High-glucose environment enhanced oxidative stress and increased interleukin-8 secretion from keratinocytes: New insights on impaired diabetic wound healing

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Short title: High-glucose increased IL-8 in keratinocytes

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Abstract

Impaired wound healing frequently occurs in patients with diabetes. Interleukin-8 (IL-8) production by keratinocyte is responsible for recruiting neutrophils during healing. Intense inflammation is associated with diabetic wounds while reduction of neutrophil infiltration is associated with enhanced healing. We hypothesized that increased neutrophil recruitment by keratinocytes may contribute to the delayed healing of diabetic wound. Using cultured human keratinocytes and diabetic rat model, the current study showed that high-glucose environment enhanced IL-8 production via epidermal growth factor receptor (EGFR) -extracellular signal-regulated kinase (ERK) pathway in a reactive oxygen species (ROS)-dependent manner in keratinocytes. In addition, diabetic rat skin showed enhanced EGFR, ERK and IL-8 expression as compared to control rats. The dermal neutrophil infiltration of the wound, as represented by expression of myeloperoxidase level, was also significantly higher in diabetic rats. Treating diabetic rats with dapsone, an agent known to inhibit neutrophil function, was associated with improved healing. In conclusion, IL-8 production and neutrophil infiltration are increased in high-glucose environment due to elevated ROS level and contributed to impaired wound healing in diabetic skin. Targeting these dysfunctions may present novel therapeutic approaches.
**Key words:** high-glucose, interleukin-8, reactive oxygen species, keratinocytes, neutrophil, wound healing
Abbreviation:

AGE: advanced glycation endproduct

CXCL-1: chemokine (C-X-C motif) ligand 1

EGFR: epidermal growth factor receptor

ERK: extracellular signal-regulated kinases

IL: interleukin

MAPK: mitogen-activated protein kinase

MDA: malondialdehyde

MPO: myeloperoxidase

NFκB: nuclear factor kappa B

ROS: reactive oxygen species
Wound healing is a dynamic, interactive process involving coagulation, inflammation, tissue formation, and tissue remodeling (1-3). Impaired skin wound healing is a common cause of morbidity and mortality among patients with diabetes mellitus (DM) (4). Dysregulation of glucose homeostasis and elevated glucose levels are the central etiologies of DM (5). With the increasing prevalence of DM around the globe (6), understanding the mechanisms responsible for poor diabetic wound healing is an important public health issue.

Normal wound healing is a carefully orchestrated process in which proper induction of inflammatory cytokines leads to neutrophil recruitment that is essential for fending off potential infections to the disrupted barrier (7). In addition to their roles in controlling microbial invasion, neutrophils had been thought to play a limited role during the healing process of uncontaminated wound (8). However, persistence of inflammation and neutrophil infiltration are characteristics associated with impaired wound healing among diabetics (9-11). Treatments that enhance diabetic wound healing are often associated with reducing inflammatory cytokines in the diabetic wound environment (12,13). Intriguingly, study by Dovi et al. (14) demonstrated that re-epithelialization after wounding is accelerated by neutrophil depletion. These findings suggested that inflammation, in particular neutrophils, has
a direct impact on wound healing. An important yet still unanswered question during diabetic wound healing is how high-glucose environment affects chemotactic activity of keratinocytes to neutrophils. Keratinocytes, the immune competent cells forming the outer layer of the skin, are important providers for neutrophil chemotactic cytokines including chemokine (C-X-C motif) ligand 1 (CXCL-1) and interleukin-8 (IL-8), both of which are identified as the major chemotactic cytokines in human wound fluid (15). It has been shown that epidermal wound generates prominent chemotactic activity toward neutrophils in the injured skin and that IL-8 production derived from keratinocytes is critically involved this process (15,16). Besides recruiting neutrophils, IL-8 has also been recognized to activate neutrophil function including increasing superoxide and hydrogen peroxide formation (17,18). These evidences indicated that epidermal keratinocytes, through interleukin-8 secretion, serve as the mediator between skin wound and neutrophil recruitment/activation during the early phase of wound healing.

