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Effects of advanced glycation end-products (AGEs) on skin keratinocytes by nuclear factor-kappa B (NF-κB) activation

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Advance glycation end-products (AGEs) are produced in patients with long-term hyperglycemia metabolic disorder and responsible for multiple symptoms including impaired wound healing. This study was designed to reveal the roles and possible mechanism of AGE in diabetic wound healing. Sixteen Sprague-Dawley (SD) rats were divided into two groups randomly; the streptozotocin (STZ) induced diabetic group and the normal group. Eight weeks later, epidermal growth factor (EGF) and AGE levels, nuclear factor-kappa B (NF-κB) localization and cell viability were measured *in vivo*. Keratinocytes from normal skin were cultured in AGE-enriched conditional media, and the cell viability, apoptosis, adhesion and migration were detected in order to find the directed evidence between AGE and keratinocytes. AGE content was higher and NF-κB expression was more localized in the nuclear of keratinocytes in diabetic skins. AGE could inhibit normal cell growth by inducing apoptosis and arresting cell division cycle, inhibiting cell adhesion and promoting migration which might be mediated by NF-κB *in vitro*. Blocking NF-κB activity could reverse effects of AGE on cell proliferation and migration, but not adhesion. Therefore, AGE could damage the skin keratinocytes function *in vivo* and *in vitro*, and the activation of NF-κB is involved in this process.

Key words: AGE, NF-kappaB, keratinocytes, diabetes, wound healing.

INTRODUCTION

Glucose represents a major fuel for most mammalian cells, and a wide range of factors regulates its utilization. However, abnormally high levels of glucose as seen in diabetes, lead to the development of chronic complications. Noteworthily, chronic impaired wounds leading very often to ulceration, necrosis and amputation are one of the severe consequences of diabetes, which result in significant morbidity. Impaired wound healing is a common vascular complication of diabetes mellitus and constitutes a major clinical problem worldwide. Several hypotheses were proposed to explain the various pathological changes induced by hyperglycemia, including advanced glycation end products (AGEs), hyperosmolarity, abnormal sorbitol and myoinositol metabolism, oxidant formation, and protein kinase C (PKC) activation by diacylglycerol (Wautier and Guillausseau, 1998; Friedman, 1999; Porte and Schwartz, 1996).

Advanced glycation end-product (AGE) results from non-enzymatic glycation of the amino groups of proteins with reducing sugars. AGE accumulate on skin elastin and collagen, interfering with normal skin function (Wondrak et al., 2002). The formation of AGE on skin

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collagen favors collagen cross-linking, resulting in impaired dermal regeneration. Increased AGE levels in skin collagen are found in diabetes (Meng et al., 2001) and play important roles in diabetic complications (Sensi et al., 1995). The migration and proliferation of keratinocytes is critical to wound re-epithelialization and defects in this function are associated with the clinical phenomenon of chronic non-healing wounds. AGE enhances expression of pro-apoptotic genes and stimulates fibroblast apoptosis through cytoplasmic and mitochondrial pathways (Alikhani et al., 2005). AGE was also reported to be responsible of impaired retinal angiogenesis in diabetes (Stitt et al., 2005). However, specific roles for AGE in keratinocyte migration and proliferation and the underlying molecular mechanisms, have not been fully established.

Nuclear factor kappa B (NF-κB) is a member of the Rel family proteins, which include p50/p105, p52/p100, p65 (RelA), RelB, and c-Rel. These proteins form homodimers or heterodimers with other Rel family proteins (Verma et al., 1995). NF-κB is involved in a wide spectrum of cellular functions, including cell cycle control, stress adaption, inflammation and control of apoptosis. High glucose is found to inhibit endothelial cell migration through NF-κB (Hamuro et al., 2002). High glucose-induced NF-κB DNA binding activity may mediate this inhibition of migration by regulating intracellular nitric oxide (Hamuro et al., 2002). Tumour necrosis factor (TNF)-alpha stimulates activation of pro-MMP2 in human skin through NF-κB mediated induction of MT1-MMP, and thus prevent normal wound healing (Han et al., 2001). NF-κB can trigger cell cycle arrest in epithelial cells in association with selective induction of a cell cycle inhibitor (Seitz et al., 2000).

However, another group demonstrates that insulin stimulates haptotactic migration of human epidermal keratinocytes through activation of NF-κB transcription factor (Benoliel et al., 1997). NF-κB is a transcription factor involved in a number of signaling pathways such as keratinocyte proliferation and differentiation in rat experiments. Several papers have reported that NF-κB has been associated with the accumulation of AGE in the tissues. AGE activates NF-κB nuclear translocation by inducing intracellular reactive oxygen species (ROS) generation in cultured endothelial cells (Bierhaus et al., 1997). Increased NF-κB p65 was observed in vascular endothelial cells of diabetic rats (Bierhaus et al., 2001). However, the role of NF-κB in regulating the function of keratinocytes remains unclear.

In the present study, we focused on the possible effects of AGE on skin keratinocytes. We first studied the direct effects of AGE on skin cells by using a rat diabetic model, and then we used a model system of primary rat skin keratinocytes in culture.

In this model system, the dose and time of AGE treatment on cells can be controlled manually and facilitates our observation of the effects of AGE. By *in vivo* and *in vitro* models, we demonstrated the involvement of NF-κB in mediating the effect of AGE in diabetes.

MATERIALS AND METHODS

Experimental animal model

Experimental diabetes (group DM) was induced in male Sprague-Dawley rats (240 to 270 g) by intraperitoneal injection of streptozotocin (STZ; 65 mg/kg, Sigma, USA). Control (group Ctrl) animals were intraperitoneally injected with sodium citrate buffer pH 4.5 followed concurrently. One-Touch Ultra was used for monitoring the blood glucose level of rats and the weights were weighed simultaneously once a week. Animals with plasma glucose concentration >15 mmol/L 1 week post induction of diabetes were included in the study as diabetic. The treatment was maintained for 8 weeks to establish late-stage diabetic model. All experimental procedures were in compliance with the institutional guidelines for the care and use of laboratory animals and conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Cell culture and chemicals

The epidermal specimens from dorsal shaved rats were harvested after 8 weeks post STZ-induction and the keratinocytes were isolated from those specimens. The skins from diabetic and control rats were removed under sterile conditions, cut into 1 cm² and digested by 0.5% dispase at 4°C overnight. The dispase was removed and the skin was digested using 0.25% trypsin. The cells were then maintained in serum-free keratinocyte-SFM medium (Gibco-BRL, Grand Island, NY, U.S.A) supplemented with bovine pituitary extract (30 μg/ml) and antibiotics (penicillin, streptomycin and fungizone). Keratinocytes, cultured into two to six generations were used for the experiments. Pyrrolidine dithiocarbamate (PDTC; Sigma, St Louis, MO, USA), an inhibitor of NF-KB, was used for cell culture to inhibit the activity of NF-kB (0.5×10^{-4} mol/L).

