Advanced Glycation End Products (AGEs) Induce Apoptosis of Fibroblasts by Activation of NLRP3 Inflammasome via Reactive Oxygen Species (ROS) Signaling Pathway

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Background: Type 2 diabetes impairs the healing process and induces apoptosis of fibroblasts, which are thought to be involved in this process. We investigated the possible mechanisms involved in AGEs-induced apoptosis of human dermal fibroblasts.

Material/Methods: We examined the expression of apoptosis-related proteins in fibroblasts isolated from human diabetic wounds. Human dermal fibroblasts exposed to AGEs were used to study the links among apoptosis, ROS, and NLRP3 inflammasome activation. Signaling mechanisms were evaluated by preincubating the cells with appropriate inhibitors. Cleaved caspase-8, cleaved caspase-3, BAX, Bcl-2, and NLRP3 inflammasome expression were measured by Western blot analysis. ROS generation, cell viability, and cell apoptosis were assessed.

Results: We observed a higher level of cleaved caspase-8 and cleaved caspase-3 expression in fibroblasts isolated from human diabetic wounds compared with controls. AGEs decreased the proliferation of cells in a concentration-dependent and time-dependent manner. The exposure of fibroblasts to AGEs significantly increased the number of cells in early and late apoptosis stages. AGEs-induced human dermal fibroblasts showed high expressions of cleaved caspase3, cleaved caspase8, and Bax. Treatment with AGEs induced the expression of NLRP3, caspase-1, and ASC. AGEs-induced apoptosis was blocked by BAY 11-7082, an inhibitor of the NLRP3 inflammasome. AGEs increased the production of ROS in fibroblasts, and its apoptogenic effect was blocked by NAC.

Conclusions: AGEs cause apoptosis of fibroblasts by inducing the generation of ROS and activating the NLRP3 inflammasome. In vivo experiments are needed to confirm these results.

MeSH Keywords: Apoptosis • Fibroblasts • Glycosylation End Products, Advanced • Inflammasomes • Reactive Oxygen Species

Abbreviations: AGEs – advanced glycation end products; ROS – reactive oxygen species; ASC – the adapter molecule apoptosis-associated speck-like protein containing a caspase recruitment domain; BAX – Bcl2-associated X protein; Bcl-2 – B cell leukemia 2; BSA – bovine serum albumin; NAC – N-Acetyl-L-cysteine; PAMPs – pathogen-associated molecular patterns; DAMPs – damage-associated molecular patterns

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**Background**

Diabetes is a public health problem of considerable magnitude. The characteristic element is high blood glucose concentrations promoting loss of insulin-producing pancreatic β-cells, which is type 1 diabetes, or through loss of insulin responsiveness in its target, like adipose and muscle tissue, which is type 2 diabetes [1]. Macro- and microvascular complications are common in long-standing type 2 diabetes and reduce the quality of life and life expectancy of patients [2]. Impaired wound healing is a serious complication of diabetes, but the mechanism by which type 2 diabetes impairs wound healing remains unknown. Skin wound healing requires a series of coordinated processes, including fibroblast cell proliferation and migration. Accumulating evidence has indicated that the mechanism of impaired diabetic wound healing is associated with increased fibroblast apoptosis [3].

In recent years, substantial attention has been paid to the role that advanced glycation end products (AGEs) might play in the pathophysiology of diabetes mellitus [4]. AGEs are a group of heterogeneous compounds formed by irreversible adducts from glucose-protein condensation reactions, as well as lipids and nucleic acids exposed to reducing sugars [5]. In animal models, AGEs have been linked to many diabetic complications, including diabetes-associated nephropathy, retinopathy, neuropathy, and impaired dermal healing [6]. Zhu et al. [7] also reported that increased AGEs concentrations impair wound healing and re-epithelialization in mice.

AGEs increase the formation of intracellular reactive oxygen species (ROS). Elevated and sustained ROS have been observed in vivo and have been associated with impaired wound repair in chronic non-healing wounds [8]. Excessive ROS production in cells causes the loss of cellular homeostasis, oxidative stress, and eventual cell destruction in organs [9]. Thus, excessive ROS generation clearly is a major potential mechanism for the development of impaired wound healing, possibly via the apoptosis of fibroblasts or their precursors.