As aforementioned, common denominator frequently observed among poor diabetic healing wound is the presence of prolonged inflammation consisting of neutrophils and macrophages (9-11). Intriguingly, neutrophils derived from diabetic patients have been shown to display reduced migration in response to IL-8 chemotaxis (19). Therefore, we hypothesized that a strong chemotactic factor is present at the
diabetic wound site that allows for effective recruitment of the chemotactic-response defective neutrophils. Using cultured normal human keratinocytes and diabetic rat model, the current study was launched to explore the effect of high-glucose environment on epidermal keratinocytes in the context of neutrophil recruitment and propose novel therapeutic option for treating acute diabetic wounds.
Research Design and Methods

Keratinocyte culture and treatment

Keratinocytes were cultivated as described previously (20). The treatments of keratinocytes included 1) cultivation with 6mM D-glucose, 26mM D-glucose, or 20mM mannitol for 7 days; 2) cultivation with or without advanced glycation endproduction (AGE) -modified bovine serum albumin BSA (BioVision, Mountain View, CA, USA) for 2 days; 3) cultivation with 6mM or 26mM D-glucose for 7 days with addition of 50µM L-ascorbic acid (Sigma) on the 7th day 4) cultivation with 6mM D-glucose for 7 days, followed by EGFR siRNA transfection.

Real-time quantitative PCR detection for IL-8 mRNA

Total RNA was extracted using the Trizol method (Gibco BRL, Gaithersburg, MD, USA) and processed as recommended by the manufacturer. Five µg of RNA was reverse-transcribed to cDNA as the PCR template. The primer sequences used were listed in table 1. Amplification and detection were performed with an ABI Prism 7500 sequence detection system (Applied Biosystems, New Jersey). The fold of gene change was calculated as $2^{\Delta\Delta CT}$.

Measurement of IL-8 and CXCL-1 in cultured keratinocytes
The supernatants derived from keratinocytes cultivated at indicated conditions were collected and stored at -20°C. The concentrations of IL-8 and CXCL-1 were determined using commercially available ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s instructions. In blocking experiments, the keratinocytes were treated with 10 µM MEK1/2 inhibitor (U0126, Calbiochem) and incubated for 24 hrs.

**Western blotting analysis**

Total cellular proteins from cultured keratinocytes were extracted with RIPA buffer (0.1% SDS, 50 mM Tris-HCl, 150 mM NaCl, 0.5% sodium deoxycholate, and 1% NP-40, pH7.5) containing protease inhibitor cocktail (Roche, Mannheim, Germany). One hundred µg of proteins were loaded into 8% SDS-PAGE and transferred onto a PVDF membrane. After blocking and washing, the membranes were incubated with first antibodies against phosphorylated extracellular signal-regulated kinases (p-ERK), total ERK, (Cell Signaling, Beverly, MA, USA) and α-tubulin (Santa Cruz Biotechnology, Santa Cruz, USA). The membranes were then incubated with HRP-labeled secondary antibody (Millipore Corporation, Billerica, MA, USA) and developed with the ImmobilonTW Western Chemiluminescent HRP Substrate (Millipore Corporation, Billerica, MA). The blots were analyzed by digital imaging.
Flow cytometry analysis of membrane and intracellular p-epidermal growth factor receptor (EGFR)

For EGFR detection, the cultured keratinocytes were harvested, washed with PBS, and incubated with phosphorylated EGFR (p-EGFR) antibody (Cell Signaling) dissolved in 1% BSA/PBS solution at room temperature for 30 min. After washing, the cells were then incubated with Alexa Fluor 488 secondary antibody (Invitrogen, Carlsbad, CA, USA) for 30 min. Subsequently, the keratinocytes were washed again with PBS and fixed with 2% paraformaldehyde at 37°C for 10 min. After centrifugation, the cell pellets were resuspended in 90% methanol for permeabilization at 4°C for 15 min. Following centrifugation and discarding the supernatants, the cells were again incubated with p-EGFR and Alexa Fluor 488 secondary antibody. The expressions of p-EGFR were analyzed on a FACScan (Becton Dickinson, CA, USA) at FL1 channel with CELLQuest Pro Software.

Small Interfering RNA (siRNA) experiment

The treated keratinocytes were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Western blotting was performed to
confirm the success of transfection. The supernatants were collected and subjected to IL-8 ELISA assay as described previously.

**Lipid peroxidation (MDA) assay**

Malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) are natural products of lipid peroxidation. Measurement of MDA is recognized as an important indicator of cell oxidative damage. The commercially available MDA assay kit (BioVision, Mountain View, CA, USA) was used to detect the MDA level according to the manufacturer’s instructions. The fluorescent intensity of each sample was determined with a microplate reader FLx800 (Bio-TEK, Vermont, USA). The absorbance was recorded at 532 nm and 553 nm, and the results were analyzed with Kcjunior software.

