Advanced glycation end-products increase lipocalin 2 expression in human oral epithelial cells

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Abstract

Background and Objectives: Diabetes mellitus (DM), a risk factor of periodontal diseases, exacerbates the pathological condition of periodontitis. A major factor for DM complications is advanced glycation end-products (AGEs) that accumulate in periodontal tissues and cause inflammatory events. Lipocalin 2 (LCN2) is an antimicrobial peptide and inflammation-related factor, and LCN2 levels increase in DM. In the present study, the effects of AGEs and lipopolysaccharide of *Porphyromonas gingivalis* (*P.g*-LPS) on LCN2 expression in human oral epithelial cells (TR146 cells) and the role of secreted LCN2 in periodontitis with DM were investigated.

Material and Methods: TR146 cells were cultured with AGEs (AGE2) and control BSA and cell viability was estimated, or with *P.g*-LPS. Conditioned medium and cell lysates were prepared from cultures of epithelial cells and used for western blotting and ELISA to analyze LCN2, RAGE, IL-6, MAPK and NF-κB. RNA was isolated from AGE-treated TR146 cells and differentiated HL-60 (D-HL-60) cells and used for quantitative real-time PCR to examine the expression of LCN2 and interleukin-6 (IL-6) mRNAs. RAGE- and LCN2-siRNAs (siRAGE, siLCN2) were transfected into epithelial cells,

and AGE-induced LCN2 expression was investigated. D-HL-60 cells were co-cultured with TR146 cells that were transfected with siLCN2 and treated with AGEs, IL-6 mRNA expression in D-HL-60 cells and cell migration were investigated.

Results: AGEs increased the expression levels of LCN2 and IL-6 in oral epithelial cells. siRAGE and a neutralizing antibody for RAGE inhibited AGE-induced LCN2 expression. AGEs stimulated the phosphorylation of ERK, p38 and NF-κB in epithelial cells, and their inhibitors suppressed AGE-induced LCN2 expression. In contrast, *P.g*-LPS did not show a significant increase on LCN2 level in TR146 cells that expressed toll-like receptor 2. In co-culture experiments, AGE-induced LCN2 inhibited IL-6 mRNA expression in D-HL-60 cells, and LCN2 knockdown in epithelial cells suppressed HL-60 cell migration.

Conclusion: These results suggested that AGEs increase LCN2 expression via RAGE, MAPK, and NF-κB signaling pathways in oral epithelial cells, and secreted LCN2 may influence the pathological condition of periodontitis with DM.

1 | INTRODUCTION

Diabetes mellitus (DM) is a metabolic disease involving hyperglycemia, and it causes complications including nephropathy, neuropathy, retinopathy, and periodontal diseases.[1] DM patients have a high prevalence of periodontal diseases, and periodontitis in DM patients sometimes shows severe inflammation with multiple gingival abscesses and destruction of periodontal tissues and resorption of alveolar bone.[2,3] Advanced glycation end-products (AGEs) are a major factor that cause DM complications and induce inflammatory responses in some systemic tissues.[4,5] AGEs accumulate at greater levels in periodontal tissues of DM patients compared to non-DM individuals,[6] and AGEs were observed in endothelial cells, epithelial cells, fibroblasts and inflammatory cells in periodontal tissues of DM-associated periodontitis.[7] AGEs bound to the receptor of AGE (RAGE), and increased interleukin-6 (IL-6) expression and reactive oxygen species (ROS) activity, and they decreased type I collagen expression in gingival fibroblasts.[8-10] The expression of vascular endothelial growth factor (VEGF) and inflammatory responses were induced by AGEs in human synoviocytes,[11] and the secretion of IL-1β and TNF-α was increased by AGEs in human macrophages.[12]

Furthermore, AGEs inhibited osteoblastic cell differentiation, increased sclerostin expression in osteocytes, weakened bone structure, activated osteoclasts, and induced bone resorption.[13-15] On the other hand, gingival epithelial cells express RAGE,[16] and AGEs increased the expression of calprotectin (S100A8 and S100A9), which is an antimicrobial peptide and damage-associated molecular patterns in human gingival epithelial cell line.[17] AGEs are thought to aggravate inflammation and tissue degradation by regulating the expression levels of inflammation-related factors in periodontal tissues, whereas AGEs are involved in the regulation of factors related to innate immunity. However, there are few studies of AGEs in epithelial cells and their effect on oral epithelial cells is not well known.

Lipocalin 2 (LCN2), neutrophil gelatinase-associated lipocalin (NGAL), and 24p3, a secreted glycoprotein with a molecular weight of 24-25 kDa, are detected in organs including lung, breast, trachea prostate colon and kidney.[18] LCN2 is expressed in immune cells and epithelial cells, and LCN2 level increased in inflammatory bowel and skin diseases, kidney disorders and obesity.[19-20] Serum LCN2 levels in DM patients with kidney diseases were higher than those of healthy individuals, and plasma LCN2 levels were

elevated in patients with gestational diabetes,[22,23] suggesting that LCN2 is related to pathological conditions in DM. LCN2 expression was increased by IL-1β in a human alveolar basal epithelial cell line, but IL-6, TNF-α and LPS did not show significant effects.[24] In contrast, TNF-α and IFN-γ induced LCN2 expression and secretion in cultured human and mouse adipocytes,[25] and LPS elevated LCN2 expression in retinal glial cells,[26] and IL-6 and LPS stimulated LCN2 production in podocytes in kidney.[27] Furthermore, glycated human albumin increased LCN2 expression in human aortic smooth muscle cells.[28] Differential regulation of LCN2 production by several inflammation-related factors are reported in some cell species.

LCN2 binds to a specific receptor called 24p3R,[19,26,29] and shows multiple functions, including antimicrobial activity, modulating inflammation and cell growth/migration/invasion.[19,20,21] For example, LCN2 bound bacterial siderophores and inhibited bacterial growth by depletion of iron and showed an antibacterial action.[30,31] LCN2 modulates inflammatory responses by inducing the expression of inflammation-related cytokines such as IL-6, IL-8 and TNF-α.[32] In contrast, LCN2 expressed anti-inflammatory actions that suppressed LPS-induced expressions of TNF-α and IL-6 in

murine retinal cells.[26] Furthermore, LCN2 induced chemotaxis of neutrophils,[33,34] but knockdown of LCN2 promoted migration and its overexpression reduced the migration of colorectal cancer cells,[35] and the over-expression of LCN2 was observed in more grown breast tumor, and a complex with LCN2 and matrix metalloproteinase-9 (MMP-9) influenced tumor growth and invasion.[36] LCN2 plays complex roles in cell migration and proliferation. Regarding periodontal diseases, LCN2 and MMP-9 were observed in polymorphonuclear leukocytes and connective tissues of adult and juvenile periodontitis,[37] and LCN2 levels in periodontitis patients were significantly higher than those in healthy individuals.[38] Although LCN2 may influence inflammation in periodontal diseases, the exact role of LCN2 in periodontitis is not well known.

In the present study, to elucidate a role of LCN2 in DM-associated periodontitis, the effects of AGE and *Porphyromonas gingivalis*-lipopoly-saccharides (*P.g*-LPS) on LCN2 expression were investigated in human oral epithelial cells, and the regulatory mechanisms of AGE-induced LCN2 expression and functions of the induced LCN2 were investigated.

2 | MATERIAL AND METHODS

2.1 | AGEs and reagents

AGEs (AGE2) were prepared in accordance with a modified method of Okazaki *et al.*.[39] In brief, DL-glyceraldehyde (0.1 M; Sigma-Aldrich, St. Louis, MO, USA) and bovine serum albumin (BSA; 50 μg/ml; Sigma-Aldrich) were mixed in a sterile phosphate buffer (0.2 M, pH 7.4) containing penicillin (100 U/ml) and streptomycin (100 μg/ml), and incubated on shaking at 37°C for 7 days. The mixture was dialyzed against phosphate-buffered saline (PBS; pH 7.4) for 3 days. Non-glycated BSA as a control was prepared from a mixture without glyceraldehyde in the same conditions. The fluorescence of prepared AGE2 and non-glycated BSA solutions was determined at an excitation/emission wavelengths of 370/440 nm to assess AGE activity, and the fluorescence strength of AGE solution was 40-fold greater than that of non-glycated BSA.

Ham's F-12, Dulbecco's modified Eagle's medium (DMEM) and RPMI1640 media were obtained from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Two *P.g*-LPS were purchased from InvivoGen (*P.gingivalis* LPS;TLR2 ligand, LPS-PG;TLR2/TLR4 ligand, San Diego, CA,

USA).

2.2 | Cell cultures

Human oral epithelial cells (TR146 cells) were kindly supplied by Dr. Mark Herzberg (Minnesota University). Human gingival epithelial cells (Ca9-22 cells) and HL-60 cells were obtained from the National Institutes of Biomedical Innovation, Health and Nutrition (Osaka, Japan). TR146 cells were seeded at 5000 cells/cm^2 and cultured in Ham's F-12 supplemented with 10% fetal bovine serum (FBS), penicillin and streptomycin for 5 days. Epithelial cells were cultured with 50 -1000 μg/ml AGEs or control BSA for 24-72 h and then used for the cell viability assay and extraction of RNA and protein (cell lysate and conditioned medium). Sub-confluent epithelial cells were treated with AGEs (500 μg/ml) or control BSA (500 μg/ml) for 15-60 min, and the cellular fraction was collected for western blotting of MAPK and NF-κB phosphorylation. In experiments with MAPK and NF-κB inhibitors, confluent cells were pre-cultured with SB203580 (15 μM), U0126 (10 μM), SP600125 (10 μM) or Bay11-7082 (10 μM) for 1 h and cultured with AGEs (500 μg/ml) or control BSA (500 μg/ml) for 48 h. Conditioned medium was

used for LCN2 ELISA. Ca9-22 cells were seeded at $1.5x10^4$ cells/ml and cultured in Eagle's Minimum Essential Medium (E-MEM) supplemented with 10% FBS, penicillin and streptomycin for 4 days. After reaching sub-confluency, Ca9-22 cells were cultured with AGEs (500 μg/ml) or control BSA (500 μg/ml) for 24 h and 48 h, and total RNA was isolated from the treated cells for quantitative real-time polymerase chain reaction (qRT-PCR) and the conditioned medium was used for enzyme-linked immunosorbent assay (ELISA). In an experiment with a neutralizing antibody, sub-confluent TR146 cells were pre-cultured with a neutralizing antibody against RAGE (1/20 dilution, GeneTex, Hsinchu City, Taiwan) for 2 h and then cultured with AGEs (500 μg/ml) or control BSA (500 μg/ml) for 48 h. In the experiment using two *P.g*-LPSs, TR146 cells were cultured with *P.g*-LPS for TLR2 ligand (0.2-5 μg/ml), *P.g*-LPS for TLR2/TLR4 ligand (0.25-1 μg/ml), and AGEs (500 μg/ml) or BSA (500 μg/ml) for 48 h. The collected conditioned medium was used for LCN2 ELISA and the cell lysate was used for western blotting of RAGE and TLR2.

