Original Research

Nuclear translocation of β-catenin induced by E-cadherin endocytosis causes recurrent erosion of diabetic cornea

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Impact statement

A recurrent and refractory corneal ulcer is the most typical clinical manifestation of diabetic keratopathy. The mechanism of repeated exfoliation of corneal epithelium caused by diabetes is not clear. Our study is the first to show the loss of intercellular junctions in diabetic keratopathy, which might be one of the principal key mechanisms responsible for the recurrent corneal erosions of diabetic keratopathy. Specifically, we found that high glucose reduced the expression of E-cadherin and transfer β-catenin from cytoplasm to nucleus, resulting in decreased stability of β-catenin/E-cadherin complex and disturbance of junction between corneal epithelial cells. These findings provide a new theoretical basis for further treatment of diabetic keratopathy. In addition, high glucose destroyed the adhesion of corneal epithelial cells to the basement membrane, which may be caused by the up-regulation of snail and MMP10 expression induced by β-catenin nuclear translocation.

Abstract

Recurrent epithelial erosion and refractory corneal ulcer are the clinical features of diabetic keratopathy (DK), which eventually lead to corneal scar and visual disturbance. In this study, we sought to determine the abnormalities of cell junction in diabetic corneal epithelial cells and the effect of high glucose on the β-catenin/E-cadherin complex. Corneal histology showed that corneal epithelial cells of high glucose mice were loosely arranged, and the immunohistochemistry showed that the expression of E-cadherin decreased, the levels of β-catenin increased in nuclear. High glucose-induced degradation and endocytosis of E-cadherin of corneal epithelial cells reduce the formation of β-catenin/E-cadherin complex and promote the nuclear translocation of β-catenin. Moreover, high glucose also activated the transcription and expression of matrix metallopeptidase and snail, which interfered with the adhesion of corneal epithelial cells to the basement membrane. These findings reveal that DK is associated with the dissociation of cell junctions. The maintenance of the stability of the β-catenin/E-cadherin complex may be a potential therapeutic target of refractory corneal ulcers in patients with diabetes.

Keywords: β-Catenin, E-cadherin, cornea, diabetes, cellular junction

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Introduction

Abnormal corneal epithelium is the most typical lesion of DK. The clinical manifestations include fragile epithelium, superficial punctate keratitis, recurrent erosion, unhealed ulcers, and corneal edema caused by changes in epithelial barrier function.[1] Chronic relapsing corneal erosion results in corneal scarring, corneal ulcers and neovascularization, which eventually leads to visual impairment in patients with diabetes.[2] Current therapies to treat DK are mainly in promoting the proliferation of limbal stem cells, improving the regeneration of corneal nerve and anti-inflammatory. There has been no satisfactorily effective method for the clinical treatment of DK, aiming at the cause of DK is the guarantee of the success of the treatment.[3–6] The corneal epithelial cellular junction is the key proceeding to remain the structure and function integrality of corneal epithelium, which plays a vital role in resisting pathogens and maintaining normal corneal transparency.[7]

In the corneal wound healing, epithelial sheets formed a tight connection between cells to migrate directionally and collectively.[8] This collective movement ensures that the epithelial cells form the external barrier and maintain the basic function of the cells.[9,10] The abnormal connection between cell–cell and cell to basement membrane will lead to repeated corneal epithelial exfoliation and chronic refractory corneal ulcers. E-cadherin belongs to the cadherin

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superfamily, which takes part in the calcium-dependent adhesion in epithelial cells.[8] E-cadherin-mediated adhesion junctions are crucial for cell sheets' directional migration. β-Catenin is recognized as an important component of intercellular junction protein complex, which interacts with the cytoplasmic domain of E-cadherin and connects with α-catenin to mediate the anchoring of the complex to cortical actin cytoskeleton.[11–15] The stability of β-catenin/E-cadherin complex is vital for the formation of epithelial barriers. In tumor cells, β-catenin detaches from the β-catenin/E-cadherin complex and migrates into the nucleus, which is the key to the spread and metastasis of tumor cells.[16] Indeed, in the canonical Wnt pathway, β-catenin moves to the nucleus and recruits transcription factors such as snail to regulate cell cycle, migration, and invasion.[17] Snail is the earliest discovered negative regulator of E-cadherin and binds to the E-cadherin promoter to inhibit its transcriptional activity, resulting in the partial or total loss of E-cadherin expression.[3,18,19]

Matrix metalloproteinases (MMPs) belong to the proteolytic family and play key roles in corneal wound healing and tissue remodeling.[20] Since DK has the characteristics of delayed corneal healing and repeated exfoliation, protease may be involved in these changes.[21] Research by Matsumura *et al.* showed that corneal epithelial cells from high-glucose and diabetic mice showed excessive MMPs activity during wound healing.[22] Gene microarray, immunostaining, and zymogram analyses showed that the expression of MMPs was upregulated in human diabetic corneal epithelium and keratinocytes *in vitro*.[23]

In this study, we sought to investigate the mechanism of abnormal corneal epithelial repair caused by high glucose. We explored the impacts of high glucose on the formation of corneal epithelial cell junction, as well as the formation and localization of intercellular junction complex in diabetic mice *in vivo* and *in vitro*.

