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Glycated matrix up-regulates inflammatory signaling similarly to *Porphyromonas gingivalis* lipopolysaccharide

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Background and Objective: Hyperglycemia and advanced glycation end-products (AGEs) have been hypothesized as the etiologic factors of diabetic periodontitis. The aim of this study was to clarify in greater detail the patterns of AGE-mediated periodontal inflammation under various physiological conditions.

Material and Methods: The deposition of AGEs and expression of the receptor for AGEs (RAGE) were identified by immunohistochemistry in Sprague–Dawley rats with experimentally induced periodontitis or diabetes. Human periodontal ligament cells (PDLCs) and mesenchymal stem cells (MSCs) were cultured under simulated conditions of hyperglycemia, *Porphyromonas gingivalis* lipopolysaccharide (LPS) stimulation and matrix glycation. Cell viability and expression of toll-like receptors (TLRs), *Rage*, an inflammatory signaling initiator (nuclear factor kappa light chain enhancer of activator β cells), an oxidative stressor (heme oxygenase-1) and collagen synthesis (type I and type IV) genes were evaluated.

Results: The deposition of AGEs and the expression of *Rage* were evident in the inflamed periodontal tissues in all rats and appeared to be enhanced in rats with diabetes. Matrix glycation augmented cytotoxicity, up-regulated RAGE and TLRs in both PDLCs and MSCs, and significantly activated downstream inflammatory signaling in MSCs. Oxidative stress was significantly increased under matrix glycation in both PDLCs and MSCs and was significantly increased at a high-glucose concentration in MSCs. A consistent decrease in expression of type I and type IV collagens was observed in MSCs, but a delayed reduction was noted in PDLCs.

Conclusions: Matrix glycation modulated cell behavior to induce inflammation equivalent to that produced by incubation with *P. gingivalis* LPS. Periodontal inflammation also led to matrix glycation, thus demonstrating a definite interaction between diabetes and periodontitis.

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Periodontitis is a bacteria-induced inflammatory condition that causes the destruction of tooth-supporting structures; this results in tooth loss and in significantly reduced function and esthetics. Periodontal ligament cells (PDLCs) and mesenchymal stem cells (MSCs) are predominant cell types contributing to the supportive and regenerative potential of the periodontium (1,2). Under constant bacterial insults, the activities of PDLCs and MSCs are modified, resulting in activation of the inflammatory process, which leads to tissue destruction (3,4). It has been demonstrated that lipopolysaccharide (LPS), the main virulence factor of

Porphyromonas gingivalis, up-regulates the expression of toll-like receptor (TLR)-2 and TLR-4 in both PDLCs and MSCs. This stimulates the production of inflammatory cytokines through activation of the transcription factor, nuclear factor-kappaβ (NF-κβ) (5,6), leading to the overproduction of inflammatory cytokines, such as interleukin (IL)-1β, IL-6 and tumor necrosis factor-α (TNF-α), which facilitate the recruitment of lymphocytes and the release of oxygen free-radicals, resulting in destruction of the surrounding tissue. As such, this exaggerated inflammatory response to LPS may play an important role in the pathogenesis of periodontitis (7).

The close relationship between diabetes and periodontitis has been demonstrated in a number of clinical investigations (8,9). Hyperglycemia and the accumulation of advanced glycation end-products (AGEs) appear to be the main factors responsible for modulating periodontal inflammation in diabetes (7,10). AGEs are the irreversible products of the nonenzymatic glycosylation of proteins or lipids that occurs via the Maillard reaction under chronic hyperglycemic conditions. Accumulation of AGEs brings about alterations in matrix–matrix interactions and interferences in matrix–cell interactions (11). They can also trigger cellular responses and change cellular functions by binding to their receptor (RAGE), a member of the immunoglobulin surperfamily characterized by multiple proinflammatory ligand-binding sites (10). This interaction induces receptor-mediated reactive oxygen species and activates translocation of the transcription factor, NF-κβ, to the nucleus, resulting in amplification of inflammation (12). Therefore, it is speculated that the accelerated destruction of periodontal tissues in diabetic subjects can be caused by oxidative stress induced by a hyperglycemic state and the accumulation of AGEs, which are found to be abundantly expressed in the peripheral tissues of patients with diabetes mellitus (12).

The regulatory effects of AGEs and hyperglycemia on the periodontium have been investigated in previous studies. AGEs were reported to induce the apoptosis of MSCs and to prevent osteogenic differentiation (13), and prolonged hyperglycemia was able to reduce the proliferation, mineralization and migration of PDLCs (14,15). The apoptosis of PDLCs and MSCs in inflamed periodontal tissues of diabetic animals was more prominent than in nondiabetic animals (16). Furthermore, evident expression of RAGE in inflamed gingival epithelium of both diabetic and nondiabetic patients implied that RAGE might also be involved in periodontal destruction (17). However, the underlying mechanism by which hyperglycemia or AGEs affect periodontal destruction has not been clearly demonstrated.

In this study, formation of AGEs is hypothesized as a crucial event linking diabetes and periodontitis. The accumulation of AGEs and the expression of RAGE during periodontal breakdown was identified *in vivo* (in an animal model). The viability and the activation of periodontitis-related inflammatory signaling of human PDLCs and MSCs under hyperglycemia, the presence of *P. gingivalis* LPS and matrix glycation were investigated using a novel *in-vitro* model. The results obtained from this study will enable clinicians to better understand the pathogenesis of periodontitis as well as the interplay between diabetes and periodontitis.