HL-60 cells were seeded at $150x10^4$ cells/ml in RPMI-1640 medium supplemented with 20% FBS and antibiotics and grown, and then cultured

with 1.25% dimethyl sulfoxide (DMSO; FUJIFILM Wako Pure Chemical Corp.) for 5 days to differentiate granulocyte-like cells according to a modified method of Manda-Handzlik *et al.*.[40]

2.3 | Cell viability assay

The effect of AGEs on the viability of TR146 cells was examined using a Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) in accordance with the manufacturer's instructions. Briefly, epithelial cells were seeded at cell density of 5000 cells/cm^2 on 96-well plates, and were cultured for 5 days and further cultured with AGEs (500 μg/ml) or control BSA (500 μg/ml) for 24-72 h, and then incubated with CCK-8 solution for 2 h. The absorbance of a well was measured at wavelength of 450 nm using a microplate reader.

2.4 | RNA isolation and qRT-PCR

Total RNAs were isolated from TR146 cells, Ca9-22 cells and HL-60 cells using a RNeasy Mini Kit (QIAGEN, Hilden, Germany). Complementary DNAs were synthesized from total RNA using the DNase I Amplification Grade (Invitrogen, Life Technologies Corp./Thermo Fisher Scientific,

Carlsbad, CA, USA) and PrimerScript® II 1st strand cDNA Synthesis Kit (TaKaRa Bio., Otsu, Japan) in accordance with the manufacturer's instructions. In qRT-PCR, the PCR mixture contained a synthesized cDNA, primers of BD-2, IL-1β, IL-6, LCN2, RAGE, SLPI, TNF-α, and β-actin and SYBR Green (Sso Advanced Universal SYBR Green Supermix, Bio Rad). PCR reaction was performed at 95°C for 30 sec once, at 95°C 10 sec and 60°C 30 sec for 40 cycles, and at 65°C for 5 sec and 95°C for 50 sec each once using a CFX96™ Real-time PCR Detection System (Bio-Rad). The levels of mRNA expression were normalized to β-actin mRNA. The sequences of the forward and reverse primers used in the present experiments were as follows: β-actin (*ACTB*); 5'-CGTCCACCGCAAATGCTT-3' and 5'-GTTTTCTGCGCAAGT TAGGTTTTG-3', BD-2(*DEFB4A*); 5'-GGTGTTTTTGGTGGTATA GGCG-3' and 5'-AGGGCAAAAGACTGGATGACA-3', *IL1B*; 5'-ACAGATGAAGTGCTC CTTCCA-3' and 5'-GTCGGAGATTCGTAGCTGGAT-3', *IL6*; 5'-AGGGCTCTT CGGCAAATGT-3' and 5'-GAAGAAGGAATGCCCATTAAC AAC-3', *LCN2*; 5'-CCACCTCAGACCTGATCCCA-3' and 5'-CCCCTGGAATTG GTTGTCCTG-3', *RAGE*; 5'-TCCAGGATGAGGGGATTTTC-3' and 5'-CCAAGTGCCAGCTAA GAGTC-3', *SLPI*; 5'-GCATCAAATGCCTGGATCCT-3' and 5'-GCATCAAAC

ATTGGCCATAAGTC-3', *TNFA*; 5'-TCAATCGGCCCG ACTATCTC-3' and 5'-ACAGGGCAATGATCC CAAAGT.

2.5 | ELISA

Sub-confluent TR146 cells were cultured with AGEs or control BSA at concentrations of 50-1000 μg/ml for 24-72 h. In experiments using *Pg.*-LPS, oral epithelial cells were treated with *P.g*-LPS (1 μg/ml) in the presence or absence of AGEs (500 μg/ml) or BSA (500 μg/ml) for 48 h. In co-culture experiments, AGE-stimulated TR146 cells and differentiated HL-60 (D-HL-60) cells were co-cultured for 24 h. After each culture, the conditioned medium was collected and mixed with phenylmethylsulfonyl fluoride (PMSF, Sigma-Aldrich). LCN2 and IL-6 in the conditioned medium were determined using a Human Lipocalin-2/NGAL Immunoassay (R&D Systems, Minneapolis, MN, USA) and a Human IL-6 Immunoassay (R&D Systems), respectively, in accordance with each manufacturer's instructions.

2.6 | Western blotting

Oral epithelial cells at sub-confluency were cultured with AGEs (500 μg/ml)

or control BSA (500 μg/ml) for 48 h, and cellular fractions were collected. In experiments with MAPK and NF-κB phosphorylation, cells were treated with AGEs (500 μg/ml) or BSA (500 μg/ml) for 15-60 min, and cell samples were isolated. The cellular fractions were dissolved in RIPA Lysis Buffer containing protease inhibitor cocktail and sodium orthovanadate (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and incubated on ice for 15 min and centrifuged at 10,000x*g* at 4°C for 10 min. The supernatant was used for western blotting analysis. Total protein in the supernatant was determined using a BCA Protein Assay Kit (TaKaRa Bio.). The proteins (2.5-12.5 μg) were electrophoretically separated on SDS-polyacrylamide gel (10%) and transferred to a polyvinylidene difluoride membrane (Immobilon®-P, Merck Millipore Ltd., Carrigtwohill, Co. Cork, ILR). The membranes were blocked using PVDF Blocking Reagent for CanGet Signal® (TOYOBO, Osaka, Japan) at room temperature for 1 h and washed in Tris-buffered saline-Tween 20 three times. The blocking membranes were reacted with an anti-RAGE antibody (1/200 dilution, Abcam, Cambridge, MA, USA), anti-p38 and anti-phospho-p38 (1/1000 dilution, Cell Signaling Technology, Danvers, MA, USA), anti-p44/42 (ERK) and anti-phospho-ERK (1/1000 dilution, Cell Signaling

Technology), anti-SAPK/JNK (JNK) and anti-phospho-JNK (1/1000 dilution, Cell Signaling Technology,), and anti-NF-κB p65 and anti-phospho-NF-κB p65 (1/1000 dilution, Cell Signaling Technology) in CanGet Signal solution 1 (TOYOBO) at 4°C overnight. After washing, the membranes were reacted with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Cell Signaling) at room temperature for 1 h. The membrane was treated with stripping buffer, blocked, and reacted with anti-β-actin antibody at room temperature for 1 h and then with HRP-conjugated secondary antibody. The membranes reacted with antibodies were developed using ECL™ Western Blotting Detection Reagents (GE Healthcare) and visualized using Image Quant LAS 500 (GE Healthcare). To detect TLR2 in TR146 cells treated with *P.g*-LPS and 24p3R in D-HL-60 cells, the cellular fraction was isolated from each cell lysate. In contrast, conditioned media were collected from the cultures of TR146 cells and D-HL-60 cells to detect LCN2. These fractions were separated by SDS-PAGE, and the cellular protein (10-20 μg total protein) and medium fraction (10 μl) on gels were transferred to PVDF membrane and reacted with anti-TLR2 antibody (1/2000 dilution, GeneTex), anti-LCN2 antibody (1/1000 dilution, LSBio, TE Huissen, The Netherlands)

or anti-BOCT (lipocalin 2 receptor; 24p3R) antibody (1/1000 dilution, St John's Laboratory Ltd., London, UK) at room temperature for 2-3 h, and then reacted with HRP-conjugated anti-rabbit polyclonal antibody (1/15000 dilution, GeneTex) at room temperature for 1 h. After washing, the membranes were developed by ECL™ Western Blotting Detection Reagents (GE Healthcare), and the reactive signals were visualized using Image Quant LAS 500 (GE Healthcare) and analyzed using Image J software (NIH, USA).

2.7 | siRNA transfection and co-culture with TR146 and D-HL-60 cells

TR146 cells were seeded at 5.4×10^4 cells/cm^2 and cultured in Ham's F-12 medium with 10% FBS for 24 h and reached to 70% confluency, and transfected with RAGE siRNA (siRAGE, 6.25 nM; Sigma-Aldrich/Merck KGaA, Darmstadt, Germany) and Control siRNA (siControl, 6.25 nM) dissolved in Opti-MEM medium (Thermo Fisher SCIENTIFIC, Waltham, MA, USA) using Lipofectamine RNAiMax (Thermo Fisher SCIENTIFIC) in accordance with the manufacturer's instructions. Cells were further cultured with AGEs (500 μg/ml) or control BSA (500 μg/ml) for 24 h to isolate RNA and for 48 h to collect conditioned medium. RNA from the transfected

cells was used for qRT-PCR and the conditioned medium was for ELISA in order to investigate the expression levels of RAGE and LCN2.