**STZ-induced diabetes**

Male Wistar rats with initial weight of 200-300 g were acclimated for 1 week. Diabetic condition was induced by intravenous injection of STZ (streptozotocin) with 50 mg/kg dosage. After 24 h, the blood glucose levels were determined among rats that received injection. A glucose concentrations >250 mg/dl in heparinized tail vein blood (measured by Glucometer) was considered as successful induction. All treated
and control rats were maintained with ad libitum access to water and diet. For indicated experiments, the diabetic rats were fed with Dapsone (U Chiu Pharmaceutical Co., Taoyuan, Taiwan) 30 mg/kg by oral gavage twice a day two days before wounding and throughout the healing process.

**Wound healing model**

After 1 week of diabetes induction, the normal and diabetes rats were used for wound healing studies. Prior to injury, rats were anesthetized by intraperitoneal injection of a Zoletil 50 solution (50mg/kg body weight). After shaving the dorsal hair and cleansing the skin with 70% ethanol, full-thickness excisional wounds were created using 6-mm biopsy stamps (Stiefel, Offenbach, Germany). Each wound region was digitally photographed and wound sizes at indicated time points were recorded. In addition, 1 and 3 days after wounding, the perilesional rats skin was harvested with 8-mm punch for further analysis. The STZ-induced diabetic rat model used in this study reflects healing of acute wound under hyperglycemic conditions. Since the gist of the study is to investigate the effect of high-glucose environment on keratinocyte during the initial stage of acute wound healing in terms of neutrophil recruitment, this animal model fulfills our purpose.
Analysis of tissue myeloperoxidase (MPO)

The measurement of MPO levels in tissue has been shown to reflect neutrophil content. MPO content in wound tissue was determined as previously described by Dovi et al. (14). Briefly, the wounds were homogenized in 2 ml 20 mM phosphate buffer, pH 7.4. The homogenates were then centrifuged at 12,000 \( \times \) g for 45 min, and the supernatants were decanted. The pellets were resuspended in 1 ml 50 mM phosphate buffer containing 10 mM EDTA and 0.5% hexadecyltrimethylammonium bromide (HTAB). After a freeze-thaw cycle, the samples were briefly sonicated and incubated at 60°C for 2 hrs. The samples were centrifuged at 500 \( \times \) g for 10 min, and the supernatants were transferred to 1.5 ml tubes. For analysis, a standard curve ranging from 0 to 3.0 units/ml MPO (Sigma) was generated. Aliquots of samples (50 \( \mu \)L) or standards were placed in 12 \( \times \) 75 mm glass tubes with 500 \( \mu \)l assay buffer (0.1 M phosphate buffer, pH 5.4, 1% HTAB, 0.43 mg/ml 3,3’,5,5’-tetramethylbenzidine). The reactions began by the addition of 50 \( \mu \)l 15 mM \( \text{H}_2\text{O}_2 \), followed by incubation at 37°C for 15 min, and finished with addition of 1.0 ml cold 0.2 M sodium acetate, pH 3.0. The absorbance of each sample and standard were read at 655 nm within 10 min.

Immunohistochemical staining
Three-micron paraffin sections were deparaffinized in xylene and rehydrated in graded alcohol dilutions. Endogenous peroxidase activity was blocked by incubation with 3 % H₂O₂ for 5 min. Antigen retrieval was performed by pressure cooking for 10 min (121 °C, 1.2 kg/cm²) in 0.01M citrate buffer (pH 6.0). The slides were then incubated with mouse anti-p-EGFR and p-ERK antibodies (1: 400 dilution; Cell Signaling) at room temperature for 60 min. Antibody reactions were detected with biotinylated link anti-mouse antibody (Biocare Medical, California, USA) for 20 min at room temperature followed by incubation with Trekavidin-HRP (Biocare Medical) for 20 min. The color was developed using DAB substrate-chromogen solution (Biocare Medical). The slides were then counterstained with hematoxylin.

Real-time quantitative Polymerase Chain Reaction analysis of rat skin IL-8 mRNA

The methods were described previously (21), and the primer sequence used were listed in table 1.