Material and methods

Animal models

Procedures performed on all animals followed protocol 032/10 approved by the Institutional Animal Care and Use Committee of the National University of Singapore. Ten male Sprague–Dawley rats were utilized, five of which were treated with one-time intraperitoneal injection of streptozotocin (STZ) (65 mg/kg; diluted in citrate buffer, pH 4.5) to induce diabetes. Blood glucose (BG) measurements (Accutrend® Plus; F. Hoffman-La Roche Ltd., Basel, Switzerland) and analysis of glycated hemoglobin (HbA1c) (Cobas® C111 Analyzer; F. Hoffman-La Roche Ltd.) were performed, whereby animals with BG levels > 250 mg/dL and HbA1c > 5% were considered diabetic. Three weeks after injection with STZ, in one randomly selected side of each animal, a 4–0 silk ligature was submerged in the gingival sulcus, and 10 μL of 1.0-mg/mL LPS from *P. gingivalis* (Invivogen, San Diego, CA, USA) was injected at the base of the interproximal papilla of the maxillary second molar (M2) to induce experimental periodontitis, as previously described (18). Suture replacement and LPS injection were performed twice per week. The animals were killed after 7 d of periodontitis induction and their maxillae were harvested, fixed in 10% formalin for 3 d and decalcified with 12.5% EDTA (pH 7.4) for 3 wk. The specimens were finally embedded in paraffin and cut into 5-μm-thick sections for histological and immunohistochemical evaluations.

Histology and immunohistochemistry

Specimens were stained with hematoxylin and eosin (Polysciences Inc., Warrington, PA, USA) for descriptive histology, while the distribution of AGEs and the expression of RAGE were evaluated by immunohistochemistry using the Cell & Tissue Staining Kit (R&D Systems, Minneapolis, MN, USA). Briefly, specimens were incubated with 0.05% trypsin/EDTA (Invitrogen Co., Carlsbad, CA, USA) for 20 min at room temperature to expose the antigens and epitopes before being immersed in 3% H_2O_2 for 10 min to eliminate endogenous peroxidase activity. The sections were incubated, overnight at 4°C, with the following rabbit polyclonal antibodies: anti-AGE (dilution 1 : 2000; Abcam PLC., Cambridge, UK) and anti-RAGE (dilution 1 : 100; Abcam PLC.) and were subsequently incubated with biotinylated secondary antibodies for 1 h at room temperature. The color was developed by 3,3-diaminobenzidine and counterstained with hematoxylin. All histological and immunohistochemical images were acquired with a Leica DMD108 system (Leica Microsystems GmbH, Wetzlar, Germany). Periodontal bone loss was determined by measuring the

distance from the cemento–enamel junction to the alveolar bone crest at the palatal surface of the mid-mesiopalatal root region of M2 under 100× magnification. Quantification of RAGE-expressing [RAGE(+)] cells was performed in five randomly selected areas under 400× magnification, and the result was presented as a percentage [(no. of RAGE(+) cells/total number of cells) × 100)].

Fabrication and characterization of glycated collagen gel

Glycated collagen gels were prepared as previously described (19). Soluble rat-tail collagen type I (BD Bioscience, Franklin Lakes, NJ, USA) was diluted with phosphate-buffered saline to a final concentration of 2.5 mg/mL and incubated at 37°C in a 5% carbon dioxide (CO_2) atmosphere for 24 h. After gelation, the gels were incubated for 5 d in 0 or 375 mM glucose 6-phosphate (G6P; Sigma-Aldrich, St Louis, MO, USA) equilibrated to pH 7.4 in phosphate-buffered saline.

The structure of glycated collagen gels was examined using a total internal reflection fluorescence microscope (Fv1000 FD/EVA; Olympus, Tokyo, Japan) at 488 nm excitation under 100× power. Depletion of the free amine group on the collagen gels was examined using 2,4,6-trinitrobenzenesulfonic acid (Sigma-Aldrich) (20). Glycated collagen gels were freeze-dried and weighed. For 2–4 mg of lyophilized collagen, 1 mL of 4% (weight by volume) sodium bicarbonate and 1 mL of 0.5% (volume by volume) 2,4,6-trinitrobenzenesulfonic acid were added and incubated at 40°C. After 2 h, 6 M hydrochloric acid was added to the solution and incubated at 60°C for 90 min. The final solution was diluted with 5 mL of deionized water and absorbance at 345 nm was examined.

Cellular viability

Immortalized human PDLCs and MSCs were cultured in a growth medium composed of Dulbecco's modified Eagle's medium (Life Technologies, Carlsbad, CA, USA), 10% fetal bovine serum (Thermo Scientific, Waltham, MA, USA), 100 U/mL of penicillin and 100 μg/mL of streptomycin (PANTM Biotech GmbH, Aidenbach, Germany). The PDLCs and MSCs were seeded in a 96-well dish at a density of 4×10^3 cells/well. They were subjected to a range of treatments to simulate different physiologic conditions. The cells were exposed to nonglycated or glycated collagen gels to simulate matrix glycation by the deposition of AGEs, were treated with a growth medium containing 1 or 4.5 g/L of glucose to simulate hyperglycemia (the dose-dependent effect in the cellular viability is shown in Figure S1) or were subjected to treatment with 10 μg/mL of *P. gingivalis* LPS. After 4 d of incubation at 37°C in an atmosphere of 5% CO_2, cell viability was examined using the CellTier® 96 AQ_{ueous} One Solution Cell Proliferation Assay (MTS; Promega, Madison, WI, USA) and the Cytotoxicity Detection Kit^{plus} (LDH; Roche, London, UK) according to the manufacturer's instructions. Both tests measured the absorbance at 490-nm wavelength using a QuantTM microplate spectrophotometer (BioTek, Winooski, VT, USA). All experiments were performed in triplicate and were repeated three times.