Strong associations of chronic organ injury with dysregulated inflammasome activity highlight the importance of the inflammasome in regulating immune responses [10]. Inflammasomes are multiprotein oligomers that respond to inflammatory stimuli by initiating an intracellular inflammatory cascade [11]. Numerous inflammasomes have been identified, including NLRP1, NLRP2, NLRP3, double-stranded DNA (dsDNA) sensors absent in melanoma 2 (AIM2), and NLRC4 [12]. The most extensively studied inflammasome is NLRP3 inflammasome, a multiprotein complex consisting of the NOD-like receptor NLRP3, the adapter molecule apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), and procaspase-1 [13]. The NLRP3 inflammasome senses endogenous and exogenous dangers, such as LPS and high glucose, resulting in the activation of caspase-1, followed by activation of cytokines IL-1β/IL-18 [14]. ROS play a critical role in this process [15]. Previous studies have demonstrated that the activation of ASC induces caspase-8-dependent apoptosis in human cancer cell lines [16]. Apoptotic cell death, the major form of cell death during development, involves cell shrinkage, nuclear condensation and fragmentation, membrane blebbing, and exposure of phosphatidylserine on the outer membrane leaflet as a phagocytic stimulus [17]. Therefore, we hypothesized that AGEs induce caspase-mediated apoptosis by activating the ROS/NLRP3 inflammasome in human dermal fibroblasts.

Although previous studies have reported activation of the NLRP3 inflammasome and IL-1β secretion in wounds of humans [18] and diabetic mice [11], the role of NLRP3 inflammation in the induction of cell apoptosis is unclear. In this study, we tested the hypothesis that ROS generation activates NLRP3 inflammasome signaling to promote caspase-8/3-dependent apoptosis in AGEs-induced fibroblasts.

**Material and Methods**

**Preparation of AGEs**

BSA (25 mg/ml) was incubated under sterile conditions with 0.1 M glyceraldehyde in 0.2 M NaPO₄ buffer (pH = 7.4) for 7 days. Unincorporated sugars were removed by PD-10 column chromatography and dialysis against phosphate-buffered saline. Control nonglycated BSA was incubated in the same conditions except for the absence of reducing sugars. Preparations were tested for endotoxin using the Endospecy ES-20S system (Seikagaku Co., Tokyo, Japan); no endotoxin was detectable. The extent of chemical modification was determined as described with 2,4,6-trinitrobenzenesulfonic acid as a difference in lysine residues of modified and unmodified protein preparations [19]. The extent of lysine modification (%) of modified BSA preparations was 65% for AGEs-BSA.

**The experimental specimens and cell culture**

The experimental specimens were obtained from the Shanghai Sixth People’s Hospital affiliated to Shanghai Jiao Tong University. The study protocol was approved by the Ethics Review Board of Shanghai Sixth People’s Hospital affiliated to Shanghai Jiao Tong University. Human dermal fibroblasts were obtained from patients without diabetes who underwent plastic surgery, and were used as an experimental control (nFB) group. Patients were 18 to 60 years of age and did not have any known comorbid malignancy or history of radiation or chemotherapy. We also isolated fibroblasts from chronic human diabetes wounds (dFB). We included type 2
diabetic patients with chronic wounds on the lower leg that had lasted at least 3 months. During sharp debridement, biopsies were taken from inflamed skin located near the center of the wound. Written informed consent was obtained from all of the enrolled participants.

The isolation and primary culture of human dermal fibroblasts were performed as described previously [20]. Briefly, skin was washed 3 times in PBS containing 1% penicillin and streptomycin sulfate, then cut into small (2x2 mm) pieces and digested with 1 mg/mL type II collagenase at 4°C for 16 h. The epidermis and subcutaneous tissues were removed from the dermis, and the dermis was finely minced. The tissue was later digested with 2.5 mg/mL trypsin for 15 min and placed in 10-cm cell culture dishes (pre-coated with FBS, HyClone) in an atmosphere of 5% CO₂ at 37°C. Dulbecco's modified Eagle’s medium (DMEM) containing 5.5 mM D-glucose, 10% fetal bovine serum (FBS), 1% penicillin, streptomycin sulfate, and 2 mM L-glutamine was used as the growth medium. The medium was replaced every 3 days, and on day 7 of culture. Cells were passaged by trypsinization. Cells at passages 2 to 3 were used for all of our experiments.

