

High-Glucose Environment Inhibits p38MAPK Signaling and Reduces Human β -3 Expression in Keratinocytes

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Diabetes mellitus is characterized by elevated plasma glucose and increased rates of skin infections. Altered immune responses have been suggested to contribute to this prevalent complication, which involves microbial invasion. In this study we explored the effects of a high-glucose environment on the innate immunity of keratinocytes by focusing on β defensin-3 (BD3) using *in vivo* and *in vitro* models. Our results demonstrated that the perilesional skins of diabetic rats failed to show enhanced BD3 expression after wounding. In addition, high-glucose treatment reduced human BD3 (hBD3) expression of cultured human keratinocytes. This pathogenic process involved inhibition of p38MAPK signaling, an event that resulted from increased formation of advanced glycation end products. On the other hand, toll-like receptor-2 expression and function of cultured keratinocytes were not significantly affected by high-glucose treatment. In summary, high-glucose conditions inhibited the BD3 expression of epidermal keratinocytes, which in turn contributed to the frequent occurrences of infection associated with diabetic wounding.

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INTRODUCTION

Patients with diabetes mellitus (DM) are predisposed toward developing complications such as increased rates of skin infections and chronic foot ulcerations that frequently lead to amputation (1–4). The rate of nontraumatic lower extremity amputation for patients with DM is more than 40 times the rate for individuals who do not have DM (1). Immune dysfunctions that affect leukocyte adherence, chemotaxis and phagocytosis occur under hyperglycemic conditions and may make DM patients more prone to infections (5–7). We recently demonstrated that the physiologic functions of keratinocytes, the immune-competent

cells that form the outermost layer of the skin, were also affected by high-glucose environments (8,9).

As the major constituents of the skin, keratinocytes generate a number of antimicrobial peptides that are involved in innate immunity, providing the first defense against potential microbial invasion from the environment. The major antimicrobial peptides found in human beings are defensins and cathelicidins (10). Human β -defensins (hBD) found in the skin were the focus of this study (10–12). Currently, four hBD of different characteristics have been identified (13–19). These defensins have demonstrated the capability to kill and/or inactivate a spec-

trum of bacteria, fungi and enveloped viruses *in vitro* (12). Results of previous studies have shown decreased BD levels in different tissues of diabetic rats (11); however, the possible alterations of hBD expression in the diabetic skin, especially during the wound-healing process, have not been thoroughly investigated.

In terms of antimicrobial activity, hBD1 and hBD2 have shown effects against Gram-negative bacteria. On the other hand, in addition to effects on Gram-negative bacteria, hBD3 has also shown antimicrobial effects against Gram-positive bacteria, including *Staphylococcus aureus*, the most common pathogen found in diabetic wounds (20). Results of previous studies demonstrated that hBD3 is the key molecule by which keratinocytes kill *S. aureus* (21). The current study was launched to investigate the biological and molecular effects of a high-glucose environment on epidermal hBD3 expression.

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ANIMAL MODEL

STZ-Induced Diabetic Rats

Male Wistar rats (200–300 g; BioLASCO Taiwan, Taipei, Taiwan) were acclimatized for 7 d before the experimental procedures and had free access to standard laboratory chow and water. Diabetes was then induced by single 55-mg/kg intraperitoneal injection of streptozotocin (STZ; Sigma, St. Louis, MO, USA). The induction was considered successful if the blood glucose levels measured were greater than 350 mg/dL 2 d after STZ injection.

Wound-Healing Model

Samples of rat skin were obtained under the protocols approved by the ethics committee of Kaohsiung Medical University Hospital. Prior to skin injury, the rats were anesthetized by a single 50-mg/kg intraperitoneal injection of Zoletil (Virbac, Carros, France). After the dorsal hair was shaved and the exposed skin was cleaned with 75% ethanol, full-thickness wounds were made on the rat dorsum by using a 6-mm biopsy punch. Perilesional biopsies were performed immediately and at 1, 3 and 5 d after wounding. The specimens were then divided into two sections. The first section was preserved with 4% neutral buffered formalin and kept at 4°C overnight. The skin section was then preserved at –20°C for subsequent immunohistochemical (IHC) studies. For the second section, the dermal component was immediately removed. The epidermis was then incubated with RNAlater solution (Ambion, Austin, TX, USA) at 4°C for 24 h followed by preservation at –80°C for subsequent quantitative polymerase chain reaction (PCR) analyses.

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 0.01 mol/L citrate buffer (pH 6.0). The

slides were then incubated with goat anti-BD3 antibody (1:50 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at room temperature for 60 min. Antibody reactions were detected with biotinylated link antigoat antibody (Biocare Medical, Concord, CA, USA) for 20 min at room temperature followed by incubation with Trekavidin-HRP (Biocare Medical) for 20 min. The color was developed by using DAB substrate-chromogen solution (Biocare Medical). The slides were then counterstained with hematoxylin.