In co-culture experiments with TR146 cells and HL-60 cells, epithelial cells ($8x10^4$ cells /cm^2) were seeded into the outer-well of Transwell® Permeable Supports (Corning, Kennebunk, ME, USA) and cultured in Ham's F-12 supplemented with 10% FBS for 24 h, and then transfected with LCN2 siRNA (siLCN2, 6.25 nM; Sigma-Aldrich/Merck) and siControl (6.25 nM) using Lipofectamine RNAiMax (Thermo Fisher SCIENTIFIC). The transfected TR146 cells were cultured with AGEs (500 μg/ml) or BSA (500 μg/ml) for 48 h, and washed with PBS (pH 7.4) to remove AGEs and BSA. D-HL-60 cells ($350x10^4$ cells/ml) were seeded in the inserts of Transwell® Permeable Supports (3 μm polycarbonate membrane, Corning) and co-cultured with TR146 cells that were transfected with siLCN2 in AGE-free medium (RPMI1640 and Ham's F-12 medium) with 10% FBS for 24 h. Total RNA was isolated from TR146 cells and D-HL-60 cells, and used for qRT-PCR to examine the expression levels of *LCN2* and *IL6* mRNAs, respectively. The conditioned medium was collected from the co-culture system and used for ELISA of LCN2 or IL-6.

2.8 | Cell migration assay

D-HL-60 cells ($350x10^4$ cells/ml) and TR146 cells transfected with siLCN2 or siControl, and treated with AGEs (500 μg/ml) or BSA (500 μg/ml) were co-cultured for 24 h using Transwell® plates (3 μm pore size-polycarbonate membrane, Corning), and the migration of D-HL-60 cells was assayed in accordance with the modified method of Justus *et al.*.[41] Briefly, D-HL-60 cells were removed from Transwell inserts after co-culture of D-HL-60 cells and TR146 cells, and an insert membrane was carefully washed in PBS, dipped in 70% ethanol solution to fix D-HL-60 cells that migrated to the undersurface of the Transwell insert for 10 min. The membrane of the Transwell insert was dried for 30 min and stained in 0.2% crystal violet solution for 10 min at room temperature. After remove excessive staining solution, the membrane with migrated HL-60 cells was very carefully dipped into distilled water many times, and dried for over 30 min. The cells migrated to an undersurface of Transwell insert were observed using a phase-contrast microscope (magnification x 100), and the number of migrated cells was counted in four microscopic field of three separated insert membranes in

each experimental group.

2.9 | Statistical analysis

The number of samples in all experiments was more than four (n=4-10). Statistical analyses were performed using Excel Analysis 2012 for Windows (SSRI, Tokyo, Japan). The comparisons among multiple groups were performed by a one-way analysis of variance (ANOVA) followed by Tukey-Kramer analysis, and the significance of differences between two groups was estimated using Student's *t*-test. *P* values less than 0.05 were considered to be significant.

3 | RESULTS

3.1 | Effect of AGEs on cell viability of TR146 cells

The viability of TR146 cells was not significantly changed when epithelial cells were cultured with AGEs (500 μg/ml) for 72 h (Supplementary figure 1). Their percentages were 100% at 24 h, 92.3% at 36 h, 92.1% at 48 h, and 85.1% at 72 h. Significant differences in cell viability between the AGE and control BSA groups were not observed during 24-72 h.

3.2 | Effects of AGEs on the expression of LCN2 and IL-6 in TR146 cells

AGE treatment significantly increased the expression of LCN2 mRNA to approximately 1.8-fold that of control BSA when TR146 cells were stimulated by AGEs (500 μg/ml) for 24 h (Figure 1A). *IL6* mRNA expression was also increased by AGEs (500 μg/ml) approximately 2-fold in TR146 cells (Figure 1B). AGEs (500 μg/ml) increased LCN2 mRNA expression by approximately 1.8-fold in Ca9-22 human gingival epithelial cells (Figure 1C). In contrast, the expression levels of BD-2 (β-defensin 2) and SLPI, other antimicrobial peptides, were not influenced by AGEs in TR146 cells (Supplementary Figure 2A and 2B).

3.3 | Effects of AGEs on the production of LCN2 and IL-6 in TR146 cells

LCN2 production was significantly increased on 48-72 h when TR146 cells were cultured with AGEs (500 μg/ml) for 24-72 h, and its level was approximately 1.8-fold that of control BSA at 48 h, and 1.9-fold at 72 h (Figure 2A). When epithelial cells were stimulated by 50-1000 μg/ml AGEs for 48 h, AGEs significantly increased LCN2 production at concentrations of 100-500

μg/ml, and the elevated LCN2 level reached approximately 1.6-fold of that of control with 500 μg/ml AGEs (Figure 2B). AGEs (500 μg/ml) significantly increased the productions of IL-6 as well as LCN2 in TR146 cells (Figure 2C), and similarly up-regulated LCN2 production in Ca9-22 cells; however, the level of LCN2 concentration in Ca9-22 cells was low, approximately 5% of its level in TR146 cells (Figure 2D).

3.4 | Inhibitory effect of siRAGE and neutralizing antibody on AGE-induced LCN2 expression

TR146 cells expressed RAGE, but its level was not changed by AGE stimulation (Figure 3A). When RAGE siRNA was transfected into TR146 cells, the expression of RAGE mRNA in epithelial cells was significantly inhibited (Figure 3B). RAGE siRNA significantly inhibited AGE-induced LCN2 mRNA expression and its protein production to similar level by BSA induction (Figure 3C, 3D). Furthermore, anti-RAGE neutralizing antibody significantly inhibited AGE-stimulated LCN2 production (Figure 3E).

3.5 | Involvements of MAPK and NF-κB in AGE-induced LCN2 expression

To investigate the involvement of MAPK in AGE-stimulated LCN2 expression, western blotting analyses of MAPK phosphorylation were performed. The phosphorylation of ERK and p38 was increased by AGE stimulation for 15 or 60 min, respectively, however AGEs did not influence JNK phosphorylation (Figure 4A). When epithelial cells were cultured with AGEs after pre-treatment with MAPK inhibitors, AGE-induced LCN2 production was significantly inhibited by p38 inhibitor (SB203580) and ERK inhibitor (U0126), but not by JNK inhibitor (SP600125) (Figure 4B). With regard to NF-κB, AGEs stimulated NF-κB phosphorylation compared with control BSA (Figure 4C), and NF-κB inhibitor (Bay11-7082) significantly decreased AGE-induced LCN2 production (Figure 4D).

3.6 | Effect of AGEs and *P.g*-LPS on LCN2 production in TR146 cells

TR146 cells expressed TLR2, however, its level was not changed by stimulation with *P.g*-LPS, with or without AGEs or BSA (Figure 5A). When TR146 cells were cultured with *P.g*-LPS as a TLR2 ligand at concentrations of 0.2-5 μg/ml and *P.g*-LPS as a TLR2/TLR4 ligand at concentrations of 0.25-1 mg/ml for 48 h, two *P.g*-LPSs did not show a significant change in LCN2

production (Supplementary Figure 3A and 3B). Furthermore, LCN2 production in epithelial cells stimulated with *P.g*-LPS as a TLR2 ligand (1 μg/ml) and AGEs (500 μg/ml) was not increased more than that with AGE stimulation (Figure 5B).

3.7 | Effect of AGE-induced LCN2 from TR146 cells on IL-6 expression in D-HL-60 cells

TR146 cells secreted LCN2 into the cultured medium, but D-HL-60 cells did not (Figure 6A). The signals of 24p3R-long and -short type expressed in D-HL-60 cells, but both types of LCN2 receptor were not expressed in TR146 cells (Figure 6A). The expression levels of LCN2 mRNA were significantly inhibited in TR146 cells transfected with siLCN2 and then stimulated with AGEs and BSA (Figure 6B). LCN2 secretion was significantly increased by AGE treatment in TR146 cells transfected with siControl and siLCN2, and AGE- and BSA-induced LCN2 secretion was significantly decreased by LCN2 mRNA knockdown in a similar manner to a change in *LCN2* mRNA (Figure 6C). When D-HL-60 cells were co-cultured with TR146 cells transfected with siLCN2 or siControl, the level of *IL6* mRNA expression in D-HL-60 cells in

the siLCN2 group was significantly higher than that in the siControl group (Figure 6D). *IL6* mRNA expression in HL-60 cells, which were co-cultured with AGE-stimulated TR146 cells, was significantly reduced as compared with BSA-treated TR146 cells in both groups with siControl and siLCN2. Furthermore, IL-6 protein levels in the conditioned medium from the co-culture with TR146 cells transfected with siLCN2 and D-HL-60 cells also was higher than that in siControl group (Supplementary figure 4). These results suggested that LCN2 from epithelial cells suppressed IL-6 expression in neutrophils and AGEs promoted this down-regulation of IL-6 expression through the LCN2 pathway. In contrast, *IL1B* mRNA expression in D-HL-60 cells co-cultured with siLCN2-transfected and BSA-treated TR146 cells slightly increased compared with that of siControl-transfected TR146 cells, but *IL1B* mRNA expression in the AGE-treatment group did not significantly change between siLCN2 and siControl (Supplementary figure 5A). In addition, there were no differences in *TNFA* mRNA expression in D-HL-60 cells between siLCN2 and siControl, and between AGEs and BSA (Supplementary figure 5B).

3.8 | Effect of AGE-induced LCN2 from TR146 cells on the migration of D-HL-60 cells

The migration of D-HL-60 cells co-cultured with TR146 cells transfected with siLCN2 was significantly inhibited as compared with that of siControl in BSA- and AGE-treated TR146 cell groups (Figure 7). When D-HL-60 cells were co-cultured with AGE-treated TR146 cells that was transfected with siControl, the migration of D-HL-60 cells significantly increased as compared with BSA-treated TR146 cells, but an AGE-induced increase was not observed in the group with TR146 cells transfected with siLCN2 (Figure 7E). The results in Figure 6C and 7E suggested that LCN2 from epithelial cells enhanced the migration of neutrophils.

4 | DISCUSSION

LCN2 is expressed in some cells including neutrophils, macrophages, several epithelial cells, adipocytes and kidney cells, and LCN2 expression increased in several inflammatory diseases including psoriasis, periodontitis and ulcerative colitis.[19,20,21] LCN2 (NGAL) was detected in polymorphonuclear leukocytes infiltrated into gingival connective tissues with periodontitis,[37]

and LCN2 levels in gingival crevicular fluid samples from the site with periodontitis were higher than that of healthy samples.[38] We previously showed that primary human gingival epithelial cells expressed LCN2 mRNA and produced LCN2 protein,[42] and that oral epithelial cells (TR146 cells) and gingival epithelial cells (Ca9-22 cells) also produced and secreted LCN2 in the present study. These reports suggested that LCN2 that is derived from cells in oral and periodontal tissues is related to periodontitis.

