

Aged garlic extract enhances the production of β -defensin 4 via activation of the Wnt/ β -catenin pathway in mouse gingiva

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Abstract. Periodontal disease is recognized as a chronic multifactorial inflammatory condition initiated by dysbiosis within subgingival plaque biofilms. Antimicrobial peptides exhibit a wide spectrum of antimicrobial action, and thus, provide one of the first lines of host defense against oral pathogens. Aged garlic extract (AGE) is effective for preventing the progression of periodontal disease. The present study examined whether AGE affects the production of antimicrobial peptides in mouse gingiva. Reverse transcription-quantitative PCR analysis demonstrated that oral administration of AGE in mice increased the mRNA level of *Defb4* in gingival tissue, while the levels of *Defb1*, *Defb14* and *Cramp* remained unaffected. AGE also upregulated the protein levels of β -defensin 4. To explore the underlying mechanism of the increased β -defensin 4 production induced by AGE, a comprehensive phosphoproteomic analysis in gingival tissues was performed. Proteomic profiling revealed activation of the canonical Wnt/ β -catenin pathway in gingiva of mice treated with AGE. Treatment of mouse gingival epithelial GE1 cells with AGE resulted in an increase of β -defensin 4 in the culture medium. In support of proteomics experiments, LF3, a specific inhibitor of Wnt/ β -catenin signaling, suppressed the AGE-induced production of β -defensin 4. In addition, β -catenin protein was found to accumulate within the nucleus in cells treated with AGE. In conclusion, the present findings suggested that AGE enhanced the production of β -defensin 4 in mouse gingiva through the canonical Wnt signal transduction pathway.

Introduction

Periodontal disease is one of the most common oral disorders caused by an imbalance between dysbiosis of dental plaque biofilm and host defense (1,2). Chronic inflammatory responses induced by periodontal pathogens lead to the destruction of gingiva and alveolar bone (3), thereby increasing the risk for tooth loss in patients with periodontal diseases, which subsequently impairs mastication and nutrient intake (4). Furthermore, a recent study using the dental data repository has reported that patients with periodontal diseases exhibit a higher risk for systematic disorders such as bronchitis, diabetes, and hypertension (5). Therefore, preventing periodontal disease is not only crucial for addressing oral health issues but also for maintaining systemic quality of life and health.

Antimicrobial peptides are ubiquitous host defense substances against pathogenic infection, and possess a broad antimicrobial spectrum (6). In addition, antimicrobial peptides are important in diverse functions such as neutralization of virulence factors derived from pathogens and immunomodulation (6). In the oral environment, antimicrobial peptides are secreted by oral epithelial cells, salivary glands, and neutrophils (7,8). In particular, epithelial antimicrobial peptides, such as β -defensin family and cathelicidin, play the pivotal role in the first line of host defense, as gingival epithelium is in close proximity to dental plaque that resides around the tooth and root surfaces (9). Therefore, promoting the production of endogenous antimicrobial peptides to achieve enhanced antimicrobial defense is considered to be one of the valuable strategies for combating periodontal infections.

Aged garlic extract (AGE) is one of the garlic (*Allium sativum* L.)-derived products manufactured by soaking garlic in a water-ethanol mixture for more than 10 months (10). Clinical trials on patients with mild to moderate periodontitis reported that daily intake of AGE at 2,400 mg/day for 4 and 18 months improved gingival bleeding index (11) and probing pocket depth (12). Furthermore, we have shown that AGE and its sulfur constituents suppressed the tumor necrosis factor- α -induced intracellular adhesion molecule-1 and interleukin-6 in human gingival epithelial cell line Ca9-22 cells (13). In addition, it was found that S-1-propenyl-L-cysteine, a major sulfur bioactive compound in AGE, inhibited the *Porphyromonas gingivalis*-derived lipopolysaccharide-induced matrix metalloproteinase-1 in

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human gingival fibroblast cell line HGF-1 cells (14). More recently, it was reported that feeding of AGE (18 mg/kg/day) to Beagle dogs with mild gingivitis for 8 weeks resulted in the improvement of gingival index score and halitosis, that is possibly due to an increase in salivary antimicrobial peptide, cathelicidin (15). Taken together, these results suggest that the therapeutic effects of AGE on periodontal disease involve not only its anti-inflammatory action but also antimicrobial action mediated by the production of antimicrobial peptides. In this study, we investigated whether AGE influences the production of antimicrobial peptides in mouse gingiva, which would help prevent the onset or progression of periodontal disease.

