ORIGINAL ARTICLE

High-Glucose Environment Enhanced Oxidative Stress and Increased Interleukin-8 Secretion From Keratinocytes

New Insights Into Impaired Diabetic Wound Healing

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Impaired wound healing frequently occurs in patients with diabetes. Interleukin (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 wounds. Using cultured human keratinocytes and a diabetic rat model, the current study shows that a 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 compared with 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 a 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.
***Diabetes* 62:2530–2538, 2013**

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 (4). Dysregulation of glucose homeostasis and elevated glucose levels are the central etiologies of diabetes (5). With the increasing prevalence of diabetes 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 patients with diabetes (9–11). Treatments that enhance diabetic wound healing are often associated with reducing inflammatory cytokines in the diabetic wound environment (12,13). Intriguingly, a study by Dovi, He, and DiPietro (14) demonstrated that reepithelialization 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 a 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 of neutrophil chemotactic cytokines including chemokine (C-X-C motif) ligand 1 (CXCL-1) and interleukin (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 in 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 IL-8 secretion, serve as the mediator between skin wound and neutrophil recruitment/activation during the early phase of wound healing.

As aforementioned, a common denominator frequently observed among poor diabetic healing wounds 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. With use of cultured normal human keratinocytes and a diabetic rat model, the current study was launched to explore the effect of a high-glucose environment on epidermal keratinocytes in the context of neutrophil recruitment and propose a novel therapeutic option for treating acute diabetic wounds.

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Received 11 December 2012 and accepted 14 February 2013.
DOI: 10.2337/db12-1714
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RESEARCH DESIGN AND METHODS

Keratinocyte culture and treatment. Keratinocytes were cultivated as previously described (20). The treatments of keratinocytes included *1*) cultivation with 6 mmol/L D-glucose, 26 mmol/L D-glucose, or 20 mmol/L mannitol for 7 days; *2*) cultivation with or without advanced glycation end production (AGE)-modified BSA (BioVision, Mountain View, CA) for 2 days; *3*) cultivation with 6 or 26 mmol/L D-glucose for 7 days with addition of 50 μmol/L L-ascorbic acid (Sigma) on the seventh day; and *4*) cultivation with 6 mmol/L D-glucose for 7 days, followed by epidermal growth factor receptor (EGFR) small interfering RNA (siRNA) transfection.

Real-time quantitative PCR detection for IL-8 mRNA. Total RNA was extracted using the TRIzol method (Gibco BRL, Gaithersburg, MD) and processed as recommended by the manufacturer. Five micrograms of RNA were reverse transcribed to cDNA as the PCR template. The primer sequences used are listed in Table 1. Amplification and detection were performed with an ABI Prism 7500 sequence detection system (Applied Biosystems, Hammonton, NJ). The fold of gene change was calculated as 2^-ΔΔCT.

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 a commercially available ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. In blocking experiments, the keratinocytes were treated with 10 μmol/L mitogen-activated protein kinase (MAPK) kinase (MEK)1/2 inhibitor (U0126; Calbiochem) and incubated for 24 h.

Western blotting analysis. Total cellular proteins from cultured keratinocytes were extracted with radioimmunoprecipitation assay buffer (0.1% SDS, 50 mmol/L Tris-HCl, 150 mmol/L NaCl, 0.5% sodium deoxycholate, and 1% NP-40, pH 7.5) containing protease inhibitor cocktail (Roche, Mannheim, Germany). One hundred micrograms of proteins were loaded into 8% SDS-PAGE and transferred onto a polyvinylidene fluoride membrane. After blocking and washing, the membranes were incubated with first antibodies against phosphorylated (p-) extracellular signal–regulated kinases (ERKs), total ERK, (Cell Signaling, Beverly, MA), and α-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA). The membranes were then incubated with horseradish peroxidase (HRP)-labeled secondary antibody (Millipore, Billerica, MA) and developed with the ImmobilonTW Western Chemiluminescent HRP Substrate (Millipore). The blots were analyzed by a digital imaging system (Alpha Imager 2000; Alpha Innotech, San Leandro, CA).

Flow cytometry analysis of membrane and intracellular p-EGFR. For EGFR detection, the cultured keratinocytes were harvested, washed with PBS, and incubated with 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) 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. After centrifugation and discarding of 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, Mountain View, CA) at FL1 channel with CELLQuest Pro Software.

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 previously described.