Gene-expression level determined by real-time PCR

PDLCs and MSCs were seeded in 12-well plates at 1.2×10^5 cells/well and 1.6×10^5 cells/well, respectively. Both cell types were seeded on nonglycated and glycated collagen gel, were bathed with growth medium containing a normal or an elevated level of D-glucose and were treated with or without LPS for 6 and 24 h. Total RNA was isolated using the RNeasy Mini kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. Thereafter, complementary DNA was synthesized from 300 ng of the total RNA using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA). The mRNA expression levels of the following genes were quantitatively analyzed using a real-time PCR system (StepOnePlus; Applied Biosystems, Carlsbad, CA, USA) using sequence-specific TaqMan gene-expression assays (Applied Biosystems): glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), collagen type I alpha-1 (type I collagen), collagen type IV alpha-1 (type IV collagen), *Tlr2*, *Tlr4*, *Nf-κβ*, heme oxygenase-1 (Ho-1) and *Rage*. The details of the primers and probes used are provided in Table S1. *Gapdh* served as the housekeeping gene. Data were analyzed using StepOne Software v2.1 (Applied Biosystems) and calculated using the $2^{-\Delta\Delta C_t}$ method. All experiments were performed in triplicate and were repeated three times.

Statistical analysis

Statistical analyses were performed using GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA). Data were pooled in their experimental groups and the results presented as mean ± standard deviation. Differences in the levels of BG and HbA1c, in the degree of periodontal bone loss and in the ratio of RAGE(+) cells, between STZ-injected and non-STZ-injected rats were compared using the unpaired *t*-test, and cellular viability titers and the gene-expression level at each time-point were compared, among treatments, using one-way analysis of variance followed by Tukey's *post-hoc* test; the level of significance (*p*-value) was < 0.05.

Results

AGE deposition and RAGE expression in periodontal tissues

Before STZ injection, no significant differences were found for BG (93.67 ± 21.43 mg/dL in non-STZ injected rats, and 91.67 ± 17.61 mg/dL in STZ-injected rats) and HbA1c (3.19 ± 0.02% in non-STZ injected rats, and 3.25 ± 0.29% in STZ-injected rats) levels between the groups. In STZ-injected rats, significantly higher levels of BG (362.50 ± 107.50 mg/dL) and HbA1c (6.02 ± 0.32%) were noted 1 wk after STZ injection and through-

out the entire study period. Elevation of BG (163.83 ± 36.07 mg/dL) and HbA1c (3.66 ± 0.12%) were also noted in rats without STZ injection, at the time of periodontitis induction, and the levels were consistent until the rats were killed (data not shown). The BG and HbA1c levels were significantly lower in STZ-injected rats at these two time points ($p < 0.01$).

Intra-orally, there was no obvious inflammatory cell infiltration, with a similar periodontal attachment level observed in the control side of both STZ- and non-STZ-injected rats (Table 1). However, prominent accumulation of supragingival plaque and some degree of gingival connective tissue degeneration were observed in most STZ-injected rats (Fig. 1A and 1D). Scanty deposition of AGEs and a low level of RAGE expression were noted in non-STZ-injected rats (Fig. 1B and 1C). In STZ-injected rats, a slight increase in the deposition of AGEs was observed in the extracellular space of gingival connective tissue with increased expression of RAGE (Table 1), especially in gingival connective tissue, periodontal ligament cells and bone surface-lining cells (Fig. 1E and 1F).

In contrast, strong inflammatory cell infiltration in subepithelial connective tissue, prominent enlargement of epithelium and a reduced alveolar bone crest level, were consistently observed at the ligature + LPS-treated sites (Fig. 2 and Table 1). Although the level and alignment of residual periodontal ligament was similar, inflammation and periodontal abscess with progressive bone resorption appeared to be more prominent in STZ-injected rats (Fig. 2A and 2D). Bone sequestrum surrounded with lymphocytes was also occasionally seen (data not shown). Pronounced accumulation of AGEs in the extracellular matrix was noted in the connective tissue of all ligature + LPS-treated sites (Fig. 2B and 2E). Significant *Rage* expression was noted in cells within the periodontal ligament and the gingival connective tissue in all the ligature + LPS-treated sites ($p < 0.05$), and the expression was slightly stronger in STZ-injected rats (Table 1 and Fig. 2C and 2F).

Table 1. Quantitative assessments from histology and immunohistochemistry

	Nondiabetes		Diabetes	
	Control	Periodontitis	Control	Periodontitis
Periodontal bone loss (μm)	250.66 ± 20.04	481.14 ± 54.45*	254.83 ± 19.46	497.43 ± 9.04*
Ratio of RAGE(+) cells (%)	8.56 ± 2.12	18.84 ± 1.87*	12.22 ± 3.67	22.97 ± 3.04*

Data are presented as mean ± standard deviation, and significant differences from the control (without periodontitis induction) specimens under the same systemic conditions (diabetes or nondiabetes) were calculated using the unpaired *t*-test (*$p < 0.05$).
RAGE, receptor for advanced glycation end-products.