Materials and methods

Ethics statement and induction of diabetes

All animal experiments were conducted in accordance with the statement of the Association for Research in Vision and Ophthalmology on the use of animals in ophthalmology and visual research and approved by the Animal Ethics Committee of Southern Medical University. In this study, eight-week-old male C57BL/6 mice were purchased from the Experimental Animal Center of Southern Medical University, and diabetic mice were induced with multiple low doses of streptozotocin[24] (STZ; 40 mg/kg dissolved in 0.1% mmol/L citric acid buffer, pH 4.5). STZ was injected into the abdominal cavities of diabetic mice for five days. On the 14th day of the experiment (9th day after the last injection of STZ), all mice fasted for 6 h (from 7:00 to 13:00). Blood glucose levels were measured by obtaining samples for measurement from the caudal vein. Measurements were obtained with the One Touch basic blood glucose-monitoring system. Mice treated with STZ with blood glucose levels ≥16.6 mmol/L were classified as diabetes mellitus (DM).

Corneal epithelial debridement and the hematoxylin-eosin (HE) staining of cornea

Mice were subjected to corneal epithelial debridement at 30 d after injection. Age-matched DM mice and normal mice were anesthetized by intraperitoneal injection of xylazine (6 mg/kg) and ketamine (80 mg/kg), while local anesthesia was performed with proparacaine hydrochloride eye drops (Alcon-Couvreur, Belgium). First, a 1.5-mm circular wound was drilled in the center of the cornea. Then the corneal epithelium was removed with a blunt blade under an operating microscope (Zeiss, Peabody, MA, USA). All mice were treated with .5% levofloxacin eye drops three times a day after surgery. To analyze the time-course of corneal epithelial wound closure, corneal epithelial defects were stained with 1% fluorescein in normal saline at 12, 24, 36, and 48 h, and photographed under a digital slit lamp (Nidek, Japan).

After 48 h, mice were euthanized with excessive chloral hydrate, and corneas were taken for use in Western blotting, hematoxylin–eosin (HE) staining. The removed eyes were fixed in Davidson's fixative, and 3-μm paraffin sections were prepared and stained with HE. The experiments were repeated at least three times.

Immunohistochemistry of mice corneas

The removed eyeballs were fixed in Davidson's fixator, and the central cornea ($d = 5$ mm) was taken to prepare 3 μm thick paraffin sections. There were three sections of each eyeball, a total of 24. Glass slides were dried at 60°C for 1 h, separated in xylene, and rehydrated in graded ethanol solution. Tris-EDTA buffer was used for antigen retrieval, and 3% H_2O_2 solution was used to quench the activity of endogenous peroxidase. Slides and antibodies were incubated overnight at 4°C. Slides were evaluated with the Polink-2 plus polymer horseradish peroxidase (HRP) detection system (ZSGB Bio, China), then stained with heme. Images were obtained under a microscope (Leica, Germany).

Primary culture of mouse corneal epithelial cells

After 48 h of therapy, the mice were killed. The head and periocular tissue were sterilized with povidone iodine solution to avoid contamination, then washed with aseptic Dulbecco's PBS. Mice corneas were examined under an operating microscope. The corneal epithelium was placed face-down in an aseptic petri dish and incubated with Dispase II in DMEM/F12 (Gibco-BRL, Grand Island, NY) at 37°C for 40 min. Epithelial cells were gently scraped off with a cell scraper, then washed, inoculated into a cell petri dish (Corning, New York), and incubated in a moist incubator at 37°C, 95% air/5% CO_2. Culture media were changed daily. The conventional culture medium was DMEM/F12, which contains 10% fetal bovine serum (Every Green, China), 1% L-glutamine (Gibco-BRL, Grand Island, NY, USA), 1% non-essential amino acids (Gibco-BRL, Grand Island, NY, USA), 1% penicillin-streptomycin (HyClone, USA), 0.1% insulin (Sigma-Aldrich, USA), 0.1% LOT121K0186 (Sigma-Aldrich, USA), and 0.1% transfer medium (Sigma-Aldrich, USA). The cells of the control

group and HCG group were derived from the corneal epithelium of mice in the control group and DM group, respectively. The cells of control and HCG were cultured in standard medium supplemented with 5 mmol/L glucose (Sigma-Aldrich, USA) and 20 mmol/L mannitol (Sigma-Aldrich, USA), or 25 mmol/L glucose, respectively (Supplementary Table S1).

Cell adhesion

Matrigel (No. 354230, Corning, USA) was thawed at 4°C overnight and diluted to 1 mg/mL with blood-free cold DMEM. Diluted matrigel was added to a 96-well plate using an aseptic pipette, incubated at 37°C for 1 h, then washed with PBS and sealed at 37°C for 1 h. Cells from the confluent 10 cm petri plate were digested with 0.05% trypsin-EDTA and resuspended in serum-free DMEM; 3×10^4 cells were implanted into a 96-well plate. Plates were centrifuged at 300 rpm for 5 min and incubated in a 5%-CO_2 humidification incubator at 37°C for 1 h. Petri dishes were then removed and centrifuged (upside down) for 5 min at 500 rpm to remove non-adherent cells. Cells were dyed with 0.5% crystal violet and 0.1% formaldehyde, then washed with PBS. After the PBS had been discarded, 40 μL 2% Triton X100 (T8200, Solarbio, China) was added to each hole. The relative number of cells in each hole was measured, and absorbance was measured at 595 nm in the microplate reader (Bio-Rad, USA). All experiments were repeated three times.

Scratch assay

The cell migration was measured by scratch assay. Cells were harvested and plated in complete medium at 10×10^4 cells/well in six-well dishes, incubated overnight to yield confluent monolayer for wounding. Wounds were made by a pipette tip of 10 μL and images were taken immediately of all wells. Then, the cell culture medium was changed to the medium containing 2% serum and D-glucose of 5 or 25 mmol/L, respectively. The wound was closed by the cell monolayer in 48 h, and the images were taken 0, 12, 24, 36, and 48 h after the wound formation. The experiment was carried out in triplicate. The samples were tested in triplicate, and the data are expressed as the mean ± SD.