Total RNA Extraction and Quantitative Real-Time PCR Analyses

The total RNA of the epidermal specimens was extracted using TRI Reagent (Invitrogen, Carlsbad, CA, USA). The total RNA was DNase treated by using TUBRO DNase (Applied Biosystems) for 25 min at 37°C. Two micrograms of TUBRO DNase-treated RNA were reverse transcribed using High Capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA, USA). The cDNA samples were then diluted with nuclease-free water to a final concentration equivalent to 20 ng/μL RNA input; 6 μL of these dilutions were then subjected to 40 cycles of quantitative real-time PCR analyses using the SYBR Green Master (Roche Applied Science, Indianapolis, IN, USA) and the ABI Prism 7500 Sequence Detection System (Applied Biosystems). Primers for rBD-3 (sense: 5'-CAATG GAGGAATATGCTGGCTTA-3'; antisense: 5'-GAGCTGACGAGTGCCACAAC-3') were tested alongside the normalizing gene β-actin (β-actin-F5'-TCTGTGTGGA TTGGTGGCTCT-3'; β-actin-R5'-GACTC ATCGTACTCCTGCTTGCT-3'). The data were analyzed by use of the Delta-Delta CT method (Applied Biosystems).

Cell Proliferation and Cell Morphology

Normal human keratinocytes were obtained from adult foreskin through routine circumcision as previously described (22). Briefly, the skin specimens were washed with phosphate-buffered saline (PBS), cut into small pieces and incubated in medium containing 0.25%

trypsin (Gibco BRL, Gaithersburg, MD, USA) overnight at 4°C. The epidermal sheet was then lifted from the dermis by using fine forceps. The epidermal cells were then pelleted by centrifugation (500g, 10 min) and dispersed into individual cells by repeated aspiration using a pipette. The keratinocytes were then gently resuspended in 5 mL of keratinocyte-SFM medium (Gibco BRL), which contained 25 ng/mL bovine pituitary extract and 5 ng/mL recombinant human epidermal growth factor. The rationale for glucose levels used was described in our previous reports (8–9). Seven days after 6 mmol/L and 26 mmol/L D-glucose treatments, the keratinocytes were subjected to a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) cell proliferation assay for determination of viability. In addition, a 5-bromo-2'-deoxyuridine (BrdU)–enzyme-linked immunosorbent assay (ELISA) was used to evaluate cell proliferation. The morphology of keratinocytes after D-glucose treatment was observed under inverted microscope. The MTS (Cell-Titer 96 aqueous proliferation assay kit; Promega, Madison, WI, USA) was performed according to the manufacturer's instructions. The proliferation of keratinocytes after D-glucose treatment was assessed by a BrdU incorporation assay using a commercially available BrdU-ELISA cell proliferation assay kit (Roche, Basel, Switzerland). The treated keratinocytes were fixed and stained according to the manufacturer's instructions. The optical density was measured at 450 nm (Model 450; Bio-Rad, Hercules, CA, USA).

Evaluation of Antibacterial Activity

The paper-disc agar diffusion method with slight modification described by Jarboe *et al.* (23) was employed. Briefly, the concentration of *S. aureus* (BCRC No. 10781) was adjusted to approximate a 0.5 MacFarland standard by adding nutrient broth (Merck, Darmstadt, Germany). Suspensions of 0.5 MacFarlands were swabbed evenly onto the Mueller–Hinton

agar plates (DIFCO, Becton Dickinson, Sparks, MD, USA). Immediately after inoculation, two paper discs impregnated with 20 μ L of supernatants derived from keratinocytes treated with 6 mmol/L or 26 mmol/L D-glucose for 7 d were dropped onto both sides of the agar plate and incubated in ambient air at 35°C. Sixteen hours later, the diameters of the inhibition zones were measured.

Measurement of hBD3 in Keratinocytes

The treatments of keratinocytes included: (a) 6 mmol/L D-glucose; (b) 12 mmol/L D-glucose; (c) 26 mmol/L D-glucose; (d) incubation with 6 mmol/L or 26 mmol/L D-glucose, followed by a stimulation with a TLR-2 agonist [20 μ g/mL Pam3 Cys-Ser-(Lys)₄, hydrochloride (Calbiochem, Merck KGaA, Darmstadt, Germany)]; (e) incubation with 6 mmol/L D-glucose followed by treatment with 10 μ mol/L p38 inhibitor (SB203580, Calbiochem); (f) incubation with 26 mmol/L D-glucose followed by treatment with 10 μ mol/L SB203580 (Calbiochem) for 2 h, then stimulated with TLR-2 agonist; (g) incubation with 6 mmol/L D-glucose with or without advanced glycation endproduct-modified bovine serum albumin (AGE-BSA) for 2 d; and (h) incubation with 6 mmol/L D-glucose with 20 mmol/L mannitol. The supernatants were collected and stored at -20°C. The concentrations of hBD3 were determined by using a commercially available ELISA kit (PeproTech, Rocky Hill, NJ, USA) according to the manufacturer's instructions. The detectable concentration range of hBD-3 was 64–4000 pg/mL.

