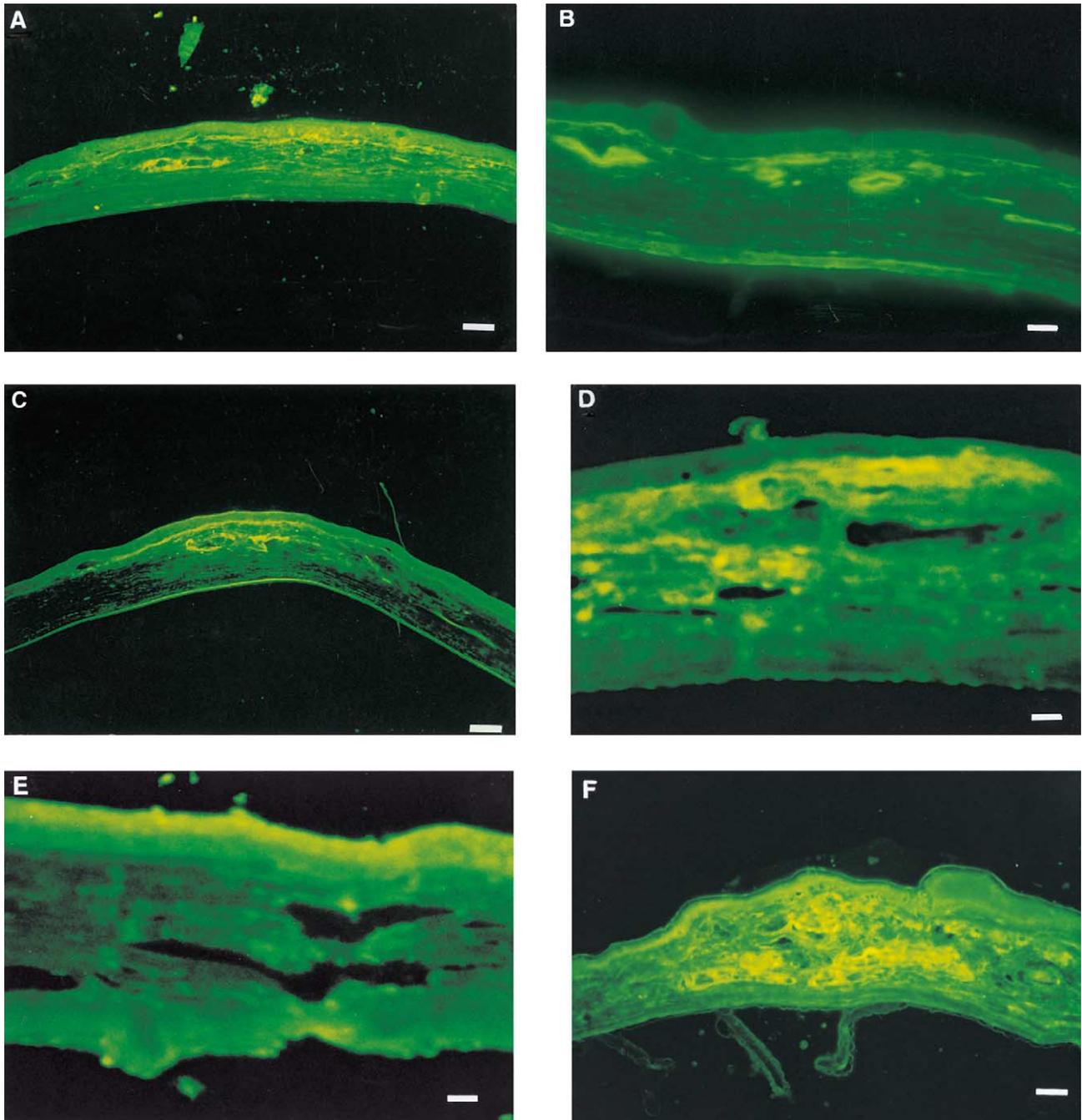
Figure 4. Immunohistochemical studies of 40- and 56-week KKAy mice using anti-type IV collagen antibody (**A**: At 40 weeks, **B**: At 56 weeks), anti-laminin antibody (**C**: 40 weeks), anti-pyrraline antibody (**D**: 40 weeks, **F**: 56 weeks), and anti-pyrraline antibody with free hapten (**E**: 40 weeks). Compare with Figure 3D for negative control. At 40 weeks of age, type IV collagen staining was intense around the basement membrane (BM), stroma and Descemet's membrane. At 56 weeks of age, staining with type IV collagen was also observed in the walls of newly formed blood vessels (**B**). Laminin staining was also intense at the BM around the center of cornea (**C**). Note that the stroma reacted with anti-pyrraline antibody in the 40- and 56-week KKAy mice (**D**,**F**). This immunoreactivity was blocked by preincubation of the monoclonal antibody with free hapten caproyl pyrraline (**E**). Bar = 100 μm (**A**,**C**,**F**), 50 μm (**B**,**D**,**E**).