Immunohistochemistry

Rat skin samples were collected from different groups and immediately put into 4% formaldehyde for fixation. Immunohistochemical assays were performed on 5 μM, formalin fixed and paraffin-embedded tissue sections (performed by Sunteambio Biotechnology ShangHai, China) using the streptavidin-biotin supersensitive method. Briefly, 5-μM thick tissue slides were deparaffinized in xylene and rehydrated serially with alcohol and water. Endogenous peroxidase was blocked with 3% hydrogen peroxide for 10 min, followed by microwave antigen retrieval in 10 mM citrate buffer (pH 6.0). The slides were blocked with 2% bovine serum albumin (BSA) in phosphate buffered saline (PBS) for 30 min and incubated overnight with specified diluted rabbit anti-AGE or NF-κB polyclonal antibody (1:50 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight. The LSAB+ kit (Dako, Carpinteria, CA) was used according to the manufacturer's instructions for detection. Normal rabbit purified IgG serum was used instead of first antibody as negative control. Antibody against AGE was purchased from Trans Genic Inc (Japan).

Preparation of AGE-albumin

AGE-albumin was prepared by incubating bovine serum albumin

(BSA) with 800 mmol/L glucose-6-phosphate at 37°C for 8 weeks in 100 mmol/L phosphates (pH 7.4) with 100 µg/ml ampicillin, 50 µg/ml gentamicin and 1.5 mmol/L phenylmethylsulfonyl fluoride (PMSF). At the end of incubation, AGE-albumin preparations were dialyzed against 5 L of 100 mmol/l phosphate and 10 mmol/L ethylenediaminetetraacetic acid (EDTA) for 24 h and 0.9% NaCl for 12 h. Control albumin was also dialyzed against these buffers. The concentration of AGE-albumin was measured by fluorescence spectrophotometry (Unoki et al., 2007).

Preparation of nuclear extracts

Frozen skin was weighed, minced and placed in 10 volumes of cold buffer A (10 mmol/L HEPES, pH 7.9, 10 mmol/L KCl, 0.1 mmol/L EDTA, 0.1 mmol/L ethylene glycol tetraacetic acid (EGTA), 0.1 mmol/L dithiothreitol and 1 mmol/L PMSF). IGEPAL (Sigma Chemical) was added to a final concentration of 0.7%. The homogenates were centrifuged at 7,000 g for 30 s and resuspended in 1 ml fresh buffer A. After centrifugation, the samples were resuspended in 0.3 ml buffer B (20 mmol/L HEPES, pH 7.9, 0.4 mol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L dithiothreitol and 1 mmol/L PMSF) and left on ice for 20 min. Finally, the samples were centrifuged at 10,000 ×g for 30 min and the supernatant removed. Protein concentration was determined by the Bicinchoninic method (Pierce).

Electrophoretic mobility shift assay

Biotin end-labeled double stranded oligonucleotides containing consensus NF-κB binding sites 5′-biotin-AGTTGAGGGGACTTTCCCAGGC-3′ and 5′-biotin-GCCTGGGAAAGTCCCCTCAACT-3′, non-labeled oligonucleotides as well as mutant non-labeled oligonucleotides 5′-AGTTGAGGCGACTTTCCCAGGC-3′ and 5′-biotin-GCCTGGGAAAGTCGCCT CAACT -3′. The binding reactions contained 5 µg of nuclear extract protein, buffer (50 mM KCl, 5 mM MgCl$_2$, 10 mM EDTA, 0.05% Nonidet P-40, and 2.5% glycerol), 1 µg of poly (dI-dC) and 2 nM of biotin-labeled DNA. The binding reactions were incubated at room temperature for 20 min, and the competition reactions were performed by adding 100-fold excess unlabeled double-stranded NF-κB consensus oligonucleotide to the reaction mixture. Then reactions were subjected to electrophoresis on a 6% electrophoresis gel at 100 V for 1 h in 0.5 ×TBE buffer. For supershift assays, nuclear extracts were incubated with antibodies (1 µg) against p65 (C-20) for 30 min at room temperature. The reactions were transferred to a nylon membrane (Pierce). The biotin-labeled DNA was detected with LightShift chemiluminescent electrophoretic mobility shift assay kit (Pierce).

Transmission electron microscopy (TEM)

Cells were fixed in 2.5% glutaraldehyde, post-fixed in 1% osmium tetroxide (OsO$_4$) for 45 to 60 min, dehydrated and embedded in Epoxy resin. Ultrathin sections (60 nm) were cut using a Leica Ultracut UCT (Leica Microsystems Inc, Bannockburn, IL) microtome, and the majority of sections counter-stained with 2% uranyl acetate and lead citrate. TEM sections were viewed and photographed with a HITACH500 transmission electron microscope.

MTT viability assay

To determine the effect of AGE on cell proliferation, MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) cell viability assays were performed. Rat keratinocytes cells were plated

in flat-bottomed 96-well plates at 5×103 cells/well concentration. To determine cell growth, after 48 h, 50 µg MTT (Sigma-Aldrich, USA) was added to each well and the plates were incubated an additional 4 h at 37°C and 5% CO$_2$. After 4 h, the supernatant was removed and the formed crystals were dissolved in 100 µL dimethyl sulfoxide (DMSO). The plates were then analyzed at 570 nm with a microplate reader (Bio-Rad, UK). All assays were performed in quadruplicate and the mean values for each data point was calculated from the combined data.

Cell adhesion assays

Cells were trypsinized and resuspended in culture medium. Same amount of keratinocytes from DM and C group were plated into 6-well plate. After 48 h, the supernatant was removed and adhesive cells were trypsinized and counted. The was calculated as

$$\text{Adhesive rate (\%)} = \frac{\text{Number of adhesive cells}}{\text{Total number}} \times 100$$

Cell migration assays

The modified Boyden Chamber technique was used to quantify skin keratinocytes chemokinesis in response to 24 h incubation with fetal bovine serum (FBS). A 24-well transwell apparatus (Costar, Corning, NY) was used, with each well containing a 6.5-mm polycarbonate membrane with 8-µM pores. Skin keratinocytes were incubated in serum-reduced medium for 24 h before experiments. Cells were trypsinized, resuspended in serum-free medium and plated at a density of 3×104 per membrane. Cells migrating through the membrane to the underside of the apparatus for 24 h were then fixed and stained. The migrated cells were quantified as number of cells migrating per low-power microscopic field.

Enzyme-linked immunosorbent assay (ELISA)

The epidermal growth factor (EGF) concentration of diabetic rat skin and cell culture supernatant was tested using EGF ELISA kit (Sunteambio Biotechnology ShangHai China). Cell EGF consumption is the content in the cell culture medium minus cell culture supernatants content.