Western Blotting Analysis

The treatments of keratinocytes included (a) 6 mmol/L D-glucose, (b) 12 mmol/L D-glucose, (c) 26 mmol/L D-glucose, (d) 6 mmol/L or 26 mmol/L D-glucose followed by stimulation with a toll-like receptor 2 (TLR2) agonist and (e) incubation with 26 mmol/L D-glucose and 2 mmol/L aminoguanidine (AG) for 7 d. Total cellular proteins were extracted

with lysis buffer (0.1% sodium dodecyl sulfate [SDS], 20 mmol/L Tris-HCl, 5 mmol/L EDTA, 2 mmol/L EGTA, and 1% Triton X-100, pH7.4) containing protease inhibitor cocktail (Roche, Mannheim, Germany). Proteins (50 μ g) were loaded into 10% SDS-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. After being blocked and washed, the membranes were incubated with first antibodies including transglutaminase-1 (Santa Cruz Biotechnology), involucrin (Santa Cruz Biotechnology), TLR-2 (Imgenex, San Diego, CA, USA), phosphorylated p38MAPK (pp38MAPK) (Cell Signaling, Beverly, MA, USA), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Millipore, Billerica, MA, USA). Subsequently, the membranes were incubated with horseradish peroxidase (HRP)-labeled secondary antibody (Millipore) and developed in the ImmobilonTW Western Chemiluminescent HRP Substrate (Millipore). The blots were then analyzed by digital imaging system (α Imager 2000; α Innotech, San Leandro, CA, USA).

Real-Time Quantitative PCR Detection for hBD3 and TLR2 mRNA

The treatments of keratinocytes included (a) 6 mmol/L D-glucose, (b) 12 mmol/L D-glucose, (c) 26 mmol/L D-glucose, and (d) incubation with 6 mmol/L or 26 mmol/L D-glucose, followed by stimulation with TLR2 agonist. Total RNA was extracted by using the Trizol method (Gibco BRL) and processed as recommended by the manufacturer. As the PCR template 5 μ g of RNA was reverse-transcribed to cDNA. The target genes analyzed were *hBD3* and *GAPDH*. The primers used for *hBD-3* were 5'-TAGCAGCTATGAGGATCCA-3' (forward) and 5'-CTTCGGCAGCATTTTC GG-3' (reverse); the primers for *TLR2* were: 5'-TCTCCCATTTCCGTC TTTT-3' (forward) and 5'-GGTCTTGGTG TTCATTATCTTC-3' (reverse); and for *GAPDH* were 5'-CCACCCATGGCAAAT TCC-3' (forward) and 5'-GGGATTTCCA TTGATGACA-3' (reverse). Amplification

and detection were performed with an ABI Prism 7500 sequence detection system (Applied Biosystems). The data were analyzed as described above.

Small Interfering RNA Experiment

The cultured keratinocytes were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Briefly, 1 μ L of hBD3 siRNA or control small interfering RNA (siRNA) and 1 μ L of Lipofectamine 2000 were each diluted with 50 μ L of Opti-MEM[®]I reduced serum medium (Invitrogen) without supplements and incubated at room temperature for 5 min. The sequences of double-strand siRNA directed against HBD3 were A: 5'-CUUACGACGACAGCACUUACGACCA-3', and B: 5'-ACUUGCCGAUCUGUCCUCCUUUGG-3' (Invitrogen). The Stealth RNAi duplexes (Invitrogen) were used as a negative control. Subsequently, they were mixed and incubated at room temperature for 20 min. One milliliter of the mixture (oligomer-Lipofectamine 2000 complex) was added to each well and incubated at 37°C for 6 h. The reaction medium was changed into keratinocyte culture medium and incubated at 37°C for 72 h. Quantitative PCR was performed to confirm the success of transfection. The supernatants were then collected and subjected to the antimicrobial activity experiment as described previously.

Statistical Analysis

For each experiment, at least three independent experiments were performed. Quantitative results are expressed as mean \pm SD. Statistical analysis was determined using the Student *t* test. A *P* value less than 0.05 was considered as significant.

RESULTS

Diabetic Rats Showed Delayed Wound Healing and Poor BD3 Induction Compared with Control Rats

As expected, the wounds of diabetic rats (*n* = 9) showed significantly delayed healing time compared with the wounds

of control rats ($n = 9$). More specifically, the healing times for the control and diabetic rats were 9.3 ± 0.8 d and 11.8 ± 1.9 d, respectively. Because the wounding process has been reported to induce BD expression (24), the IHC staining was performed on the perilesional skin in a sequential manner. The expressions of BD3 appeared 1 d after skin wounding on the upper layers of the perilesional skin of the control rats, whereas the perilesional specimen obtained from the diabetic rats demonstrated negligible BD3 expression even after wounding. The BD3 staining of the skin obtained from the control rats became more intense 3 d after wounding (Figure 1A–D). On the other hand, throughout the experiment, the BD3 expression was not observed on the perilesional diabetic rat skin. Corroborating with the results of IHC staining, the results of quantitative PCR analyses of the control perilesional skin showed significant increases of BD3 mRNA expression (an approximately six-fold increase at day 1 and day 3 compared with day 0) after wounding, whereas no significant increase was observed in the diabetic rat skin (Figure 1E).

