

Advanced glycation end products (AGEs) and their receptor (RAGE) induce apoptosis of periodontal ligament fibroblasts

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Abstract

Diabetics have an increased prevalence of periodontitis, and diabetes is one of the causative factors of severe periodontitis. Apoptosis is thought to be involved in this pathogenic relationship. The aim of this study was to investigate apoptosis in human periodontal ligament (PDL) fibroblasts induced by advanced glycation end products (AGEs) and their receptor (RAGE). We examined the roles of apoptosis, AGEs, and RAGE during periodontitis in diabetes mellitus using cultured PDL fibroblasts that were treated by AGE-modified bovine serum albumin (AGE-BSA), bovine serum albumin (BSA) alone, or given no treatment (control). Microscopy and real-time quantitative PCR indicated that PDL fibroblasts treated with AGE-BSA were deformed and expressed higher levels of RAGE and caspase 3. Cell viability assays and flow cytometry indicated that AGE-BSA reduced cell viability ($69.80 \pm 5.50\%$, $P < 0.01$) and increased apoptosis ($11.31 \pm 1.73\%$, $P < 0.05$). Hoechst 33258 staining and terminal-deoxynucleotidyl transferase-mediated nick-end labeling revealed that AGE-BSA significantly increased apoptosis of PDL fibroblasts. The results showed that the changes in PDL fibroblasts induced by AGE-BSA may explain how AGE-RAGE participates in and exacerbates periodontium destruction.

Key words: RAGE; Apoptosis; Diabetes mellitus; Periodontal diseases

Introduction

Periodontal disease is a common complication of diabetes (1), but the underlying pathogenesis and internal relationship of the two diseases is not yet clear (2). Previous studies suggest that advanced glycation end products (AGEs) and the receptor for advanced glycation end products (RAGE), which form during chronic hyperglycemia, play roles in the pathogenesis of diverse diseases (3,4), and may also play a key role in periodontitis in the diabetic patient (5).

RAGE was first described in 1992 by Neeper et al. (6), and in 2005, RAGE was identified in gingival tissues of patients with type 2 diabetes (7). Since then, there has been considerable interest in the role RAGE plays in periodontitis. RAGE is a member of the immunoglobulin receptor superfamily and is expressed by diverse cells, including endothelial and smooth muscle cells, lymphocytes, monocytes, and neurons (8). In addition to AGEs, a number of other ligands can bind to RAGE, including amyloid fibrils, amphoterin, and S100/calgranulins. For all tissues so far examined, overexpression of RAGE is associated with accumulation of RAGE ligands. The accumulation of RAGE ligands in

diseased tissues leads to sustained receptor expression through positive feedback; this prolongs cell activation and plays a significant role in diverse chronic disorders (9).

The role of AGEs in induction of apoptosis has been extensively investigated, however many of these studies were performed on various cell types, including neuronal and endothelial cells (10-12), and used soluble AGEs, thus resulting in differing apoptotic responses (13-15). Soluble AGEs induce apoptosis through activation of RAGE (10,12,16). This ligand-receptor activation promotes the upregulation of reactive oxygen species (ROS) resulting in apoptosis (17). ROS activate transcription factors to induce the growth arrest and DNA damage 153 (*GADD153*) gene, which in turn causes the cell to undergo apoptosis through the upregulation of caspase-3 (18,19). At the same time, RAGE binding could also activate multiple signaling pathways such as MAPK, JNKs, or Cdc42/Rac, together with activation of transcription factors AP-1 or NF- κ B that regulate many important cellular functions (20-22).

The periodontal ligament is a structure connecting teeth to the alveolar bone. It is a key contributor to the process of

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regeneration of the periodontium. Due to the proximity of the periodontal ligament to alveolar bone, cytokine production by PDL fibroblasts might influence the processes of periodontium destruction in periodontal disease. PDL fibroblasts are fibroblast-like cells and are the predominant mesenchymal cells of the periodontal ligament. They are mainly involved in metabolism of the periodontal ligament, synthesis of collagen fibers, and other extracellular matrix components. In addition, PDL fibroblasts produce several proinflammatory mediators and apoptosis factors when stimulated by AGEs.