TABLE 1
Primer sequence of genes investigated in the study

Gene	Sequence (primer)
IL-8	5′-CGTGGCTCTCTTGGCAGCCTTCCTGAT-3′ (forward)
	5′-TCAAAAACTTCTCCACAACCCTCTGCA-3′ (reverse)
GAPDH	5′-CCACCCATGGCAAATTCC-3′ (forward)
	5′-GGGATTTCCATTGATGACA-3′ (reverse)
EGFR siRNA	A, 5′-UUAACUUUCUCACCUUCUGGGAUCC-3′
	5′-GGAUCC CAGAAGGUGAGAAAGUUAA-3′
	B, 5′-AAAUCUGUGAUCUUGACAUGCUGCG-3′
	5′-CGCAGCAUGUCAAGAUCACAGAUUU-3′
β-actin (rat)	5′-tctgtgtggattggtggctct-3′ (forward)
	5′-gactcatcgtactcctgcttgct-3′ (reverse)
IL-8 (rat)	5′-tcaacgggcagaatcaaagag-3′ (forward)
	5′-ctcagacagcgaggcacatc-3′ (reverse)

Lipid peroxidation (malondialdehyde) assay. Malondialdehyde (MDA) and 4-hydroxynonenal 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) 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, Winooksi, VT). The absorbance was recorded at 532 and 553 nm for excitation and emission, respectively, and the results were analyzed with Kcjunior software.

Streptozotocin-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 streptozotocin with 50 mg/kg dosage. After 24 h, the blood glucose levels were determined among rats that received injection. Glucose concentration >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 30 mg/kg dapsone (U Chiu Pharmaceutical, Taoyuan, Taiwan) by oral gavage twice a day 2 days before wounding and throughout the healing process.

Wound-healing model. After 1 week of diabetes induction, the normal and diabetic rats were used for wound-healing studies. Prior to injury, rats were anesthetized by injection of a Zoletil 50 solution (50 mg/kg body wt i.p.). After shaving of the dorsal hair and cleansing of 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 rat skin was harvested with 8-mm punch for further analysis. The streptozotocin-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 a 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. The measurement of myeloperoxidase (MPO) levels in tissue has been shown to reflect neutrophil content. MPO content in wound tissue was determined as previously described by Dovi, He, and DiPietro (14). Briefly, the wounds were homogenized in 2 mL 20 mmol/L phosphate buffer, pH 7.4. The homogenates were then centrifuged at 12,000*g* for 45 min, and the supernatants were decanted. The pellets were resuspended in 1 mL 50 mmol/L phosphate buffer containing 10 mmol/L EDTA and 0.5% hexadecyltrimethylammonium bromide. After a freeze-thaw cycle, the samples were briefly sonicated and incubated at 60°C for 2 h. The samples were centrifuged at 500*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 μL) or standards were placed in 12 × 75 mm glass tubes with 500 μL assay buffer (0.1 mol/L phosphate buffer, pH 5.4; 1% hexadecyltrimethylammonium bromide; and 0.43 mg/mL 3,3′,5,5′-tetramethylbenzidine). The reactions began by the addition of 50 μL 15 mmol/L H_2O_2, followed by incubation at 37°C for 15 min and finished with addition of 1.0 mL cold 0.2 mol/L 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% H_2O_2 for 5 min. Antigen retrieval was performed by pressure cooking for 10 min (121°C, 1.2 kg/cm^2) in 0.01 mol/L 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, Concord, CA) 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-eosin.

Real-time quantitative PCR analysis of rat skin IL-8 mRNA. The methods have previously been described (21). The primer sequence used is listed in Table 1.

Statistical analysis. SPSS system for Windows, version 12.0 (SPSS, Chicago, IL), was used for statistical analysis. For each experiment, at least three independent experiments were performed. The results are expressed as means ± SD. Student *t* test was used for statistical evaluation between control and experimental groups in the study. In addition, one-way ANOVA followed by post hoc Fisher's least-significant-difference test was used for comparison of wound healing between different animal groups. A P value <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, and inhibition tests suggested that ERK

signaling is critically involved in the high glucose–induced upregulation process. The IL-8 mRNA expression of keratinocytes cultivated in a high-glucose environment for 7 days was approximately 1.5-fold compared with their normal-glucose counterpart. Similarly, the IL-8 concentration in the supernatants derived from the high-glucose keratinocyte cultures was ~50% higher than their normal glucose counterpart. Osmolarity control with 20 mmol/L mannitol showed no significant effect on IL-8 production. (Fig. 1*A* and *B*). The levels of CXCL-1 in the culture supernatants were not significantly different between the normo-glucose– and high glucose–cultivated keratinocytes. (Fig. 1*C*) It has been reported that nuclear factor-κB (NF-κB), p38 MAPK, and ERK are involved in IL-8 secretion of keratinocytes (22,23). Since we had previously demonstrated that upregulation of NF-κB and p38 MAPK was not observed in high glucose–cultivated keratinocytes (21), we focused on ERK signaling. As demonstrated in

