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Original Article

Dendritic epidermal T cells facilitate wound healing in diabetic mice

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Abstract: The impairment of skin repair in diabetic patients can lead to increased morbidity and mortality. Proper proliferation, apoptosis and migration in keratinocytes are vital for skin repair, but in diabetic patients, hyperglycemia impairs this process. Dendritic epidermal T cells (DETCs) are an important part of the resident cutaneous immunosurveillance program. We observed a reduction in the number of DETCs in a streptozotocin-induced diabetic mouse model. This reduction in DETCs resulted in decreased IGF-1 and KGF production in the epidermis, which is closely associated with diabetic delayed wound closure. DETCs ameliorated the poor wound-healing conditions in diabetic mice by increasing keratinocyte migration and proliferation and decreasing keratinocyte apoptosis in diabetes-like microenvironments. Our results elucidate a new mechanism for diabetic delayed wound closure and point to a new strategy for the treatment of wounds in diabetic patients.

Keywords: Diabetes, wound healing, dendritic epidermal T cells

Introduction

The most common dermatological complications of diabetic patients are wound-healing deficits [1], which can impose significant medical and socioeconomic burdens [2]. Indeed, among diabetic patients, there is an increased incidence of foot ulcers and amputations that are caused by wound-healing deficits [3]. The mechanisms that underlie compromised wound healing are not completely understood [4, 5], and the development of effective treatments for diabetic wounds is a challenging problem in clinical practice [6].

The dynamic process of wound repair primarily involves inflammatory cell recruitment, angiogenesis, re-epithelization and fibroblast proliferation, which are regulated by cytokines and growth factors [7]. However, diabetes impairs the orderly sequence of cellular and molecular events, which results in delayed wound repair [8]. Therefore, to elucidate the mechanisms of diabetic-induced wound-healing deficits, it is important to identify the key cells and factors that regulate wound repair. For example, IGF-1 plays an important role in epidermal development and maintenance, and also serves as a key growth factor in regulating the migration and proliferation of keratinocytes as well as protecting keratinocytes from apoptosis [9-13]. KGF also contributes to keratinocyte proliferation and migration [14-16].

Furthermore, dendritic epidermal T cells (DETCs) are the exclusive producer of IGF-1 and KGF in the epidermis [17, 18]. DETCs are an interdigitating population of epidermal resident lymphocytes that express the Vγ3Vδ1 T cell receptor (TCR) and compose the majority of T cells in the epidermis [19]. DETCs serve as primary responders to epithelial damage and are important for wound healing [20, 21]. Following skin damage, DETCs respond to an unidentified self-antigen expressed on damaged keratinocytes in a major histocompatibility complex (MHC)-independent way [22, 23]. Then, IGF-1 and KGF are released from DETCs at the wound edge and play an important role in facilitating wound healing [17, 18]. Impaired homeostasis

and activation of epidermal γ δ T cells are both observed in the diabetic epidermis [24]. However, there is limited evidence on whether DETCs are associated with diabetic wound-healing defects.

The data from the present study indicates that there is a decreased presence of DETCs at the wound site in diabetic mice, which results in reduced IGF-1 and KGF production in the epidermis near the wound. DETC application could improve wound-healing defects in diabetic mice. Thus, we performed functional analyses to assess the effect of DETCs on keratinocytes in diabetes-like microenvironments and found that DETCs could enhance the migration and proliferation of keratinocytes as well as reduce apoptosis in diabetes-like microenvironments. These results demonstrate that the reduced number of DETCs in diabetic mice was associated with delayed wound closure. This may be an important mechanism that results in diabetes-induced wound-healing deficits.

Materials and methods

Animals

C57BL/6J (B6) mice were purchased from the Experimental Animal Department of the Third Military Medical University in Chongqing, China. All animals were maintained under specific pathogen-free conditions and used at 6 to 8 weeks of age.

STZ-induced diabetic animal model

C57BL/6J (B6) mice were injected i.p. with 150 ul of STZ (100 mg/kg, Sigma-Aldrich, USA) or the vehicle control for 6 consecutive days. Venous blood glucose levels were measured in non-fasted animals using a glucometer. Mice were evaluated every 2 days at 2:00 p.m. and were considered diabetic when the blood glucose levels were sustained above 250 mg/dL.