Statistical analysis

SPSS system for Windows version 12.0 (SPSS Inc., Chicago, IL) was used for statistical analysis. For each experiment, at least three independent experiments were performed. The results are expressed as mean ± standard deviation (SD).
Student’s t test was used for statistical evaluation between control and experimental groups in the study. In addition, one-way Anova followed by post-hoc LSD test was used for comparison of wound healing between different animal groups. A p-value of < 0.05 is considered to be statistically significant.
Results

High-glucose environment increased IL-8 expression at both mRNA and protein levels in cultured keratinocytes. Inhibition tests suggested ERK signaling is critically involved in the high-glucose induced upregulation process.

The IL-8 mRNA expression of keratinocytes cultivated at high-glucose environment for 7 days was approximately 1.5 fold as compared to their normal glucose counterpart. Similarly, the IL-8 concentration in the supernatants derived from the high-glucose keratinocyte cultures was approximately 50% higher than their normal glucose counterpart. Osmolarity control with 20mM mannitol showed no significant effect on IL-8 production. (Fig. 1A,B) The levels of CXCL-1 in the culture supernatants were not significantly different between the normo- and high-glucose cultivated keratinocytes. (Fig. 1C) It has been reported that nuclear factor kappa B (NFκB), p38 mitogen-activated protein kinase (p38MAPK), and ERK are involved in IL-8 secretion of keratinocytes (22,23). Since we had previously demonstrated that upregulation of NFκB and p38MAPK was not observed in high-glucose cultivated keratinocytes (21), we focused on ERK signaling. As demonstrated in Fig. 1D, addition of U0126 induced significant IL-8 reduction. Therefore, we hypothesized that the activation of ERK signaling is critically involved in elevating IL-8 level of high-glucose cultivated keratinocytes. We next determined the expression of ERK in
keratinocytes cultivated under different glucose conditions. As demonstrated in Fig. 1E and 1F, the p-ERK expression was significantly increased in high-glucose treated keratinocytes while the expression of total ERK was not significantly altered. This result validated the notion that high-glucose environment increased IL-8 production in keratinocytes via an ERK-related pathway.

**High-glucose cultivation increased the expression of p-EGFR in cultured keratinocytes.** Gene silencing of EGFR significantly reduced p-ERK expression and decreased IL-8 production in cultured keratinocytes.

Epidermal injuries were associated with EGFR activation, an event that is associated with enhanced ERK signaling. We next determine the expression of p-EGFR under different glucose cultivation. As demonstrated in Fig. 2A, high-glucose treatment significantly increased the expression of p-EGFR by approximately 30%. To determine the functionality of increased p-EGFR expression, a gene-silencing approach was employed. Accordingly, EGFR gene-silenced keratinocytes showed significantly reduced p-ERK expression (Fig. 2B,C) and approximately 30% and 70% reduction in IL-8 protein and mRNA expression, respectively (Fig. 2D,E). These results indicated that high-glucose environment enhanced the expression of EGFR, an event that contributed to increased p-ERK and IL-8 expression in cultured...
Advanced glycation endproduct (AGE) has limited effect on IL-8 expression and p-ERK expression in cultured keratinocytes.

High-glucose treatment is associated with AGE formation, an event that may modify physiologic functions of keratinocytes. As demonstrated in Fig. 3, exogenous AGE treatment showed no significant effect on p-ERK and IL-8 expression of cultured keratinocytes. These results indicated that AGE plays limited role in excessive IL-8 level observed in our experimental conditions.

Lipid peroxidation product was increased in high-glucose cultivated keratinocytes. Ascorbic acid treatment reduced p-EGFR and p-ERK expressions as well as normalized IL-8 secretion in high-glucose cultivated keratinocytes.

Increased oxidative stress has been associated with high-glucose environment. In support with this notion, the level of malondialdehyde (MDA), a surrogate marker for estimation of damage induced by reactive oxygen species (ROS), was significantly increased in high-glucose cultivated keratinocytes by about 3 fold as compared to control (Fig. 4A). Treating high-glucose cultivated keratinocytes with ascorbic acid
normalized the expression of p-EGFR and p-ERK (Fig. 4B-D). In addition, the IL-8 concentration in culture supernatants derived from high-glucose cultivated keratinocytes was 146.14 ± 25.21% of the control without ascorbic acid treatment while after exogenous ascorbic acid treatment, the supernatants from high-glucose cultures contained 107.84 ± 18.37% of IL-8 as compared to control. No significant difference in IL-8 concentration was found between normal- and high-glucose cultivated keratinocytes after addition of ascorbic acid into the culture media. These results indicated that increased oxidative stress is associated with enhanced IL-8 production in high-glucose treated keratinocytes. The perilesional skin of the diabetic rats demonstrated significantly higher IL-8 expression as compared to control rats after wounding. In addition, the level of neutrophil infiltration, as reflected by levels of myeloperoxidase (MPO), was significantly higher in diabetic wounds as compared to control.