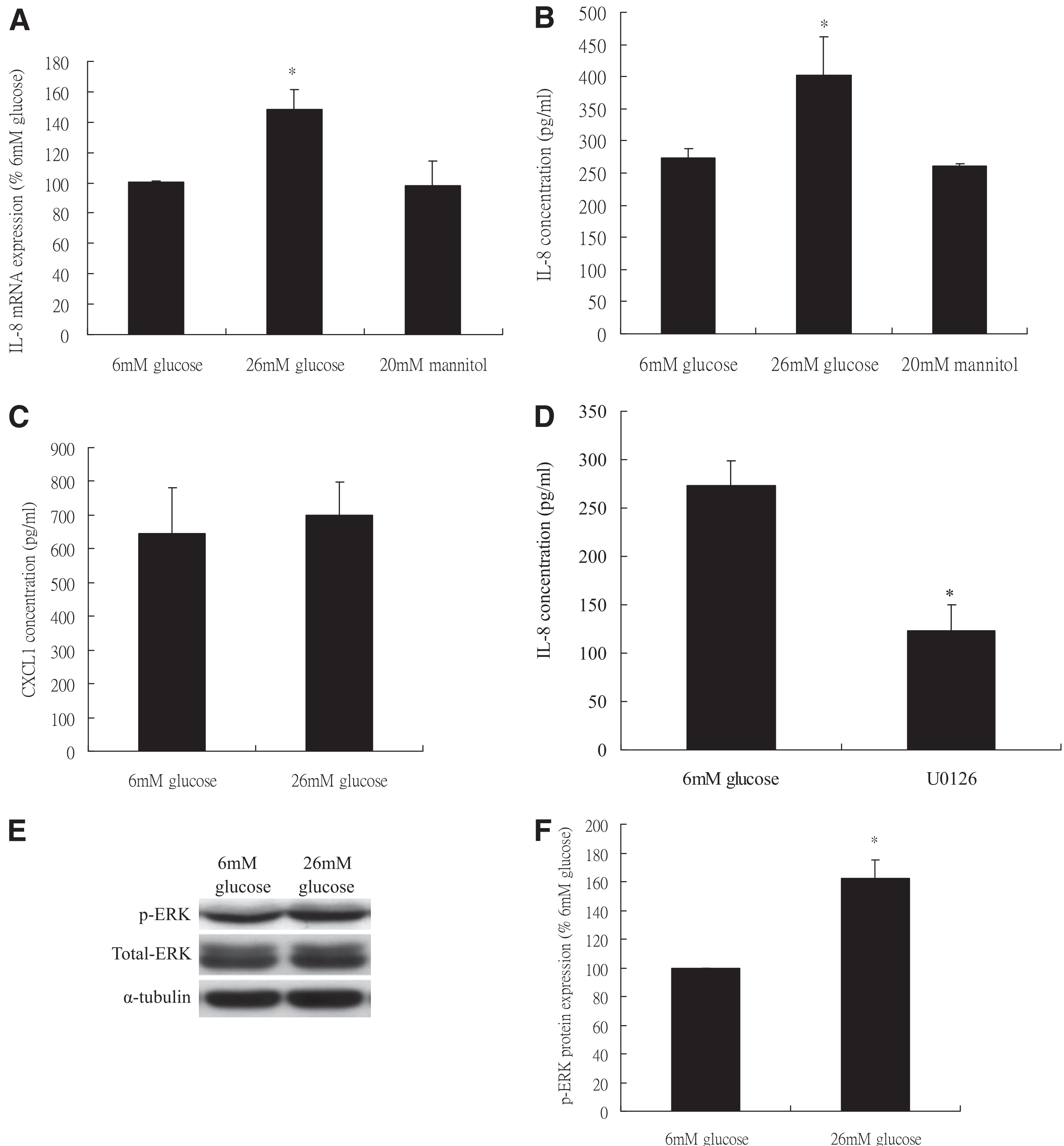

FIG. 1. High-glucose environment increased the expression of IL-8 in cultured human keratinocytes. Human keratinocytes showed increased expressions of IL-8 mRNA (*A*) and protein (*B*) after 7 days of high-glucose cultivation. The levels of CXCL-1 (*C*) were not significantly altered by high-glucose cultivation. *D*: Inhibition tests indicated that U0126 significantly reduced the IL-8 production from cultured keratinocytes. *E*: Representative blot showing increased p-ERK expression in high glucose–cultivated keratinocytes. *F*: Densitometric analyses of *E* with $n = 3$. *$P < 0.05$ compared with 6 mmol/L glucose.