**Cell treatment protocol**

For stimulation, cells were incubated with 100 μg/mL, 200 μg/mL, or 300 μg/mL AGEs for 72 h. For treatment, cells were pre-treated with BAY 11-7082 (10 μM) or NAC (10 mM) for 4 h, and then incubated with 300 μg/mL AGEs for 72 h. Protein expression in lysates was analyzed by Western blotting using antibodies specific for NLRP3, ASC, Caspase-1, cleaved Caspase-3, Bax, Bcl-2, and cleaved Caspase-8. The Cellular Reactive Oxygen Species Detection Assay Kit was used for intercellular ROS assay. Cell viability and cell apoptosis were assessed.

**Cell viability analysis**

Cell viability was determined using the Cell Counting Kit-8 (CCK-8; Dojindo, CK04) according to the manufacturer's protocol [21]. Cells were digested into cell suspension and the concentration of the cells was adjusted to 1x10⁵/mL. Cells were seeded in 96-well plates and incubated for 24 h. We examined cell viability at 0, 24, 48, and 72 h after AGEs treatment. Cells were exposed to 10 ml of CCK-8 solution for 4 h at 37°C. Subsequently, the optical density was measured at 450 nm using a microplate reader.

**Cell apoptosis analysis**

Apoptosis was assessed using flow cytometry (FACS Calibur, BD Biosciences, USA) with the Annexin V: FITC Apoptosis Detection Kit II (BD Biosciences) according to the kit instructions [22]. The cells were washed with Dulbecco's phosphate-buffered saline (PBS, pH=7.4) twice (2000×g, 5 min), resuspended in 500 μL binding buffer with 5 μL Annexin V-FITC in the dark for 10 min, and then stained with 5 μL propidium iodide (PI) for 5 min. Apoptosis was analyzed at an excitation wavelength of 488 nm using the FL1 channel for Annexin V-FITC and the FL2 channel for PI.

**Western blot analysis**

Total protein was extracted from the cells by using M-PER mammalian protein extraction reagent (Pierce, IL, USA) and estimated using a bicinchoninic acid protein assay kit (Pierce). Equal amounts of 15 μg protein were loaded onto (11%) SDS-PAGE gels and transferred onto nitrocellulose membranes using a wet-style transfer tank. The membranes were incubated with multiple antibodies, including antibodies against NLRP3 (1: 500) (Abcam, Cambridge, UK), ASC (1: 400), Caspase-1 (1: 400), Caspase-3 (1: 250), Caspase-3 p17 (1: 600), Bax (1: 350), Bcl-2 (1: 400), Caspase-8 (1: 600), Caspase-8 p18 (1: 400), and β-actin (1: 1200) (Santa Cruz, CA, USA), followed by the secondary HRP-conjugated anti-rabbit/mouse antibody (Santa Cruz). After washing with Tris-buffered saline Tween (TBST), the bands were detected by chemiluminescence (ECL) and imaged with X-ray films. β-actin was used as an endogenous reference for normalization.

**Measurement of ROS production**

Cellular ROS generation was measured with the Cellular Reactive Oxygen Species Detection Assay Kit (Red Fluorescence), which was purchased from Abcam (Cambridge, UK) [23]. Cells were seeded at 1×10⁵ cells/100 μL per well in a 96-well plate. ROS Red Working Solution (100 μL) was added and incubated in a 37°C/5% CO₂ incubator for 60 min. Cells were then treated with test compounds for 60 min to induce ROS production. An increase in the fluorescence intensity at an excitation wavelength of 535 nm and an emission wavelength of 590 nm was monitored in bottom read mode.