Taken together, many previous studies indicate a potential relationship of AGEs, RAGE, diabetes, and periodontitis. Although many publications have described the relationship between apoptosis and RAGE in other cell types, no studies have yet examined the role of RAGE in periodontal cellular apoptosis, an important aspect in the pathogenesis of periodontitis. In the present study, we used an AGE-RAGE model comprising cultured human PDL fibroblasts treated with AGE-modified bovine serum albumin (AGE-BSA) (23) to measure the accumulation of AGE-BSA, the apoptosis ratio of AGE-BSA-activated cells, and the expression of RAGE.

Material and Methods

AGE-BSA preparation

The AGEs used in the present study were prepared from 50 mg/mL BSA (Sigma, USA) with 0.5 M glucose (Sigma) in 0.02 M PBS buffer (phosphate buffered saline, Sigma), pH 7.4. The solution was passed through a 0.22 μm filter (Millipore, USA), and incubated in the dark at 37°C for 12 weeks. Unmodified BSA (without glucose) was treated identically and used as a control. Following incubation, extensive dialysis against glucose was performed to remove unincorporated glucose. AGEs were identified by fluorescence spectrophotometry (Hitachi V-2001, Japan) (24). The AGE content of our AGE-BSA was 81.8 U/mg protein, and was <0.9 U/mg in the unmodified BSA. The endotoxin content of these preparations was negligible as determined by the limulus amoebocyte lysate assay (Endos, USA).

Cell culture

Human PDL fibroblasts used in the present study were obtained from periodontal ligament tissue harvested from the roots of freshly extracted, uninfected, impacted third molar teeth. Immediately after extraction and under sterile conditions, the teeth were washed in a PBS solution with 200 U/mL penicillin and 200 $\mu\text{g}/\text{mL}$ streptomycin sulfate. Then, the periodontal ligament tissue was gently scraped from the middle third of the root. The tissue was cultured in Dulbecco's Modified Eagle's Medium (Sigma), with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin, and 100 $\mu\text{g}/\text{mL}$ streptomycin. Gelatin-coated flasks were used for incubation at 37°C in a

humidified atmosphere (5% CO₂, 95% air). The medium was replaced every 2 days until confluence at 3-5 days. At the third passage, the FBS concentration was reduced to 5% and the resulting confluent PDL fibroblasts were used for experiments. The research was approved by the appropriate human ethics committee of Air Force General Hospital PLA.

Cell treatment protocol

PDL fibroblasts were randomly divided into 3 groups: AGE-BSA (group A), BSA (group B), and control (group C). PDL fibroblasts in group A were incubated for 48 h with AGE-BSA (final concentration of 200 $\mu\text{g}/\text{mL}$). PDL fibroblasts in group B were incubated for 48 h with BSA (final concentration of 200 $\mu\text{g}/\text{mL}$). PDL fibroblasts in group C were incubated under normal growth conditions. The cells were treated with AGE-BSA or BSA for 48 h in all experiments except for the cell viability assay (described below), in which viability was assessed continuously in the cells of each group treated with AGE-BSA or BSA for 24, 48, and 72 h. The concentration of AGE-BSA (200 $\mu\text{g}/\text{mL}$) was determined by the results of relevant, published studies (25-27). All experiments were performed 5 times and results are reported as means \pm SE.

Analysis of cell numbers

For the cell counts, PDL fibroblasts in each group were cultured at 1.0×10^5 cells/mL in 24-well plates, and then observed with an inverted microscope (ECLIPSE TS-100, Nikon, Japan) at 10 \times magnification. Photomicrographs were recorded with a digital camera (Nikon). In every well, the software overlaid a grid of evenly spaced horizontal and vertical lines (Adobe Photoshop, version CS3) over the digital image. This allowed cells to be counted on the surface of four quadrants in each well of the culture plate. Using these standardized conditions, counts of PDL fibroblasts were performed by a single examiner who was blinded to the study procedures.