Real-time reverse-transcription polymerase chain reaction (PCR)

Cells were seeded at 30×10^4 cells/well in six-well plates and incubated at 37°C for 24 h, at which point the cell culture medium was changed to medium containing 5 mmol/L or 25 mmol/L D-glucose. After 48 h, the cells were harvested, and total RNA was extracted using Trizol (Takara, Japan). The concentration of RNA was detected with a NanoDrop 2000 spectrophotometer (Thermo Fisher, USA). Real-time PCR assays were performed using a CFX96 real-time PCR detection system (Bio-Rad, USA) in accordance with the manufacturer's instructions. All assays were calculated on the basis of the ΔΔCq method, and mRNA expression was measured by n-fold change according to the $2^{-\Delta\Delta Ctq}$ method. All reactions were performed in triplicate, and mouse β-tubulin served as the endogenous control. The PCR primers were designed by Blast (Supplementary Table S2).

Western blotting

Total protein was extracted by RIPA lysis buffer. Nuclear protein was extracted with a Cellytic Nuclear Extraction Kit (Sigma, Germany), and cytoplasmic protein was extracted with a ProteoPrep Universal Extraction Kit (Sigma-Aldrich, USA). The extracted proteins were separated by SDS-PAGE gel and transferred to polyvinylidene difluoride membranes. The membrane was blocked at room temperature for 1 h, then incubated overnight with primary antibody at 4°C. On the second day, the membrane was incubated with HRP-coupled secondary antibody (Invitrogen, USA) for 1 h, and the immuno-conjugate was detected by a chemiluminescence imaging system (BioRad, USA) with use of an enhanced chemiluminescence substrate (Millipore, Germany). And each experiment was repeated at least three times. The antibodies used are shown in Supplementary Table S3.

Co-immunoprecipitation

Total protein was extracted using a Byotime protein extraction kit (Byotime, China) and incubated with antibody overnight at 4°C. A+G agarose protein (CwBiotech, China) was added to incubate for 3 h. Immunoprecipitation analysis was carried out by Western blotting, and each experiment was repeated at least three times.

Immunocytochemical imaging

Cells were cultured in a 96-well plate for 48 h according to the culture conditions of each group, then fixed with 200 μL 4% methanol-free solution (Sigma-Aldrich, USA) at room temperature for 15 min. Then, cells were blocked with 0.3% Triton-X-100 (Sigma-Aldrich, USA) at room temperature, and the first antibody was added to incubate overnight in a humidification box at 4°C. The following day, fluorescent secondary antibody was added in a darkroom. Cells were incubated at room temperature for 1 h. Nuclei were stained with DAPI (Invitrogen, USA). Each experiment was repeated three times. The primary antibodies used were as follows: anti-β-catenin (1:100), anti-Snail (1:100), anti-keratin 12 (1:100), anti-cytokeratin 3 (1:100), anti-E-cadherin (1:50), and anti-MMP10 (1:100). The secondary antibodies used were as follows: goat anti-mouse IgG (H + L) highly cross-adsorbed secondary antibody (1:100).

Statistical analysis

The wound area of the scratch assay, the gray value of Western-blot bands, and the fluorescence intensity were measured and analyzed by ImageJ. The SPSS 20.0 statistical package and GraphPad Prism software were used for the statistical analysis. The adhesion of corneal epithelial cells, the expression level of protein and mRNA were statistically analyzed by Student's paired two-tailed *t*-test. $P < 0.05$ was considered significant. The area of corneal epithelial defect was measured and analyzed by ImageJ software. Quantitative data are

presented as mean ± SD and were compared among groups with Dunnett's post hoc test following a one-way ANOVA. $P < 0.05$ was considered statistically significant.

Results

Diabetic mice exhibit delayed corneal epithelial trauma repair, and the histopathological section showed loose arrangement and abnormal differentiation of corneal epithelial cells

On the 14th day after STZ injection, blood glucose levels in DM group were significantly higher than control group (32.28 ± 1.58 mmol/L, compared with 8.65 ± 1.09 mmol/L, $P < 0.05$). On a parallel with the elevated blood glucose, the DM group showed general characteristics of diabetes such as weight loss, increased food consumption, and increased water intake, compared with the control group (Table 1). The corneal epithelium of mice were scraped to simulate the post-traumatic repair process of corneal epithelium in patients with diabetes and observed their wound repair within 48 h. The area of the epithelial defect decreased with time in all groups, However, compared with the control group, the wound repair of corneal epithelium in DM group was slower (12 h: $P < 0.01$; 24, 36, 48 h: $P < 0.001$), and recurrent epithelial defect appeared at 48 h (Figure 1(a) and (b)).

The histopathological results showed that the epithelial cells in control group showed multilayer structure, the superficial layer was neatly arranged, and the basal cells were high columnar, cells remaining intact and lined up tightly. The cells in the DM group were arranged loosely, wing cells were flat and elongated. Most parts of the superficial cells showed sloughing. The basal cells lost their columnar shape and were flat and creeping, and the cells were disordered (Figure 1(c)).

Immunohistochemical results showed that E-cadherin was expressed in the cytoplasm and membrane of central corneal epithelial cells. There was a strong expression in the control group, but a weak expression in the DM group. β-Catenin was moderately expressed in the cytoplasm in control group, but was strongly expressed in the cytoplasm and nucleus of DM group. MMP10 was mainly expressed in the cytoplasm and Snail was expressed in the nucleus. The immunohistochemical staining of both was increased in DM group (Figure 1(d)).