Table 2. ^{3}H-Thymidine Uptake* in Cornea and Limbus

	Cornea		Cornea + Limbus	
	8 Weeks	16 Weeks	8 Weeks	16 Weeks
KKAy	14.59 ± 7.41	5.41 ± 3.31	20.57 ± 7.35	9.74 ± 2.75
	n = 9	n = 6	n = 7	n = 5
C57BL/6J	14.68 ± 4.44	11.26 ± 5.23	16.48 ± 4.76	16.33 ± 6.09
	n = 7	n = 6	n = 7	n = 6

*Cell/mm
$^†P < 0.05.$

causes. Mild cataract progression in KK mice similar to human diabetic cataract was also reported.[18] However, there have been no reports regarding corneal changes in KKAy mice.

In this study, we investigated corneal lesions in KKAy mice and present some new interesting findings. Histological examination of the 10-week KKAy mice showed poor adhesion of corneal basal cells to the BM, with the presence of subepithelial deposits.

Our preliminary data also showed periodic acid-Schiff positive deposits in the BM and basal cells (data not shown). With aging, the corneal opacities became prominent, particularly in the central area, which is the area most exposed to outside stimuli. At 28 weeks of age, a large number of activated keratocytes were observed in the stroma beneath the damaged corneal epithelium. In the anterior stroma, remnants of cell components and deposits with lacuna formation were observed, which were probably caused by the continuous existence of poor adhesion and detachment of corneal epithelial cells. Beyond 50 weeks of age, the lesions extended to the peripheral limbus, more keratocytes were activated and greater amounts of subepithelial and stromal deposits with microvessel formations were observed in the stroma. The overlying corneal epithelial cell layer was thin, atrophic, and detached from the stroma in the central cornea, with goblet cell invasion noted in the peripheral cornea.
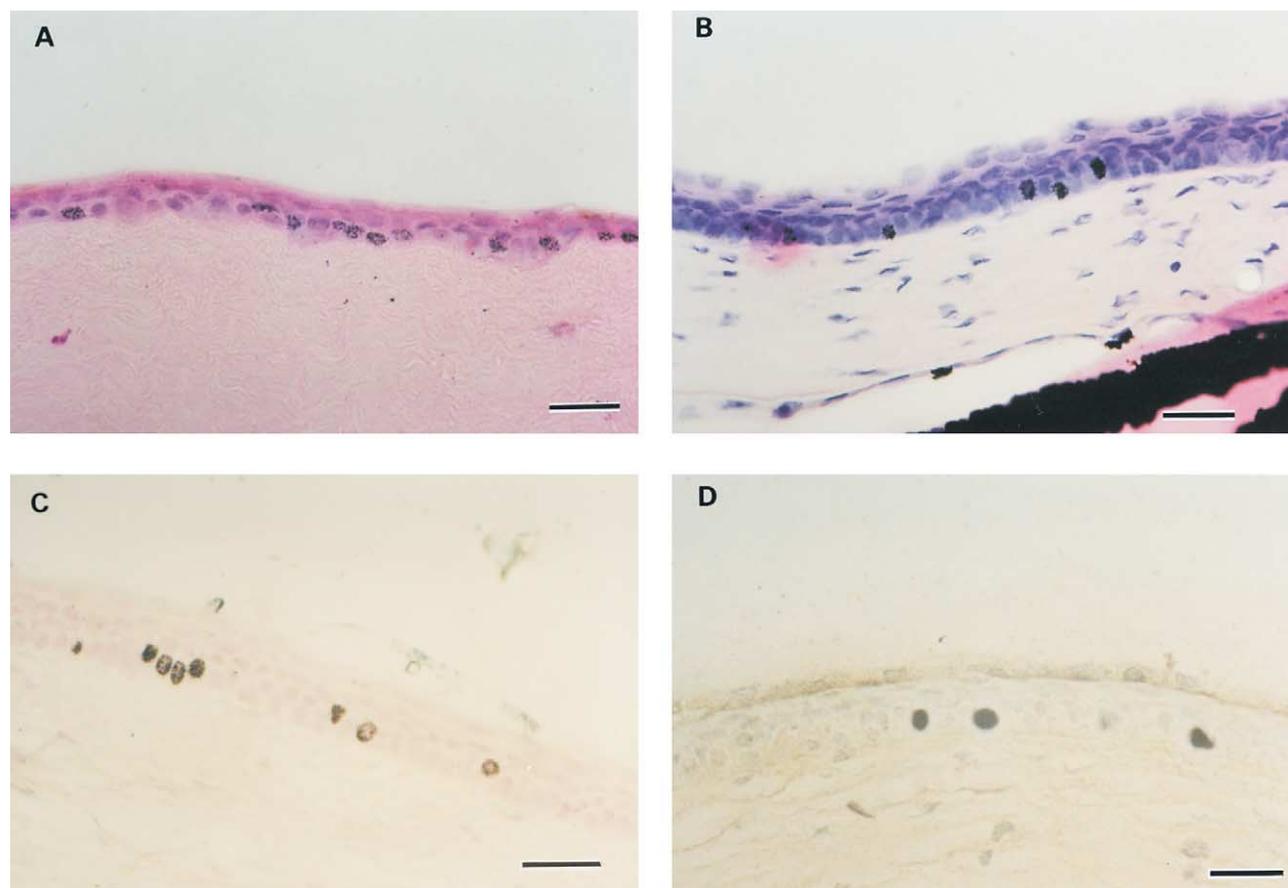