In order to validate our in vitro results in vivo, a diabetic rat model was used. Accordingly, diabetic rats showed impaired wound healing. The days required to achieve complete wound healing were 9.0 ± 0.4 and 11.5 ± 0.8 days, respectively, for control and diabetic rats (n=12). Representative figures of healing process were shown in Fig. 5A, and the planimetry analysis was shown in Fig. 5B. The rates of healing for control and diabetic rats were 12.8% and 6.9 % healing/day, respectively.
from day 0 to day 9. It should be noted that in our wound model, a fibrin blot (crust) invariably formed after wounding. For the control rats, most crust resolved after day 3 post-wounding and wound closure proceeded smoothly afterward. For the diabetic rats, most crusts did not resolve until after 5 days. Therefore, the delayed resolution of crust may be considered as part of impaired wound healing in our experiment.

In terms of IL-8 expression, wounding increased IL-8 expression in both control and diabetic rat skin. The IL-8 increase in diabetic rat skin was significantly higher as compared to control skin. More specifically, the ratio of IL-8 increase in diabetic perilesional skin was 1.75 ± 0.08 and 1.32 ± 0.02 fold of control perilesional rat skin 1 and 3 days after wounding, respectively. Immunohistologically, the epidermis of diabetic rats showed more intense staining of p-EGFR and p-ERK as compared to control (Fig 5C). The dermal neutrophil infiltration, as reflected by MPO level, was significantly higher in diabetic rats as compared to control rats (Fig. 5D).

**Systemic dapsone treatment reduced MPO level of the wound and improved healing of diabetic rat skin.**

Since we hypothesized that increased neutrophil infiltration contributes to impaired healing in diabetic rats, systemic dapsone, an agent recognized to impair neutrophil function, was administered to diabetic rats. The time required for diabetic rats to
achieve complete wound closure was significantly shortened after systemic dapsone treatment. More specifically, the time required for complete wound closure in diabetic rats reduced from 11.5 ± 0.8 days without dapsone treatment to 8.9 ± 0.5 days after dapsone treatment (fig. 5A), and the rate of healing from day 0 to day 9 improved from 6.9% to 11.6% healing/day. The fibrin clot also resolved at earlier time (3 days) post wounding. In addition, the level of dermal MPO in control and diabetic rats with dapsone treatment 1 day after wounding were 5.55 ± 0.17 and 6.09 ± 0.13 fold, respectively, as compared to respective dermis before wounding. No difference was found between these two groups. Therefore, systemic dapsone treatment shortened the duration required for wound healing and reduced the level of MPO during wound healing in diabetic rats.
Discussion

Although neutrophils derived from diabetic patients demonstrated defective chemotactic-response to IL-8, the diabetic wounds were characterized by excessive and prolonged neutrophil infiltration. This intriguing phenomenon lead us to hypothesize that high-glucose environment may elicit keratinocytes to increase secretion of IL-8, an event which in turn results in excessive neutrophil recruitment.

Previously, it was reported that the injury-induced immune responses were mediated by activation of EGFR. Intriguingly, IL-8 production from keratinocytes in response to skin injury also occurred in an EGFR-dependent manner (16). In this study, we demonstrated that high-glucose environment renders keratinocytes prone to produce higher levels of IL-8 due to enhanced EGFR-ERK signaling. These results corroborated with previous study showing that EGFR-ERK cascade regulates the production of IL-8 in lung cancer cells (24). It should be noted that our experimental condition did not significantly alter the physiologic status (viability, growth, and differentiation) of culture keratinocytes as demonstrated in our previous study (21).