**Statistical analysis**

Data are presented as the mean ±SD and were processed using SPSS 16.0 statistical software. Statistical significance was determined using the t test for 2 experimental groups. For more than 2 groups, the statistical evaluation of data was performed using one-way analysis of variance (ANOVA). For all tests, a P value <0.05 was considered to be significant.
Results

Apoptosis analysis in human diabetic wound isolated fibroblasts

We first examined the apoptosis-related protein expression in human diabetic wound isolated fibroblasts. Higher levels of cleaved caspase-8 and cleaved caspase-3 expressions were observed in wounds of diabetic patients compared with the nFB group (P<0.05) (Figure 1A, 1D, 1I). We also examined the expression of Bcl-2 and Bax, and observed higher levels of Bax with lower levels of Bcl-2 when comparing human diabetic wound isolated fibroblasts (dFB group) with the nFB group (P<0.05) (Figure 1B, 1C, 1I).

AGES induces apoptosis in human dermal fibroblast

We treated cells with different concentrations of AGEs for different periods of time (0, 24, 48, and 72 h) and later evaluated cell viability with the CCK-8 assay to determine the effects of AGEs on fibroblasts. The results showed that AGEs decreased the proliferation of cells in a concentration-dependent manner (Figure 2A). AnnexinV-FITC/PI staining combined with flow cytometric analysis was used to test the influence of AGEs on cell apoptosis. The exposure of fibroblasts to AGEs significantly increased the number of cells in early and late apoptosis stages (Figure 2D). We also examined apoptosis-related proteins in AGES-induced human dermal fibroblasts. AGEs-induced human dermal fibroblasts showed high expressions of cleaved caspase3, cleaved caspase8, and Bax (Figure 1A, 1B, 1D, 1I). AGEs promoted the expression of cleaved caspase3, cleaved caspase8, and Bax in a concentration-dependent manner. Decreased levels of Bcl-2 were also observed in AGES-induced human dermal fibroblasts (Figure 1C, 1I).

AGES induce NLRP3 inflammasome activation and ROS generation

We accessed the expression of NLPR3 inflammasome and ROS generation in AGES-induced human dermal fibroblasts and human diabetic wound isolated fibroblasts. In these cells, we observed a concentration-dependent increase in expression of NLPR3, caspase-1, and ASC (Figure 1E–1G, 1I). We also observed a higher level of ROS generation in AGES-induced human dermal fibroblasts and human diabetic wound isolated fibroblasts (Figure 1H).

AGES-induced cell apoptosis is dependent on NLRP3 inflammasome activation

To further investigate the necessity of NLRP3 in enhanced cytokine secretion, NLPR3 expression was inhibited by BAY 11-7082 in fibroblasts. After the inhibitor treatment, a significant reduction in NLRP3 inflammasome protein expression level was found (Figure 3E–3H). Pretreatment of AGES-induced cells with the NLRP3 inhibitor significantly reduced the pro-apoptotic proteins caspase-8, caspase-3, and Bax expression but increased the expression of the anti-apoptotic protein Bcl-2 (Figure 3A–3D, 3H). We evaluated cell viability with the CCK-8 assay to determine the effects of NLRP3 inflammasome inhibitor on AGES-induced human dermal fibroblasts (Figure 2B). The results show that BAY 11-7082 treatment promoted cell proliferation in a time-dependent manner compared with the group that did not receive BAY 11-7082. Flow cytometric analysis showed a lower apoptosis rate in the BAY 11-7082 pre-treatment group when compared to the AGES alone treatment group (Figure 2D). These results suggest that the promotion of apoptosis in AGES-induced fibroblast was mediated by NLRP3 activation.

ROS-mediated NLRP3 inflammasome activation is involved in AGES-induced cell apoptosis in fibroblasts

Intracellular reactive oxygen species (ROS) play a critical role in different types of cell survival. When these cells were treated with 5 mM NAC (ROS scavenger), the elevation in the protein expression levels of NLPR3, ASC, and caspase-1 induced by AGES in fibroblast was obviously eliminated (Figure 4E–4G, 4I). Pretreatment with 5 mM NAC reversed the effect of AGES on the expression of apoptosis-related molecules. NAC pretreatment significantly decreased the expression of the pro-apoptotic proteins Bax, caspase-3, and caspase-8, but increased the expression of the anti-apoptotic protein Bcl-2, compared with the AGES alone treatment group (Figure 4A–4D, 4I). Furthermore, NAC treatment promoted cell proliferation in a time-dependent manner when assessed using the CCK-8 assay and compared to the AGES alone treatment group (Figure 2C). Pretreatment of NAC decreased the apoptotic population of fibroblasts as determined by flow cytometric analysis (Figure 2D). These results suggest that intracellular ROS plays an important role in AGES-induced inflammasome activation and apoptosis in fibroblasts.