Immunocytochemistry

Immunocytochemistry was performed using polyclonal monospecific antibodies to RAGE (Becton, Dickinson and Company, USA). Peroxidase-conjugated rat anti-human IgG (Becton, Dickinson and Company) was used to visualize antibody-antigen binding. Briefly, treated cells grown on coverslips were fixed in 95% alcohol for 15 min, permeabilized with methanol, and blocked with 3% BSA in PBS for 1 h at room temperature. Cells were incubated in polyclonal mono-specific antibody to RAGE (1:200 in PBS) overnight at 4°C. After incubation, the cells were washed with PBS and incubated with rat anti-human IgG for 2 h at room temperature, washed with PBS, and counterstained with 4'-6-diamidino-2-phenylindole (DAPI; Becton, Dickinson and Company). Expression of RAGE was graded by two investigators as no staining (–), mild

staining (+), moderate staining (++), and intense staining (+++) (28). After application of biotinylated anti-mouse secondary antibody, sections were counterstained with hematoxylin.

MTT assay

Cell viability was determined by the methylthiazolyl-diphenyl-tetrazolium (MTT) assay, as described previously (29). Briefly, cells were seeded into 200 μ L culture medium in 96-well plates at 5×10^3 cells/well. After 24, 48, and 72 h of culture, cells were treated as described in the cell treatment protocol (see above). The cell culture medium was removed, and each well was filled with 100 μ L fresh DMEM without FBS and incubated for 1 h at 37°C. Each well was then incubated with MTT (Sigma) for 4 h, and dimethylsulfoxide (Sigma) was added to dissolve the solid residue. Then, the absorbance at 490 nm of each well was determined using a microplate reader (Thermo, USA). Viability is reported as a percentage relative to untreated control samples at 24 h.

Detection of apoptosis by flow cytometry

Apoptosis was assessed by the Annexin V-FITC apoptosis kit (Franklin Lakes, USA) (30), and the cells in each group were harvested at 48 h for testing. PDL fibroblasts were harvested by trypsin treatment according to the assay manufacturer's instructions, washed twice with cold PBS, and centrifuged (300 g, 5 min). Cell pellets were resuspended in 200 μ L of $1 \times$ binding buffer at 1×10^6 cells/mL. Then, cells were incubated with 5 μ L Annexin V for 10 min at 4°C in the dark, and 5 μ L propidium iodide (PI) was added for an additional 10 min. Flow cytometry was performed 1 h later. Data were analyzed using CELLQuest software (Becton, Dickinson and Company). The results were interpreted as follows. Cells in the lower-left quadrant (Annexin V⁻/PI⁻) represented living cells; those in the lower-right quadrant (Annexin V⁺/PI⁻) represented early apoptotic cells; those in the upper-right quadrant (Annexin V⁺/PI⁺) represented late apoptotic cells; those in the upper-left quadrant (Annexin V⁻/PI⁺) represented necrotic cells.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

For detection of DNA fragmentation by TUNEL staining, cell sections were deparaffinized, rehydrated through a series of graded alcohols, and washed in distilled water followed by PBS. Subsequently, the sections were permeabilized using proteinase K (20 μ g/mL, 20 min at room temperature; Sigma), washed in PBS, incubated in 2% H₂O₂ in 0.1 M PBS for 30 min at room temperature, and treated with Triton-X (Sigma). The TUNEL reaction was performed using a cell death kit (Becton, Dickinson and Company). Briefly, the sections were incubated with TUNEL reaction mixture for 60 min, washed twice in PBS, treated with converter-POD solution for 30 min, rinsed with

PBS, and visualized by adding 3'-diaminobenzidine for 10 min at room temperature. They were then washed in PBS, counterstained with hematoxylin, and mounted for light microscopy. Apoptotic cells were identified by their dark brown nuclei under a light microscope.