Keratinocytes Cultivated under Normal- and High-Glucose Conditions Showed Comparable Viability, Growth and Differentiation after 7 Days of Treatment

Our previously reported results showed that prolonged cultivation of keratinocytes under high-glucose environmental conditions resulted in decreased cellular survival, possibly due to depletion of growth supplements and accumulation of harmful metabolic byproducts. Because we intended to evaluate the effects of a high-glucose environment on keratinocytes for a longer term, the culture medium was changed every other day. The viability of keratinocytes cultured at various glucose levels was not significantly different, as determined by the MTS assay (data not shown). In addition, cell proliferation, as determined by the BrdU-ELISA assay, showed no significant difference between the normal- and

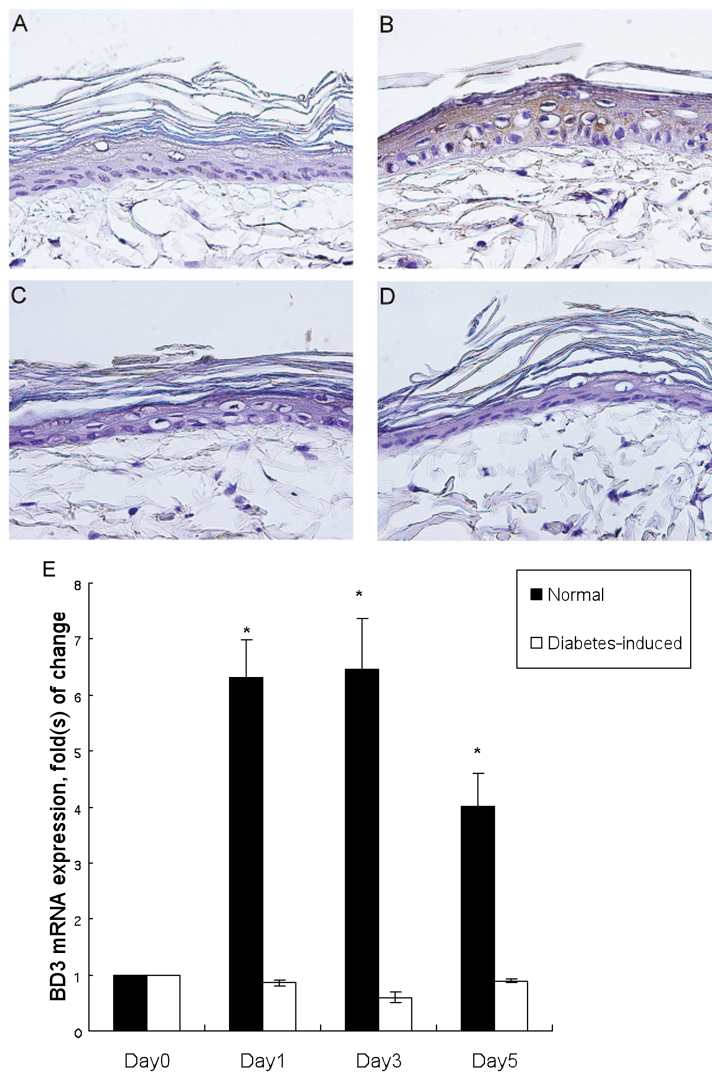


Figure 1. Expression of BD3 in wounded rat skin. (A–D) Immunohistochemical BD3 staining (brown) of the perilesional skin of the control (A, B) and the diabetic rat (C, D) at day 0 and 3 after wounding respectively. Original magnification 400x. (E) The BD3 mRNA levels of perilesional rat skin at different time points after wounding * $P < 0.05$ compared with day 0.

high-glucose cultivated groups. Moreover, microscope analysis revealed no significant differences in cellular morphology among the keratinocytes treated with different levels of glucose. Involucrin and transglutaminase-1 are two important markers for characterizing the differentiation status of keratinocytes. High-glucose treatment did not significantly alter the expression of these proteins (Figure 2). These results indicated that the cellular physiologic status was comparable between the normo- and

high-glucose groups under our experimental conditions.

Keratinocytes Cultured at High-Glucose Settings Showed Decreased hBD3 at Both mRNA and Protein Levels

Because our experimental conditions did not significantly affect the growth and the differentiation status of keratinocytes, we next evaluated the effects of high-glucose treatment on keratinocytes in terms of hBD3 production. The levels of hBD3 in the culture supernatants ob-

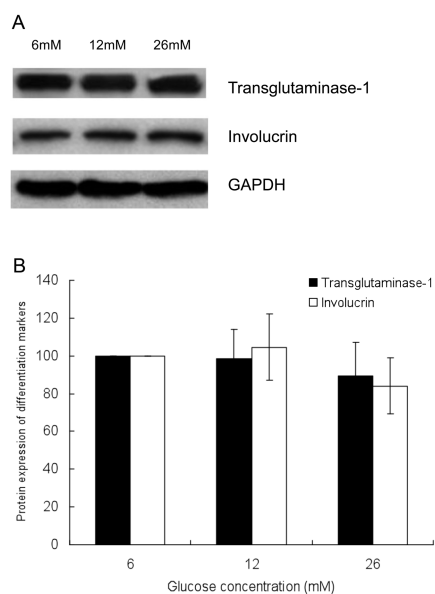


Figure 2. Effect of high-glucose treatment on human cultured keratinocyte differentiation. (A) The effect of high-glucose treatment on transglutaminase-1 and involucrin expressions of keratinocytes. The figure shown represents one experiment repeated 3x with similar results. (B) Densitometric analysis of (A), ratio of differentiation markers/GAPDH, with 6 mmol/L glucose treatment representing 100%.