Discussion

In response to hyperglycemia in patients with diabetes, various metabolic mechanisms contribute to the pathogenesis of diabetic complications [24]. The close participation of AGES in impaired diabetic wound healing has been well documented. One mechanism through which AGES may affect pathological processes is by enhanced apoptosis, as supported by in vitro studies [25]. However, the mechanisms by which AGES lead to apoptosis are unclear. In the present study, we observed that AGES significantly induced caspase-8/3-dependent apoptosis in fibroblasts. The ROS generation and NLRP3 inflammasome activation were upregulated by AGES treatment. Moreover,
Figure 1. Protein expression, ROS production, and cell viability in AGEs-induced human dermal fibroblasts and human diabetic wound isolated fibroblasts. (A) Protein expression of cleaved Caspase-3 and Caspase-3. (B) Protein level of Bax. (C) Protein level of Bcl-2. (D) Protein expression of cleaved Caspase-8 and Caspase-8. (E) Protein level of NLRP3. (F) Protein level of ASC. (G) Protein level of Caspase-1. (H) ROS production was measured with fluorometry. (I) Protein expression of Bax, Bcl-2, NLRP3, ASC, and Caspase-1 were determined by Western blot. nFB – human dermal fibroblasts; dFB – fibroblasts isolated from diabetic wounds. * p<0.05 vs. control (nFB).
AGEs-induced apoptosis was significantly suppressed by pretreating the cells with NLPR3 inhibitors. ROS inhibition showed similar inhibitory effects on apoptosis. Our results indicate that AGEs-induced apoptosis of fibroblasts might be the result of ROS generation and NLRP3 activation.

Wound healing requires the coordination of several cell types, including keratinocytes, fibroblasts, endothelial cells, and inflammatory cells [26]. Fibroblasts are the primary cellular component of connective tissues, and fibroblast cell proliferation and migration play key roles in the formation of new extracellular matrix and further wound repair [27]. This orderly progression of the healing process is severely impaired in diabetic patients, and high glucose is thought to be the primary reason for delayed wound healing [20]. The formation of AGEs is enhanced by hyperglycemia and participates in many pathological processes of diabetes. Previous studies have extensively investigated the role of AGEs in the induction of apoptosis in various cell types [28,29]. In our study, pretreatment with AGEs decreased the proliferation of fibroblasts in a concentration-dependent and time-dependent manner. We also observed high expression of cleaved caspase8, cleaved caspase3, and...
Figure 3. The role of BAY 11-7082 treatment in AGEs-induced human dermal fibroblasts. (A) Protein expression of cleaved Caspase-3 and Caspase-3. (B) Protein level of Bax. (C) Protein level of Bcl-2. (D) Protein expression of cleaved Caspase-8 and Caspase-8. (E) Protein level of NLRP3. (F) Protein level of ASC. (G) Protein level of Caspase-1. (H) Protein expression of Bax, Bcl-2, NLRP3, ASC, and Caspase-1 were determined by Western blot. nFB – human dermal fibroblasts. * p<0.05 vs. control (nFB+AGE).
Figure 4. The role of NAC treatment in AGEs-induced human dermal fibroblasts. (A) Protein expression of cleaved Caspase-3 and Caspase-3. (B) Protein level of Bax. (C) Protein level of Bcl-2. (D) Protein expression of cleaved Caspase-8 and Caspase-8. (E) Protein level of NLRP3. (F) Protein level of ASC. (G) Protein level of Caspase-1. (H) ROS production was measured with fluorometry. (I) Protein expressions of Bax, Bcl-2, NLRP3, ASC, and Caspase-1 were determined by Western blot. nFB – human dermal fibroblasts. * p<0.05 vs. control (nFB+AGE)
Bax in AGEs-induced human dermal fibroblasts. These results are in agreement with our in vivo findings that patients diagnosed with diabetic wounds have upregulated expressions of cleaved caspase8, cleaved caspase3, and Bax proteins compared to normal controls. This connection between our in vitro results and those obtained in diabetic wound patients supports the notion that diabetes has an adverse effect on the proliferation of fibroblasts.