Wounding procedure

Wounding was performed on mice anesthetized with sodium pentobarbital. Briefly, the dorsal surface of the mouse was shaved, the back skin and panniculus carnosus were pulled up, and one or two sets of sterile full-thickness wounds were generated using a sterile 4-mm punch tool. In some experiments, 10^6 DETCs were dissolved in 40 μl of phosphate buffered saline (PBS) and injected intra-dermally around the wound area at four injection sites immediately after wounding and daily thereafter, 40 μl of PBS was applied to the wounds of the control mice in the same manner.

Isolation of epidermal sheet

The skin harvested from STZ-induced diabetic and control mice was washed twice in sterile PBS. Next, the skin was cut into 5 mm × 5 mm pieces and washed again with PBS. The pieces were digested with 0.5 g/l Dispase II (Sigma, USA) at 37°C for 1-2 hours, and then the epidermis and dermis were separated carefully. The epidermal sheet was minced and digested with 0.5% trypsin at 37°C for 10 minutes, followed by cell collection via centrifugation. The cells were suspended in RPMI 1640 Medium (GIBCO BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum, 100 mg/ml of streptomycin, 100 U/mL penicillin and 2 mM glutamine (Hyclone, USA).

Isolation of DETCs

The mouse DETCs were isolated from the skin as previously described [25]. The purity of the isolated mouse DETCs was > 90%, as determined by flow cytometry.

Isolation of primary keratinocytes

Primary keratinocytes were isolated from newborn B6 mice according to the protocol mentioned in the results. The isolated cells were re-suspended in Serum-Free Keratinocyte Medium (K-SFM, GIBCO, 17005) with human recombinant epidermal growth factor (0.1-0.2 ng/ml), bovine pituitary extract (20-30 mg/ml), mouse epidermal growth factor (10 ng/ml; BD, 354001), cholera toxin (1×10^{-10} M; Sigma, C9903), calcium chloride (0.05 mM) and penicillin and streptomycin solution (100 IU/ml, GIBCO, 15140122). The cells were counted and cultured under 5% CO_2 at 37°C in an incubator. Culture medium was replenished every 2-3 days.

Culture of keratinocytes

In some experiments, primary keratinocytes was cultured in the presence or absence of DETCs in a diabetes-like microenvironment (26

mmol/L D-glucose). Co-culture of DETCs and keratinocytes was performed in combination with 8×10^5 primary keratinocytes: 10^5 DETCs in RPMI 1640 Medium (GIBCO BRL, Gaithersburg, MD) supplemented with 5% fetal bovine serum (Hyclone, USA).

In vitro migration assays

The keratinocytes were seeded on 6-well plates at a density of 10^6 cells/well. After cells grew to confluent monolayers, "wounds" were created at the center of each well by scraping, and the culture debris from each well was removed by washing with PBS. The keratinocyte migration was observed in the presence or absence of DETCs in a diabetes-like microenvironment (26 mmol/L D-glucose). The ratio of keratinocytes to DETCs was 8:1.

Antibodies and flow cytometry

PerCP CY5.5-conjugated mAb specific for γ δ TCR (GL3 Tianjin Sungene Biotech Co. Ltd) and BV605-conjugated mAbs specific for CD3e (BD Biosciences, USA) were purchased. An apoptosis kit (Invitrogen) was purchased for the reliable detection of cell apoptosis. Flow cytometry data acquisition was performed on an Attune Acoustic Focusing Cytometer (Applied Biosystems, Life Technologies, CA, USA), and the data were analyzed using FlowJo software (Tree Star Incorporation, USA). Experiments were repeated at least three times using the same conditions and settings.