Gel characterization

By reducing sugars nonenzymatically with the amino group of proteins under prolonged hyperglycemia, deposition of AGEs is able to reduce the free amine group on the extracellular matrix without significant change of the gross structure (12). It was demonstrated that 77.70 ± 3.10% of the free amine group was reduced on collagen gels incubated with G6P compared with the control. The structure of collagen gels after incubation with G6P was found to be similar to that of the control when examined using total internal reflection fluorescence microscopy (Figure S2).

Cellular behavior under hyperglycemia, LPS stimulation and matrix glycation

Cellular viability— The mitogenesis and cytotoxicity profiles under different culture conditions were examined by MTS and LDH assays, respectively, and the results are illustrated in Fig. 3. Based on the dose-dependent test of cell viability (Figure S1), under higher glucose concentrations (> 4.5 g/L), enhancement of mitogenesis and reduction of cytotoxicity was noted in PDLCs, whereby significant inhibition of mitogenesis was seen in MSCs. Thus, a glucose concentration of 4.5 g/L in the culture medium was selected to represent a hyperglycemic state, and the results are shown in Fig. 3. Furthermore, matrix glycation significantly reduced mitogenesis and increased cytotoxicity. *P. gingivalis* LPS appeared to enhance cytotoxicity in both cell types at a lower concentration of glucose in both glycated and nonglycated matrix, whereby mitogenesis was enhanced in PDLCs but suppressed in MSCs in a nonglycated matrix (Fig. 3). However, the effect was less prominent at the higher glucose concentration or with matrix glycation.

Ligand–receptor activation— There was no significant alteration in the expression of *Rage, Tlr2* and *Tlr4* among treatments in MSCs and PDLCs after 6 h of incubation (data not shown). Significant elevation in the expression of *Rage*, under treatment with G6P, was noted in MSCs after 24 h (Fig. 4A). *P. gingivalis* LPS significantly up-regulated the expression of *Tlr2*, and the effect was further augmented by glycated matrix (Fig. 4B). No significant change of *Tlr4* among treatments in MSCs was observed (data not shown). In PDLCs, *P. gingivalis* LPS appeared to up-regulate *Rage* (Fig. 4E). Glycated matrix did not significantly influence the expression of *Rage* but was able to significantly up-regulate *Tlr4* in the absence of LPS and at the low-glucose concentration (Fig. 4F). The expression of *Tlr2* was undetectable in PDLCs. The glucose concentration did not solely appear to affect ligand–receptor activation in MSCs or PDLCs.

Oxidation stress and inflammatory signaling— Ho-1, an oxidative stress-sensitive gene, was examined to evaluate the oxidative stress of cells. NF-κβ, the response transcription factor to reactive oxygen species and inflammatory cytokines, was utilized as the

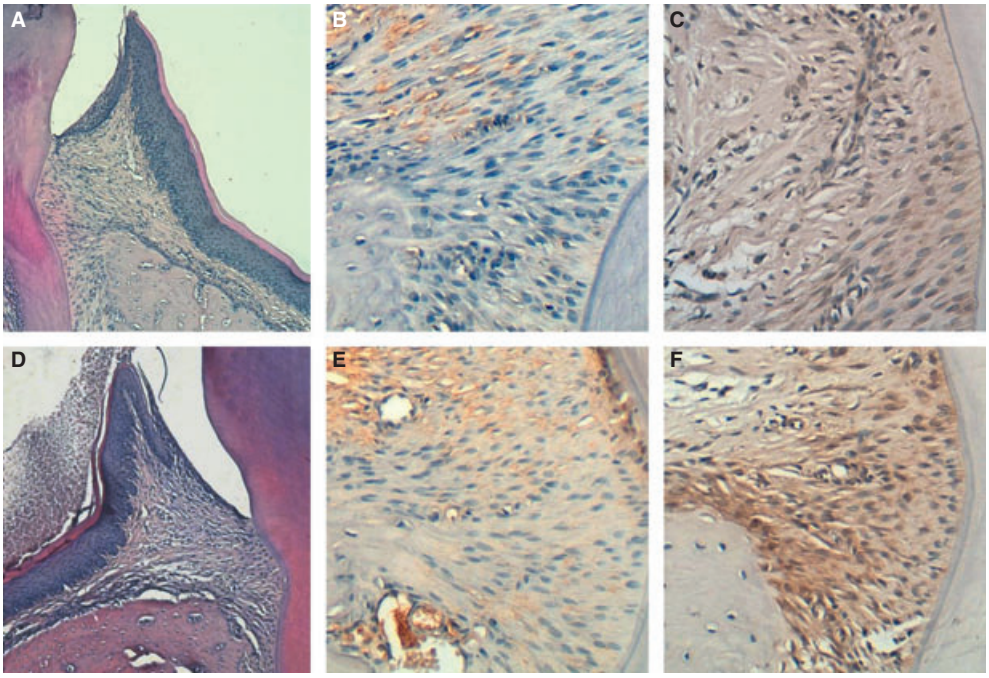

Fig. 1. Descriptive histology of the advanced glycation end-products–receptor for advanced glycation end-products (AGE–RAGE) axis in the periodontium of nondiabetic (A–C) and diabetic (D–F) rats. The palatal side of the transverse section of the mid-M2 region was selected for evaluation. (A, D) Histological observation by hematoxylin and eosin staining (magnification, 100×). (B, E) AGE deposition in the extracellular matrix, visualized by immunohistochemistry (magnification, 400×). Note the slight to moderate accumulation of AGEs in both nondiabetic and diabetic specimens. (C, F) Intracellular RAGE expression, visualized by immunohistochemistry (magnification 400×). Note that RAGE was expressed infrequently in nondiabetic rats but strongly in diabetic rats.