The cell-based model of DK was established

We established a cell-based model to mimic the corneal epithelial wound repair disorder caused by high glucose. Primary cells were authenticated using short tandem repeat (STR) analysis as described in 2012 in ANSI Standard (ASN-0002) by the ATCC Standards Development Organization. The STR results showed that no multiple alleles and no cross contamination were found in the primary corneal epithelial cells. As the cell was isolated from mouse tissues, no STR data were logged in the database ATCC, DSMZ, JCRB, EXPASY, and RIKEN (Supplementary File S1 and S2).

The morphology and growth features of corneal epithelial cells were observed by inverted microscopy. Cells were triangular or polygonal with three to six processes, and the cells had a lower nuclear–cytoplasmic ratio. When the cells grew for two days, the cells had a wide connection with adjacent cells; their prominences were thick and interlaced. Western blotting and the immunofluorescence assays showed that CK12 and CK3 (specific markers of corneal epithelial cells) were expressed in each group (Supplementary Figure S1).

The degradation of E-cadherin and nuclear translocation of β-catenin induced by high glucose resulted in corneal epithelial cell–cell detachment

In order to explore the effect of high glucose on intercellular junction, we detected the expression level of intercellular junction protein complex molecules: E-cadherin and β-catenin. In the investigation of protein expression, we found that E-cadherin showed low expression in high glucose-cultured cells but was highly expressed in normal cells (Figure 2(a) and (b), $P < 0.05$). The expression of β-catenin was slightly reduced in HCG group, but not significantly ($P = 0.7072$). We also found that the mRNA transcription of E-cadherin and β-catenin was higher in the control group than HCG group (Figure 2(c), E-cadherin: $P< 0.001$; β-catenin: $P < 0.05$). Therefore, we detected the expression of β-catenin protein in different cell components. The protein expression of β-catenin in cell components was different. β-Catenin was highly expressed in the nucleus in the HCG group ($P < 0.001$); on the contrary, it was mainly expressed in the cytoplasm in the control group (Figure 2(a), $P < 0.01$).

Under the confocal microscopy, we found that β-catenin (red) was located in the cytoplasm in the control group, while in the HCG group it was located in nucleus and perinuclear compartment. In addition, the E-cadherin-mediated intercellular contact in the HCG group was less than that in the control group. E-cadherin (green) was mainly expressed in the cell–cell connection of control group, while it was weakened in HCG group (Figure 2(d) and (e), $P < 0.01$).

Table 1. Body weight, blood glucose concentration, and food consumption for mice in the various treatment groups measured at the end of experiments (9 days after streptozotocin injection).

Group	Blood glucose (mmol/L) 0 day	14 days	30 days	Body weight (g) 0 day	14 days	30 days	Food consumption (g/d; 14 days)
Control	7.32 ± 1.25	8.65 ± 1.09	7.79 ± 1.30	28.52 ± 2.37	31.26 ± 1.69	37.38 ± 2.54	3.78 ± 0.54
DM	6.82 ± 0.73	32.28 ± 1.58*	35.5 ± 2.26*	31.25 ± 1.76	18.69 ± 2.13*	22.2 ± 3.32*	9.54 ± 1.96*

The data are presented as means and SD ($n = 6$).
*$P < 0.05$ (versus control, Dunnett's post hoc test following a one-way ANOVA).