Western blot analysis

Proteins were extracted from cells or epidermal tissue of mice by lysis kits (KeyGEN BioTECH, CA) that contained 1% protease inhibitor cocktail, 5% phenymethylsulphonyl fluoride and 5% phosphatase inhibitor cocktail according to the manufacturer's protocol. The lysed cellular samples were scraped, collected and agitated for 20 minutes followed by centrifugation at 14,000 × g for 15 minutes at 4 °C. The supernatant was collected as total cellular proteins, and protein concentrations were determined by a BCA protein assay (Thermo Scientific, Rockford, USA). Equal protein (20 μg) from each sample was loaded onto 10% SDS-PAGE gels for electrophoresis. The separated proteins were transferred to a polyvinylidenedifluoride (PVDF) membrane (Millipore Immobilon, USA). The membrane was blocked with Tris-buffered saline (TBS) containing 3% bull serum albumin (BIOSHARP, CA) for 2 hours at room temperature and then incubated with primary rabbit antibodies to IGF-1, KGF (1:200, Santa Cruz Biotechnology, USA), a rabbit antibody to PCNA (1:1000, Abcam, UK), and a mouse antibody to GAPDH (1:5000, KANGCHEN BIO-TECH, CA) at 4°C overnight. The membranes were subsequently washed 5 times with TBS containing 0.1% Tween 20 and then incubated with HRP-labeled goat anti-rabbit/mouse secondary antibody (1:5000, ZSGB-BIO, CA) for 1 hour at room temperature. Finally, the membranes were washed 5 times with TBS containing 0.1% Tween 20 and visualized using enhanced chemiluminescence (Pierce, USA) according to the manufacturer's instructions. The bound antibodies were detected using the ChemiDoc™ XRS western blot detection system (Bio-Rad, USA).

Statistical analysis

Statistical comparisons were performed with Student's t-test. Data are presented as the mean ± standard deviation (SD). In all cases, a *P* value less than 0.05 was considered to be statistically significant.

Results

Reduced DETCs around the wound lead to weakened production of IGF-1 and KGF in the epidermis of diabetic mice

Accumulating evidence has revealed that IGF-1 and KGF play an essential role in regulating the proliferation, apoptosis and migration of keratinocytes to reestablish the skin barrier following wounding. Considering diabetes-induced wound healing deficits, we investigated the expression of IGF-1 and KGF in the epidermis around the wound sites of diabetic mice. Wild-type C57BL/6J mice were administered STZ or vehicle control daily for 6 days [26], and then received full-thickness wounds in their back skin [21]. The results indicate that the levels of IGF-1 and KGF in the epidermis around the wound were reduced in the diabetic mice compared with wild-type controls (**Figure 1A**). Because IGF-1 and KGF are produced exclusively by DETCs in the epidermal compartment, we investigated whether DETCs were involved in the reduction of IGF-1 and KGF levels in the