Fig. 1*D*, 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. 1*E* and *F*, 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 a 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, and 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 determined the expression of p-EGFR under different glucose cultivation. As demonstrated in Fig. 2*A*, high-glucose treatment significantly increased the expression of p-EGFR: by ~30%. For determination of the functionality of increased p-EGFR expression, a gene-silencing approach was used. Accordingly, EGFR gene-silenced keratinocytes showed significantly reduced p-ERK expression (Fig. 2*B* and *C*) and ~30% and 70% reduction in IL-8 protein and mRNA expression, respectively (Fig. 2*D* and *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 keratinocytes.

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

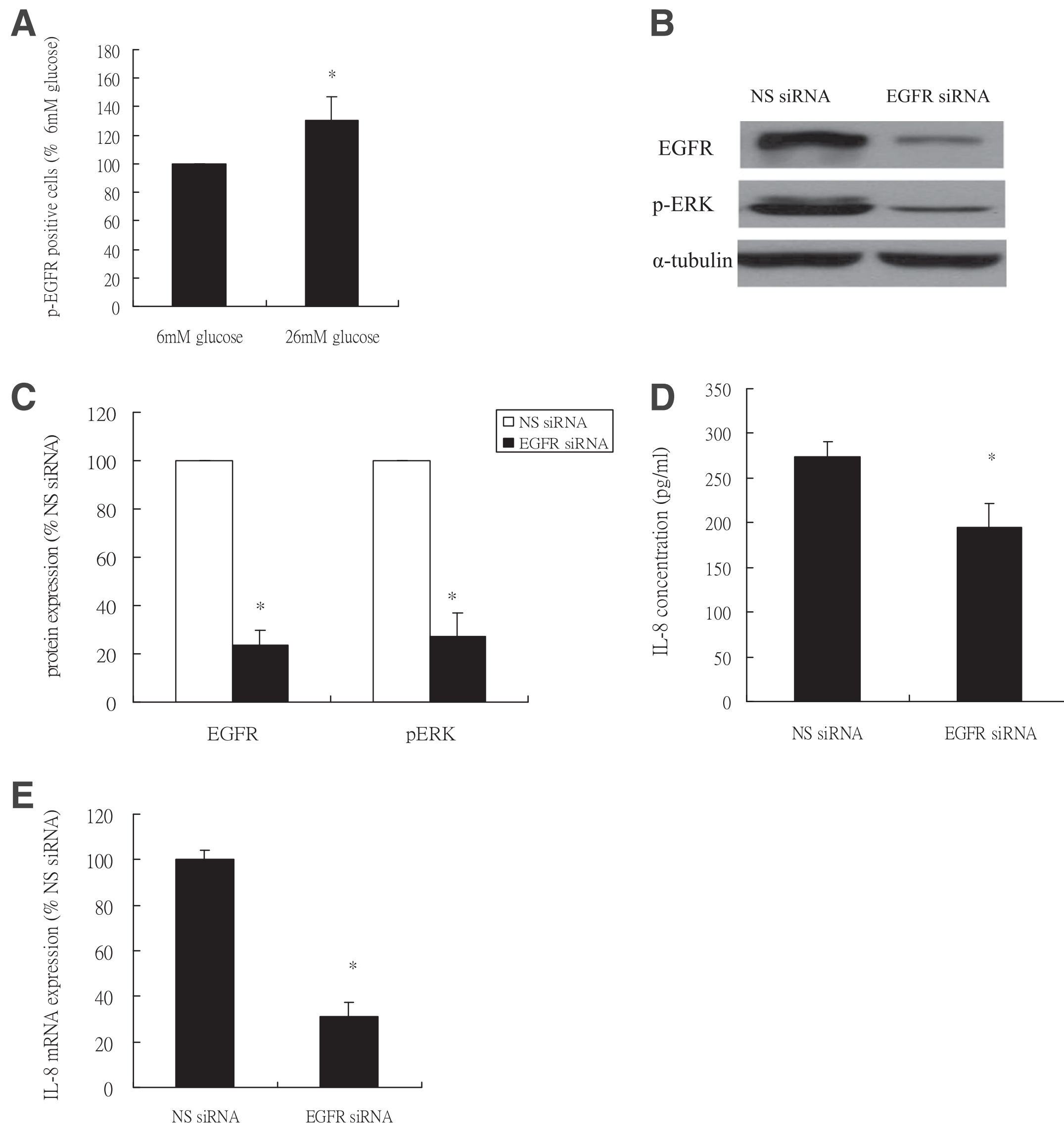

FIG. 2. High-glucose cultivation increased the expression of p-EGFR, the molecule that regulated p-ERK expression and IL-8 production in cultured keratinocytes. *A*: Human keratinocytes demonstrated increased expressions of p-EGFR after 7 days of high-glucose treatment. *B*: Representative blot showing 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 mRNA (*D*) and protein (*E*) levels. NS, nonsilencing. $^*P < 0.05$ compared with 6 mmol/L glucose (A) and compared with nonsilencing group (C–E).

cultured keratinocytes. These results indicated that AGE plays a limited role in the excessive IL-8 level observed in our experimental conditions.

Lipid peroxidation product was increased in high glucose–cultivated keratinocytes, and 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 a high-glucose environment. In support of this notion, the level of MDA, a surrogate marker for estimation of damage induced by reactive oxygen species (ROS), was significantly increased in high glucose–cultivated keratinocytes by approximately threefold compared with control (Fig. 4*A*). Treating high glucose–cultivated keratinocytes with ascorbic acid normalized the expression of p-EGFR and p-ERK (Fig. 4*B–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 compared with 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 compared with that of control rats after wounding. In addition, the level of neutrophil infiltration, as reflected by levels of MPO, was significantly higher in diabetic wounds compared with control.