Different factors may initiate the process resulting in elevated IL-8 levels and enhanced neutrophil recruitment to diabetic wound. One potential event responsible for increased levels of IL-8 involves formation of AGE. More specifically, AGE has been shown to stimulate pro-inflammatory chemokine production in endothelial cells
via activation of both MAPK and NFκB pathways (25). In our experimental conditions, however, AGE did not appear to play a significant role as neither IL-8 nor p-ERK levels were significantly altered by AGE treatment. Therefore, pathway other than AGE formation was evaluated. Previous studies demonstrated that high blood glucose induces oxidative stress that leads to generation of ROS, an event that participates in the development of diabetic complications and propagate excessive inflammatory cascade (26). It has been shown that ROS signaling may be involved in IL-8 production in different cells (27) and that increased ROS levels may activate EGFR cascade (28,29). In accordance with these reports, our results demonstrated that keratinocytes cultivated at high-glucose environment showed increased level of MDA, an end-product of lipid peroxidation resulting from excess ROS. Moreover, IL-8 production and EGFR-ERK expression of high-glucose cultivated keratinocytes were significantly reduced by ascorbic acid, a major water-soluble antioxidant known to counteract the effects of ROS on human skin (30). Therefore, increased oxidative stress may be the initiating event responsible for increased IL-8 production in keratinocytes cultivated under high-glucose environment.

In order to examine our hypothesis in vivo, a diabetic rat model for acute wound healing during hyperglycemic condition was employed. As expected, the skin wounds of diabetic rats required longer time to heal as compared to control.
Moreover, although skin wounding increased IL-8 expression of the perilesional skin from both diabetic and control rats, the increase in diabetic rats was significantly higher. Since neutrophil derived from diabetic patients showed reduced migration in response to IL-8 chemotaxis (19), the enhanced IL-8 expression from diabetic rat skin provided a reasonable explanation for increased neutrophil infiltration to the diabetic wounds. In addition, our animal diabetic model also showed increased p-EGFR and p-ERK expression in the epidermis and elevated MPO expression in the dermis as compared to control. These results indicated that the EGFR-ERK activation and neutrophil infiltration are indeed more intense in the epidermis and dermis, respectively, of the diabetic rat wounds as compared to control. The results from our in-vitro experiments suggested that antioxidants may reduce IL-8 expression from high-glucose cultivated keratinocytes and therefore improve diabetic wound healing via normalization of over-zealous inflammatory reactions. In support with this hypothesis, recent report has shown that supplementation of dietary antioxidants selectively regulates the inflammatory responses and promotes wound healing in diabetic mice (31).

The mechanism regarding neutrophil-mediated delayed healing remains elusive. In previous study using neutrophil-depleted mice, both wild type and diabetic mice showed accelerated wound closure (14). In the same study, it was shown that
neutrophils retard wound closure by impeding re-epithelialization but not the overall dermal repair. Therefore, it was proposed that excessive protease secretion by neutrophils may inhibit keratinocyte migration and proliferation via inducing keratinocyte detachment. In our experiment, crust of the diabetic rat showed delayed resolution as compared to control. It is known that neutrophils are capable of secreting various cytokines including tumor necrosis factor-alpha and vascular endothelial growth factor that are known to induce vascular permeability and promote fibrin deposition (32-34). This may provide a reasonable explanation for the delayed resolution of fibrin clot observed in our diabetic rats. To explore the functional role of increased neutrophil infiltration on diabetic wound healing in our study, dapsone was given to the diabetic rat during the wound healing process. Dapsone is an antimicrobial agent with anti-inflammatory properties and is known to inhibit the function of neutrophils (35). Accordingly, our results demonstrated that systemic dapsone treatment improved wound healing (clots were resolved 3 days after wounding) and reduced the neutrophil infiltration to the wound site of the diabetic rat skin. These results further supported the notion that excessive neutrophil infiltration contributed to the impaired wound healing in diabetic animals. Corroborating with previous studies on promoting diabetic wound repair (12,13), reduction of inflammatory cytokines in the diabetic wound environment may be an important
approach to improve healing in diabetic patients.