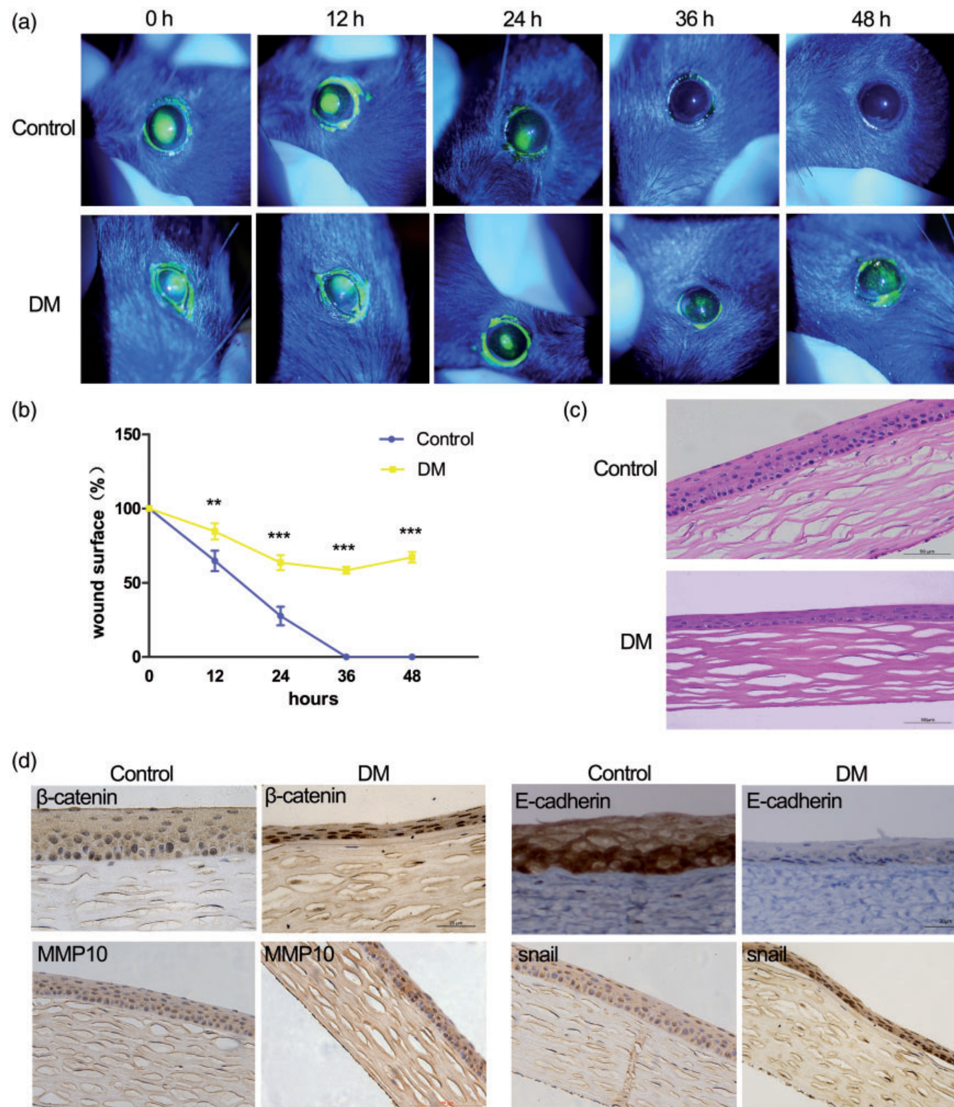

Figure 1. In diabetic mice, the repair of corneal epithelium was delayed after trauma, and the corneal epithelial cells showed abnormal morphology and adhesion. (a) Representative fluorescein staining of corneal epithelial defects in mice from the control and DM groups 0, 12, 24, 36, and 48 h after epithelial debridement. (b) Time course of corneal repair in control and DM group ($n = 6$; $^{**}P < 0.01$, $^{***}P < 0.001$, compared with control group by one-way ANOVA with post hoc Dunnett test). (c) HE staining of the cornea in control and DM group. (d) Immunohistochemistry for E-cadherin, β-catenin, MMP10, and snail in the corneal epithelium. β-Catenin was moderately expressed in the cytoplasm of the control group, while it was strongly expressed in the nucleus in the DM group. Strong staining for E-cadherin was observed in the cytoplasm and membranes of corneal epithelium cells from control group, but was expressed at negligible levels in DM group. The immunohistochemical staining of MMP10 and snail was significantly increased in DM group. (A color version of this figure is available in the online journal.)

High glucose induced the desquamation of corneal epithelial cells from basement membrane, is associated with the overexpression of MMP10 and snail

Then we detected the effect of high glucose on the adhesion of corneal epithelial cells to basement membrane by cell adhesion experiment. Compared with the control group, the adhesion of corneal epithelial cells cultured with high glucose was weakened. Corneal epithelial cells in high glucose were unable to form cell–matrix adhesion, resulting in a large number of exfoliated epithelial cells (Figure 3(a), $P < 0.001$). The scratch assay showed that the convergence rate of corneal epithelial cells cultured with high glucose was slower than control group after injury, and there was a great many of apoptosis and detachment from the basement membrane (Figure 3(b) and (c), 12 h: $P < 0.01$, 24 h: $P < 0.001$, 36 h: $P < 0.001$, 48 h: $P < 0.001$).

To work out what was driving those differences, we detected the expression and localization of MMP10 and snail. MMP10 (green) and snail (green) are located in the cytoplasm and nucleus, respectively (Figure 4(a)). Low levels of MMP10 and snail fluorescence were observed in the control group than HCG group (Figure 4(a), $P < 0.01$). We confirmed this through Western-blot and RT-PCR experiments. The mRNA transcription and protein expression of MMP10 and Snail increased in the high-glucose group but decreased in the control group (Figure 4(b) and (c), MMP10: $P < 0.01$, snail: $P < 0.001$).