Immunofluorescence staining

Cells were fixed with 4% formaldehyde and then permeabilized with PBS containing 0.2% Triton X-100. Slides were blocked by 5% BSA and incubated with a primary antibody at room temperature for 1 h, and then FITC conjugated secondary antibody was added to incubate for another 30 min and observed under microscope. As a negative control, primary specific antibodies were replaced with a control immunoglobulin G (IgG).

Flow cytometry

For cell cycle analysis, cells were trypsinized, washed with PBS and fixed in 70% ice-cold ethanol at 4°C overnight. Afterward, cells were washed with PBS, incubated with 100 µg/ml RNase at 37°C for 20 min and stained with propidium iodide (50 µg/ml). The percentage of cells in different phases of the cell cycle was analyzed using a FACScan flow cytometer. Cell apoptotic rate was measured by Annexin/PI double staining, followed by flow cytometry analysis.

Table 1. Weekly weight and blood sugar of diabetic and control group rats.

Time (week)	Group (number)	Weight (g)	Blood sugar (nmol/ml)
0	C (6)	229.3 ± 3.66	6.97 ± 0.15
	D (6)	228.7 ± 1.33	6.57 ± 0.21
1	C (6)	307.6 ± 5.8	8.3 ± 0.21
	D (6)	271.8 ± 4.67*	25.6 ± 1.12*
3	C (6)	416.6 ± 3.46	6.7 ± 0.2
	D (6)	306.7 ± 12.6*	22.53 ± 1.56*
4	C (6)	468 ± 5.41	7.12 ± 0.17
	D (6)	299.2 ± 20*	27.33 ± 1.44*
5	C (6)	445 ±5.4	6.6 ± 0.3
	D (6)	280.8 ± 17.9*	26.2 ± 1.78*
6	C (6)	492.2 ± 7.36	7 ± 0.2
	D (6)	356 ± 18.35*	H
7	C (6)	518.4 ± 3.64	7.26 ± 0.32
	D (6)	352 ± 19.28*	H

*$p < 0.05$; D, diabetic; C, control; H, above maximum detective limit.

Statistical analysis

Values represented mean ± SD of samples measured in triplicate. Each experiment was repeated 3 times, unless otherwise indicated. The significance of differences between experimental groups was analyzed using the Student's t test and 2-tailed distribution.

RESULTS

Establish animal model of diabetes

One week after treatment, diabetic animals (group DM) showed a significant increase in blood glucose compared with the control group (group Ctrl). The average blood sugar in DM group is 25.6 mmol/ml, whereas 8.3 mmol/ml in the group Ctrl. Diabetic rats also had decreased body weights at the average weight of 271.8 g, while the control animal is 307.56 g. After 8 weeks, rats treated with STZ appeared to have un-reversible skin damaged. Skin thickness in STZ induced rats reduced remarkably than in the control ones (data not shown). Weekly weight and blood sugar in DM and Ctrl group are listed in Table 1. All the rats in diabetic group showed classical symptoms of diabetes- polyurea (frequent urination), polydipsia (increased thirst) and polyphagia (increased hunger). The AGE was accumulated only in skins from DM rats within 4 weeks to 12 weeks but not in skins from normal rats (Figure 1).

***In vivo* effects of accumulated AGE in DM rat skin**

As shown in Figure 2, more NF-kb (p50/p65) nuclear localization was shown in keratinocytes from DM group. The STZ-induced diabetic rat model we built for further study is a late-stage diabetes model which reflects the symptoms of human late-stage diabetic patients. To further confirm the phenomenon we observed in DM rats, we isolated keratinocytes from the control and DM group rats, respectively. The viability (Figure 3A), the adhesive (Figure 3C) and migratory ability (Figure 3D) of the keratinocytes isolated from DM rats were dramatically inhibited compared with that in the control ones. Since EGF is one of the important growth factor in promoting cell growth and differentiation, we next examined the EGF levels in rat skin. We found that EGF level raised remarkably in long term diabetes rats (39.26 ± 0.44 pg/cm^2) compared with the normal skin (37.57 ± 0.71 pg/cm^2) (Figure 3B). In spite of the increase levels of EGF, the proliferation of keratinocytes was inhibited (Figure 3E) with increased apoptotic rate (Figure 3F) in primary cells isolated from DM rats. However, we found increased migration ability in keratinocytes from DM rats in comparison with those from the control rats. For nuclear localization of p65, the effective molecule of NF-κB, was seen in cultured keratinocytes only from DM group by immunofluorescence staining (Figure 3G). This result was further confirmed by EMSA analysis (Figure 3H). Our data suggest that AGE are abundantly
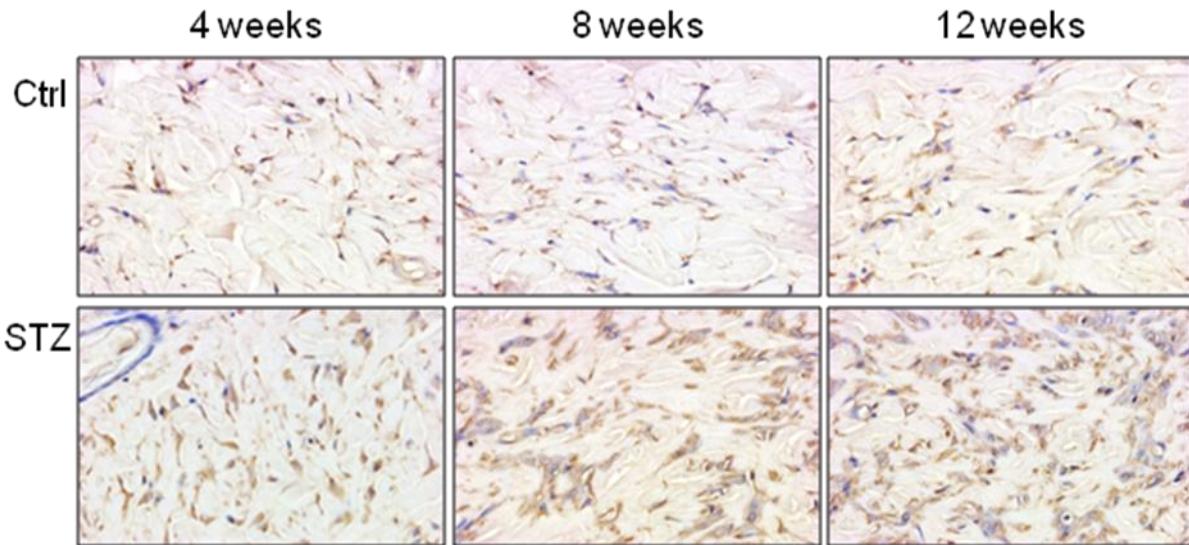
Figure 1. Immunohistochemistry analysis of AGEs in diabetic and control rats. Expression of AGE increased from 4 to 12 weeks after induction by STZ (DM model) compared to sodium citrate (Ctrl). Original magnification × 100.
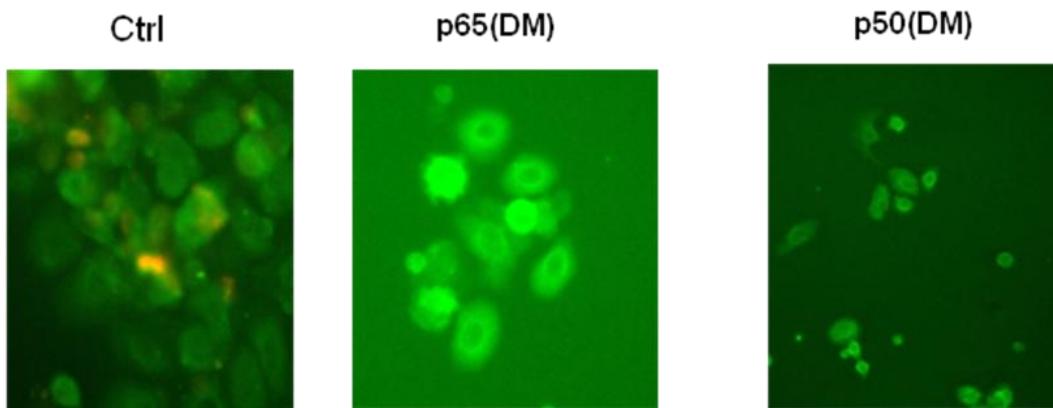