Figure 5. ^{3}H-thymidine uptake in 8-week C57BL/6J (**A**), 8-week KKAy (**B**), 16-week C57BL/6J (**C**), and 16-week KKAy mice (**D**). Each nucleus taking up ^{3}H-thymidine was colored black. At 8 weeks of age, corneal basal cells actively taking up ^{3}H-thymidine were observed in both types of mice (**A,B**). At 16 weeks of age, ^{3}H-thymidine uptake decreased in the cornea of KKAy mice (**D**). Bar = 100 μm (**A,B,C,D**).

From these findings, it can be concluded that corneal changes first occurred in the epithelium and the BM. Poor adhesion of the corneal epithelium might be the main reason for chronic epithelial detachment, which in turn induces the activation of keratocytes beneath the impaired epithelium. These activated keratocytes would generate several types of extracellular matrices (ECMs) and other substances in order to repair the wounded sites. This chronic stimulation may lead to the generation, accumulation and deposition of substances such as calcium, resulting in the development of corneal stromal opacities.

Immunohistochemical examination revealed abnormal distribution of ECMs in the aging KKAy mouse. The BM, vessel walls, and stroma stained intensely with Type IV collagen. The corneal BM and the walls of newly formed vessels in the central cornea showed intense staining with laminin, a major component of the BM. Although the mechanism to explain the production of these ECMs remains unclear, it is suggested that continuous damage to corneal epithelial cells and the BM might have led to the further activation of the already chronically activated keratocytes in order to repair the wound. The other proposed explanation is that the synthesis of ECM in tissues increases under hyperglycemic stress. Type IV collagen and laminin were reported to have increased in the diabetic kidney, resulting in thickening of the glomerular BM.[19] Type IV collagen was also detected in the vitreous matrix of diabetic individuals.[20] Pyrraline, one of the structure-characterized advanced glycation end products,[13] was also formed around the BM and the stroma in the aging KKAy mice. These end products are generated by the Maillard reaction through non-enzymatic glycation of proteins.[21] They have been reported to increase in the lens and kidneys of streptozotosin-induced diabetic rats,[22,23] and also in the kidneys, lungs, pancreas, skin, lens, retinal vessels, and peripheral nerves in human diabetics.[24–26] Pyrraline-like material in the human diabetic serum significantly increases compared to healthy individuals.[27] The level of pentosidine, another type of advanced glycation end product, was also reported to be higher in the diabetic cornea.[28] Accumulation of glucose and glycogen in the corneal epithelial cells in the diabetic animal model implies that an accelerated Maillard reaction could also occur in corneal tissues. The accumulation of advanced glycation end products changes the structure of proteins and may lead to the overexpression of cytokines, such as transforming growth factor-β, eventually leading to the production of ECMs.[29–33] Moreover, synthesized collagen could also be a good target for glycation since collagen is rich in both lysine and hydroxylysine and its turnover rate is extremely slow. This modified collagen shows abnormal intercellular reactions resulting in marked diabetic complications.[34]

Hyperglycemia and its by-products may play major roles in causing corneal abnormalities in diabetes mellitus.

In the present study, proliferative activities of corneal epithelial cells were also examined. The corneal epithelial cell proliferation in KKAy mice decreased at 16 weeks of age, when the clinical complications were not obvious. Although the reason for the decrease in corneal epithelial cell proliferation when the stroma was not severely damaged remains obscure, some evidence exists to support our results. In vitro studies have shown that the culture of endothelial cells under hyperglycemic conditions causes non-enzymatic glycosylation of intracellular proteins,[35] and decreasing cell proliferation. Our previous study using chemically induced IDDM model rats revealed that corneal epithelial cell proliferation decreased in the more advanced age groups.[4] Our preliminary study on corneal wound healing after alkali burns in younger KKAy mice showed weak epithelial cell attachment to the BM, as compared to the controls (data not shown). The present results suggest that hyperglycemia causes impaired corneal cell proliferation in the early phase, leading to decreased wound healing in diabetes.

In summary, our results suggest that the initial damage occurs in the corneal epithelial basal cells and BM in the younger period, followed by activation of underlying keratocytes, leading to the production of ECM, and the accumulation of advanced glycation end products. These diabetic metabolic changes influence the pathology of the cornea in KKAy mice. Although the corneal changes in KKAy mice are more severe than those in human beings and modified to some extent due to the genetic KK factors, we suggest that the results of the present study will help explain the pathogenesis of corneal disturbances associated with type 2 diabetes.

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