LCN2 levels in serum and plasma from patients with type 2 DM were significantly higher than that in healthy individuals.[22,43] LCN2 may influence the pathological condition of DM, but details of the function and mechanism are still being investigating. Chung *et al.*[28] first reported that AGEs, a major factor caused DM complications, increased LCN2 levels in human vascular smooth muscle cells and stimulated invasion and migration of cells by up-regulation of LCN2 expression. AGEs increased the expressions of IL-6 and ICAM-1 in human gingival fibroblasts,[9] and the expressions of IL-6 and LCN2 in oral and gingival epithelial cells, suggesting AGEs influence several cells in periodontal tissues and aggravate inflammation by regulating the expression of inflammation-related factors.

Although AGEs and IL-1α increased LCN2 levels in human oral and gingival epithelial cells,[42] the effects of pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) and *E.coli*-LPS on LCN2 expression appear to be different according to the cell species,[24-27] and their regulation of LCN2 expression is thought to be dependent on the expression of specific receptors and co-factors in various cells.[19] In the present study, although TR146 cells expressed TLR2 and TLR4, and the used two *P.g*-LPSs bound to TLR2 and TLR2/TLR4 did not significantly increased LCN2 expression in epithelial cells. Bondy-Carey *et al.*[44] showed that *P.gingivalis* did not affect the expression levels of LCN2, IL-1β and IL-8 in primary human gingival epithelial cells, but the levels of three factors increased remarkably when *P.gingivalis* was added to the co-culture with epithelial cells and primary human neutrophils, suggesting that *P.gingivalis* mainly increases LCN2 expression in the presence of neutrophils. LCN2 may be produced in AGE-stimulated epithelial cells and *P.gingivalis*-stimulated neutrophils in periodontal tissues of periodontitis with DM, and may influence inflammation in DM-associated periodontitis.

The signaling pathway in AGE-induced LCN2 expression is still not well

understood. In the present study, oral and gingival epithelial cells (TR146 and Ca9-22) expressed RAGE mRNA or its protein, but AGEs did not show a clear change in RAGE level, suggesting that the change in RAGE expression in response to AGEs may be different depending on the cell species. In any case, AGEs increased LCN2 expression through the RAGE pathway because RAGE siRNA and anti-RAGE neutralizing antibody significantly inhibited AGE-induced LCN2 expression in oral epithelial cells.

In a case of oral epithelial cells (TR146 cells), AGEs stimulated the phosphorylation of ERK and p38 and their specific inhibitors significantly inhibited AGE-induced LCN2 expression in oral epithelial cells (TR146 cells). In human aortic smooth muscle cells, AGEs increased the phosphorylation of Akt, ERK, JNK and p38, however ERK and p38 inhibitors did not significantly decrease LCN2 expression.[28] NF-κB was related to the regulatory mechanism of AGE-induced LCN2 expression in TR146 cells in the present study. The promoter region of human lipocalin 2 gene contained response elements for several transcription factors including NF-κB, AP-1 and C/EBPβ.[19,28,45] Bu *et al.*[46] showed that LCN2 expression increased in an NF-κB-dependent manner when human vascular smooth muscle cells were

stimulated with IL-1β. In contrast, Chung *et al.*[28] reported that C/EBPβ, but not NF-κB, was the transcription factor responsible in AGE-induced LCN2 expression in vascular smooth muscle cells. The contribution of C/EBPβ to AGE-induced LCN2 expression in oral epithelial cells was not elucidated, however, NF-κB is thought to be closely associated with AGE-induced LCN2 expression, and we speculated that the MAPKs and transcription factors related to AGE-induced LCN2 expression may be different in various cell types.

LCN2 has multiple functions including the fields of infection, immunity, inflammation and metabolism, and plays roles as an inflammatory mediator and anti-inflammatory regulator.[20] LCN2 secreted from oral epithelial cells suppressed IL-6 expression in neutrophils in the present study (Figure 6), and recombinant LCN2 (25 nM) decreased LPS-induced *IL6* mRNA expression in LCN2-deficient bone marrow-derived macrophages.[47] The levels of pro-inflammatory cytokines such as IL-6, TNF-α and MCP-1 elevated in retina tissue with LPS-induced uveitis, and an injection of LCN2 into the retina reduced the increases in these cytokine levels and inflammatory infiltration, and LCN2 (40 nM) further suppressed LPS-induced TNF-α and IL-6

expressions in retinal cell of neonatal rats.[26] These results showed anti-inflammatory functions by LCN2. In contrast, when human neutrophils and differentiated HL-60 cells were stimulated with recombinant lipocalin (rLCN2) at 500 nM and 1 μM, the expression levels of pro-inflammatory cytokines such as IL-6, IL-8 and TNF-α were significantly increased.[33] LCN2 (10 μM) purified from mouse uterine fluid elevated the levels of IL-6, IL-8 and MCP-1 in conditioned medium from a human endometrial cell line.[48] These reports suggested that LCN2 induced inflammatory responses, and LCN2 appears to play complex roles with regard to inflammation. We speculated that this difference of LCN2 function may be due to the concentration of LCN2. When LCN2 at comparatively high concentrations (more than 500 nM) was used, LCN2 up-regulated inflammatory responses,[33,48] but anti-inflammatory effects were induced by LCN2 at lower concentrations (25 and 40 nM).[26,47] In the present experiment of Figure 6C, LCN2 concentrations in the co-culture medium of the siLCN2 group was approximately 0.4-0.8 nM, and IL-6 expression levels in D-HL-60 cells significantly elevated in these culture conditions (Figure 6D). Furthermore, LCN2 concentrations in GCF were in the range of 2.4 to 4.4 nM and serum

LCN2 concentration was approximately 2.4-10 nM.[22,38] LCN2 at physiological concentrations in the body may play a role as an anti-inflammatory regulator because LCN2 concentrations in body fluid appear to be comparatively low. However, the pathophysiological functions of LCN2 for inflammatory responses have not been decided because LCN2 concentrations were not measured in local periodontal tissues with periodontitis.

In the present study, when differentiated HL-60 cells were co-cultured with TR146 cells that were transfected with siControl and stimulated with AGEs, the migration of D-HL-60 cells was promoted. D-HL-60 cell migration was significantly inhibited by a co-culture with TR146 cells with LCN2 knockdown by siLCN2 transfection. LCN2 is known to be implicated in cell proliferation, migration and chemotaxis of immune cells and cancer cells.[49] Schroll *et al.*[34] showed that recombinant human LCN2 at a concentration of 10 nM stimulated chemotaxis of polymorphonuclear neutrophils (PMNs), and chemotactic activity of PMNs isolated from LCN2 knockout mice reduced, and Shao *et al.*[33] reported that recombinant mouse LCN2 (20 nM) induced chemotaxis of peripheral neutrophils from psoriatic

patients, suggesting that LCN2 at lower concentrations (10-20 nM) stimulated the chemotactic activity of neutrophils and was considered to promote the infiltration of neutrophils into inflammatory tissues.

There are very few reports that have investigated the effect of AGEs on cell migration via LCN2 action. Chung *et al.*[28] demonstrated that AGE-elicited LCN2 promoted the migration of vascular smooth muscle cells and influenced diabetic vascular complications. We firstly found that LCN2 from AGE-stimulated oral epithelial cells promoted the migration of neutrophils and affected the expression of pro-inflammatory cytokines in neutrophils. LCN2 appears to regulate complex inflammatory and anti-inflammatory responses in various tissues. In the present study, LCN2 stimulated the migration of neutrophils, whereas it suppressed IL-6 expression in neutrophils. Although the pathophysiological functions of LCN2 are still not fully elucidated, the present study suggests that AGEs increased LCN2 expression in oral epithelial cells via the RAGE, MAPKs (ERK and p38) and NF-κB signal pathway, and secreted LCN2 may function as a modulator of inflammation and a regulator of the immune system by anti-inflammatory actions (Figure 8). Taken together, AGE-induced LCN2 from epithelial cells

acts directly in pathological conditions such as periodontitis with DM and shows indirect influences by regulating the functions of neutrophils.

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CONFLICT OF INTEREST

None of the authors have any conflicts of interest related to this study.

AUTHOR CONTRIBUTIONS:

Rie Kido; Practice of all experiments and writing a manuscript, Yuka Hiroshima; Instruction of experimental methods (Cell cultures, PCR, siRNA experiment etc.), Jun-ichi Kido; Making study plan, instruction of experimental methods and writing a manuscript, and summarizing study, Takahisa Ikuta; Instruction of cell cultures, Eijiro Sakamoto; Instruction of AGEs preparation, Yuji Inagaki; Instruction of staining cells, Koji Naruishi and Hiromichi Yumoto; Advice for study.

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SUPPORTING MATERIAL

Supplementary figure 1. Effect of AGEs on the cell viability of TR146 cells.

Supplementary figure 2. Effect of AGEs on β-defensin 2 and SLPI expression in TR146 cells.

Supplementary figure 3. Effect of *P.g*-LPS on LCN2 expression in TR146 cells.

Supplementary figure 4. Effect of LCN2 from TR146 cells on IL-6 protein secretion into the conditioned medium of the co-culture system with TR146 and D-HL-60 cells.

Supplementary figure 5. Effect of AGE-induced LCN2 from TR146 cells on IL-1β and TNF-α expression in co-cultured D-HL-60 cells.

FIGURE LEGENDS

Figure 1. Effects of AGEs on the expressions of *LCN2* and *IL6* mRNAs in oral and gingival epithelial cells. Total RNAs were extracted from TR146 cells (A, B) and Ca9-22 cells (C) cultured with AGEs or BSA, and analyzed by qRT-PCR. The expression levels of *LCN2* and *IL6* were normalized to that of *ACTB*. Data for *LCN2* and *IL6* in TR146 cells are expressed as the mean ± SD of eight and six RNA samples, respectively. *LCN2* data from Ca9-22 cells are the mean ± SD of five RNA samples. $^{**}P<0.01$ compared with BSA.