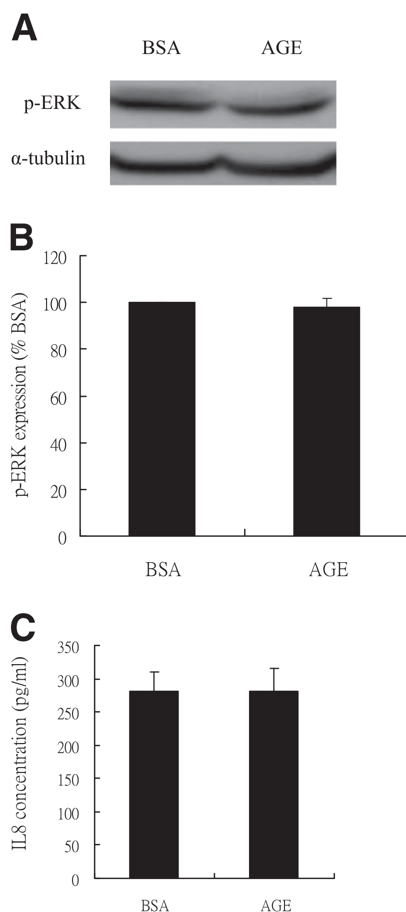

FIG. 3. AGE has limited effect on p-ERK and IL-8 expression in cultured keratinocytes. *A*: Treating cultured human keratinocytes with AGE-modified BSA did not significantly affect the p-ERK expression as demonstrated by Western blotting analyses. *B*: Densitometry analyses of *A*, n = 3. *C*: IL-8 production from cultured keratinocytes was not significantly altered by AGE treatment.

For validation of our in vitro results in vivo, a diabetic rat model was used. Accordingly, diabetic rats showed impaired wound healing. The length of time required to achieve complete wound healing was 9.0 ± 0.4 and 11.5 ± 0.8 days, respectively, for control and diabetic rats (n = 12). Representative figures of healing process are shown in Fig. 5*A*, and the planimetry analysis is shown in Fig. 5*B*. 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 postwounding, 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 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 compared with control skin. More specifically, the ratio of IL-8 increase in diabetic perilesional skin was 1.75 ± 0.08-fold 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 compared with control (Fig. 5*C*). The dermal neutrophil infiltration, as reflected by MPO level, was significantly higher in diabetic rats compared with control rats (Fig. 5*D*).

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. 5*A*), 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) postwounding. In addition, the level of dermal MPO in control and diabetic rats with dapsone treatment 1 day after wounding was 5.55 ± 0.17-fold and 6.09 ± 0.13-fold, respectively, compared with 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 led us to hypothesize that a high-glucose environment may elicit keratinocytes to increase secretion of IL-8, an event which in turn results in excessive neutrophil recruitment.