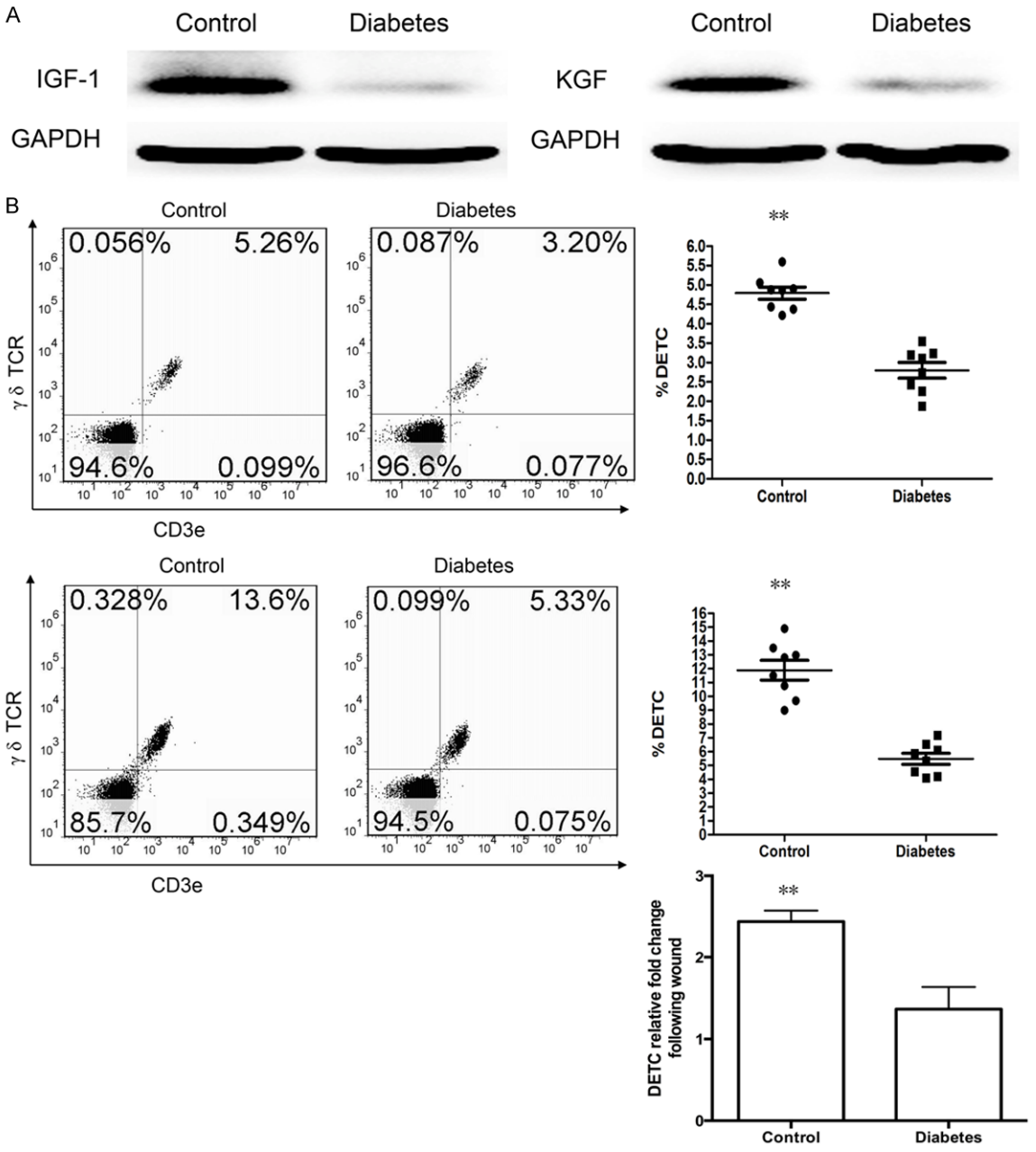

Figure 1. Reduced DETCs around a wound resulted in the weakened production of IGF-1 and KGF in the epidermis of diabetic mice. Wild-type C57BL/6J mice were administered daily i.p. injections of STZ or vehicle control for 6 days, and received full-thickness wounds in their back skin 4 weeks after STZ treatment. A. Reduced expression of IGF-1 and KGF in the epidermis around the wound of diabetic mice. On day 1 after wounding, the epidermis around wound of STZ-induced diabetic or control mice was obtained to detect the protein expression of IGF-1 and KGF by Western blot. B. Diabetic mice displayed significantly fewer DETCs in the intact epidermis and wounded epidermis compared with wild-type controls. DETCs numbers were increased upon wounding in wild-type controls compared with diabetic mice. The epidermis in the intact skin and around the wound at day 1 after wounding of STZ-induced diabetic or control mice were obtained to examine the number of DETCs by using FACS. *$p < 0.05$ and **$p < 0.005$ vs vehicle control (two-tailed, unpaired Student's t-test).

epidermis around the wound site of diabetic mice. Diabetic mice displayed fewer DETCs in the intact epidermis and wounded epidermis (**Figure 1B**) compared with wild-type controls. We also observed that the quantity of DETCs was increased upon wounding in wild-type con-