Figure 2. Immunoflourescence showing more p65 and p50 expressed in kerationocyes in skins from rats in control groups and diabetic rates (DM). Original magnification × 200 (Ctrl and p65), × 100 (p50).

accumulated and cause damages in keratinocytes from late-stage diabetic rat. Abnormal activated NF-κB may play roles in AGE caused damages.

Treating normal rat keratinocytes with AGE mimics damages in DM rats

Since complicated phenomenon was found in keratinocytes from DM rats, we next used *in vitro* cell model to reveal the possible mechanism. We previously found inhibited cell growth and cell cycle progression, increased migration ability in keratinocytes from DM rats, and positive staining of AGE in DM rat skin. Based on this and other researches, we speculated that the accumulation of AGE was the key that damaged keratinocytes. To this end, we performed *in vitro* analysis by culturing normal rat keratinocytes with AGE to mimic the damages in keratinocytes in DM rats. As expected, we first observed cell morphological changes 48 h after treatment with different concentration of AGE. Cells became flattered with dark spot in cytoplasm when treated with low dose of AGE (50 and 100 μg/ml) and abundant apoptosis were seen in cells treated with high dose (200 μg/ml) of AGE (Figure 4A). TEM analysis observed apoptosis changes and more euchromatin in nuclear of keratinocytes treated with AGE (Figure 4B). AGE inhibited the growth of normal keratinocytes in a dose- and time-dependent manner (Figure 4C). These results show that *in vitro* cultured normal keratinocytes with AGE could mimic the features that characterized those in DM rats.
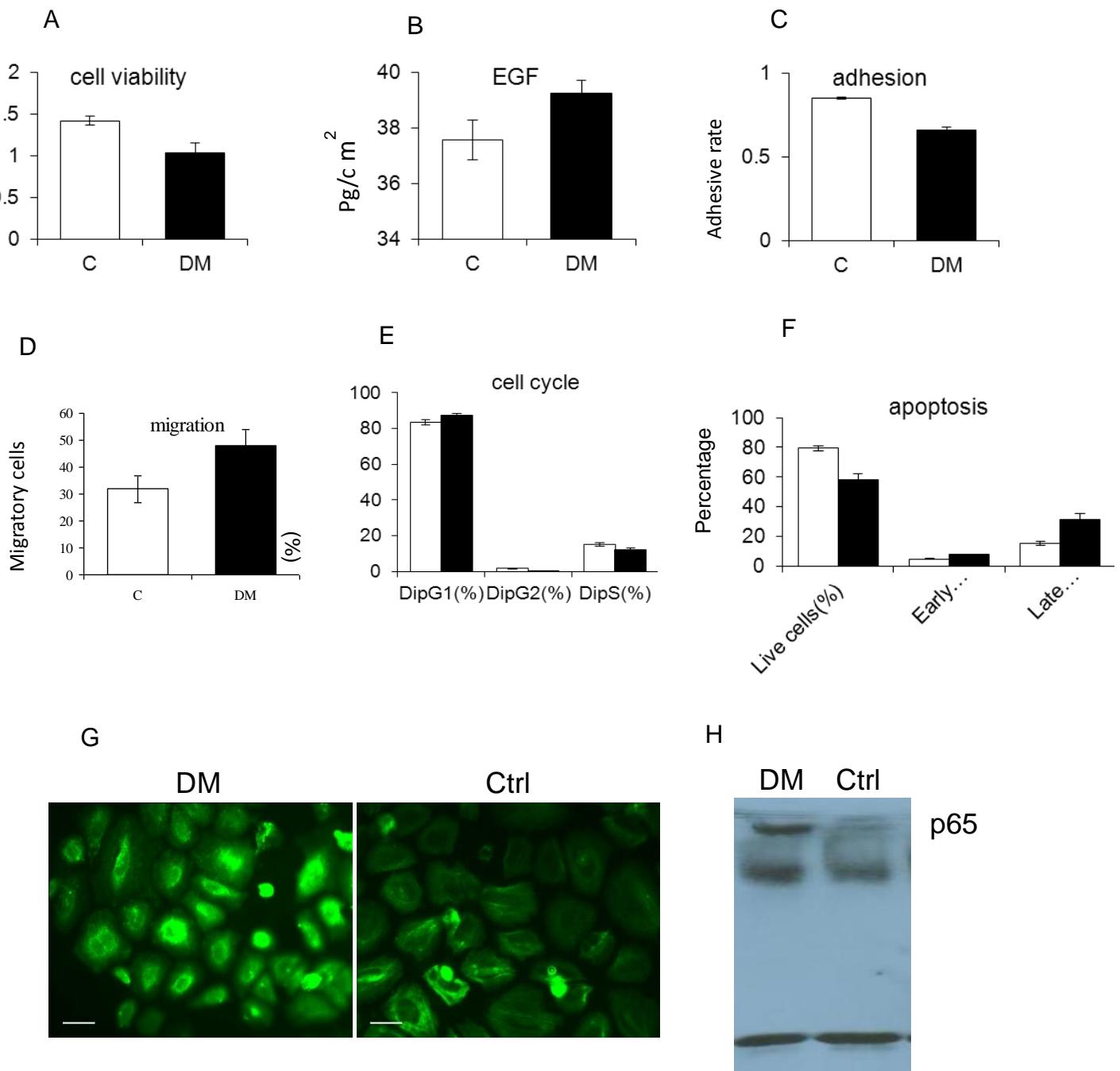
Figure 3. The effect of AGEs on keratinocytes isolated from the control (C) and diabetic (DM) rats. (A) Cell variety was measured by MTT assays; (B) the EGF in skins from different models were measured by ELISA using the same amount of protein isolated from same size skin; (C) cell adhension was mwasured 24 h after plating isolated ketatinocytes from different models; (D) Migration ability of cells isolated from control and diabetic rat skins were analyzed; (E) cell cycle analysis were performed using flow cycometry immediately after isolating cells from tissue; (F) apoptosis was analyzed by Annexin V/PI double staining using cells isolated from skin; (G) p65 immunoflourescence staining was performed using cells isolated from skin after cell attachment; bar 20 μM; (H) EMSA analysis of p65 in DM and Ctrl skin.