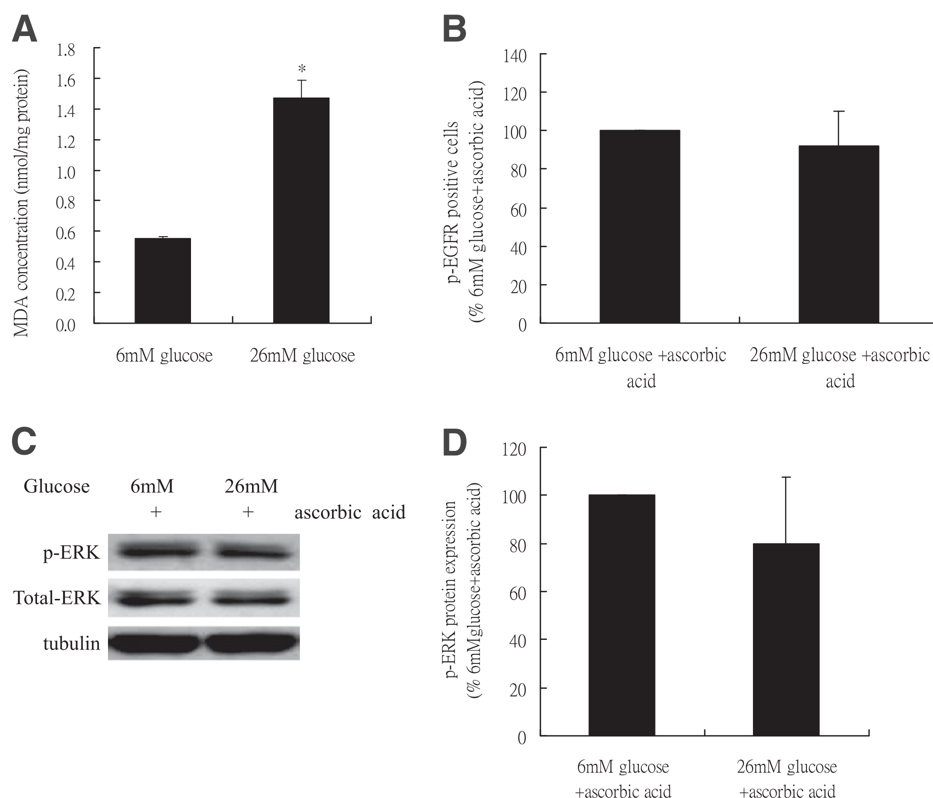

FIG. 4. Lipid peroxidation product MDA is increased in high glucose–cultivated keratinocytes, and ascorbic acid normalized the expressions of p-EGFR and p-ERK in high glucose–cultivated keratinocytes. *A*: Human keratinocytes demonstrated increased expressions of MDA after 7 days of high-glucose treatment. *$P < 0.05$ compared with 6 mmol/L glucose. Addition of ascorbic normalized the expression of p-EGFR (*B*) and p-ERK (*C*). *D*: Densitometric analyses of *C* with $n = 3$.

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 a 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 proinflammatory chemokine production in endothelial cells via activation of both MAPK and NF-κB 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, a 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 diabetes complications and propagates 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 demonstrate that keratinocytes cultivated in a 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 a high-glucose environment.

For examination of our hypothesis in vivo, a diabetic rat model for acute wound healing during hyperglycemic condition was used. As expected, the skin wounds of diabetic rats required longer time to heal compared with 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 compared with 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 compared with control. The results from our in vitro experiments suggest that antioxidants may reduce IL-8 expression from high glucose–cultivated keratinocytes and