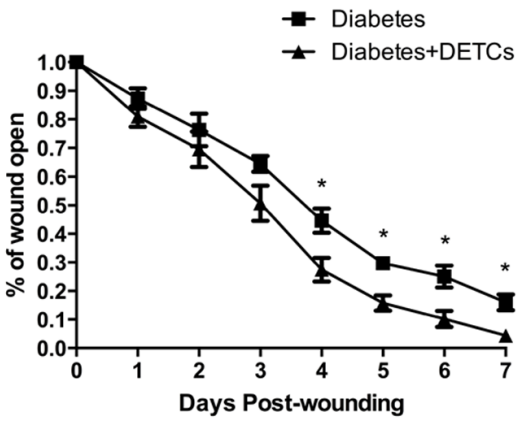

Figure 2. The application of DETCs improved wound repair in diabetic mice compared with vehicle controls. Wild-type C57BL/6J mice were administered daily injections of STZ or vehicle control for 6 days, and received full-thickness wounds in their back skin at 4 weeks after STZ treatment. The application of DETCs to the wounds promoted wound healing. Mice were injected intra-dermally with 10^6 DETCs or buffer control after wounding, and wound closure was measured over time. *$p < 0.05$ and **$p < 0.005$ vs vehicle control (two-tailed, unpaired Student's t-test).

trols; however, the quantity of DETCs was only slightly increased in diabetic mice (**Figure 1B**). Our data suggests that a reduced number of DETCs around the wound site results in markedly weakened IGF-1 and KGF levels in the epidermis around the wounds of diabetic mice.

Reduced DETCs in diabetic wounds are closely associated with delayed wound closure in diabetic mice

Because DETCs produce IGF-1 and KGF to promote wound healing and TCR $\delta^{-/-}$ mice exhibit impaired wound healing [27], we investigated whether reduced DETCs were involved in the delayed wound repair in diabetic mice. The application of DETCs improved wound repair in diabetic mice compared with controls (**Figure 2**). These data indicate that reduced numbers of DETCs in the wound margin contribute to diabetic delayed wound closure.

DETC application promotes the expression of IGF-1, KGF and PCNA in the epidermis around the wound site and inhibits the apoptosis of epidermal cells around the wound site in diabetic mice

We investigated the effects of the addition of DETCs on a diabetic wound. The results indicated that the application of DETCs enhanced the expression of IGF-1 and KGF in the epidermis around wounds of diabetic mice 4 days post-wounding compared with controls (**Figure 3A**). Proliferation activity during wound healing was analyzed by detecting PCNA (proliferating cell nuclear antigen) and we found that the level of PCNA in the epidermis around the wound site in diabetic mice was increased after the application of DETCs (**Figure 3A**). Furthermore, the apoptosis of epidermal cells around the wound in diabetic mice was evidently decreased by the addition of DETCs (**Figure 3B**). These results indicate that diabetic wound healing conditions are improved by the application of DETCs.

DETCs enhance migration and proliferation and reduce apoptosis in keratinocytes in diabetes-like microenvironments

Several studies have reported the effects of diabetes-like conditions on keratinocytes by investigating impaired proliferation and weakened cell locomotion [28-30]. Thus we investigated the effects of DETCs on the functions of keratinocytes in diabetes-like microenvironments. Keratinocytes were isolated from newborn C57 wild-type mice [31] and cultured in the presence or absence of DETCs. Our results indicated that the level of PCNA in keratinocytes under diabetes-like microenvironments was enhanced in the presence of DETCs (**Figure 4A**). Moreover, a prominent reduction in keratinocyte apoptosis was noted after 3 days of cultivation in diabetes-like conditions in the presence of DETCs (**Figure 4B**). Furthermore, an in vitro scratch wound assay was used to assess cell mobility in diabetes-like environments in the presence or absence of DETCs. A significant increase in keratinocyte motility was noted after 3 days of cultivation in diabetes-like microenvironments in the presence of DETCs (**Figure 4C**). These results indicated that DETCs reverse the negative effects of diabetes-like environments on keratinocytes.