NF-κB mediates the effects of AGE on rat keratinocytes

As indicated in DM rat model, NF-κB activation might be the key that mediate the effects of AGE on keratinocytes.

To investigate the mechanism of AGE-induced apoptosis and related alteration in NF-κB activity, we treated normal cells by using AGE with or without NF-κB inhibitor PDTC (Grandjean-Laquerriere et al., 2002). After treatment with PDTC, the inhibition of cell growth was eliminated. By

A
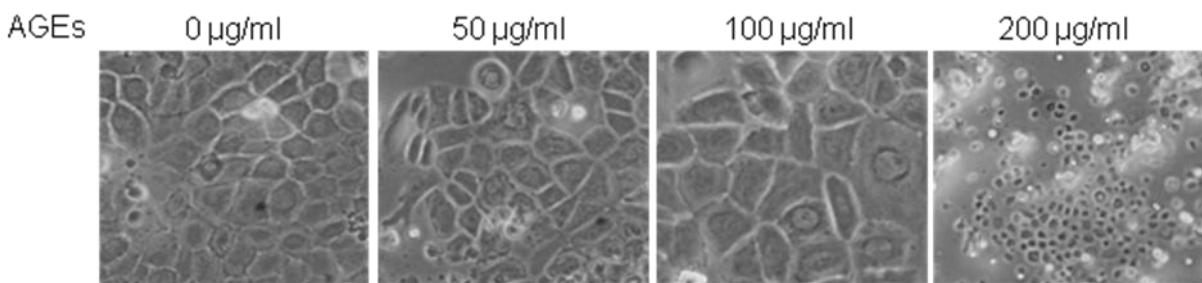
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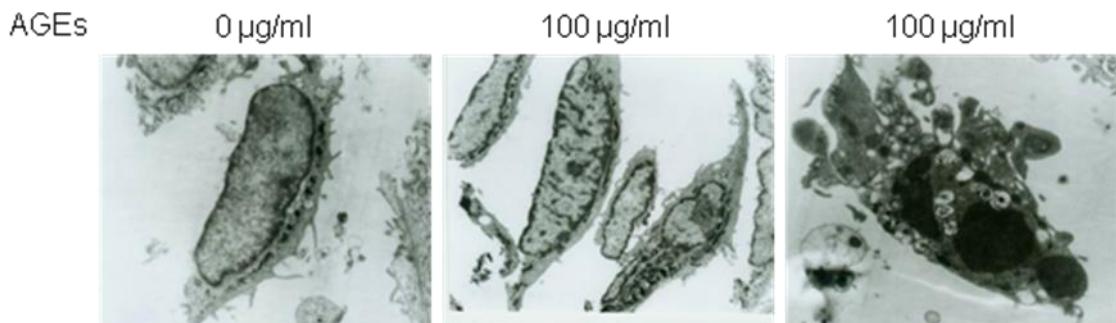
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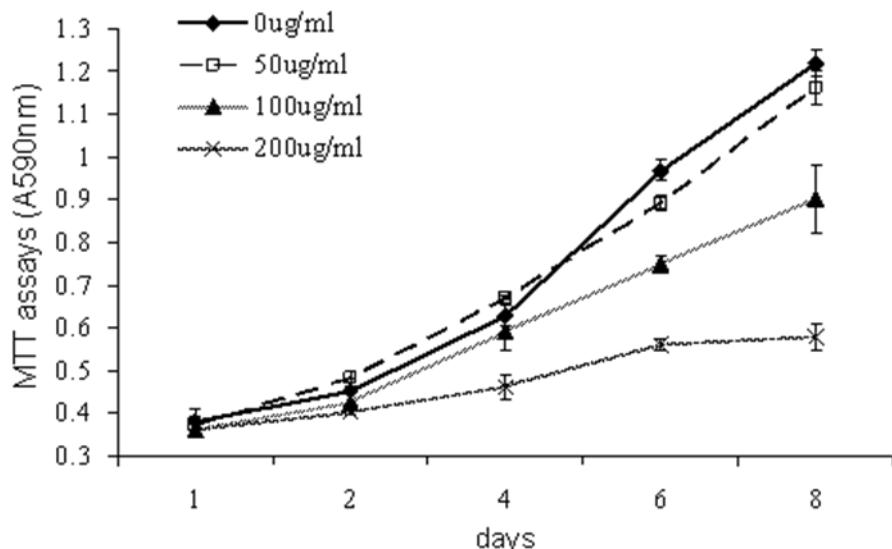
Figure 4. AGEs inhibit growth in cultured normal kerationocytes. (A) Cell morphology changes with or without AGEs treatment were observed under bright field microscope. Original pictures were taken under × 100 magnification. (B) TEM analysis of keratinocytes are shown. (C) Cell growth inhibition by AGEs were measured by MTT assays.