Hoechst 33258 fluorescent staining

PDL fibroblasts in each group were washed in ice-cold PBS, and fixed in a solution of methanol-acetic acid (3:1, v/v) for 15 min at 4°C. Apoptotic cells were identified by Hoechst 33258 (5 μ g/mL in PBS; Sigma) staining for 5 min at room temperature. The staining of cell nuclei was examined by Olympus fluorescence microscopy (Olympus, Japan) with an excitation wavelength of 340 nm and an emission wavelength of 460 nm.

Statistical analysis

Data are reported as means \pm SE and were analyzed using one-way ANOVA for comparisons of group means. For all analyses, differences were considered significant if P was less than 0.05. All statistical analyses were performed using the SPSS 10.0 software (USA).

Results

Cell counting, growth, morphology, and RAGE expression

To determine the extent of RAGE expression after AGE-BSA treatment, human PDL fibroblasts were subjected to AGE-BSA (group A), BSA (group B), or given no treatment (control group C). There were no significant differences in cell number in the 3 groups after 48 h, which indicated no mass cell mortality had occurred (group A, 215.3 ± 45.0 cells/ μ L; group B, 226.6 ± 55.6 cells/ μ L; group C, 230.5 ± 50.9 cells/ μ L; $P > 0.05$). Figure 1 shows representative photomicrographs of PDL fibroblasts treated with AGE-BSA and BSA for 48 h, and control cells. The control cells and cells treated with BSA had normal fusiform morphology. However, treatment with AGE-BSA caused notable deformation of PDL fibroblasts, and reduced the total number of cells. Figure 1 also shows the expression of RAGE, as detected by immunocytochemistry. Group A exhibited significant immunoreactivity against RAGE, whereas RAGE was basically undetectable in groups B and C.

Effects of cell viability induced by AGE-BSA

Figure 2 shows the percentage of viable cells in groups A and B relative to the control group from 24 to 72 h. The cell viability of the control group at 24 h was deemed as 100%. As time went on, the cell viability of group A was gradually reduced, but almost no change occurred in groups B and C. Comparison of the AGE-BSA and control groups indicated significantly lower cell viability in the AGE-BSA group at each time ($P < 0.01$). However, comparison of the BSA group with the control group indicated no