Materials and methods

Reagents. All chemicals were purchased from FUJIFILM Wako Pure Chemical Corporation unless stated otherwise. A canonical Wnt signaling pathway specific inhibitor LF3 and a glycogen synthase kinase-3 (GSK-3) specific inhibitor 6-bromindirubin-3'-oxime (BIO) were from Cayman Chemical (Ann Arbor). For Western blotting, the primary antibodies against β -defensin 4 (BS60360, Bioworld Technology, St. Louis Park, MN, USA) and β -actin (PM053-7, MBL Life Science), and the secondary antibodies, horse radish peroxidase (HRP)-conjugated against mouse (#7076S, Cell Signaling Technology) and rabbit (#7074S, Cell Signaling Technology) were used. A Mouse Beta-defensin 4 ELISA kit was obtained from BT LAB.

Preparation of AGE. AGE powder was prepared as previously described (16). The powder was dissolved in deionized water (DW) to obtain the AGE stock solution (20 mg/ml). The stock solution was stored at -20°C until use.

Animals and treatment. Five weeks old male ddY mice were purchased from Japan SLC Inc. (Hamamatsu, Shizuoka, Japan) and kept at $23\pm 3^{\circ}\text{C}$ and $50\pm 10\%$ humidity, under a 12 h light-dark cycle in the animal facility at Wakunaga Pharmaceutical Co., Ltd. Food (CE-2; CLEA Japan Inc.) and water were provided *ad libitum*. Mice were allowed to acclimate for 1 week, and then at 6 weeks of age, randomly divided into the DW-treated (control) group and the AGE-treated group. The control and AGE-treated groups were given DW and AGE, respectively, by oral gavage administration (10 ml/kg body weight) using a disposable feeding needle (Fuchigami). We used the dose 2 g/kg/day of AGE that has been shown to be safe and sufficiently effective in our previous studies (17-19). Gingival tissues were dissected out after the mice were euthanized by exsanguination under anesthesia with 2.5% isoflurane for induction and maintenance. Animal experiments were approved by the Wakunaga Pharmaceutical Company Institutional Animal Care and Use Committee (approval no. 360).

Cell culture. Mouse gingival epithelial GE1 cells (RCB1709, RIKEN Bioresource Research Center) were cultured in Minimum Essential Medium alpha (MEM α) with 10% fetal bovine serum, penicillin-streptomycin (x1), and 10 ng/ml recombinant murine epidermal growth factor (PeproTech) at

37°C and 5% CO_2 in a humidified atmosphere. GE1 cells were seeded at a density of 13,000 cells/cm² and grown to confluent monolayer. After confluency, the culture medium was replaced by fresh MEM α with 1% fetal bovine serum, penicillin-streptomycin (x1), and 10 ng/ml recombinant murine epidermal growth factor (20).

Reverse transcription-quantitative PCR analysis. Total RNA was extracted from mouse gingiva with acid guanidinium thiocyanate-phenol-chloroform extraction using RNAiso plus (Takara Bio Inc.). Complementary DNA was synthesized from total RNA using a PrimeScript RT reagent kit with a genomic DNA Eraser (RR047A, Takara Bio Inc.), and amplified on a CFX96 real-time PCR detection system (Bio-Rad Laboratories) with KAPA SYBR fast qPCR master mix (KAPA Biosystems). The PCR primers (Integrated DNA Technologies, Inc.) are listed in Table I. The fold change in the mRNA level relative to β -actin was calculated based on the $\Delta\Delta\text{Ct}$ method (21).

Western blotting. Gingival tissues and GE1 cells were lysed in a radio-immunoprecipitation assay buffer (Merck) with 1X PhosSTOP™ (Sigma-Aldrich) and 1X cComplete™ protease inhibitor cocktail (Roche, Basel, Switzerland) to obtain total protein. For nuclear protein extraction, two extraction buffers were used as follows; Buffer A, 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), 1X PhosSTOP™ and 1X cComplete™ protease inhibitor cocktail; Buffer B, 20 mM HEPES, 400 mM NaCl, 1 mM EDTA, 1 mM DTT, 1X PhosSTOP™ and 1X cComplete™ protease inhibitor cocktail. GE1 cells were incubated in Buffer A on ice for 15 min, and then added 1/10 volume of 10% Nonidet P-40 substitute (Nacalai Tesque, Kyoto, Japan). Lysates were centrifuged at 20,000 x g for 5 min at 4°C . The supernatant was removed, and the resultant pellet was washed twice with Buffer A. The washed pellet was resuspended in Buffer B, incubated on ice for 30 min, and subsequently centrifuged at 20,000 x g for 30 min at 4°C . After centrifugation, the supernatant was used as the nuclear protein fraction. Each extracted protein was diluted to a protein concentration of 1 mg/ml with 4X sample buffer containing 250 mM Tris-HCl (pH 6.8), 8% sodium dodecyl sulfate, 40% glycerol, 2% bromophenol blue, and 400 mM DTT, and then boiled at 98°C for 5 min. Protein extracts (20 μg) were separated on 4-20% Mini-PROTEAN TGX™ Gel (Bio-Rad Laboratories) and transferred onto Trans-Blot Turbo nitrocellulose membranes (Bio-Rad Laboratories) using a Trans-Blot Turbo Transfer System (Bio-Rad Laboratories). The membranes were treated with the primary antibody against β -defensin 4 (1:500) or β -actin (1:2,000) overnight at 4°C , and then the HRP-conjugated secondary antibody (1:20,000) for 1 h at room temperature. Immunoreactive proteins were visualized with Armasham ECL Prime peroxidase solution (Cytiva) or ImmunoStar™ LD by using ChemiDoc™ MP (Bio-Rad Laboratories). The density of each immunoreactive band was analyzed using Band/Peak Quantification Tool in ImageJ 1.54i (22).