Discussion

A cutaneous complication that is closely associated with diabetes is delayed wound closure [32]. Because DETCs are an important part of the resident cutaneous immunosurveillance program and play an important role in wound repair [33], we investigated whether these cells are associated with diabetic skin healing

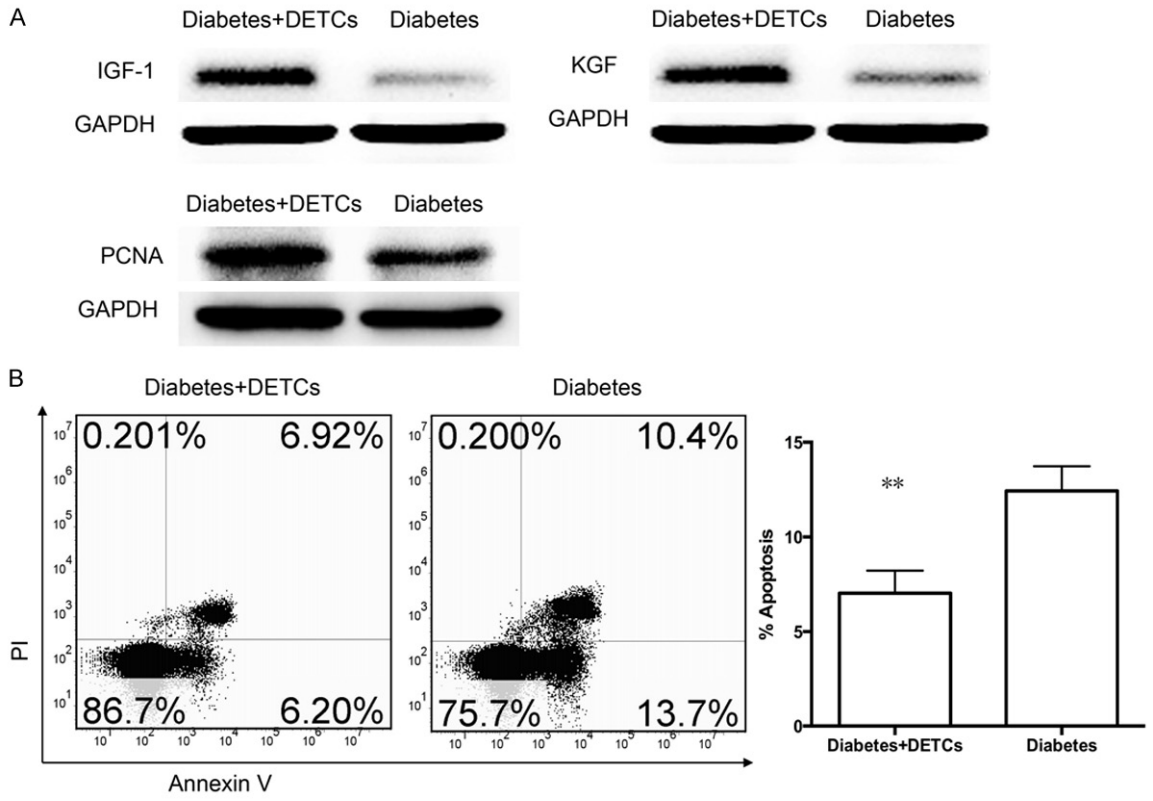

Figure 3. Diabetic wound healing conditions were improved by the application of DETCs. A. DETC application promoted the expression of IGF-1, KGF and PCNA in the epidermis around wound in diabetic mice. On day 4 after wounding, the epidermis around the wounds of STZ-induced diabetic mice was obtained to detect the expression of IGF-1, KGF and PCNA by Western blot. B. DETC application inhibited the apoptosis of epidermal cells around the wound in diabetic mice. Epidermal cells were stained with annexin V and propidium iodide (PI) to assess apoptosis and analyzed by flow cytometry. *$p < 0.05$ and **$p < 0.005$ vs vehicle control (two-tailed, unpaired Student's t-test).

defects. Strikingly, we observed that DETCs were reduced around wound sites in diabetic mice, which resulted in diminished levels of IGF-1 and KGF and delayed wound healing in the skin of diabetic mice. To our knowledge, this is the first description correlating DETCs with diabetic wound healing defects.