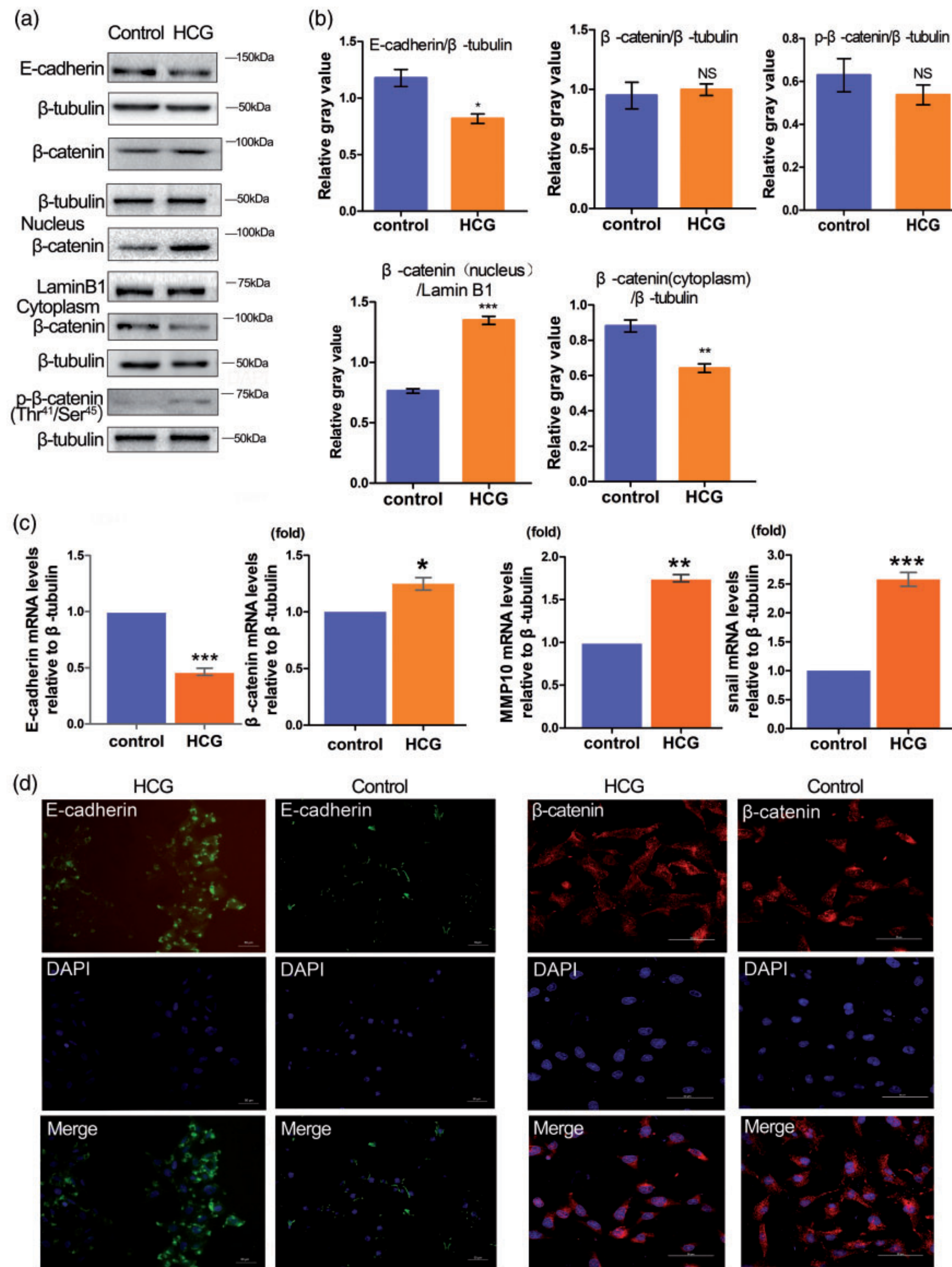

Figure 2. High glucose decreased the expression of intercellular junction protein complex molecules. (a) Western blotting analysis of β-catenin, p-β-catenin, E-cadherin, snail, and MMP10 expression in corneal epithelial cells. (b) The graph represents relative gray value of Western-blot. (c) Real-time RT-PCR analysis for mRNA expression levels of β-catenin, E-cadherin, snail, and MMP10 in corneal epithelial cells. (d) Fluorescence localization of E-cadherin in control and HCG group under confocal microscope. The scale bars indicate 50 μm. (e) Fluorescence localization of β-catenin in control and HCG group under confocal microscope. The scale bars indicate 50 μm. All data shown are the mean ± SD ($n = 6$; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, Compared to control group, two-tailed t-test). All experiments were performed in triplicate. (A color version of this figure is available in the online journal.)

The recruitment of *β*-catenin by E-cadherin was inhibited by high glucose, resulting in a decrease in complex formation

To further investigate the link between high glucose on E-cadherin and β-catenin dimers, the expression of E-cadherin and β-catenin complex was quantitatively determined by immunoprecipitation. The results showed that the binding of E-cadherin and β-catenin occurred in all groups, but the level of E-cadherin and β-catenin dimer in the high-glucose group was lower than that in the control group (Figure 5(a) and (b)).

Discussion

DK is a dysfunctional corneal disease, which leads to punctate epithelial erosions, recurrent corneal erosion, and intractable corneal ulcer.[25] Persistent corneal epithelial defects caused by diabetes usually lead to corneal opacity, corneal ulcers, blurred vision or permanent vision loss, and corneal neovascularization.[26] Although considerable progress has been made in the pathogenesis of DK, the crucial mechanisms of recurrent corneal erosion, and persistent epithelial defects caused by diabetes are still not fully understood. In this study, we found that high glucose