Figure 2. Effects of AGEs on the expression levels of LCN2 and IL-6. The amounts of LCN2 and IL-6 in the conditioned medium from TR146 cells that were cultured with AGEs (50-1000 μg/ml; closed column) and BSA (0-1000 μg/ml; open column) for 24-72 h were using each ELISA kit (A, B, C). Data are expressed as the mean ± SD of three-seven samples in (A), four-ten samples in (B) and three samples in (C). (D) LCN2 amount in the conditioned medium of Ca9-22 cells cultured with AGEs (500 μg/ml) or BSA (500 μg/ml) for 48 h was determined. Data are expressed as the mean ± SD of five samples. $^{**}P<0.01$ compared with BSA.

Figure 3. Regulation of AGE-induced LCN2 expression by inhibiting RAGE expression. (A) RAGE protein in the cell lysates from TR146 cells cultured with AGEs (500 μg/ml) or BSA (500 μg/ml) for 48 h was analyzed by western blotting. Four separate samples were analyzed, and a typical example is shown. (B, C, D) TR146 cells transfected with siRAGE or siControl were cultured with AGEs or BSA for 24 h (RNA assay) or 48 h (protein assay) as described in Materials and Methods. Data of RAGE and LCN2 mRNAs, and LCN2 protein in the conditioned medium are expressed as the means ± SD of six-ten samples (B, C) and four-six samples (D), respectively. (E) In an experiment using a neutralizing antibody against RAGE, data of LCN2 amount are expressed as the mean ± SD of four-six samples. *P<0.05, **P<0.01 comparing between BSA and AGEs in the siControl group, and comparing BSA and AGEs between siControl and siRAGE.

Figure 4. Involvement of MAPKs and NF-κB in AGE-induced LCN2 expression in TR146 cells. (A, C) Cell lysate samples were prepared from TR146 cells that were treated with AGEs or BSA for 15 or 60 min. The

phosphorylation levels of p38, ERK, JNK and p65 were examined by western blot analysis as described in Materials and Methods. In total, four-six samples were analyzed and a typical image is shown for each. The numbers in parentheses are ratios of image density determined using Image J and normalized to the Control image. (B, D) In experiments using inhibitors including SB203580 (15 μM), U0126 (10 μM), SP600125 (10 μM) and Bay11-7082 (10 μM), LCN2 amount in the conditioned medium from TR146 cells treated with AGEs or BSA was determined using a LCN2 ELISA kit. Data are expressed as the mean ± SD of nine-fifteen samples. $^{**}P<0.01$ comparing between BSA and AGEs.

Figure 5. Effect of *P.g*-LPS and AGEs on LCN2 expression in TR146 cells. (A) The cell lysate was collected from TR146 cells that were incubated with *P.g*-LPS (1 μg/ml), AGEs (500 μg/ml) or BSA (500 μg/ml) for 48 h. TLR2 in the cell lysate samples was analyzed by western blotting. Three separate samples were analyzed, and a typical example is shown. (B) LCN2 protein in the conditioned medium from TR146 cells that were cultured with *P.g*-LPS, AGEs or BSA for 48 h was determined using LCN2 ELISA kit. Data are

expressed as the mean ± SD of ten samples. $^{**}P<0.01$ comparing with BSA and *P.g*-LPS+BSA.

Figure 6. Effect of AGE-induced LCN2 from TR146 cells on IL-6 expression in co-cultured D-HL-60 cells. (A) The conditioned media were collected from the normal culture of TR146 cells and D-HL-60 cells and analyzed by western blotting using an anti-LCN2 antibody. Three medium samples were analyzed and a typical image is shown. The cell lysates (20 μg protein) were isolated from cultured TR146 and D-HL-60 cells, and analyzed by a western blotting using an anti-24p3R antibody, as described in Materials and Methods. Two signals of 24p3R-L and -S were detected in the same samples. Five cell lysate samples were analyzed and a typical image is shown. (B) Total RNA was extracted from TR146 cells transfected with siLCN2 or siControl, and then stimulated with AGEs or BSA as described in Materials and Methods. RNA samples were analyzed by qRT-PCR to examine *LCN2* mRNA expression levels. Data are expressed as the mean ± SD of eight samples. (C) After co-culture with TR146 and D-HL-60 cells for 24 h, conditioned medium without AGEs was collected and used for LCN2 ELISA. Data are expressed as the

mean ± SD of thirteen samples. (D) Total RNA was extracted from the co-cultured D-HL 60 cells (350x10^4 cell/Transwell insert) and analyzed by qRT-PCR for *IL6* mRNA. Data are expressed as the mean ± SD of eight-thirteen samples. *P<0.05, **P<0.01 comparing between BSA and AGEs in the siControl group, and comparing BSA and AGEs between siControl and siLCN2.

Figure 7. Effect of AGE-induced LCN2 from TR146 cells on the migration of D- HL-60 cells. D-HL-60 cells (350x10^4 cells/ml) were co-cultured with TR146 cells transfected with siLCN2 or siControl, and treated with AGEs or BSA for 24 h using Transwell® plates (A: siControl-BSA, B: siControl-AGE, C: siLCN2-BSA, D: siLCN2-AGE). D-HL-60 cells that migrated to the undersurface of the membrane of Transwell insert were fixed and stained with 0.2% crystal violet solution, as described in Materials and Methods. The migrated cells were observed using a phase-contrast microscope. (E) The numbers of D-HL-60 cells that migrated to undersurface of the Transwell insert membranes were counted for each experimental group. Data are expressed as the mean ± SD of four microscopic fields on three separated

membranes in each experimental group. $^{**}P<0.01$ comparing between BSA and AGEs in the siControl group, and comparing with BSA and AGEs between siControl and siLCN2. BSA siControl: 428±28 cells/field.

Figure 8. A schematic diagram showing the proposed mechanism that AGE-induced LCN2 expression in human oral epithelial cells and function of secreted LCN2. The secreted LCN2 plays a role of inflammatory modulator by down-regulating IL-6 expression in neutrophils and promoting migration of neutrophils, and may influence periodontitis with DM.

Fig. 1

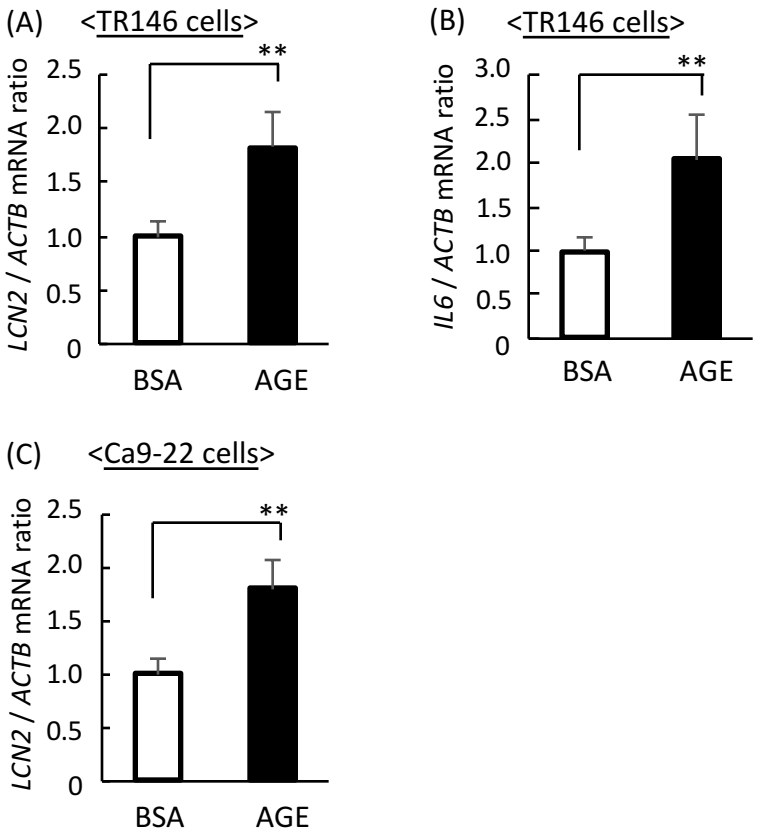

(A) <TR146 cells>
LCN2 / ACTB mRNA ratio
2.5
2.0
1.5
1.0
0.5
0
**
BSA
AGE
(B) <TR146 cells>
IL6 / ACTB mRNA ratio
3.0
2.5
2.0
1.5
1.0
0.5
0
**
BSA
AGE
(C) <Ca9-22 cells>
LCN2 / ACTB mRNA ratio
2.5
2.0
1.5
1.0
0.5
0
**
BSA
AGE