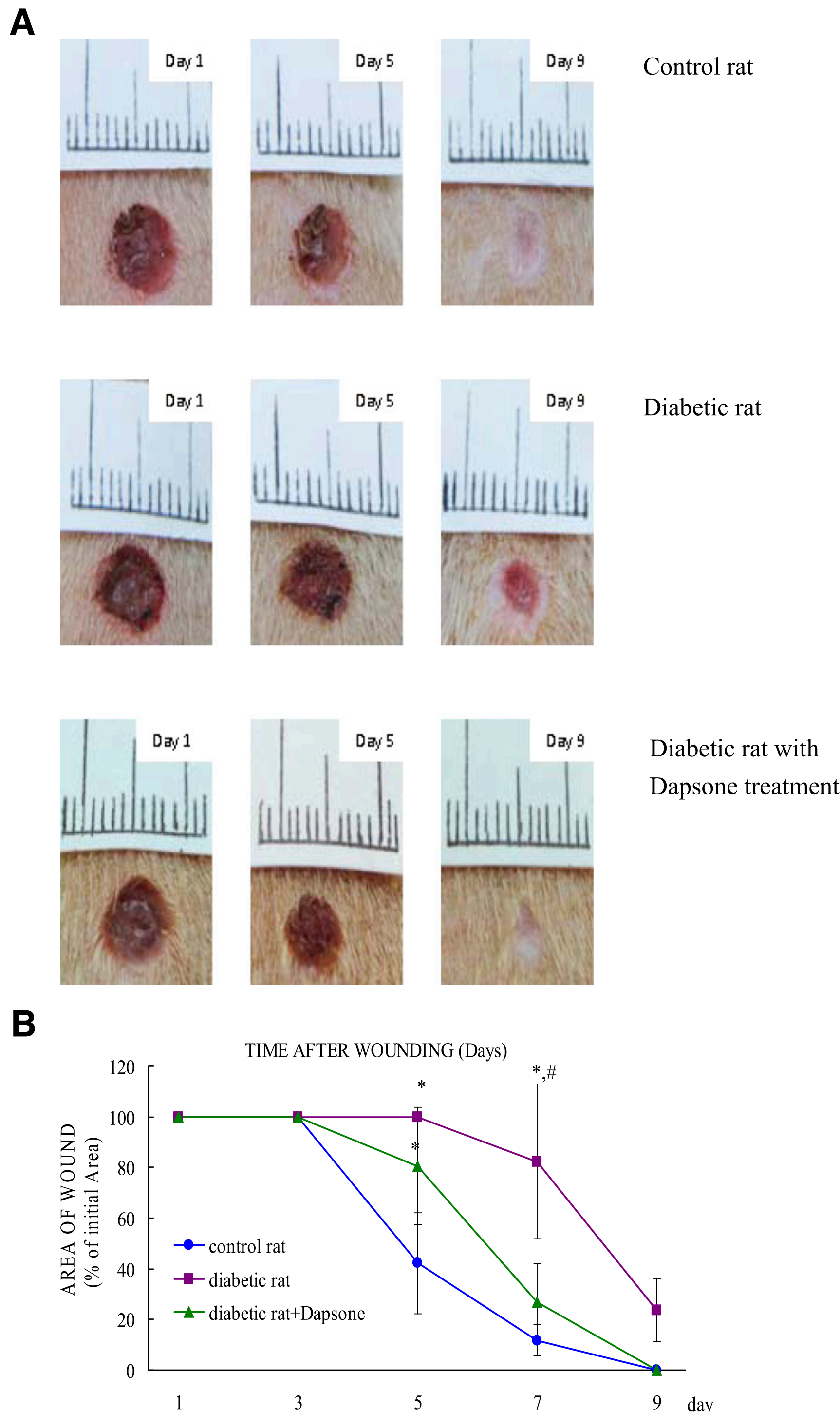

FIG. 5. Diabetic rats showed impaired wound healing and increased dermal MPO expression compared with control. *A*: At 9 days after wounding, control rats showed healing with complete reepithelialization. On the other hand, diabetic rats showed incomplete wound healing. Diabetic rats receiving dapsone treatment also showed adequate healing 9 days after wounding. *B*: Wound closure in different groups as expressed by percent of remaining wound area compared with initial wound. *$P < 0.05$ compared with control; #$P < 0.05$ compared with diabetic rat treated with dapsone. *C*: Expression of p-EGFR and p-ERK in control and diabetic rat skin. *Left panel*: original magnification ×400. *Right panel*: original magnification ×1,000. *D*: The increase in dermal MPO expression of diabetic rats was significantly higher than control rats at 1 and 3 days after wounding. *$P < 0.05$ compared with control rat.

therefore improve diabetic wound healing via normalization of overzealous inflammatory reactions. In support of this hypothesis, a 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 a 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 reepithelialization but not the overall dermal