tained from the keratinocytes treated with 12 mmol/L and 26 mmol/L glucose were significantly lower (approximately 67% and 60%, respectively) than their normo-glucose (6 mmol/L) counterpart. Corroborating with the protein expression, the hBD3 mRNA expression of the keratinocytes cultivated in 12 mmol/L and 26 mmol/L glucose were also significantly lower (approximately 70% and 54%, respectively) compared with the 6 mmol/L control group (Figure 3). Treating cultured keratinocytes with the p38 inhibitor SB203580 significantly decreased the levels of hBD3 to $56.5 \pm 4.1\%$ of the control. The osmolarity control treatment group showed an insignificant reduction of hBD3 from 583.5 ± 15.0 pg/mL to 483.7 ± 35.1 pg/mL, indicating that the changes in glucose and not osmolarity levels are responsible for reduction of hBD3 expression.

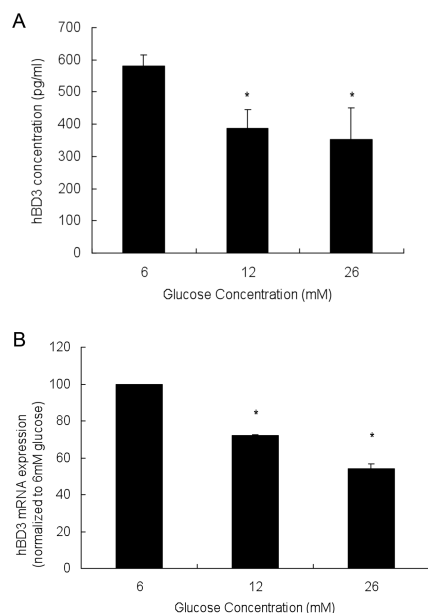


Figure 3. Effects of different glucose levels on hBD3 production of cultured human keratinocytes. (A) The protein expressions of hBD3 in the supernatants of keratinocytes cultivated under different levels of glucose. (B) The hBD3 mRNA expressions of the keratinocytes cultivated under different glucose levels. The expression of hBD3 for keratinocytes cultivated with 6 mmol/L glucose representing 100% in the figure. * $P < 0.05$.

Culture Supernatants from Keratinocytes Cultivated in Normal Glucose Concentrations Showed Better Anti-*S. Aureus* Activity than Keratinocytes Cultivated in High Glucose

Results of a previous study showed that hBD3 is the key molecule with which keratinocytes kill *S. aureus* (21). Therefore, we examined the anti-*S. aureus* activity of the culture supernatants derived from different culture conditions. The supernatants from keratinocytes cultured with high glucose exhibited only approximately 80% of the anti-*S. aureus* capacity of keratinocytes cultured with normal glucose (Figure 4). This result indicates that high-glucose treatment rendered keratinocytes less capable of fending off *S. aureus*. To confirm the functional role of hBD3 on

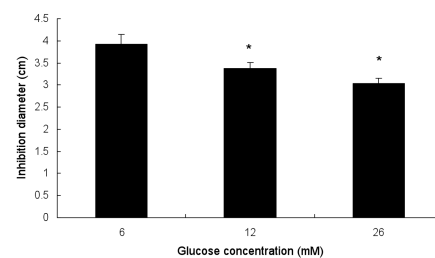


Figure 4. The anti-*S. aureus* activity of the supernatants obtained from keratinocytes cultivated under different glucose conditions. The diameters of the inhibition zones were shown. * $P < 0.05$.

anti-*S. aureus* capacity in our experiment, we used gene silencing of *hBD3* by siRNA. Accordingly, the supernatants from the siRNA-treated keratinocytes showed only $70\% \pm 8.0\%$ capacity against *S. aureus* growth compared with the control vehicle. This result indicated that hBD3 was at least partially responsible for inhibiting *S. aureus* growth in our experiments. The remaining antimicrobial activity noted in the experiment was likely due to antibiotics present in the medium we used for cell culture and possibly due to other antimicrobial peptides released by keratinocytes. To confirm this hypothesis, supernatants were collected from keratinocytes cultured without the addition of antibiotics, and similar experiments were repeated. However, none of the supernatants obtained under these conditions demonstrated significant antimicrobial activity, regardless of the glucose levels during culture.

No significant differences in the expression of TLR-2 were observed between the normal- and high-glucose-cultivated groups. TLR-2 is an important regulator of hBD3 expression. High-glucose treatment may reduce TLR-2 expression, which can lead to reduced hBD3 production. Therefore, we evaluated the expression of TLR-2 in keratinocytes under high-glucose cultivation. As demonstrated by the data presented in Figure 5, high-glucose treatment did not significantly alter the expression of TLR-2 at the protein and mRNA levels.