The mechanisms of wound healing are complicated and the secretion and concentration of local growth factors can affect the process of epidermal regeneration. IGF-1 is an important growth factor that is closely associated with wound healing. IGF-1 stimulates keratinocyte migration, proliferation and inhibits keratinocyte apoptosis to accelerate wound repair. Additionally, mIGF-1 transgenic mice have a hyperplastic epidermis and accelerated wound closure [34]. Keratinocyte growth factor (KGF) belongs to the FGF family and is also called as fibroblast growth factor-7 (FGF-7), which has been shown to be a potent stimulator of keratinocyte migration, proliferation and adhesion [35]. In the present study, we found reduced IGF-1 and KGF levels in the epidermis around the wound sites of diabetic mice.

The epidermal cells serve as a vital barrier to protect the body against environmental harms, and this function is mediated partly by resident DETCs [36]. DETCs are typically in a pre-activated state in the epidermis and are activated upon interactions with damaged keratinocytes [37, 38]. DETCs facilitate wound repair by expressing growth factors, including IGF-1 and KGF [17, 18]; TCR $\delta^{-/-}$ mice show delayed wound closure [39]. Considering that in the epidermal compartment, IGF-1 and KGF are exclusively produced by DETCs [18, 39], we investigated whether diabetes impacts DETCs around wounds. Our results indicate that DETCs are reduced both in the intact and wounded epider-