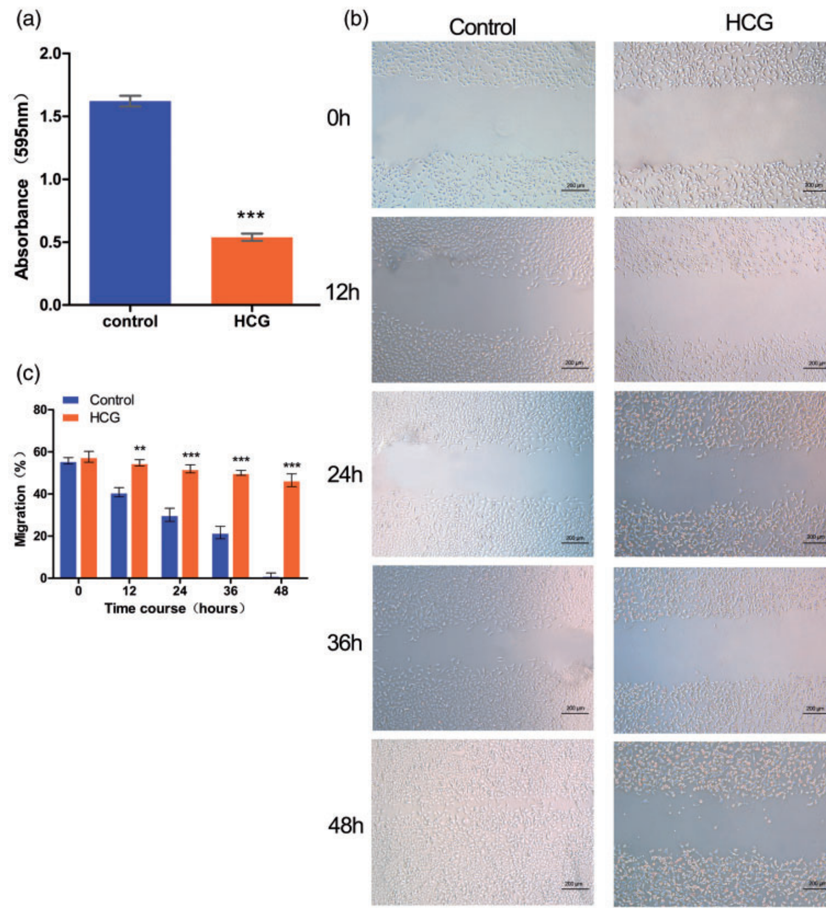

Figure 3. High glucose inhibited the adhesion of corneal epithelial cells to the basement membrane. (a) Adhesion of control and HCG cells to matrigel-coated plates at 37°C. The extent of cell adhesion expressed as absorbance values were quantified using MTT dye. (b) Scratch assay was performed with control and HCG cells. The scale bars indicate 200 μm. (c) The graph represents time kinetics of wound confluence percentage, calculated by Image J software. All data shown are the mean ± SD ($n = 6$; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, Compared to control group, two-tailed t-test). All experiments were performed in triplicate. (A color version of this figure is available in the online journal.)

affected the localization and expression of E-cadherin, resulting in the transport of β-catenin from cytoplasm to nucleus. Abnormal intercellular junction caused by unstable E-cadherin/β-catenin complex is an important mechanism of recurrent corneal erosion and intractable corneal ulcer in diabetes. Moreover, we provide evidence and mechanism of high glucose inhibiting corneal epithelial repair after trauma in diabetic mice, which depends on depolymerizing E-cadherin/β-catenin complex, finally suppressing formation of intercellular junction in diabetic corneal epithelial cells. In addition, high glucose also promoted the expression of snail and MMP10, affecting the stability of cell basement membrane adhesion.

DK often deviates from the normal wound healing, resulting in persistent corneal epithelial defects, and is not responsive to the treatment of general corneal ulcers in a hyperglycemic environment. The normal repair of corneal epithelium adheres to the classical XYZ theory of corneal epithelial repair.[27] In the early repair stage, the epithelial cells migrate as a coherent sheet to cover the wound.[28,29] Corneal epithelial cells always maintain a tight intercellular junction in wound repair and remained cohesive adhesion of cell to the basement membrane.[26,30] In corneal epithelial wound healing, the priority of epithelial cells is to restore the epithelial barrier. Therefore, maintaining appropriate cell adhesion when epithelial cells migrate on the wound is crucial to prevent further compromised of cornea.[31,32] The E-cadherin/β-catenin complex is involved in the adhesion of corneal epithelial cells and is considered to be an essential structure for maintaining the integrity of stratified epithelium.[33,34] It is obvious that the binding of β-catenin to E-cadherin is a prerequisite for adhesion. It is noticeable that the stability of corneal epithelial cell adhesion is of vital importance for corneal wound repair in corneal epithelial wound healing. E-cadherin/β-catenin adhesion system is implicated in corneal epithelial cell adhesion, and the β-catenin signal transduction plays an integral role in the junctional complex. E-cadherin is a $Ca2^{+}$-dependent glycoprotein with a single transmembrane domain, which is essential for the function of intercellular junction.[35] The E-cadherin-mediated cellular junction is essential for the directional and collective migration of corneal epithelial cells.[8] In our study, immunofluorescence, Western-blot, and co-immunoprecipitation showed that β-catenin and E-cadherin complex were depolymerized, and the expression of E-cadherin protein was