In summary, excessive neutrophil infiltration contributed to the impaired healing process in acute wounds associated with hyperglycemic environment. In addition, oxidative stress created by the high-glucose environment contributed to elevated p-EGFR expression that subsequently resulted in enhanced ERK signaling and increased IL-8 production in epidermal keratinocytes. As impaired wound healing in diabetic patients is still an important clinical condition that frequently imposes therapeutic challenges to physicians and poses serious complications for patients, potential therapies targeting oxidative stress-dependent EGFR-ERK signaling-induced IL-8 secretion in keratinocytes may be a potential therapeutic strategy for ameliorating delayed healing of acute diabetic wound,
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Contributors

"C.L wrote the manuscript and researched data. C.W. researched data and contributed to discussion. S.H. researched data and contributed to discussion. I.W. researched data and contributed to discussion. G.C. contributed to discussion and reviewed/edited the manuscript."
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Legends for figures

Fig. 1  **High-glucose environment increased the expression of interleukin-8 (IL-8) in cultured human keratinocytes.** Human keratinocytes showed increased expressions of IL-8 mRNA (A) and protein (B) after 7 days of high-glucose cultivation. The levels of CXCL-1 (C) were not significantly altered by high-glucose cultivation. (D) Inhibition tests indicated that U0126 significantly reduced the IL-8 production from cultured keratinocytes. (E) Representative blot showing increased phosphorylated-extracellular signal-regulated kinases (p-ERK) expression in high-glucose cultivated keratinocytes. (F) Densitometric analyses of (E) with n=3.

Fig. 2  **High-glucose cultivation increased the expression of p-epidermal growth factor receptor (EGFR), the molecule that regulated p-ERK expression and IL-8 production in cultured keratinocytes.** (A) Human keratinocytes demonstrated increased expressions of p-EGFR after 7 days of high-glucose treatment. (B) Representative blot showing gene-silencing of EGFR reduced the p-ERK expression in cultured keratinocytes. (C) Densitometric analyses of (B) with n=3. Gene-silencing of EGFR significantly reduced the IL-8 production from cultured keratinocytes at (D) mRNA and (E) protein levels. NS: Non-Silencing
Fig. 3  Advanced glycation endproduct (AGE) has limited effect on p-ERK and IL-8 expression in cultured keratinocytes.  (A) Treating cultured human keratinocytes with AGE modified bovine serum albumin (BSA) did not significantly affect the p-ERK expression as demonstrated by western blotting analyses. (B) Densitometry analyses of (A), n=3.  (C) IL-8 production from cultured keratinocytes was not significantly altered by AGE treatment.

Fig. 4  Lipid peroxidation product malondialdehyde (MDA) is increased in high-glucose cultivated keratinocytes, and ascorbic acid normalized the expressions of p-EGFR and p-ERK in high-glucose cultivated keratinocytes.  (A) Human keratinocytes demonstrated increased expressions of MDA after 7 days of high-glucose treatment.  Addition of ascorbic normalized the expression of (B) p-EGFR and (C) p-ERK.  (D) Densitometric analyses of (C) with n=3.

Fig. 5  Diabetic rats showed impaired wound healing and increased dermal myeloperoxidase (MPO) expression as compared to control.  (A) At 9 days after wounding, control rats showed healing with complete re-epithelialization.  On the
other hand, diabetic rats showed incomplete wound healing. Diabetic rats receiving
dapsone treatment also showed adequate healing 9 days after wounding. (B) Wound
closure in different groups as expressed by percent of remaining wound area
compared to initial wound. *indicated p<0.05 as compared to control and # indicated
p<0.05 as compared to diabetic rat treated with Dapsone. (C) The expression p-EGFR
and p-ERK in control and diabetic rat skin. Left column: original magnification
400x; Right column: original magnification 1000x. (D) The increase in dermal MPO
eexpression of diabetic rats was significantly higher than control rats at 1 and 3 days
after wounding.
| Gene          | Sequence (Primer)                                                                 |
|--------------|----------------------------------------------------------------------------------|
| IL-8         | 5’-CGTGGCTCTCTTTGGCAGCCTTCTTGATGAT-3’ (forward)                                  |
|              | 5’-TCAAAACTTCTCCAAACCACTTCTGCA-3’ (reverse)                                      |
| GAPDH        | 5’-CCACCCATGGCAATATCC-3’ (forward)                                               |
|              | 5’-GGGATTTCATTGATGACA-3’ (reverse)                                               |
| EGFR siRNA   | A: 5’-UUAACUUUCUCACCUCUUUCUGGGAUCC-3’                                           |
|              | 5’-GGAUCC CAGAAGGUGAGAAAGUUAA-3’                                                |
|              | B: 5’-AAAUCUGUGAUCUUGACAUUC GCG-3’                                               |
|              | 5’-CGCAGCAUGUCAAGAUCACAGAUUU-3’                                                 |
| β-actin (rat)| 5’-TCTGTGTGGATTGTTGGCTTCT-3’ (forward)                                          |
|              | 5’-GACTCATCGTACTCTGCTTGCTTGTGCT-3’ (reverse)                                     |
| IL-8 (rat)   | 5’-TCAACGGGCAGAATCAAAAGAG-3’ (forward)                                          |
|              | 5’-CTCAGACAGCGAGGCGACATC-3’ (reverse)                                           |
Figure 1A