marker of inflammatory signaling. In MSCs, the high-glucose concentration significantly elevated oxidative stress from 6 h, and matrix glycation further augmented the stress at 24 h (Fig. 4C). Activation of *Nf-κβ* following treatment with *P. gingivalis* LPS or G6P was evident at 24 h, whereas the combination treatment only significantly up-regulated *Nf- κβ* expression compared with treatment with *P. gingivalis* LPS or G6P alone at the low-glucose concentration (Fig. 4D). In PDLCs, up-regulation of *Ho1* following treatment with G6P was not significant until 24 h, whereas the high-glucose concentration did not significantly elevate oxidative stress (Fig. 4G). The activation of inflammatory signaling was not evident, even at 24 h, in PDLCs (Fig. 4H).

Collagen synthesis— The effect of matrix glycation on collagen was evaluated by monitoring the gene-expression levels of type I and type IV collagens. Significant down-regulation of type I and type IV collagens was evident at 6 h in MSCs seeded on the glycated matrix, and the high-glucose concentration could significantly suppress the expression level (Fig. 5A and 5B). However, *P. gingivalis* LPS did not significantly affect the expression level. A similar tendency of further down-regulation of gene expression was noted at 24 h.

In PDLCs, however, under matrix glycation, a transient elevation of type I and type IV collagens was evident at 6 h but appeared to decrease at 24 h. The glucose concentration and *P. gingivalis* LPS did not significantly alter the expression of matrix genes (Fig. 5C and 5D).

Discussion

It has been widely accepted that certain diabetic complications can augment inflammatory responses, and periodontitis is one of the manifestations of diabetes (7). In this study, the accumulation of plaque was evident in STZ-injected animals as a result of alterations in the ecologic environment favoring the growth of gram-negative anaerobic and black-pigmented periodontal pathogens (21,22). With ligature + LPS treatment, STZ-injected rats demonstrated a more exaggerated inflammatory response, indicating that diabetes mellitus may augment periodontitis in the presence of virulence factors. In chronic diabetes, accumulation of AGEs and enhanced expression of RAGE has been hypothesized to be a major cause of exacerbation of tissue injury (23,24). The presence of AGEs and RAGE was evident within the connective tissue of STZ-injected animals and was even more prominent in ligature + LPS-treated sites (Figs 1E, 1F, 2E and 2F). Significant expression of AGEs and RAGE in periodontally inflamed tissues was also observed even in non-diabetic (non-STZ-injected) rats (Fig. 2B and 2C). Similar results were reported by Katz *et al.* (17). They found no obvious difference in the expression of RAGE in periodontally inflamed tissue of

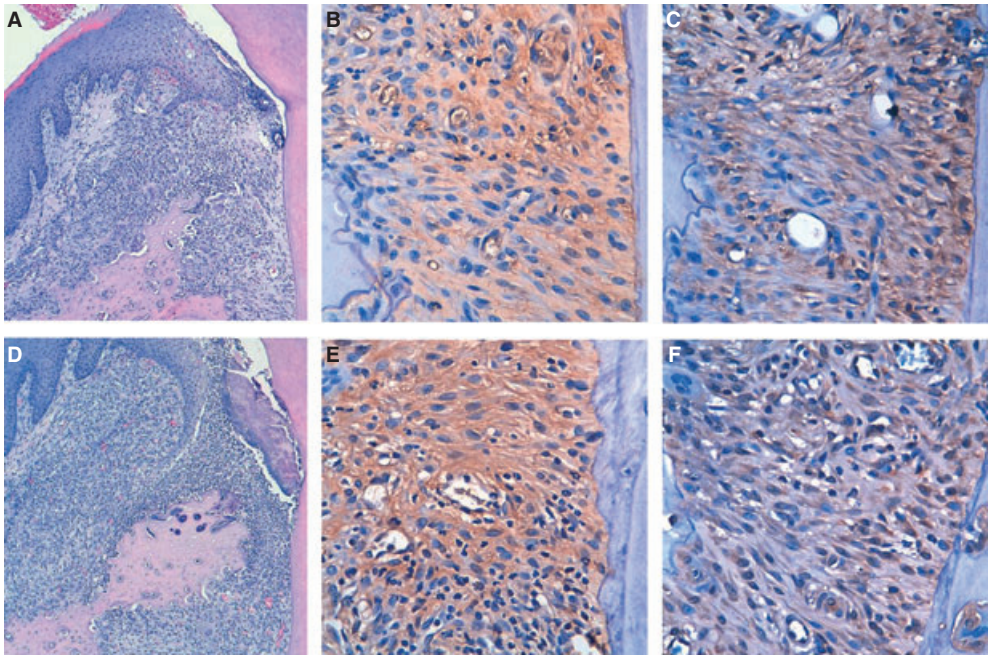

Fig. 2. Descriptive histology of the advanced glycation end-products–receptor for advanced glycation end-products (AGE–RAGE) axis in the experimental periodontitis of nondiabetic (A–C) and diabetic (D–F) rats. The palatal side of the transverse section of the mid-M2 region was selected for evaluation. (A, D) Histological observation by hematoxylin and eosin staining (magnification, 100×). Note the inflammatory infiltrate in the gingival connective tissue and prominent epithelial enlargement. The level of attachment of native periodontal ligament was similar in both nondiabetic and diabetic specimens. (B, E) Immunohistochemical visualization of AGE deposition in the extracellular matrix (magnification, 400×). (C, F) Immunohistochemical visualization of the intracellular expression of RAGE (magnification, 400×). Note that significant deposition of AGE and expression of RAGE could be seen in both nondiabetic and diabetic specimens.