electrophoretic mobility shift assay, we also observed that PDTC pretreatment can prevent the activation of NF-κB induced by AGE (Figure 5A). Reduced consumption of EGF in cells treated with AGE was obtained by inhibiting NF-κB activation using PDTC (Figure 5B). Using flow cytometry-based cell cycle analysis, we also observed that the frequency of cells in G0/G1 phase decreased to 76% and G2/M phase increased to 15% when the cells were cultured in both AGE and PDTC, which was similar to the control groups of keratinocytes treated with PDTC alone (Figure 5C). These results indicate that AGE induced keratinocytes growth was arrested by activation of NF-κB signal pathway. The results showed that 100 µg/ml AGE induced early apoptosis in keratinocytes
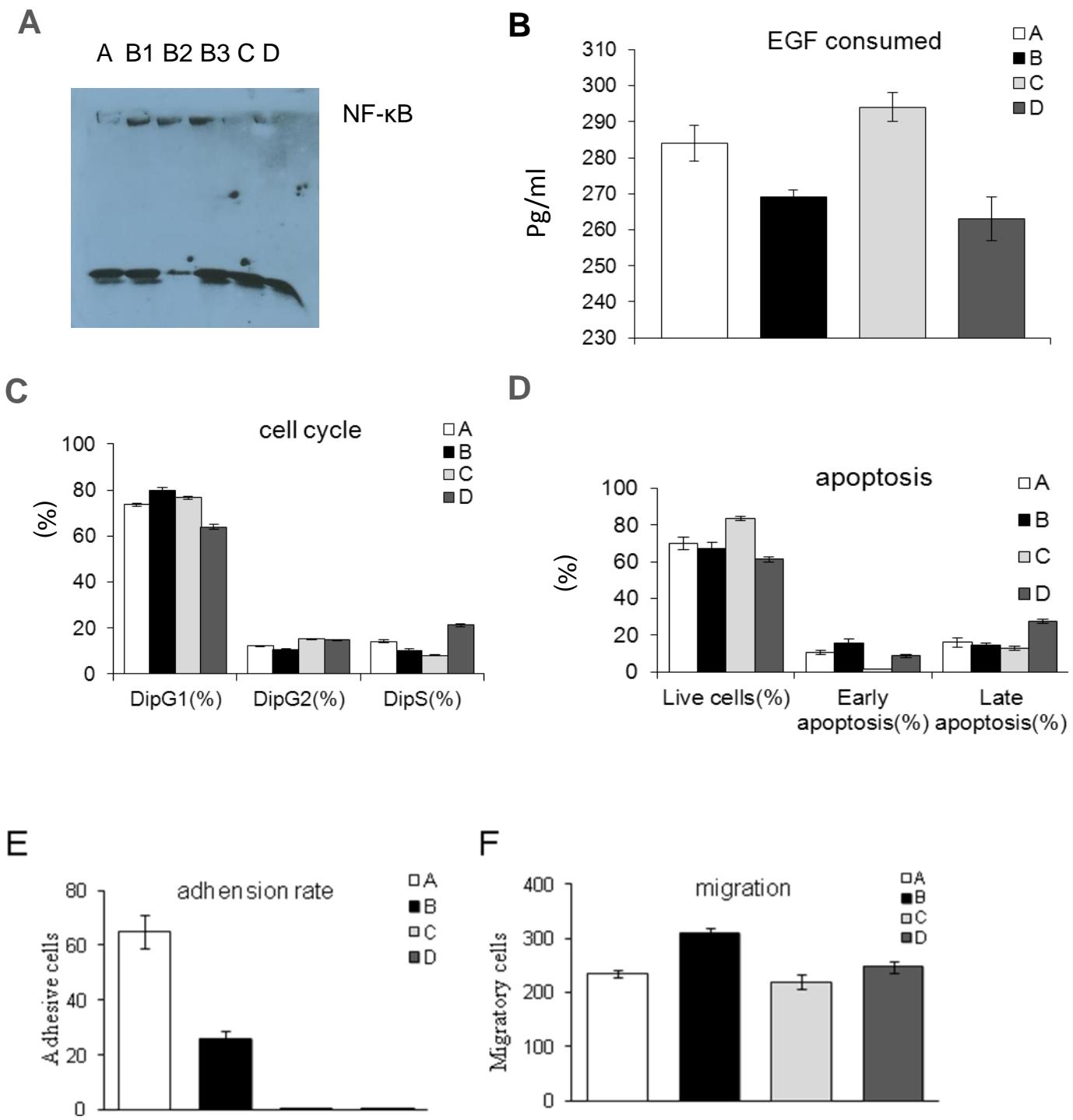
Figure 5. Effects of AGEs on cultured normal keratinocytes. (A) EMSA analysis of p65 activation in control group (group A).

(Figure 5D). The initiation of cell migration is critical to repair mechanisms that restore the normal integrity of cuticular and for the process of wound healing (Usui et al., 2005, 2008).

Therefore, our results clearly indicated that AGE irritated keratinocytes migration by inducing activation of NF-κB (P<0.05). Furthermore, NF-κB inhibitor PDTC attenuated AGE induced irritation of keratinocytes migration (Figure 5F). Surprisingly, the blocked NF-κB activity failed to rescue the adhesive ability by AGE. As a matter of fact, blocking NF-κB activity completely shut down the adhesive ability (Figure 5E). This phenomenon might suggest that the activation of NF-κB played positive role in cell adhesion. Taken together, our *in vitro* data

suggest that NF-κB mediated AGE had effect on cultured normal keratinocytes by both positive and negative way.

DISCUSSION

Hyperglycemia has an important role in the pathogenesis of diabetic complications by increasing protein glycation and the gradual build-up of AGE in body tissues. The formation of AGE has been recognized as an important pathophysiological mechanism in the development of diabetic complications (Miyata and Dan, 2008). Recently, many experimental studies have shown that these harmful effects of AGE may play roles in the pathogenesis of micro- and macrovascular complications of diabetes (Peppa et al., 2003), diabetic neuropathy and impaired wound healing. As one of important repair cells during wound healing, what and how AGE impairs functions of keratinocytes are still unclear. As in our interest, we performed *in vivo* and *in vitro* rat diabetic models to examine the effect of AGE on skin keratinocytes and its possible downstream signaling pathway. We revealed a key molecule NF-κB, as a major effecter of AGE.

In order to confirm the pre-clinical relevance of AGE and diabetic impaired skin would-healing, we first used a STZ-induced diabetic rat as model to reveal whether the AGE was accumulated in skin. We found AGE abundantly accumulated in the skin of diabetic rats after 8-week STZ treatment, but few in the early stage of diabetes (at week 4). This result was same as our previous study, we considered that this is because the formation of AGE is a long-time process and may start damage at late stage of diabetes. This phenomenon actually matches the human diabetes, which showed irreversible damages only at late stage. Furthermore, we observed that during the 8-week treatment, the rat in DM group exhibited similar symptoms as shown in human. These included maintenance of high level of blood sugar, continuing weight loss, increasing amount urine and thinner skin. Eventually, the accumulated AGE will cause irreversible damages on skin and other vital organs. Our rat DM model successfully showed the pathological features of human DM, and thus can be used for further research.

Since wound healing is a complicate process, it consists of four highly integrated and overlapping phases: hemostasis, inflammation, proliferation and tissue remodeling or resolution. Keratinocytes play important role in wound healing, including cell division and cover of the wound surface. Enhanced cellular proliferation several cell diameters away from the edge is associated with wound re-epithelialization (Coulombe, 2003). The formation of AGE under hyperglycemia and the interaction with their receptors (RAGE) are associated with impaired wound healing in diabetic mice (Goova et al., 2001, Huijberts et al., 2008). Our study discovered that AGE inhibited growth, adhesion and induced apoptosis in keratinocytes *in vivo* and *in vitro*. Notably, cell cycle analysis revealed that treating cells with AGE blocked cell from going into mitosis and stayed in G0/G1 phase.