[Bar chart showing IL-8 mRNA expression (% 6mM glucose) for 6mM glucose, 26mM glucose, and 20mM mannitol conditions.]

* Denotes significant difference.
Figure 1B

![Bar chart showing IL-8 concentration (pg/ml) for different glucose and mannitol concentrations.

- 6mM glucose
- 26mM glucose
- 20mM mannitol

The chart indicates a significant increase in IL-8 concentration at 26mM glucose compared to 6mM glucose and 20mM mannitol.]
Figure 1C

![Bar graph showing CXCL1 concentration (pg/ml) for 6mM and 26mM glucose conditions. The graph displays higher CXCL1 concentration in 26mM glucose compared to 6mM glucose.](image-url)
Figure 1D

- IL-8 concentration (pg/ml)

- 6mM glucose
- U0126

* Indicates statistical significance.
Figure 1E

|                | 6mM glucose | 26mM glucose |
|----------------|-------------|--------------|
| p-ERK          |             |              |
| Total-ERK      |             |              |
| α-tubulin      |             |              |
Figure 1F

[Bar chart showing p-ERK protein expression (% 6mM glucose) for 6mM and 26mM glucose. The chart indicates a significant increase in p-ERK protein expression at 26mM glucose compared to 6mM glucose, marked with an asterisk (*).]
Figure 2A

- **p-EGFR positive cells (% 6mM glucose)**

- **Graph:**
  - x-axis: Glucose concentration (6mM vs. 26mM)
  - y-axis: Percentage of p-EGFR positive cells
  - Comparison between 6mM glucose and 26mM glucose
  - Significance indicated by asterisk (*)
Figure 2B

| Protein          | NS siRNA | EGFR siRNA |
|------------------|----------|------------|
| EGFR             |          |            |
| p-ERK            |          |            |
| α-tubulin        |          |            |
Figure 2C

![Bar chart showing protein expression (% NS siRNA) for EGFR, pERK.](chart)

- NS siRNA
- EGFR siRNA

* Significant difference.
Figure 2E

IL-8 mRNA expression (% NS siRNA)

NS siRNA  EGFR siRNA

*
|        | BSA | AGE |
|--------|-----|-----|
| p-ERK  |     |     |
| α-tubulin |    |     |
Figure 3C

IL6 concentration (pg/ml)

BSA

AGE
Figure 4A

![Bar graph showing MDA concentration (nmol/mg protein) for 6mM glucose and 26mM glucose. The graph indicates a significant difference (*).]
Figure 4B

![Bar graph showing p-EGFR positive cells (% 6mM glucose + ascorbic acid) for 6mM glucose + ascorbic acid and 26mM glucose + ascorbic acid.](image_url)
Figure 4C

| Glucose | 6mM | 26mM | ascorbic acid |
|---------|-----|------|---------------|
| p-ERK   |     |      |               |
| Total-ERK |    |      |               |
| tubulin |     |      |               |
Figure 4D

The bar chart illustrates the p-ERK protein expression (% 6mM glucose + ascorbic acid) for different glucose concentrations. The chart shows a comparison between 6mM glucose + ascorbic acid and 26mM glucose + ascorbic acid.
Figure 5A

Control rat

Diabetic rat

Diabetic rat with Dapsone treatment
Figure 5B

TIME AFTER WOUNDING (Days)

AREA OF WOUND (% of initial Area)

- control rat
- diabetic rat
- diabetic rat + Dapsone

* *, #
Figure 5C
Figure 5D

![Bar graph showing MPO (fold of day 0) for control and diabetic rats on day 1 and day 3.](image-url)

- **Control rat**
- **Diabetic rat**

- * indicates a statistically significant difference between the two groups.

MPO (fold of day 0) vs. Day:
- **Day 1**
- **Day 3**