Fig. 2

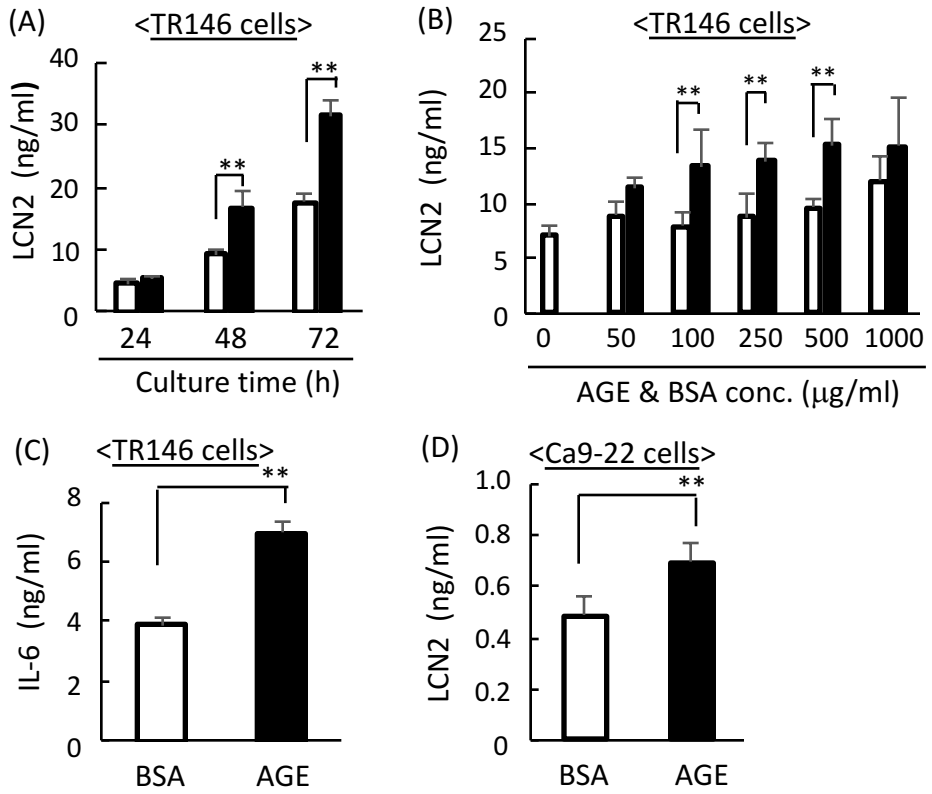
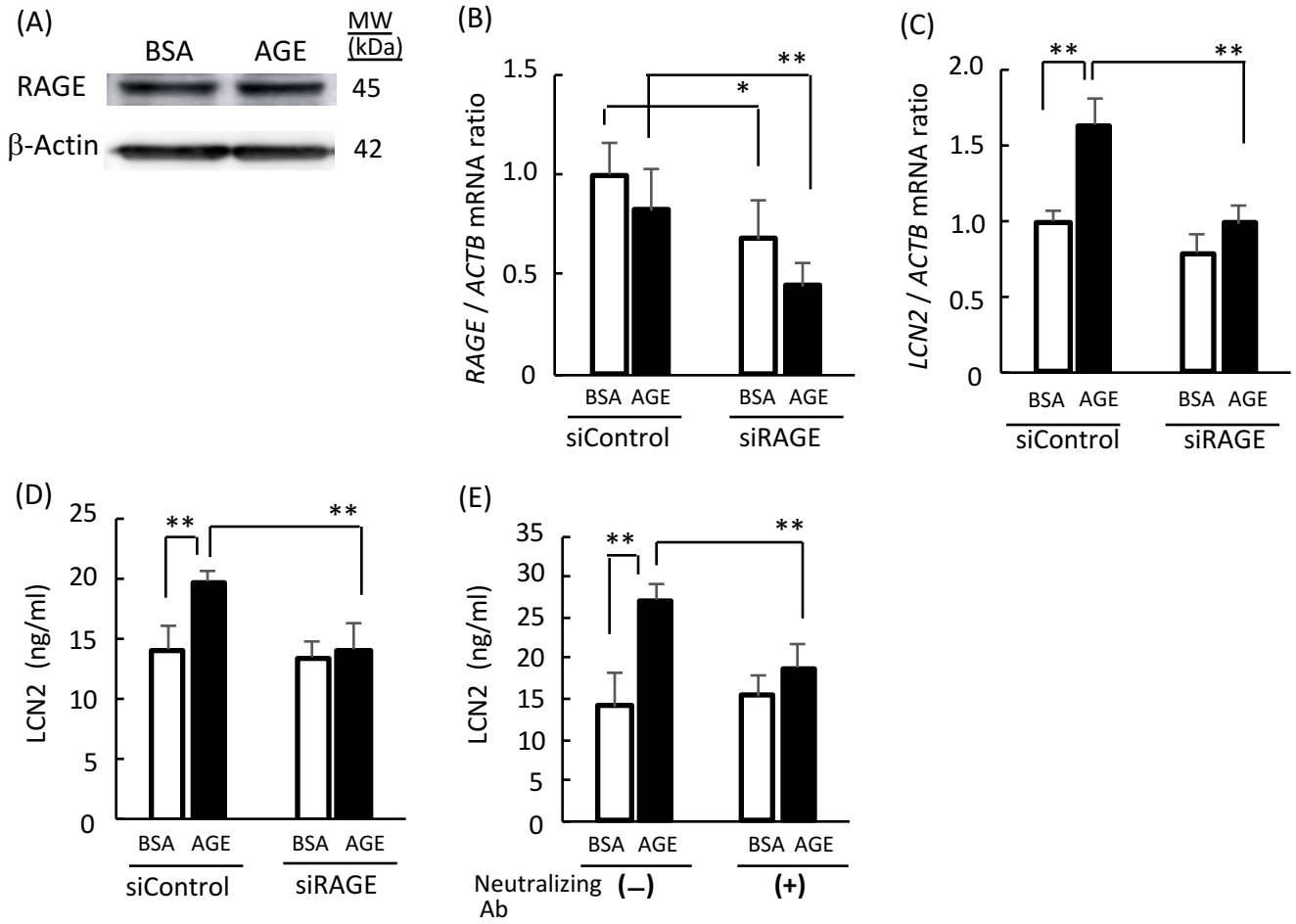

(A)
<TR146 cells>
LCN2 (ng/ml)
40
30
20
10
0
**
**
24
48
72
Culture time (h)
(B)
<TR146 cells>
LCN2 (ng/ml)
25
20
15
10
5
0
**
**
**
0
50
100
250
500
1000
AGE & BSA conc. (μg/ml)
(C)
<TR146 cells>
IL-6 (ng/ml)
8
6
4
2
0
**
BSA
AGE
(D)
<Ca9-22 cells>
LCN2 (ng/ml)
1.0
0.8
0.6
0.4
0.2
0
**
BSA
AGE

Fig. 3


(A)
BSA
AGE
MW (kDa)
RAGE
45
β-Actin
42
(B)
RAGE / ACTB mRNA ratio
1.5
1.0
0.5
0
**
*
BSA AGE
BSA AGE
siControl
siRAGE
(C)
LCN2 / ACTB mRNA ratio
2.0
1.5
1.0
0.5
0
**
**
BSA AGE
BSA AGE
siControl
siRAGE
(D)
LCN2 (ng/ml)
25
20
15
10
5
0
**
**
BSA AGE
BSA AGE
siControl
siRAGE
(E)
LCN2 (ng/ml)
35
30
25
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15
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5
0
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BSA AGE
BSA AGE
Neutralizing Ab
(–)
(+)

Fig. 4

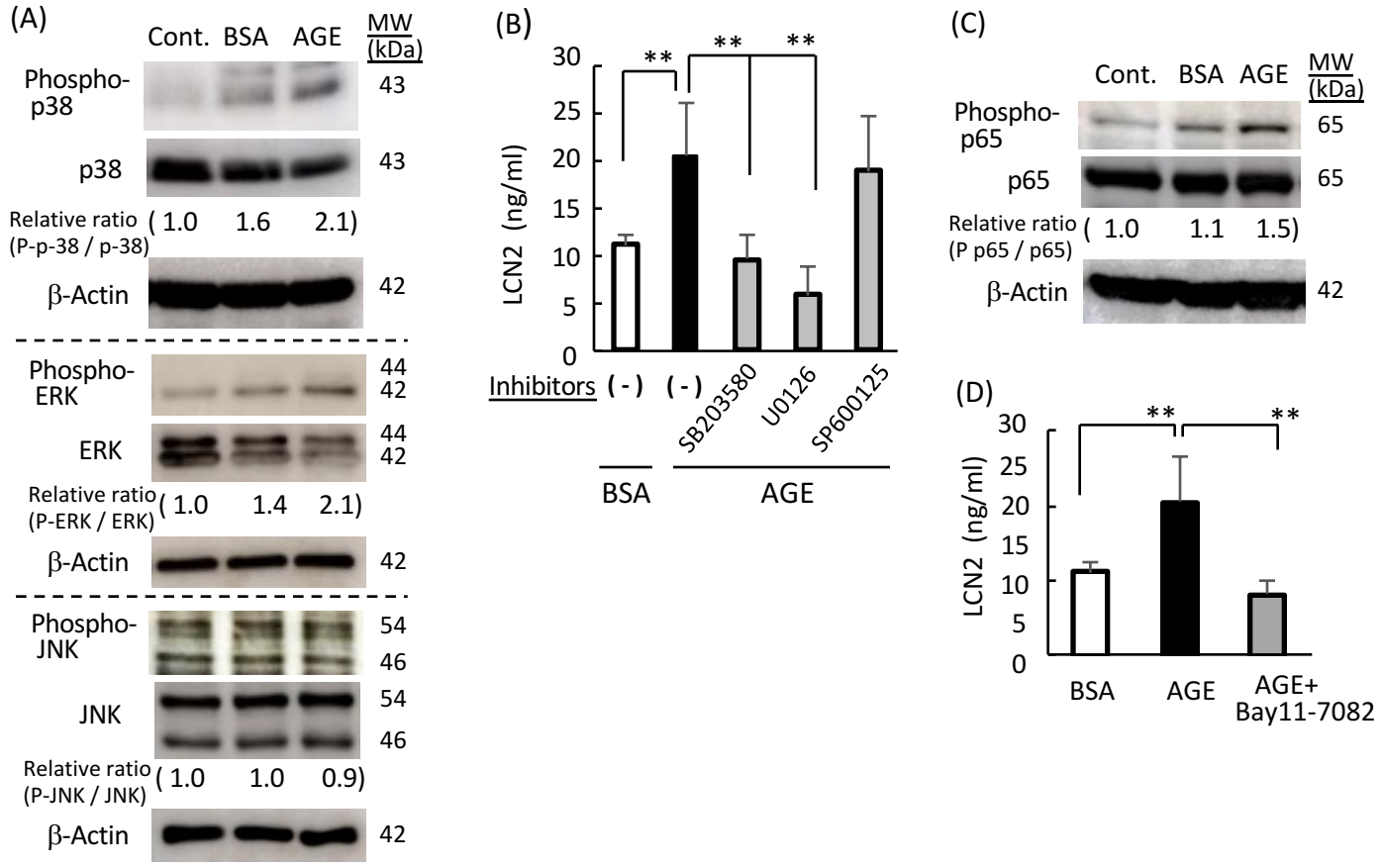

(A)
Cont. BSA AGE
MW (kDa)
Phospho-p38
43
p38
43
Relative ratio (P-p-38 / p-38) (1.0 1.6 2.1)
β-Actin
42
Phospho-ERK
44
42
ERK
44
42
Relative ratio (P-ERK / ERK) (1.0 1.4 2.1)
β-Actin
42
Phospho-JNK
54
46
JNK
54
46
Relative ratio (P-JNK / JNK) (1.0 1.0 0.9)
β-Actin
42
(B)
LCN2 (ng/ml)
30
25
20
15
10
5
0
**
**
**
Inhibitors (-) (-) SB203580 U0126 SP600125
BSA
AGE
(C)
Cont. BSA AGE
MW (kDa)
Phospho-p65
65
p65
65
Relative ratio (P p65 / p65) (1.0 1.1 1.5)
β-Actin
42
(D)
LCN2 (ng/ml)
30
25
20
15
10
5
0
**
**
BSA
AGE
AGE+ Bay11-7082