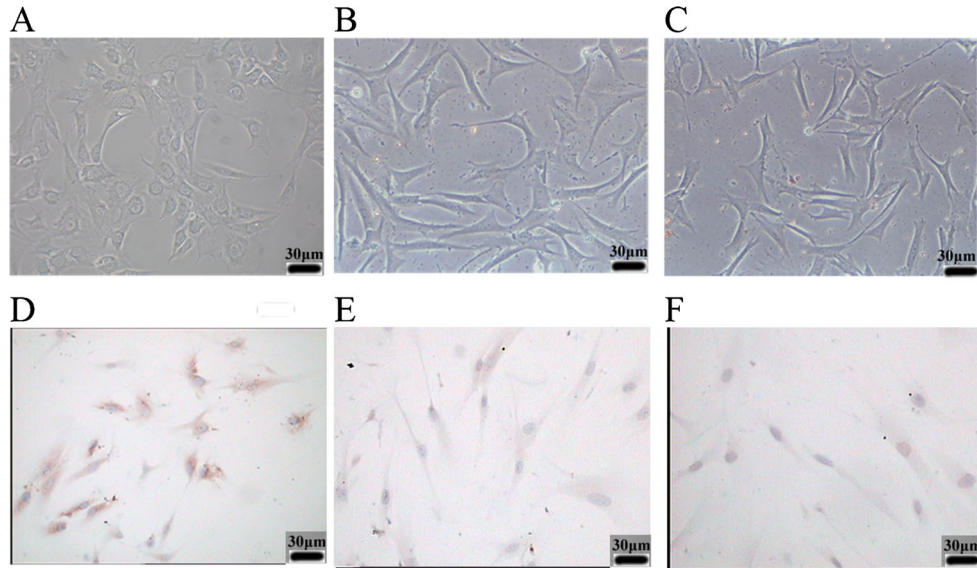


Figure 1. Effect of AGE-BSA on cell morphology and RAGE expression of human periodontal ligament fibroblasts (PDL fibroblasts). Relative to control cells (C), 48-h treatment with AGE-BSA altered cell morphology and reduced the number of cells (A). BSA-treated cells (B) were similar to control cells (C). Similarly, immunocytochemistry indicated that cells treated with AGE-BSA for 48 h had significantly greater expression of RAGE (D) than BSA-treated cells (E) and control cells (F). Magnification, 400 \times .

significant differences in cell viability ($P>0.05$).

Cell apoptosis measured by flow cytometry, Hoechst 33258 staining, and TUNEL staining

The flow cytometry experiments (Figure 3) indicated that control cells underwent almost no apoptosis or necrosis, with only $1.1 \pm 0.38\%$ of cells classified as apoptotic. Group B was nearly the same as the control ($P>0.05$), with only $1.5 \pm 0.73\%$ of cells classified as apoptotic. In contrast, group A had a significantly greater percentage of apoptotic cells ($11.31 \pm 1.73\%$; $P<0.05$).

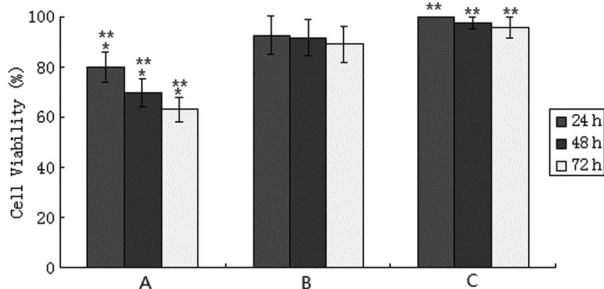


Figure 2. Viability of HPDL fibroblasts treated with AGE-BSA, BSA and control for 24, 48, and 72 h. Pooled analysis indicated significant differences in viability at 24, 48, and 72 h in group A ($*P<0.05$), and significant differences between group A and group C ($**P<0.01$), but no significant difference in the viability of group B and group C at any time ($P>0.05$). One-way ANOVA was used for statistical analyses.

To further confirm the ability of AGE-BSA to elicit apoptosis *in vitro*, TUNEL staining was carried out on sections. As illustrated in Figure 4, AGE-BSA treatment caused a significantly higher percentage of TUNEL-positive apoptotic cells compared with the other two groups. These results indicated that AGE-BSA is a potent apoptosis-inducing agent *in vitro*.

The PDL fibroblasts in the control and BSA groups stained with Hoechst 33258, exhibited small, round normal-appearing nuclei. In contrast, cells treated with AGE-BSA showed signs of apoptosis, including highly condensed and fragmented nuclei (Figure 5).

Discussion

RAGE is expressed in diverse cell types (8) and has been implicated in periodontal complications that accompany diabetes (7). Previous studies of cultured cells have shown that an interaction of AGEs with RAGE modulates certain cellular properties (31,32). In the present study, we hypothesized that RAGE expression is in some way related to PDL apoptosis. Thus, the RAGE pathway appears to be one of the pathogenic mechanisms that connect diabetes and periodontitis.

Injury of PDL fibroblasts induced by the AGE-RAGE system seems to play an important role in the development of advanced stages of diabetes mellitus. The AGE-RAGE system may cause PDL activation, dysfunction, or death depending on dose and exposure time. Therefore, in this study, AGE-BSA was used to develop a model. Cell