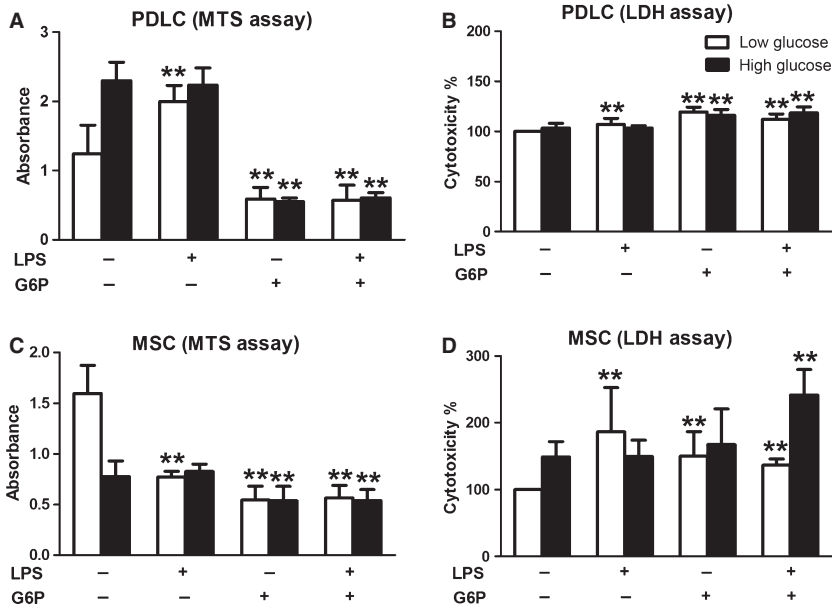

Fig. 3. Viability of mesenchymal stem cells (MSCs) (A, B) and periodontal ligament cells (PDLCs) (C, D). Cells were seeded on nonglycated [0 mM glucose 6-phosphate (G6P)] or glycated (375 mM G6P) collagen gels and were cultured with 10 μg/mL of lipopolysaccharide (LPS) in the presence of 1 g/L (low concentration, white bars) or 4.5 g/L (high concentration, black bars) of glucose for 4 d. Mitogenesis and cytotoxicity were determined by MTS (Promega) and LDH (Roche) assays, respectively. Data are expressed as mean ± standard deviation of three independent experiments. Significant differences from control were calculated by one-way analysis of variance followed by Tukey's *post-hoc* test (*$p < 0.05$; **$p < 0.01$).