Fig. 5

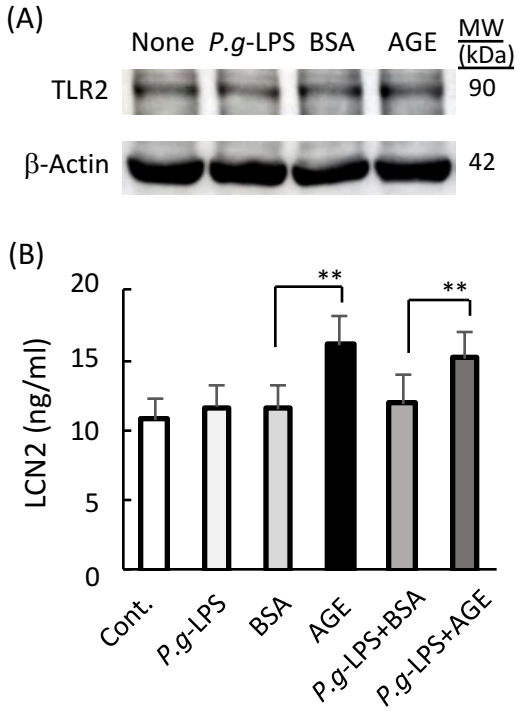

(A)
None
P.g-LPS
BSA
AGE
MW (kDa)
TLR2
90
β-Actin
42
(B)
LCN2 (ng/ml)
20
15
10
5
0
**
**
Cont.
P.g-LPS
BSA
AGE
P.g-LPS+BSA
P.g-LPS+AGE

Fig. 6

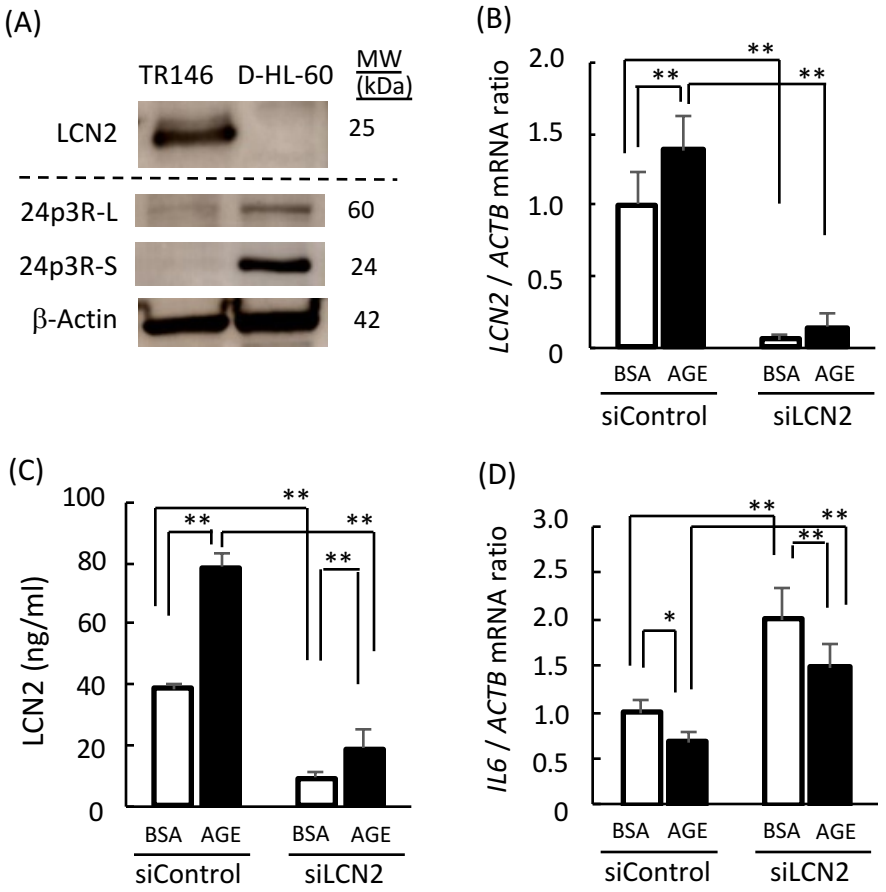
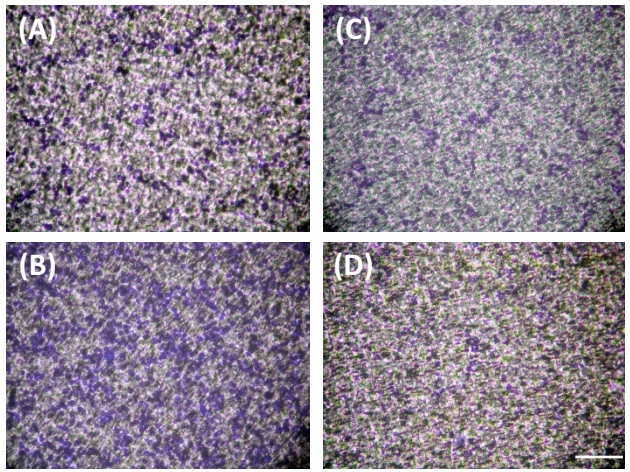

(A)
TR146
D-HL-60
MW (kDa)
LCN2
25
24p3R-L
60
24p3R-S
24
β-Actin
42
(B)
LCN2 / ACTB mRNA ratio
2.0
1.5
1.0
0.5
0
**
**
**
BSA AGE
BSA AGE
siControl
siLCN2
(C)
LCN2 (ng/ml)
100
80
60
40
20
0
**
**
**
**
BSA AGE
BSA AGE
siControl
siLCN2
(D)
IL6 / ACTB mRNA ratio
3.0
2.5
2.0
1.5
1.0
0.5
0
**
**
**
*
BSA AGE
BSA AGE
siControl
siLCN2

Fig. 7



(Bar=100 μm)

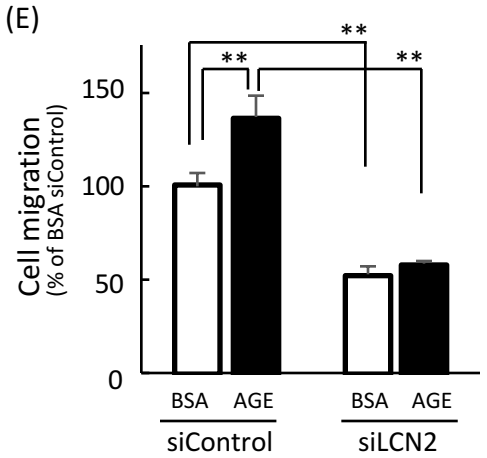
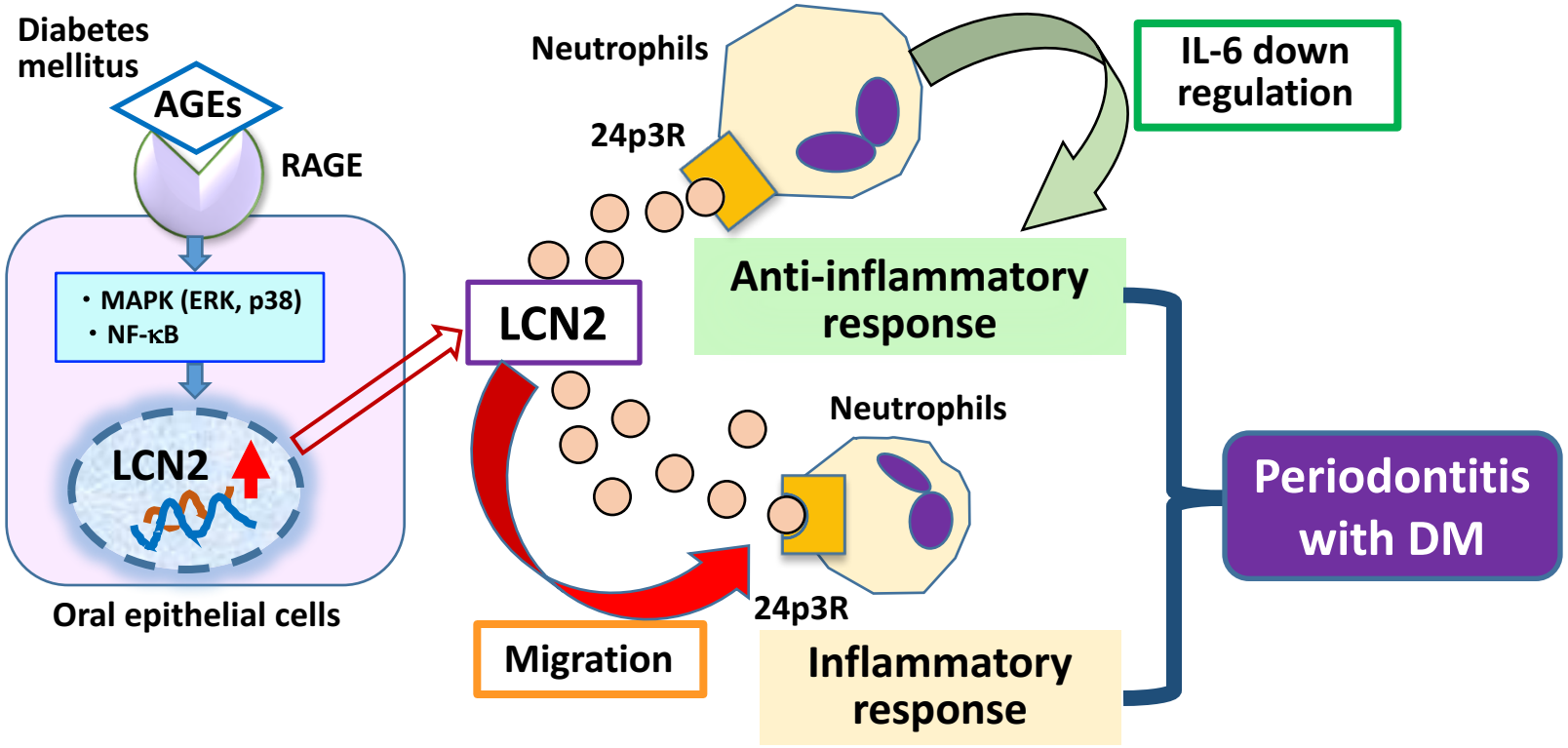
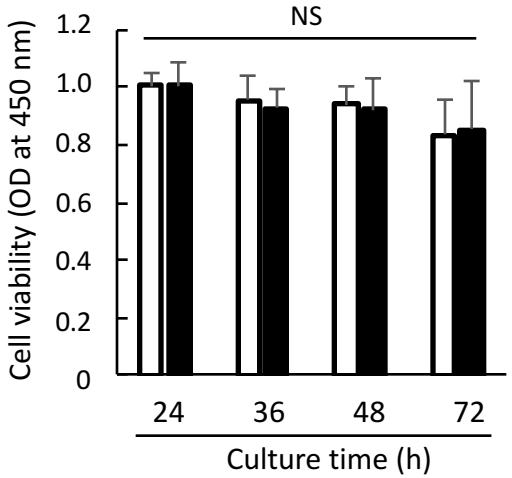