Furthermore, we also found that the inhibition of growth and induction of apoptosis of AGE on cells were dose- and time-dependant. The low dose of AGE short-term could even stimulate growth, but long-term and high dose would inhibit growth and induce apoptosis. AGE is shown to regulate a variety of inflammatory cell responses and growth-promoting events, which to a certain extent occur normally during wound healing (Imani et al., 1993; Bernheim et al., 2001). Excessive AGE, however, could promote overt oxidant stress which could in turn impair normal cellular functions and wound tissue remodeling (Ido et al., 2001). It also explained that the body had the adjustment in order to maintain homeostasis. This may be due to the counterpart mechanism that when AGE is low, the cells can overcome the effect and on the country stimulate growth to help maintain the balance. However, when AGE levels are above the limit, the damage would be sustained. Accordingly, EGF consuming in AGE treated and from DM rat keratinocytes remains low compared with the control. In consistence with former studies (Guo and Dipietro, 2010), our result showed that the usage of AGE treatment revealed another possible mechanism that why EGF is high in patients but the wound still cannot be healed. Our study also suggested that impaired intake and use of growth factors may explain why clinical studies on growth factors have failed to show beneficial effects (Smiell et al., 1999).

There are several cellular functions critical for wound healing, such as proliferation, adhesion and migration. Enhanced keratinocyte proliferation and migration could facilitate wound closure and vice versa. Re-epithelialization is a critical component of wound healing in which keratinocytes become activated to proliferate and migrate as an invading sheet to resurface the wound (Coulombe, 2003). The directed migration of keratinocytes is critical to wound re-epithelialization and defects in this function are associated with the clinical problem of chronic non-healing wounds (Raja et al., 2007). Many factors have been reportedly to affect the migration of keratinocytes towards the wound and inhibit epithelialization (Linke et al., 2010; Brem et al., 2009). In our case, we want to know whether AGE inhibits the adhesion and migration of keratinocytes. Surprisingly, we first found increased migration of cells isolated from late stage DM rats, and further confirmed by *in vitro* culture of normal cells with AGE. Considering the activation of NF-κB in keratinocytes from DM rat skin and cultured normal keratinocytes treated with AGE, we speculated that the activation of NF-κB played a role in positive regulation of migration in DM condition.

The involvement of NF-κB activity is often documented in inflammation and cancer. NF-κB proteins promote cellular proliferation, inhibit apoptosis, and activate

cellular stress responses. The activation of NF-κB is implicated in skin wounds (Hayden and Ghosh, 2004; Hanada and Yoshimura, 2002). The activation of NF-κB in STZ-induced DM rat was also reported (Kameyama et al., 2008; Kelleher et al., 2010). In consistent with previous studies, we found activation of NF-κB in keratinocytes from DM rat and also in normal keratinocytes treated with AGE. The consequences of activation of NF-κB are complicated, neither over- nor under-activated NF-κB activity is good for cell viability and motility. Overexpression of active p65 subunit in transgenic epithelium produces hypoplasia and growth inhibition (Seitz et al., 1998). The activity of proinflammatory transcription factor NF-κB is induced and contributed to cell damage and death in tissues (Kern, 2007). We found nuclear localization of p65 NF-κB in DM rat skin which suggested the NF-κB was activated. To elucidate the role of NF-κB in AGE treated keratinocytes, we used specific NF-κB inhibitor PDTC to down-regulate its activation in cell cultured with AGE. We hypothesized that NF-κB played negative role in response to AGE.

However, *in vitro* study revealed more complicated phenomenon. Increased cell proliferation, decreased early apoptosis, recovery of EGF consuming were observed in cells treated with AGE and PDTC compared to those with AGE. In addition, inhibiting NF-κB activity also decreased adhesion and migration of cells treated with AGE and PDTC compared to those with AGE. These results suggested that activation of NF-κB by AGE might negatively regulate cell proliferation but positively regulate cell mobility. On the contrary, normal keratinocytes showed decreased EGF consumption, increased apoptosis and inhibited cell proliferation when treated with PDTC. Considering that NF-κB activation under AGE treatment is the response of cells to harmful environment, proper levels of NF-κB activity is therefore critical to maintain homeostasis in normal cells. Hence, based on our findings by using *in vitro* and *in vivo* models, it appears that NF-κB activation contributes to cell growth inhibition and increased motility in DM keratinocytes. Thus, optimization NF-κB activity is required during the process of diabetic wound-healing.

ACKNOWLEDGEMENT

REFERENCES

Alikhani ZM, Boyd CM, Nagao K, Trackman PC Graves DT (2005). Advanced glycation end products enhance expression of pro-apoptotic genes and stimulate fibroblast apoptosis through cytoplasmic and mitochondrial pathways. J Biol Chem., 280(13): 12087-95.

Benoliel AM, Kahn-Perles B, Imbert J, Verrando P (1997). Insulin stimulates haptotactic migration of human epidermal keratinocytes through activation of NF-kappa B transcription factor. J Cell Sci., 110 (17): 2089-2097.

Bernheim J, Rashid G, Gavrieli R, Korzets Z, Wolach B (2001). *In vitro* effect of advanced glycation end-products on human polymorphonuclear superoxide production. Eur. J. Clin. Invest. 31(12): 1064-1069.

Bierhaus A, Chevion S, Chevion M, Hofmann M, Quehenberger P, Illmer T, Luther T, Berentshtein E, Tritschler H, Muller M, Wahl P, Ziegler R, Nawroth P. P (1997). Advanced glycation end product-induced activation of NF-kappaB is suppressed by alpha-lipoic acid in cultured endothelial cells. Diabetes, 46(9): 1481-1490.

Bierhaus AS, Schiekofer M, Schwaninger M, Andrassy PM, Humpert J, Chen M, Hong T, Luther T, Henle I, Kloting M, Morcos M, Hofmann H, Tritschler B, Weigle M, Kasper M, Smith G, Perry AM, Schmidt DM, Stern HU, Haring E, Schleicher PP, Nawroth (2001). Diabetes-associated sustained activation of the transcription factor nuclear factor-kappaB. Diabetes, 50(12): 2792-2808.

Brem HA, Kodra MS, Golinko H, Entero O, Stojadinovic VM, Wang CM, Sheahan AD, Weinberg SL, Woo HP, Ehrlich M, Tomic-Canic (2009). Mechanism of sustained release of vascular endothelial growth factor in accelerating experimental diabetic healing. J. Invest. Dermatol., 129(9): 2275-2287.

Coulombe PA(2003). Wound epithelialization: accelerating the pace of discovery. J. Invest. Demerol., 121(2): 219-230.

Friedman EA. (1999) Advanced glycosylated end products and hyperglycemia in the pathogenesis of diabetic complications. Diabetes Care. 22 Suppl 2: B 65-71.

Goova MT, Li J, Kislinger T, Qu W, Lu Y, Bucciarelli LG, Nowygrod S, Wolf BM, Caliste X, Yan SF, Stern DM Schmidt AM (2001). Blockade of receptor for advanced glycation end-products restores effective wound healing in diabetic mice. Am. J. Pathol., 159(2): 513-525.

Grandjean-Laquerriere A, Gangloff SC, Le Naour R, Trentesaux C, Hornebeck W, Guenounou M (2002). Relative contribution of NF-kappaB and AP-1 in the modulation by curcumin and pyrrolidine dithiocarbamate of the UVB-induced cytokine expression by keratinocytes. Cytokine, 18(3): 168-177.