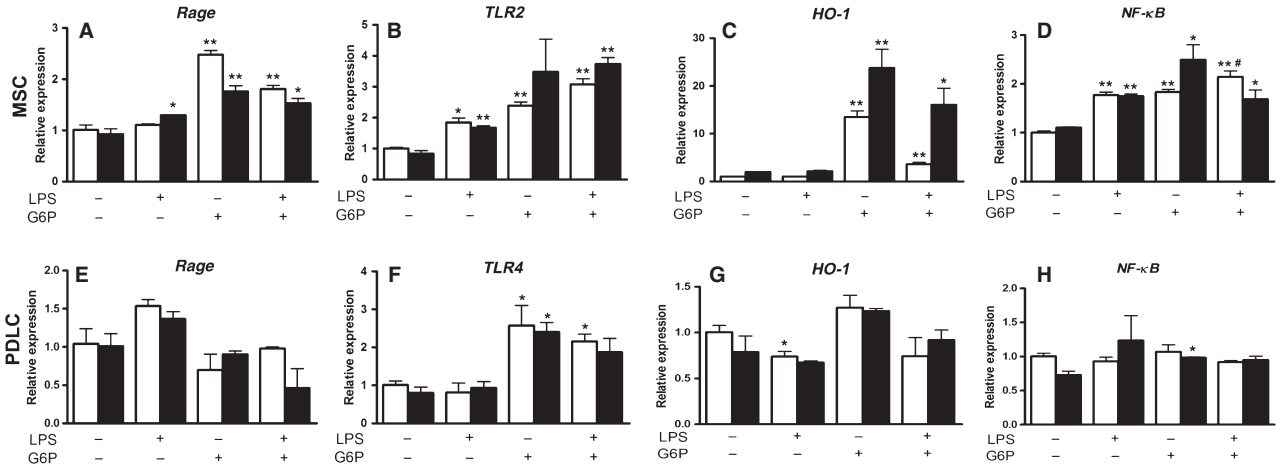

Fig. 4. Ligand–receptor and inflammatory signaling of mesenchymal stem cells (MSCs) (A–D) and periodontal ligament cells (PDLCs) (E–H). The gene-expression profiles of receptor for advanced glycation end-products (*Rage*) (A, E), toll-like receptor *Tlr2* and *Tlr4* (B, F), heme oxygenase-1 (Ho-1) (C, G) and nuclear factor kappa light chain enhancer of activator β cells (*Nf-κβ*) (D, H) were examined in simulated periodontal diabetic conditions, including treatment with 10 μg/mL of lipopolysaccharide (LPS) or seeding on glycated collagen [glucose 6-phosphate (G6P)] gels. Cells were cultured for 24 h in either low-glucose Dulbecco's modified Eagle's medium (DMEM) (white bars) or high-glucose DMEM (black bars), and total RNA was extracted and analyzed by real-time PCR. Expression of mRNA was quantified in relation to a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*). Data are expressed as mean ± standard deviation of three independent experiments. Significant differences from the control were calculated by one-way analysis of variance followed by Tukey's *post-hoc* test (*$p < 0.05$; **$p < 0.01$). Note that in MSCs, the expression of *Nf-κβ* was significantly up-regulated under the combination of *P.gingivalis* LPS and glycated matrix compared with *P.gingivalis* LPS alone or glycated matrix alone at a lower glucose concentration only, whereas the up-regulation was not significant at higher glucose concentrations (panel D; #$p < 0.05$ compared with *P.gingivalis* LPS and glycated matrix, respectively, at a lower glucose concentration).

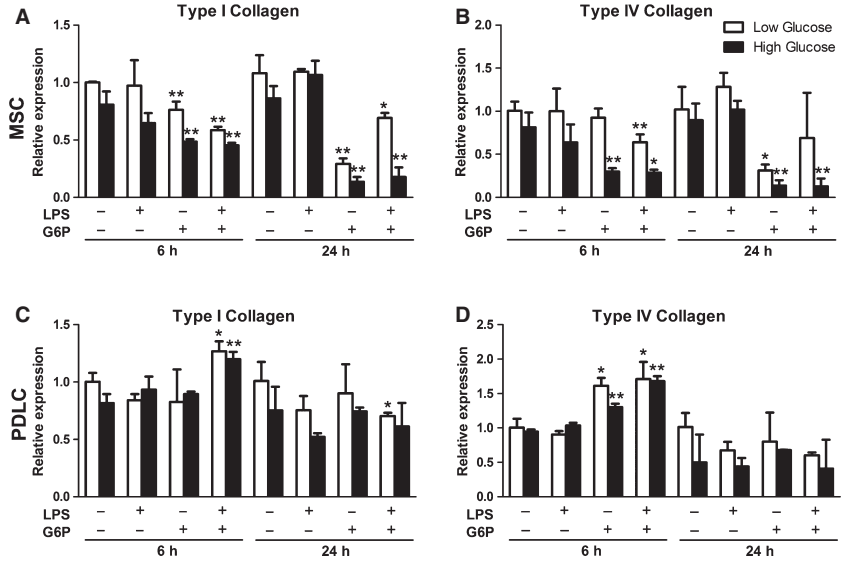

Fig. 5. Matrix synthesis ability of mesenchymal stem cells (MSCs) (A, B) and periodontal ligament cells (PDLCs) (C, D). Gene-expression profiles of type I collagen (A, C) and type IV collagen (B, D) were examined in the simulated periodontal diabetic conditions, including treatment with 10 μg/mL of lipopolysaccharide (LPS) or seeding on glycated collagen [glucose 6-phosphate (G6P)] gels. Cells were cultured for 6 and 24 h in low-glucose Dulbecco's modified Eagle's medium (DMEM) (white bars) or in high-glucose DMEM (black bars), and the total RNA was extracted and analyzed by real-time PCR. The expression of mRNA was quantified in relation to a housekeeping gene [glyceraldehyde-3-phosphate dehydrogenase (GAPDH)]. Data were expressed as mean ± standard deviation of three independent experiments. Significant differences from the control at the same time-point were calculated by one-way analysis of variance followed by Tukey's *post-hoc* test (*$p < 0.05$; **$p < 0.01$).

diabetic and nondiabetic patients, but the level of *Rage* mRNA was significantly higher in the former group. Up-regulation of RAGE and TLRs in diabetic patients with periodontitis was also reported (25), and an investigation further demonstrated that the expression of RAGE, TLRs and inflammatory mediators was more related to chronic periodontitis than to diabetes (26). Taken together, current evidence not only addresses the intimate relationship between diabetes mellitus and RAGE expression but also suggests that the AGE–RAGE axis can be induced as a consequence of periodontal inflammation, perhaps because of increasing oxidative stress and proinflammatory cytokines facilitating nonenzymatic glycation, leading to the accumulation of AGEs in the extracellular matrix to activate RAGE (27).

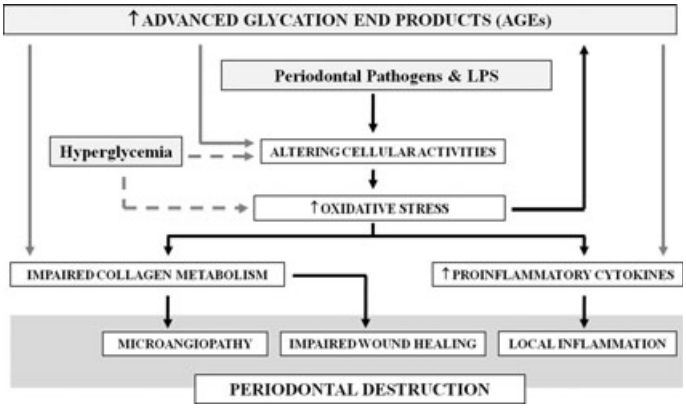

Fig. 6. Proposed mechanism for the inter-relationship among periodontitis, diabetes and advanced glycation end-products (AGEs). In response to periodontal microorganisms, cellular activities are altered to elevate oxidative stress, leading to impaired collagen metabolism, the production of proinflammatory cytokines and consequently to periodontal destruction (solid dark lines). The evaluated oxidative stress may induce extensive glycation of protein and lipid and form AGEs. Accumulation of AGEs may alter cellular activity, impair collagen metabolism and elicit cytokine production (solid grey lines). Hyperglycemia may directly influence the cell behavior and increase oxidative stress to induce the formation of AGEs (dashed grey lines).