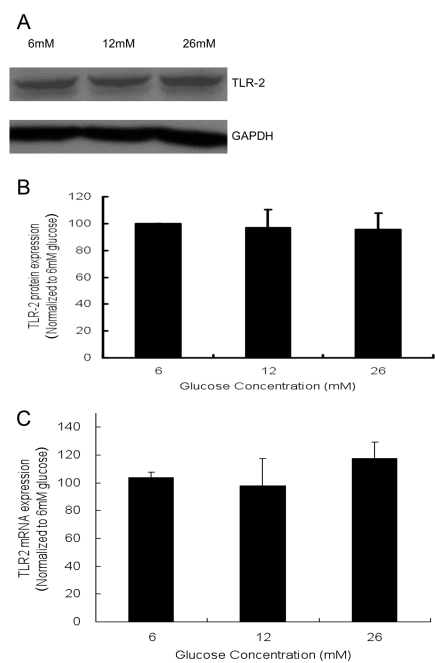


Figure 5. Effect of high glucose cultivation on TLR-2 expressions of keratinocytes. (A) The protein expressions of TLR-2 in keratinocytes cultivated under different levels of glucose. A representative blot from three independent experiments with similar result is shown. (B) Densitometric analysis of (A), ratio of TLR2/GAPDH, with 6 mmol/L glucose treatment representing 100%. (C) The TLR-2 mRNA expressions of the keratinocytes cultivated under different levels of glucose with keratinocytes cultivated with 6 mmol/L glucose representing 100% in the figure. * $P < 0.05$.

Treating Keratinocytes with a TLR-2 Agonist Significantly Increased hBD3 Expression

Because TLR-2 is an important regulator of hBD3 expression and its expression was not affected by high-glucose treatment, we next evaluated the functionality of TLR-2 on keratinocytes cultivated under high-glucose conditions. Treating keratinocytes with a TLR-2 agonist increased the protein and mRNA expression of hBD3 more than two-fold regardless of the glucose treatment. More specifically, the hBD3 levels of the keratinocytes cultivated in 6 mmol/L and 26 mmol/L glucose increased from 583.48 ± 76.40 pg/mL to $1148.24 \pm$

109.83 pg/mL and 341.72 ± 7.68 pg/mL to 627.39 ± 107.21 pg/mL, respectively. However, after TLR-2 stimulation the absolute levels of hBD3 from the group cultivated in normal glucose remained significantly higher than the levels of their high-glucose counterpart. These results indicated that the keratinocyte TLR-2 remained functional after high-glucose treatment, and a certain constitutive inhibitory effect was imparted by the high-glucose treatment that resulted in reduced hBD3 production.

Phosphorylated p38MAPK Expression Was Significantly Reduced in Keratinocytes Cultivated in High Glucose

Treatment with a TLR2 agonist significantly increased the expression of phosphorylated p38MAPK. Both p38MAPK and nuclear factor κ B (NF κ B) signaling are known to regulate hBD3 expressions. As demonstrated in Figure 6, both 12-mmol/L and 26-mmol/L glucose treatment significantly decreased the constitutive expression of phosphorylated p38MAPK in cultured keratinocytes. On the other hand, the NF κ B-binding activities of keratinocytes treated with 26 mmol/L glucose were $1.12 \pm .19$ -fold greater than those of the 6-mmol/L glucose group, with no significant differences noted between the two groups. Treating cultured keratinocytes with a TLR-2 agonist resulted in a clear increase of phosphorylated p38MAPK expression, even in the high-glucose-cultivated keratinocytes, as shown in Figure 7A.

SB203580, a p38MAPK Inhibitor, Abrogated the hBD3-Enhancing Effect of TLR2 on High-Glucose-Treated Keratinocytes

Because the TLR-2 agonist increased phosphorylated p38MAPK expression and hBD3 production of the high-glucose-cultivated keratinocytes, SB203580 was added to determine the functional role of enhanced phosphorylated p38MAPK in TLR-2 induced hBD3 increase. As expected, treating the high-glucose-cultivated keratinocytes with

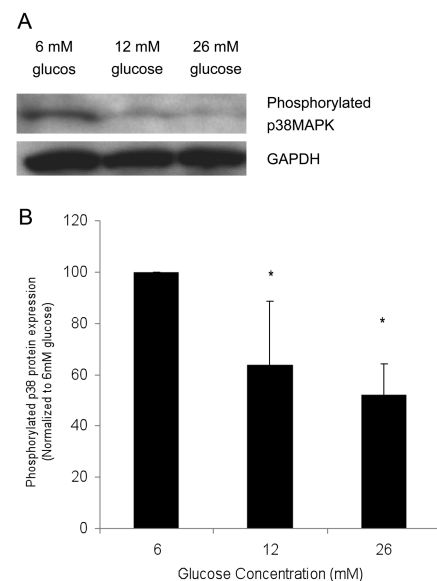


Figure 6. The phosphorylated p38MAPK expression of cultured human keratinocytes after indicated treatment condition. (A) The protein expressions of phosphorylated p38MAPK in keratinocytes cultivated with 6 mmol/L, 12 mmol/L and 26 mmol/L glucose. A representative blot from three independent experiments with similar results is shown. (B) The densitometric analysis of (A), ratio of phosphorylated p38MAPK/GAPDH, with 6 mmol/L glucose treatment representing 100%. * $P < 0.05$.