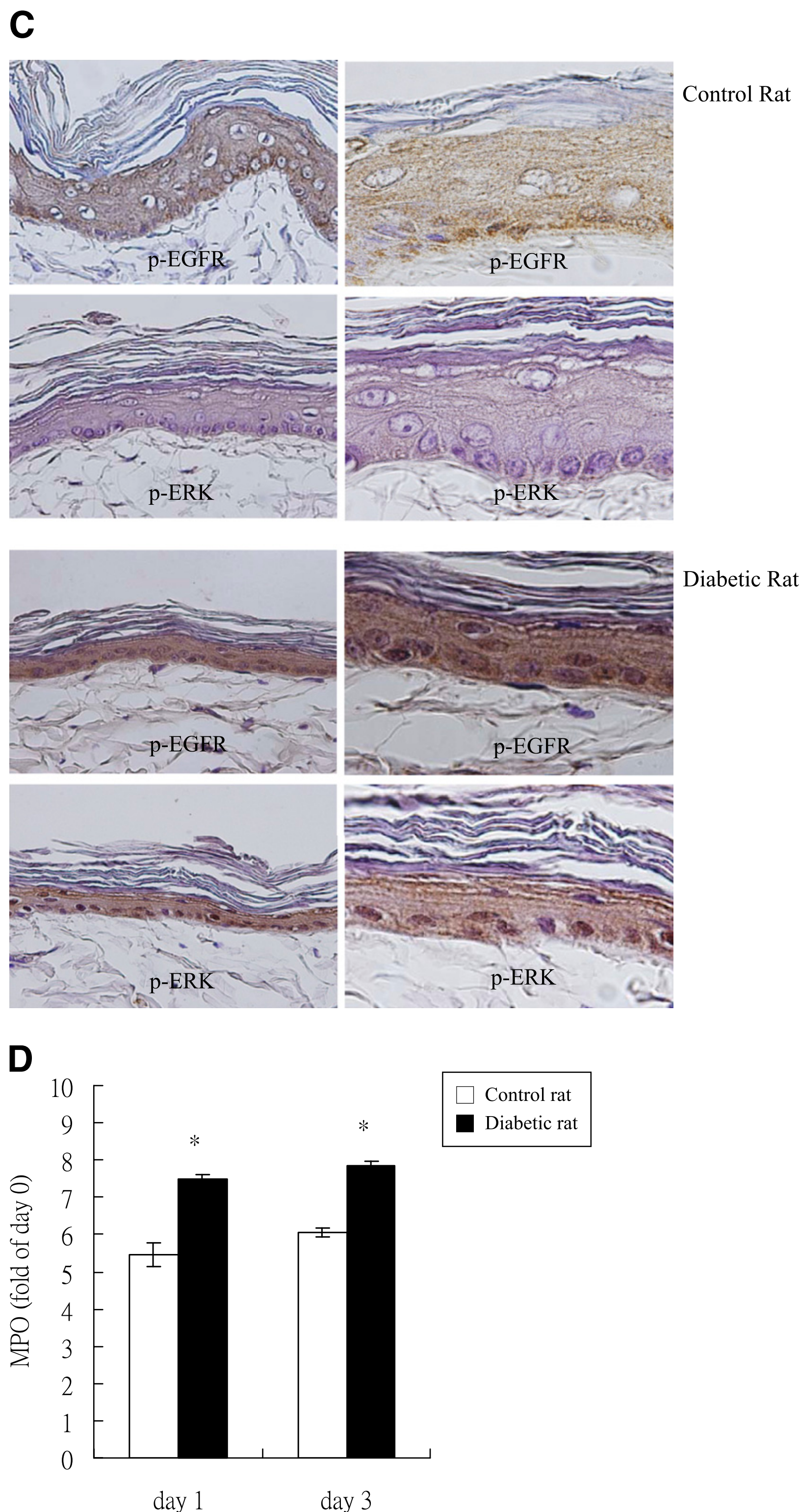

FIG. 5. Continued.

repair. Therefore, it was proposed that excessive protease secretion by neutrophils may inhibit keratinocyte migration and proliferation via induction of keratinocyte detachment. In our experiment, crust of the diabetic rat showed delayed resolution compared with control. It is known that neutrophils are capable of secreting various cytokines, including tumor necrosis factor-α 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 improving 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.

ACKNOWLEDGMENTS

This study was supported by National Science Council, Taiwan (grants NSC98-2314-B-037-016-MY3 and NSC101-2314-B-037-020-MY3).

No potential conflicts of interest relevant to this article were reported.

C.-C.E.L. wrote the manuscript and researched data. C.-S.W., S.-M.H., and I.-H.W. researched data and contributed to discussion. G.-S.C. contributed to discussion and reviewed and edited the manuscript. G.-S.C. is the guarantor of this work and, as such, had full access to data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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