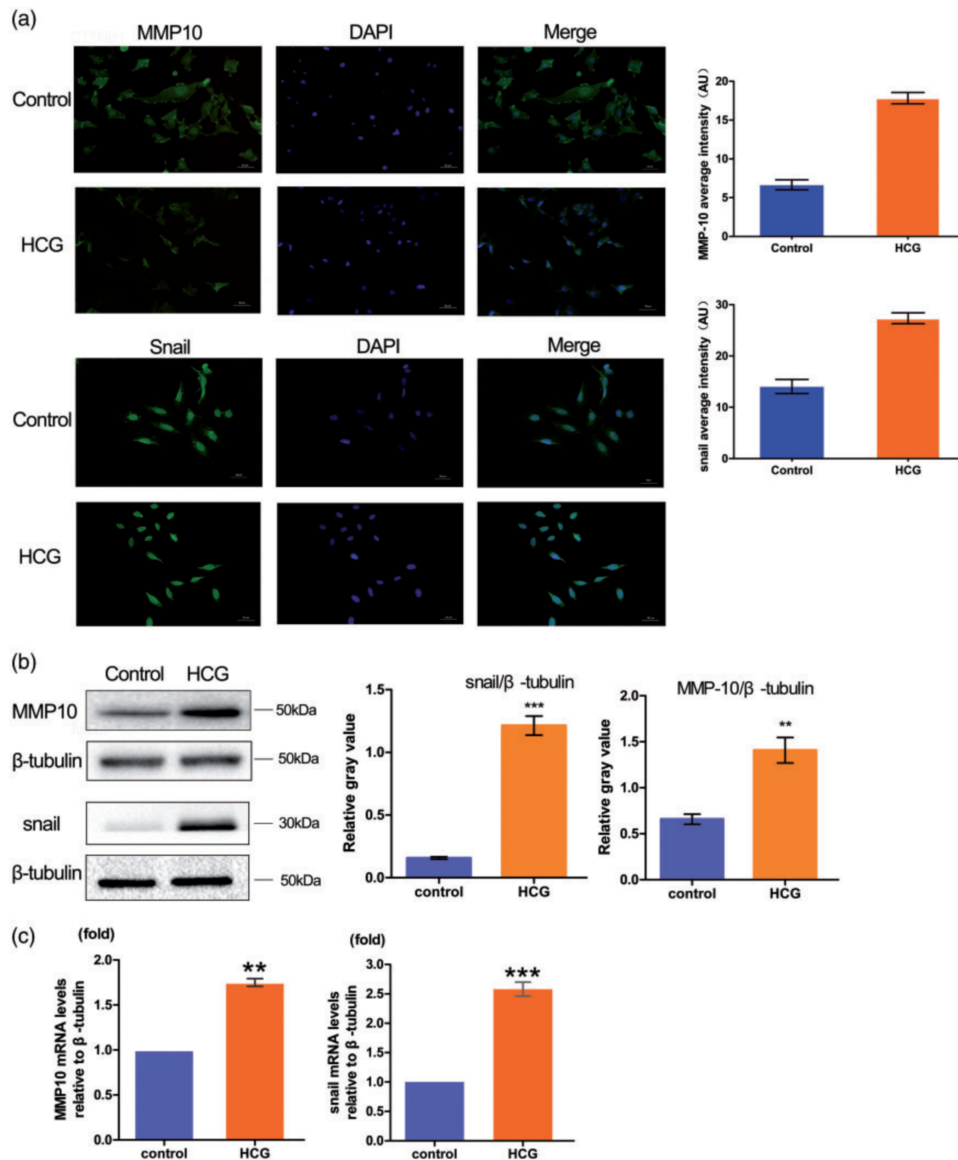

Figure 4. MMP10 and snail were activated in corneal epithelial cells of diabetic mice. (a) Immunofluorescence staining of MMP10 and snail in control group and HCG group. The scale bars indicate 50 μm. The graph represents fluorescence intensity of MMP10 and snail. (b) Western blotting analysis of MMP10 and snail expression in corneal epithelial cells. The graph represents relative gray value of snail and MMP10. (c) Real-time RT-PCR analysis for mRNA expression levels of MMP10 and snail in corneal epithelial cells. All data shown are the mean ± SD ($n = 6$; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, compared with control group, two-tailed t-test). All experiments were performed in triplicate. (A color version of this figure is available in the online journal.)

significantly decreased on the cell membrane, only weakly expressed in the cytoplasm.

The β-catenin signal transduction plays an integral role in the junctional complex.[36] As an important intercellular connexin, β-catenin is attached to intercellular junction protein complex together with cadherin.[37] Under conditions of strong adhesion, β-catenin is stable and protected within the cadherin complex, forming a strong coupling structure and maintaining tight cell junctions.[36,38,39] Our study found that E-cadherin was degraded under elevated glucose, but the β-catenin in the cytoplasm did not increase. According to previous studies, there are two metabolic pathways after β-catenin is isolated from β-catenin/E-cadherin complex:[40,41] (1) β-catenin is phosphorylated and degraded with GSK3 β to form a degradation complex, which is finally degraded by ubiquitin modification. (2) The ligand protein Wnt binds to the membrane surface receptor protein FZD and activates the intracellular protein DVL to inhibit the degradation of β-catenin and stabilize the free β-catenin protein in the cytoplasm. We had explored the expression of phosphorylated β-catenin in primary cultured corneal epithelial cells by Western-blot. It was found that the level of phospho-β-catenin (Thr41/Ser45) in HCG group was almost as low as control group. We observed that the endocytosis of E-cadherin decreased the ability to recruit β-catenin. This leads to amount of unphosphorylated β-catenin in the cytoplasm translocated into the nucleus, and triggers the transcription and translation of some molecules in the nucleus. β-Catenin participates in Wnt signal pathway and plays a key role in cell migration

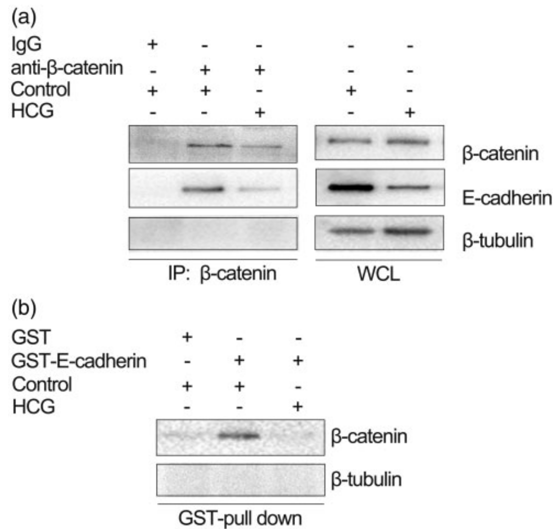

Figure 5. The binding of β-catenin and E-cadherin in the HCG group was weaker than control group. (a) Immunoprecipitation was performed with β-catenin antibodies or mouse IgG-charged protein G beads and cell lysate from corneal epithelial cells and analyzed by Western-blot, as indicated. (b) The GST or GST-cadherin proteins expressed by the bacteria were charged to glutathione-agarose beads. Pulldowns were performed as described and revealed in Western-blot analysis.

and differentiation.[42] The accumulation of β-catenin in the nucleus increases the mRNA transcription of snail and the generation of proteins.[41] Snail binds to the promoter of E-cadherin and inhibits its transcriptional activity, leading to the partial or complete loss of E-cadherin expression, which is considered to be the primary and most important step in destroying cell adhesion.[43,44]

AUTHORS' CONTRIBUTIONS

All authors participated in the design, interpretation of the studies, analysis of the data, and review of the manuscript; WBM, XHL, and SFF designed and conducted the experiments; ZYX helped revised the manuscript; HC, XHC, and LNZ provided software guidance and analyzed the data.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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SUPPLEMENTAL MATERIAL

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