SB203580 abrogated the enhancing effect of TLR-2 agonist on hBD3 expressions. More specifically, the hBD3 levels decreased from 564.5 ± 1.2 pg/mL to 330.4 ± 2.3 pg/mL (approximately 60%), confirming the importance of p38MAPK signaling in TLR-2-induced hBD3 response and validating the integrity of TLR-2 function under the experimental conditions of our study.

AGE-BSA Significantly Decreased the hBD3 and the Phosphorylated p38MAPK Expressions of Cultured Keratinocytes

High-glucose treatments are known to induce AGE accumulation. However, the effects of AGE on hBD3 production have not been documented. Treating keratinocytes with BSA and AGE-BSA resulted in hBD3 levels of 763.3 ± 16.6 pg/mL and 556.6 ± 1.3 pg/mL, respectively, indicat-

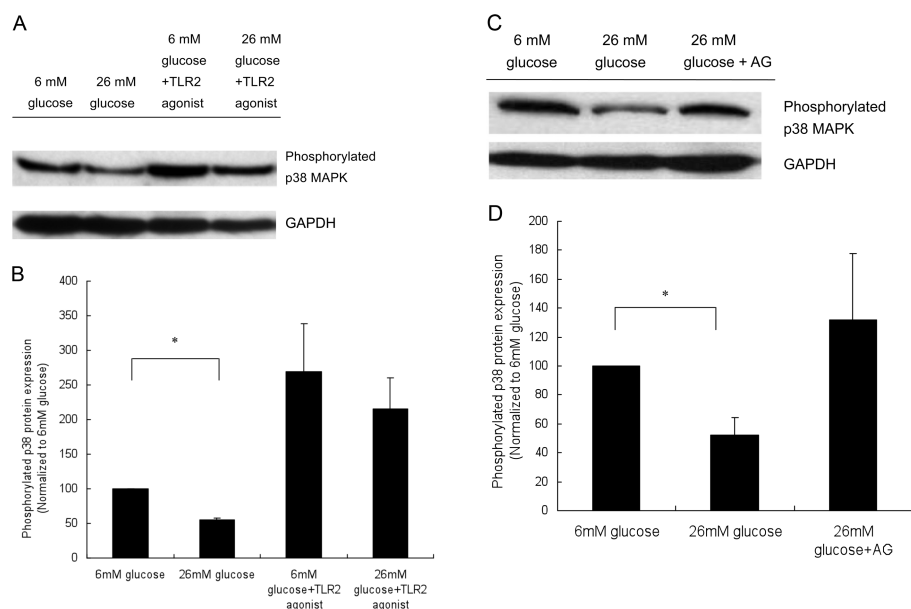


Figure 7. The phosphorylated p38MAPK expression of cultured human keratinocytes after indicated treatment condition. (A) The protein expressions of phosphorylated p38MAPK in keratinocytes with or without TLR-2-agonist stimulation were evaluated. A representative blot from three independent experiments with similar results is shown. (B) The densitometric analysis of (A), ratio of phosphorylated p38MAPK/GAPDH, with 6 mmol/L glucose treatment representing 100%. (C) The effects of AG, an inhibitor of advanced glycation end-product, on protein expression of phosphorylated p38MAPK expression of high glucose-cultivated keratinocyte. The figure shown is representative of blots from three independent experiments with similar results. (D) Densitometric analysis of (C), ratio phosphorylated p38/GAPDH, with 6 mmol/L glucose treatment representing 100%. * $P < 0.05$.

ing that AGE significantly reduced the hBD3 production of keratinocytes. To determine if AGE formation was involved in suppressing constitutive phosphorylated p38MAPK expression in the high-glucose-treated keratinocytes, AG, an inhibitor of AGE formation, was added to the high-glucose-treated keratinocyte culture. Restoration of phosphorylated p38MAPK expression was found in the high-glucose-treated keratinocytes after introduction of AG (Figure 7C).

DISCUSSION

It has been shown that wounding of the skin initiates various innate immune responses, including induction of hBD3 expression via transactivation of the epidermal growth factor receptor (EGFR) (24). Because it is impractical to perform sequential repeated biopsies on human diabetic patients, an animal model was

employed to study the effect of hyperglycemia on epidermal BD3 expression. In parallel with previous findings, our rat model showed enhanced BD3 expression at both mRNA and protein levels after skin wounding. On the other hand, the perilesional skin of the diabetic rat wound showed negligible BD3 induction even after wounding, indicating that BD3 response was dysfunctional under hyperglycemic conditions. To mimic diabetic conditions in human beings, additional *in vitro* studies were performed using cultured human keratinocytes under various glucose-treatment conditions.

As demonstrated by our results, high-glucose cultivation reduced the constitutive hBD3 expression of human keratinocytes, both at mRNA and protein levels. In addition, the supernatants obtained from keratinocytes grown in cultures containing high glucose concentrations

showed decreased bactericidal capacity toward *S. aureus* compared with keratinocytes grown in cultures containing normal glucose concentrations. These data strongly suggest that hBD3 expression and function of keratinocytes were suboptimal at hyperglycemic settings, and this particular dysfunction at least contributed to the high infection rates associated with diabetic wounds. It should be noted that the osmolarity control treatment using mannitol did not significantly affect the concentrations of hBD3. This result further validated and strengthened the notion that high glucose levels rather than elevated osmolarity were responsible for the reduced hBD3 levels observed. It should be noted that the supernatants obtained from keratinocytes cultured without antibiotics did not demonstrate significant antimicrobial activity in our experimental conditions. It has previously been shown that certain synergistic antimicrobial effects can be found when suboptimal doses of antibiotic and hBD3 are combined (25). Therefore, it is likely that the concentrations of hBD3 in our experimental conditions were not adequate to induce significant antimicrobial effects alone, but when combined with exogenous antibiotic, the levels of hBD3 present were able to induce a synergistic effect.