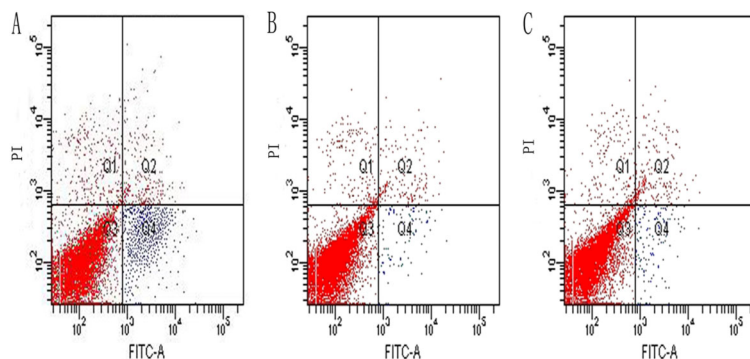


Figure 3. Apoptotic cells can be observed at a specific Q4 of the graphics for groups A, B, and C. Flow cytometry analysis of PDL fibroblasts apoptosis following treatment with AGE-BSA (A) and BSA alone (B). Group A ($11.31 \pm 1.73\%$ apoptosis) had significantly more apoptotic cells than the control group (C) ($1.1 \pm 0.38\%$, $P < 0.05$). The percentage of apoptotic cells in B ($1.5 \pm 0.73\%$) was similar to the control ($P > 0.05$). All cells were cultured for 48 h in different culture environments. One-way ANOVA was used for statistical analyses.

counts showed no statistical differences in the 3 study groups, from which it can be concluded that PDL fibroblasts did not die when treated with either AGE-BSA or BSA. However, it did not follow from this result that apoptosis had not occurred, since apoptosis occurs prior to cell death. It can be inferred from the study results that PDL fibroblasts treated with AGE-BSA could induce apoptosis. Exposure to AGE-BSA can lead to alteration of cell shape, the recombination of actin filaments, and formation of intercellular gaps. In the present study, we observed that cells treated with AGE-BSA were deformed and contracted, had increased intracellular granular material, and had a roughened profile. Alteration in cell morphology indicates damage to intercellular adhesions and junctions (33). This has implications for cell permeability, because adhesions and junctions of PDL fibroblasts are important for maintaining their function as barriers of the periodontal wall.

The AGE-RAGE interaction modulates cellular properties through the generation of proinflammatory cytokines that participate in cell apoptosis (34). Recent research indicates that apoptosis is a critical step in the destruction of periodontal tissue in diabetic periodontitis (35). For example, in a study of diabetic rats, Liu (35) demonstrated increased apoptosis of bone-lining cells and osteoblasts. Thus, diabetes is associated with a persistent inflammatory response, increased loss of cell attachment,

increased alveolar bone resorption, and impaired new bone formation. The latter may be affected by increased apoptosis of bone-lining cells and PDL fibroblasts (35). Alikhani (13) showed that diabetes significantly increases the apoptosis of osteoblast cells *in vivo* and that this contributes to diabetes-mediated impairment of new bone formation. However, those studies found that apoptosis participated in destruction of periodontal tissue in diabetic patients. Until now, none of the studies fully elucidated the pathway or potential role of apoptosis induced by AGE-RAGE. In this paper, we hypothesized that the high expression of RAGE in cell membranes stimulated by AGE could activate some enzymes or cell factors that could increase the proportion of apoptotic cells. Based on the results of the present study, the AGE-RAGE system may stimulate an increase in apoptosis. The predominant apoptotic pathway involves caspase-8 activation of caspase 3, and is independent of NF- κ B activation (13). Al-Mashat (36) provided direct evidence that increased apoptosis of fibroblasts and osteoblasts caused by diabetes contributes to impaired healing processes, and this can be explained, in part, by diabetes-mediated up-regulation of proapoptotic genes and caspase activity.