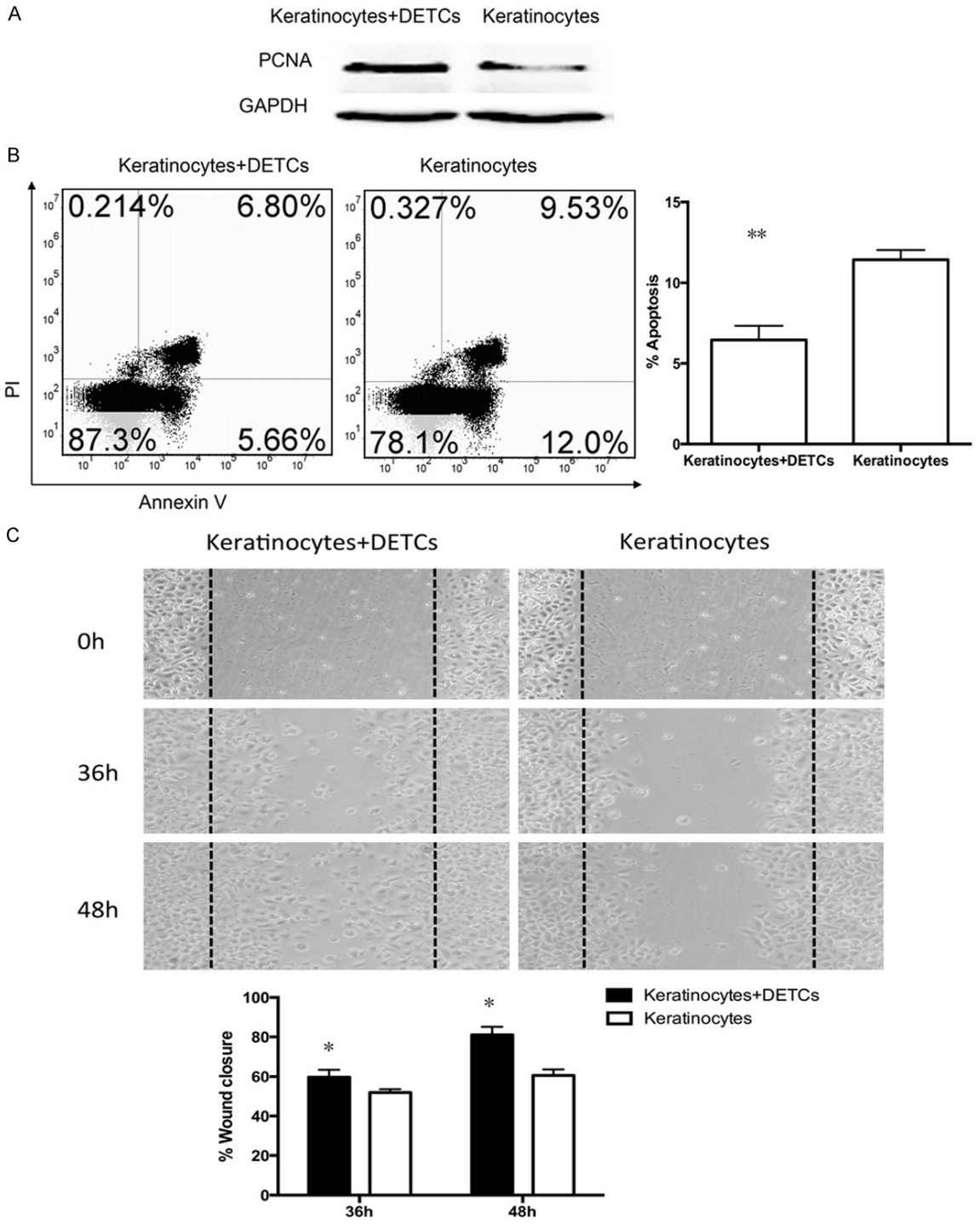

Figure 4. DETCs reversed the negative effects of diabetes-like environments on keratinocytes. Keratinocytes were isolated from newborn C57 wild-type mice and cultured in the presence and absence of DETCs. A. The proliferation of keratinocytes under diabetes-like microenvironments was enhanced in the presence of DETCs after 3 days of cultivation. The cells were obtained to detect the expression of PCNA by western blot. B. The apoptosis of keratinocytes in diabetes-like microenvironments was reduced in the presence of DETCs after 3 days of cultivation. Keratinocytes were stained with annexin V and propidium iodide (PI) to assess apoptosis and analyzed by flow cytometry. C. DETCs enhanced the migration of keratinocytes in diabetes-like microenvironments. An in vitro scratch wound assay was used to assess the cell mobility in diabetes-like environments in the presence or absence of DETCs. *$p < 0.05$ and **$p < 0.005$ vs vehicle control (two-tailed, unpaired Student's t-test).

mis. More importantly, in the wild-type controls DETCs were significantly increased upon wounding, but these cells were only slightly increased in diabetic mice. These results suggest that reduced proliferation and/or impaired recruitment of DETCs occurs following a wound in diabetic mice, which results in fewer DETCs around the wound site that can participate in wound healing. Furthermore, we observed that impaired wound healing could be improved by the application of DETCs to the wounds of diabetic mice. Taken together, these results suggest that a reduction in DETCs is closely associated with compromised wound repair in diabetic mice.

We also performed further investigations on the effect of DETCs on wounds in diabetic mice. The results indicated increased levels of IGF-1 and KGF in the epidermis of diabetic mice after the addition of DETCs. The proliferation of epidermal cells was also enhanced and the apoptosis of epidermal cells was significantly reduced in diabetic mice after DETC application. Therefore, these factors synergize to accelerate wound healing in diabetic mice after DETC application.

Disruption of glucose homeostasis is also associated with compromised wound repair in diabetic patients. Recent studies reveal that hyperglycemia is deleterious to the proliferation and migration of keratinocytes [28-30]. However, it is not known whether DETCs have a beneficial effect on keratinocytes in a diabetic microenvironment. Therefore, we explored the effects of DETCs on the functions of keratinocytes in diabetes-like microenvironment in vitro. Our results show that DETCs enhanced keratinocyte proliferation and locomotive capacity in diabetes-like conditions. There was also a reduction in the apoptosis of keratinocytes due to the presence of DETCs. Therefore, DETCs appear to accelerate wound healing by enhancing keratinocyte proliferation and migration and reducing keratinocyte apoptosis in a diabetic microenvironment.

Taken together, our results emphasize the importance of IGF-1 and KGF-producing DETCs in diabetic wound healing. We showed that DETCs are reduced in the epidermis around a wound in diabetic mice. Additionally, the application of DETCs to the wound can ameliorate poor wound-healing conditions and improve the migration and proliferation of keratinocytes and decrease their apoptosis in diabetes-like conditions. Our results suggest that reduced DETCs around a wound may contribute to the diabetes-induced deficits in wound closure. Furthermore, our observations point to a new strategy for treating wounds in diabetic patients.

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Disclosure of conflict of interest

The authors declare no competing financial interests. The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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