Our results revealed that TLRs, as well as RAGE, were up-regulated by seeding the cells on glycated matrix, which consequently activated NF-κβ signaling (Fig. 4). To the best of the authors' knowledge, no *in-vitro* study has confirmed this phenomenon, and only a recent *in-vivo* study appeared to report equivalent results (28). These results demonstrated that AGEs facilitated inflammatory and fibrotic effects of the proximal tubular epithelial cells, suggesting that AGEs mediate tissue inflammation. On the other hand, we also demonstrated that *P. gingivalis* LPS could activate RAGE expression in PDLCs seeded on the nonglycated matrix (Fig. 4E), implying that periodontopathogens potentially activate signaling pathways in parallel to AGEs. The major effect of hyperglycemia in tissue destruction appears to be the induction of expression of reactive oxygen species to increase oxidative stress (Fig. 4C) and subsequently to activate RAGE signaling and tissue inflammation (29). However, the effect on the activation of receptors and inflammatory signaling was not pronounced compared with *P. gingivalis* LPS stimulation or tissue glycation in this study, indicating that hyperglycemia might not be the direct etiological factor to modulate periodontal inflammation. According to the results of the present study, the involvement of AGEs in periodontitis is illustrated in Fig. 6. Taken together, the results confirm that the existence of the AGE–RAGE axis could augment periodontal inflammation, and that AGEs and stimulation with *P. gingivalis* LPS might converge via the same ligand–receptor pathway. Controlling matrix glycation might be considered as a potential treatment alternative in the management of periodontitis.

Differences in cell viability and in gene expression in PDLCs and MSCs were also observed in this study. The high glucose concentration appeared to support the viability of PDLCs but inhibit the activity of MSCs (Figure S1). The results obtained for PDLCs appeared to contradict previous investigations (14,15), perhaps because of the cell–matrix interaction on seeded collagen matrix. In this study, the suppression of cathepsin activity potentially prevented the degradation of the collagen matrix and up-regulated adhesion receptors in PDLCs (30,31). In MSCs, however, abruptly elevated oxidative stress under high-glucose concentration (Fig. 4B) might increase the cytotoxicity, consequently reducing the viability of cells. Therefore, under the same conditions of matrix glycation or stimulation with *P. gingivalis* LPS, PDLCs also revealed a delayed and relatively modest response in the activation of the ligand–receptor interaction, inflammatory signaling and matrix synthesis compared with MSCs (Figs 3 and 4). Overall, PDLCs were not as sensitive as MSCs in response to *P. gingivalis* LPS, G6P or a high-glucose concentration. This could explain the findings in this experimental periodontitis model, where bone destruction in diabetic rats appeared more progressive but the level of periodontal ligament attachment was equivalent to that of nondiabetic rats (Table 1). Studies also demonstrated that the differentiation capability of MSCs could be significantly suppressed under matrix glycation or hyperglycemia (32), further highlighting the importance of inflammation and glycemic control in recovering the regenerative capability of MSCs in the periodontium.

This study had limitations. First, only cells with regenerative potential (i.e. PDLCs and MSCs) were examined *in vitro*, whereby the response of gingival fibroblasts, epithelial cells and inflammatory cells was not investigated. Second, although the artificial glycated matrix presented a structure similar to that of the glycated extracel-

lular matrix, the deposition of G6P in the collagen matrix might not fully reflect the cell–matrix interaction in diabetic subjects. Third, *P. gingivalis* LPS was the only virulence factor *in vitro*. Although *P. gingivalis* is the predominant periodontopathogen in chronic periodontitis, the activation of inflammatory signaling by *P. gingivalis* LPS appeared to be weaker than that induced by the LPS from *Aggregatibacter actinomycetemcomitans* or *Escherichia coli*, and the effect in fibroblasts or endothelial cells was not as evident as in macrophages or dendritic cells (33–35). *P. gingivalis* LPS may also interfere with the signaling mediated by *E. coli* LPS (33). Furthermore, there is a lack of direct mechanistic evidence for the formation of AGEs under inflammatory conditions and for the modulation of periodontal inflammation by AGEs in physiologically healthy conditions.

Within the limitations of the study, we demonstrated a close inter-relationship between the AGE–RAGE axis and periodontitis, whereby the AGE–RAGE axis was activated in experimental periodontitis, and matrix glycation might further augment inflammation. Future investigations will aim to confirm the role of AGEs in modulating periodontal inflammation and to validate the effect of anti-glycation agents in controlling periodontal inflammation.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Details of the primers and probes.

Figure S1. Viability of MSCs (A, B) and PDLCs (C, D).

Figure S2. Confocal reflectance microscopic image for the fiber arrangement in the seeding collagen matrix.

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