This study demonstrated that AGE-BSA increased RAGE expression by PDL fibroblasts. Our immunocytochemistry studies indicated that RAGE expression in AGE-BSA-treated cells, while greater than that of control

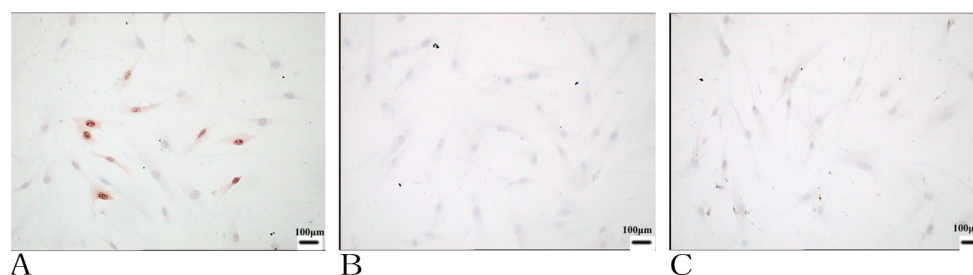


Figure 4. Evaluation of apoptosis in cultured PDL fibroblasts using the TUNEL method. Forty-eight hours after culture, apoptosis was detected using an apoptosis detection kit after fixation. Cells with dark brown nuclei were considered apoptotic. The number of apoptotic cells increased in the AGE-BSA group (A) compared with BSA treatment (B) and the control (C). Magnification, $400\times$.

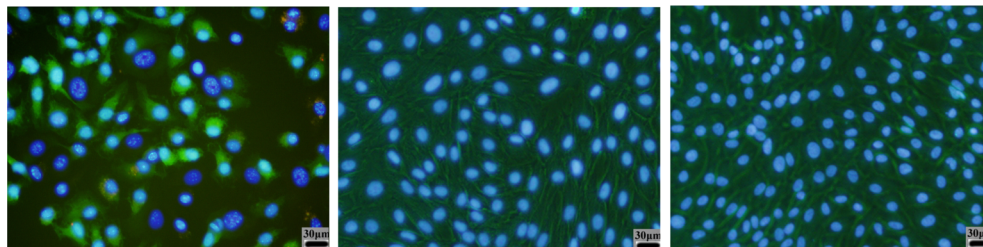


Figure 5. Nuclei were counterstained with Hoechst 33258 and observed under a fluorescence microscope. All cells were cultured for 48 h in different culture environments. Cells in the AGE-BSA group (*left panel*) showed bright blue dots in the nuclei, which represented nuclear fragmentation in the apoptotic cells. Cells in the BSA group (*middle panel*) and control group (*right panel*) were uniformly blue in viable cells. Magnification, 400 \times .

cells, was still somewhat weak. This could be partially attributable to the brief AGE-BSA stimulus, or because of other unknown biological processes that depleted the amount of RAGE in our cultured cells.

Previously, RAGE had been identified in diverse epithelial cells, including the gingival tissue of diabetic patients (7). RAGE has some biological characteristics that suggest it plays a pivotal role in the pathogenesis of periodontal disease. First, it is a cell receptor associated with proinflammatory responses. Second, increased expression of various receptors is associated with the severity of periodontal disease (37). Apoptosis has been found to be the prominent form of cell death in response to AGE-BSA (14). However, in that study, deficits in wound healing associated with higher rates of fibroblast apoptosis were evaluated. Clinically, apoptosis is preferred over necrosis since it is associated with less severe tissue damage. Apoptosis induced by RAGE appears to be

important in the pathogenesis of a variety of human diseases including Alzheimer's disease, rheumatoid arthritis, cancer, and inflammatory bowel disease, but there have been few reports on periodontitis in diabetes mellitus. In this study, we found that AGE-RAGE causes an increase in apoptosis of critical PDL fibroblasts. As shown in Figures 3, 4, and 5 apoptosis was the main cell-death pathway for cells treated with AGE-BSA for 48 h at a concentration of 200 $\mu\text{g}/\text{mL}$.

In conclusion, the results indicated that *in vitro* stimulation of PDL fibroblasts by AGE-BSA upregulated RAGE and the accumulation of AGE-RAGE was associated with cell apoptosis, which may contribute to deficient periodontium formation. The findings suggest a possible reason why periodontitis in diabetes mellitus is more serious and refractory than periodontitis in otherwise healthy patients.

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