Previously, it has been shown that sustained NLRP3 inflammasome activity in wounds of diabetic humans and mice contributes to the persistent inflammatory response and impaired healing characteristic of these wounds [30]. Besides the role of the NLRP3 inflammasome in the development of lytic pyroptotic death via activation of caspase-1, the present study shows that caspase-8/3-dependent apoptosis is an additional pathway resulting from inflammasome activation. Numerous lines of evidence have indicated that endogenous molecules from cell metabolism or the degradation of the extracellular matrix trigger inflammation, acting as ‘danger signals’ called damage-associated molecular patterns (DAMPs) [31]. We proposed that AGES in diabetes are a class of DAMPs that activate the NLRP3 inflammasome in a manner similar to that found in other chronic inflammatory diseases. Caspase-8 and caspase-3 were seen with the NLRP3 inflammasome in response to AGES stimulation. The involvement of the NLRP3 inflammasome in AGES-induced fibroblast apoptosis was shown by enhanced NLRP3 inflammasome activity and through inhibition of apoptosis with a specific NLRP3 inhibitor.

Several factors lead to NLRP3 inflammasome activation, including pathogen-associated molecular patterns (PAMPs), damage-associated molecular patterns (DAMPs), other exogenous invaders, or environmental stress. ROS has been identified to play a critical role in this process. All known PAMPs and DAMPs, including the NLRP3 activators mentioned above, such as AGES or high glucose, trigger the production of ROS, which leads to activation of the NLRP3 inflammasome [32]. ROS arises from the accumulation of damaged ROS-generating mitochondria. Environmental stresses such as high glucose, lipid peroxidation, and protein oxidation can dramatically increase ROS production, resulting in oxidative stress, cell damage, and death [23]. Mitochondrial dysfunction has been shown to participate in the induction of apoptosis and has even been described as playing a key role in the apoptotic pathway [33]. In this study, the exposure of fibroblasts to AGES significantly increased the ROS Red Dye fluorescence and showed increased intracellular ROS production in parallel with increased apoptosis. Furthermore, our data indicated that an ROS scavenger, NAC, can significantly prevent NLRP3 inflammasome activation and cell apoptosis during AGES stimulation. Many of the studies showing that AGES induce the apoptosis of fibroblasts have documented increased ROS production in the treated cells. To the best of our knowledge, our results are the first to demonstrate that ROS generation induced by AGES results in the apoptosis of fibroblasts through activation of NLRP3 inflammasome signaling. This finding suggests a possible priming role for ROS generation in impaired diabetic wound healing progression since the AGES are stimulated mostly in response to the environmental stresses that these diabetic patients experience.

On the basis of these results, we suggest the following pathway as one of the possible mechanisms through which apoptosis can occur. After AGES stimulation, ROS generation induces NLRP3 inflammasome activation, which leads to enhanced caspase-8/3 activity, and subsequently cleaves many cellular proteins, leading to apoptosis. Since healing in human diabetic wounds is associated with higher levels of fibroblast apoptosis, the formation of AGES may impair diabetic healing, partly by inducing fibroblast apoptosis. The identification of signaling pathways that lead to fibroblast apoptosis may provide novel therapeutic options to improve diabetic wound healing.

**Conclusions**

In summary, we found that AGES cause apoptosis of fibroblasts via induction of ROS generation and NLRP3 inflammasome activation. The apoptosis of fibroblasts by AGES may be an important mechanism for the suppression of wound healing in diabetes. Our findings indicate that targeting the NLRP3 inflammasome is a promising approach. To extend the present findings, further in vivo experiments are needed.

**Compliance with ethics guidelines**

This study was approved by the Ethics Review Board of Shanghai Sixth People’s Hospital affiliated to Shanghai Jiaotong University.

**Conflict of interest**

None.
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