Fig. 8

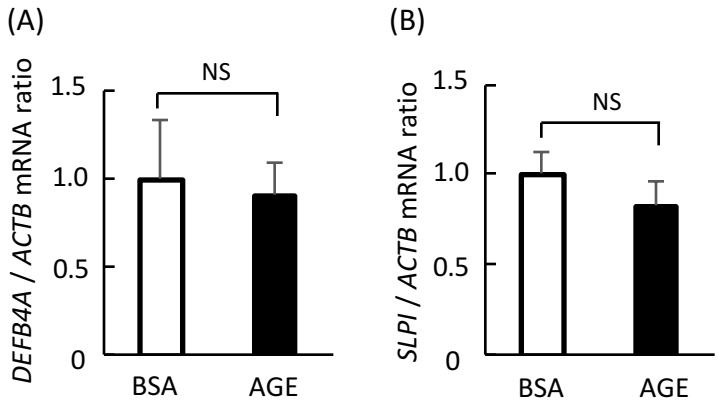

Diabetes mellitus
AGEs
RAGE
• MAPK (ERK, p38)
• NF-κB
LCN2
Oral epithelial cells
Neutrophils
24p3R
IL-6 down regulation
Anti-inflammatory response
LCN2
Neutrophils
24p3R
Migration
Inflammatory response
Periodontitis with DM

Supplementary figure 1



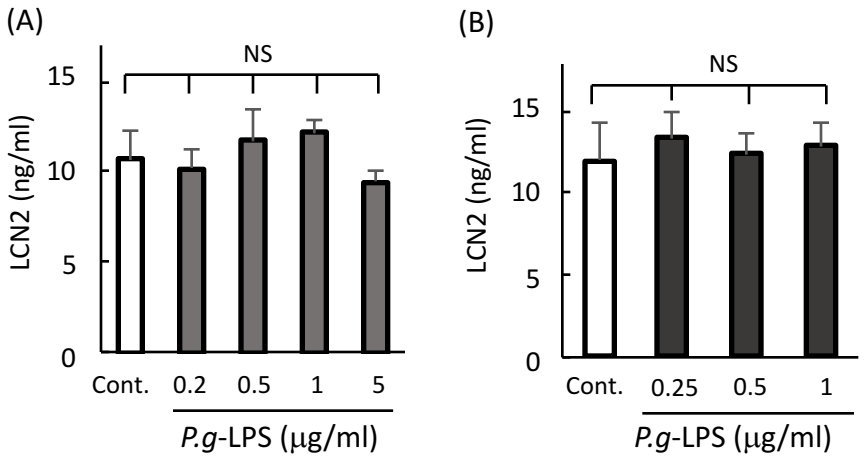
Supplementary figure 1. Effect of AGEs on the cell viability of TR146 cells.
TR146 cells were seeded at 5,000x10^4 cells/ml and cultured for 5 days, then with AGEs (500 μg/ml) or BSA (500 μg/ml) for 24-72 h. Cell viability was examined as described in Materials and Methods. Open columns and closed columns show the mean absorbance of BSA- and AGE-treated cell cultures at a wavelength of 450 nm, respectively. Data are expressed as the mean±SD of 5-7 samples. N.S. shows no significant difference.

Supplementary figure 2



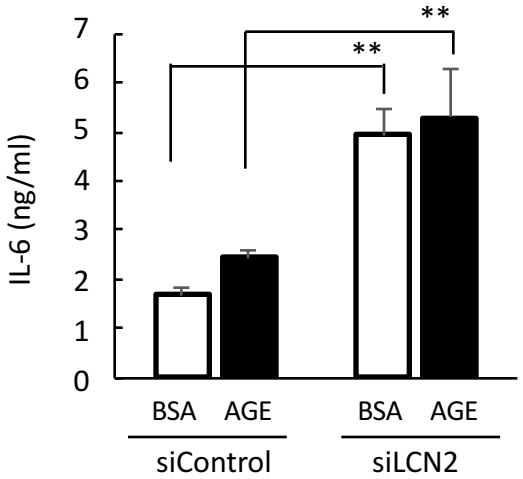
Supplementary figure 2. Effect of AGEs on β-defensin 2 and SLPI expression in TR146 cells. Sub-confluent TR146 cells were cultured with AGEs (500 μg/ml) and BSA (500 μg/ml) for 24 h. RNA was isolated from the treated cells and analyzed by qRT-PCR using specific primers of *DEFB4A* (A: β-defensin 2) and *SLPI* (B). Data of DEFB4A and SLPI are expressed as the mean±SD of 9 and 6 samples, respectively.

Supplementary figure 3



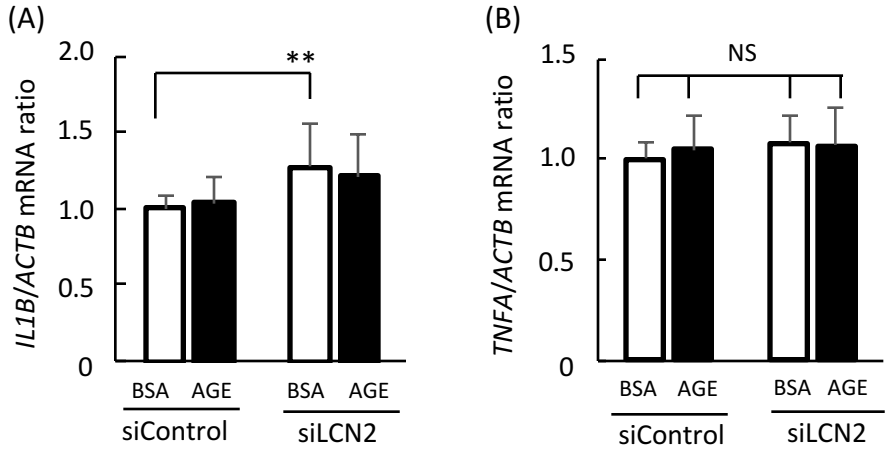
Supplementary figure 3. Effect of *P.g*-LPS on LCN2 expression in TR146 cells.
Sub-confluent TR146 cells were cultured with and *P.g*-LPS (A: TLR2 ligand type) at 0-5 μg/ml concentration and *P.g*-LPS (B: TLR2/TLR4 ligand type) at 0-1 μg/ml concentration for 48 h. Conditioned medium was collected and LCN2 amount was determined using LCN2 ELISA kit. Data of *P.g*-LPS (TLR2 ligand type) and *P.g*-LPS (TLR2/TLR4 ligand type) are expressed as the mean±SD of 4 and 7 samples, respectively.

Supplementary figure 4



Supplementary figure 4. Effect of LCN2 from TR146 cells on IL-6 protein secretion into the conditioned medium of the co-culture system with TR146 and D-HL-60 cells.
The conditioned medium was collected from the co-culture with TR146 cells transfected with siControl or siLCN2 and D-HL-60 cells. IL-6 protein from two cells in the co-culture system was determined using an ELISA kit. Data are expressed as the mean±SD of 6-7 samples. *P<0.01 comparing between siControl and siLCN2.

Supplementary figure 5



Supplementary figure 5. Effect of AGE-induced LCN2 from TR146 cells on IL-1β and TNF-α expression in co-cultured D-HL-60 cells.
Sub-confluent TR146 cells were transfected with siControl and siLCN2 for 24 h, and then with AGEs (500 μg/ml) and BSA (500 μg/ml) for 48 h, and further co-cultured with differentiated HL-60 (D-HL-60) cells for 24h. RNA was isolated from D-HL-60 cells and analyzed by qRT-PCR using specific primers of *IL1B* (A) and *TNFA* (B). Data of *IL1B* and *TNFA* are expressed as the mean±SD of 20 and18 samples, respectively. **P<0.01 as compared between siControl and siLCN2 in BSA group (A).