TLRs played an important regulatory role in hBD synthesis. Human skin expressed both TLR-2 and TLR-4, whereas cultured primary normal human keratinocytes expressed functional TLR-2, but not TLR-4 (26). TLR-2 is considered the major receptor for Gram-positive bacteria by virtue of its ability to recognize bacterial cell-wall components (27). When cultured under high-glucose conditions, TLR-2 expression on keratinocytes was not significantly altered. Moreover, addition of TLR-2 agonist was able to increase the hBD3 levels of both normal- and high-glucose-treated groups, although the absolute hBD3 levels were still significantly lower in the high-glucose-cultivated group. These results indicate that at clinical settings, the ab-

solute hBD3 levels may not be adequately expressed in response to normal physiological stress such as skin wounding or infection at hyperglycemic settings. In addition, these results also demonstrated that the TLR-2 signaling of the high-glucose-treated keratinocytes remained functional.

Induction of defensin synthesis may involve several cascades, including p38MAPK and NFκB pathways (28–31). As demonstrated by our results, high-glucose conditions reduced constitutive phosphorylated p38MAPK expression but has a limited impact on active NFκB binding. Therefore, the constitutively downregulated p38MAPK signaling contributed to the reduced hBD3 expressions under high-glucose environmental conditions. Intriguingly, that TLR-2 agonist was able to increase the expression of phosphorylated p38MAPK, resulting in restoration of hBD3 levels. These results indicated that TLR-2 signaling remained intact in a high-glucose environment and that the TLR-2 agonist may be clinically useful for promoting better diabetic wound healing through upregulation of hBD3, an effective antimicrobial peptide and important component of innate immunity.

Increased levels of advanced glycation endproducts (AGE) have been implicated in complications associated with diabetes (32). It has been recognized that glucose can react directly with protein amino groups (glycation) to form labile Schiff bases which subsequently rearranged to more stable Amadori product. In time, glycated protein yielded reactive dicarbonyl compounds that can form AGE. Under physiologic conditions, AGEs are formed constantly in the body. The rate of formation is prominently increased in the presence of high glucose and/or increased oxidative stress. Previous studies have shown that AGE imparted detrimental effects on fibroblasts and endothelial cells (33,34), the important participants of wound healing process. It has also been shown that AGE accumulation inside the cell is a rapid process as Giardino *et al.* (35) had shown that 7 days of

high-glucose (30 mmol/L) treatment resulted in a 13.8-fold increase of AGE accumulation compared with cells cultured in normal (5 mmol/L) glucose. AGE, a nucleophilic hydrazine compound, inhibits the formation of AGE and has been shown to prevent impaired wound healing in diabetic rats (36,37). Therefore, the effects of AGE on keratinocytes in terms of hBD3 production and p38MAPK signaling were examined. Treating keratinocytes with AGE significantly decreased the levels of hBD3 production. In addition, treating the high-glucose-cultivated keratinocytes with AGE showed reversal of the reduced phosphorylated p38MAPK expression, indicating that the increased formation of AGE contributed to the reduced phosphorylated p38MAPK expression observed in keratinocytes cultivated at high glucose levels. Recently reported data demonstrate that inhibition of p38MAPK activity during wounding blocked EGFR internalization (38), an event crucial for hBD3 induction. Therefore, downregulation of p38MAPK signaling by AGE may be the early event responsible for suboptimal hBD3 expression found in diabetic wounds. It should be noted that previous reports have shown that an AGE-induced transient increase of p38MAPK signaling that returned to the basal level within 45 min (39,40). Our current results suggested that after initial transient elevation, sustained AGE exposure inhibited the activity of the p38MAPK cascade. Further studies clarifying the differences between the short- and long-term effects of AGE on cellular physiology are warranted.

In summary, we demonstrated that a high-glucose environment reduced hBD3 expression in keratinocytes. This pathogenic process involved inhibition of p38MAPK signaling, an event that resulted from increased AGE formation. In addition, the TLR-2 agonist was able to elevate the hBD3 expressions in high-glucose-cultivated keratinocytes. Recently reported data suggested that enhanced hBD3 expression promoted wound healing in infected diabetic wounds (41). Our current findings provide further support

for this approach. Although hyperglycemic conditions inhibited constitutive hBD3 expression on keratinocytes and resulted in inadequate hBD3 upregulation upon stimulation, the signaling via TLR-2 appeared to be intact. The topical TLR (TLR7 and TLR8) agonist imiquimod has already been used for treatment of various dermatologic conditions, and topical TLR2 agonists treatment for managing diabetic wounds is a strategy worthy of investigation in the near future.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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