Guo S, Dipietro LA (2010). Factors affecting wound healing. J. Dent. Res., 89(3): 219-229.

Hamuro M, Polan J, Natarajan M, Mohan S (2002). High glucose induced nuclear factor kappa B mediated inhibition of endothelial cell migration. Atherosclerosis, 162(2): 277-287.

Han YP, Tuan TL, Wu H, Hughes M Garner WL (2001). TNF-alpha stimulates activation of pro-MMP2 in human skin through NF-(kappa)B mediated induction of MT1-MMP. J. Cell Sci., 114(1): 131-139.

Hanada T, Yoshimura A (2002). Regulation of cytokine signaling and inflammation. Cytokine Growth Factor Rev. 13(4-5): 413-421.

Hayden MS, Ghosh S (2004). Signaling to NF-kappaB. Genes Dev., 18(18): 2195-2224.

Huijberts MS, Schape NCr Schalkwijk CG (2008). Advanced glycation end products and diabetic foot disease. Diabetes Metab. Res. Rev., 24 Suppl 1: S19-24.

Ido Y, Chang KC, Lejeune WS, Bjercke RJ, Reiser KM, Williamson JR Tilton RG. (2001). Vascular dysfunction induced by AGE is mediated by VEGF via mechanisms involving reactive oxygen species, guanylate cyclase, and protein kinase C. Microcirculation, 8(4): 251-263.

Imani F, Horii Y, Suthanthiran M, Skolnik EY, Makita Z, Sharma V, Sehajpal P, Vlassara H (1993). Advanced glycosylation endproduct-specific receptors on human and rat T-lymphocytes mediate synthesis of interferon gamma: role in tissue remodeling. J. Exp. Med., 178(6): 2165-2172.

Kameyama N, Arisawa S, Ueyama J, Kagota S, Shinozuka K, Hattori A, Tatsumi Y, Hayashi H, Takagi K, Wakusawa S (2008). Increase in P-glycoprotein accompanied by activation of protein kinase Calpha and NF-kappaB p65 in the livers of rats with streptozotocin-induced diabetes. Biochim Biophys Acta., 1782(5): 355-360.

Kelleher AR, Fairchild TJ Keslacy S (2010). STZ-induced skeletal muscle atrophy is associated with increased p65 content and downregulation of insulin pathway without NF-kappaB canonical cascade activation. Acta Diabetol., 47(4): 315-323.

Kern TS (2007). Contributions of inflammatory processes to the development of the early stages of diabetic retinopathy. Exp. Diabetes Res., 2007: 95103.

Linke A, Goren I, Bosl MR, Pfeilschifter J, Frank S (2010). The suppressor of cytokine signaling (SOCS)-3 determines keratinocyte proliferative and migratory potential during skin repair. J. Invest. Dermatol., 130(3): 876-885.

Meng J, Sakata N, Imanaga Y, Takebayashi S, Nagai R, Horiuchi S (2001). Carboxymethyllysine in dermal tissues of diabetic and nondiabetic patients with chronic renal failure: relevance to glycoxidation damage. Nephron, 88(1): 30-35.

Miyata T, Dan T (2008). Inhibition of advanced glycation end products (AGEs): an implicit goal in clinical medicine for the treatment of diabetic nephropathy? Diabetes Res. Clin. Pract., 82(1): S25-29.

Peppa M, Brem H, Ehrlich P, Zhang JG, Cai W, Li Z, Croitoru A, Thung S, Vlassara H (2003). Adverse effects of dietary glycotoxins on wound healing in genetically diabetic mice. Diabetes, 52(11): 2805-2813.

Porte Jr D, Schwartz MW (1996). Diabetes complications: why is glucose potentially toxic? Science, 272(5262): 699-700.

Raja K, Sivamani M, Garcia S, Isseroff RR (2007). Wound re-epithelialization: modulating keratinocyte migration in wound healing. Front Biosci., 12: 2849-2868.

Seitz CS, Deng H, Hinata K, Lin Q, Khavari PA (2000). Nuclear factor kappaB subunits induce epithelial cell growth arrest. Cancer Res., 60(15): 4085-4092.

Seitz CS, Lin Q, Deng H, Khavari PA (1998). Alterations in NF-kappaB function in transgenic epithelial tissue demonstrate a growth inhibitory role for NF-kappaB. Proc. Natl. Acad. Sci. USA, 95(5): 2307-2312.

Sensi M, Pricci F, Pugliese G, De Rossi M, Petrucci AFG, Cristina A, Morano S, Pozzessere G, Valle E, Andreani D Mario UD (1995). Role of advanced glycation end-products (AGE) in late diabetic complications. Diabetes Res. Clin. Pract., 28(1): 9-17.

Smiell JM, Wieman TJ, Steed DL, Perry BH, Sampson AR, Schwab BH (1999). Efficacy and safety of becaplermin (recombinant human platelet-derived growth factor-BB) in patients with nonhealing, lower extremity diabetic ulcers: a combined analysis of four randomized studies. Wound Repair Regen., 7(5): 335-346.

Stitt AW, McGoldrick C, Rice-McCaldin A, McCance DR, Glenn JV, Hsu DK, Liu FT, Thorpe SR Gardiner TA(2005). Impaired retinal angiogenesis in diabetes: role of advanced glycation end products and galectin-3. Diabetes, 54(3):785-794.

Unoki H, Bujo H, Yamagishi S, Takeuchi M, Imaizumi T Saito Y (2007). Advanced glycation end products attenuate cellular insulin sensitivity by increasing the generation of intracellular reactive oxygen species in adipocytes. Diabetes Res. Clin. Pract., 76(2): 236-244.

Usui ML, Mansbridge JN. Carter WG, Fujita M. Olerud JE (2008). Keratinocyte migration, proliferation, and differentiation in chronic ulcers from patients with diabetes and normal wounds. J. Histochem. Cytochem., 56(7): 687-696.

Usui ML, Underwood RA, Mansbridge JN, Muffley LA,. Carter WG Olerud JE (2005). Morphological evidence for the role of suprabasal keratinocytes in wound reepithelialization. Wound Repair Regen., 13(5): 468-479.

Verma IM, Stevenson JK, Schwarz EM, Van Antwerp D Miyamoto S (1995). Rel/NF-kappa B/I kappa B family: intimate tales of association and dissociation. Genes Dev., 9(22): 2723-2735.

Wautier J, Guillausseau P (1998). Diabetes, advanced glycation endproducts and vascular disease. Vasc. Med., 3(2): 131-137.

Wondrak GT, Roberts MJ, Jacobson MK Jacobson EL (2002). Photosensitized growth inhibition of cultured human skin cells: mechanism and suppression of oxidative stress from solar irradiation of glycated proteins. J. Invest. Dermatol., 119(2): 489-498.