Enzyme-linked immunosorbent assay (ELISA). Quantification of β -defensin 4 in culture medium was

Table I. Primer sequences used for reverse transcription-quantitative PCR.

Gene name	Sequence (5'-3')
<i>Defb1</i>	Forward: ATT CAA GCC TCA TCT GTC AGC C Reverse: TTG TGA GAA TGC CAA CAC CTG C
<i>Defb4</i>	Forward: GGT GCT GCT GTC TCC ACT TG Reverse: TTC ATC TTG CTG GTT CTT CGT CT
<i>Defb14</i>	Forward: GTA TTC CTC ATC TTG TTC TTG Reverse: AAG TAC AGC ACA CCG GCC AC
<i>Cramp</i>	Forward: TGT GAG GTT CCG AGT GAA GG Reverse: TGT GCA CCA GGC TCG TTA C
<i>Gapdh</i>	Forward: CCA GCA AGG ACA CTG AGC AA Reverse: ATT CAA GAG AGT AGG GAG GGC T

performed using a Mouse Beta-defensin 4 ELISA kit according to the manufacturer's protocol. Culture medium was collected and centrifuged at 13,200 x g for 10 min at 4°C. The supernatants were stored at -80°C until use. The colorimetric absorbance was measured at a test wavelength of 450 nm using Multiskan GO Microplate Spectrophotometer (Thermo Scientific).

Proteomics analysis. The lysis of gingival tissues resected from mice after 6 h treatment with AGE was performed by incubating with tris(2-carboxyethyl)phosphine hydrochloride for 1 h at 55°C, alkylating with iodoacetamide for 30 min at room temperature, and digesting overnight with Pierce™ Trypsin Protease MS-Grade (Thermo Fisher Scientific) at a trypsin-protein ratio of 1:50 (w/w). Phosphorylated peptides were enriched using Titansphere Phos-TiO Tip (GL Sciences Inc.). Residual detergents and salts in the samples were removed using a HiPPR Detergent Removal Spin Column Kit (Thermo Fisher Scientific) and GL-Tip SDB columns (GL Sciences Inc.), respectively. The clean-up peptides were analyzed on a Q-Exactive Mass Spectrometer equipped with a Vanquish Neo LC System (Thermo Fisher Scientific). Phosphorylated peptides were identified using the Proteome Discoverer software (Thermo Fisher Scientific), and then compared with the Uniprot curated *M. musculus* proteome database (release 2023.6). As a result, only 'Annotated Sequence' passing a cut-off of 5% false discovery rate (FDR Confidence: 'Medium') was considered for further analysis. Functional enrichment analysis was performed by Gene Ontology (GO) biological process database using GeneCodis 4 (23).

Statistical analysis. Data analyses and graphical visualization were performed using KyPlot Free ver. 6.0.2 (KyensLab Inc.). Data are expressed as mean ± standard deviation. Unpaired Student's t test, Welch's t test, Mann-Whitney U test (for 2 groups) or one-way analysis of variance (for more than 3 groups), followed by post hoc Dunnett's test or Holm-Bonferroni test, were used to assess statistical significance. Differences at P<0.05 were considered statistically significant.

Results

Effect of daily administration of AGE on the mRNA and protein level of β -defensin 4 in mouse gingiva. We administered AGE (2 g/kg/day) to mice for 2 weeks and examined the production of antimicrobial peptides, specifically β -defensin 1, β -defensin 4, β -defensin 14, and cathelicidin, in gingiva. As shown in Fig. 1A, the mRNA level of *Defb4* was significantly increased in AGE-treated mice compared to DW-treated (control) mice. In contrast, the mRNA level of other epithelial antimicrobial peptides, including *Defb1*, *Defb14*, and *Cramp*, remained unchanged (Fig. S1). We next performed Western blot analysis to examine the effect of AGE on the protein level of β -defensin 4, and found that AGE induced the significant increase (Fig. 1B).

Effect of single administration of AGE on the mRNA and protein level of β -defensin 4 in mouse gingiva. We next gave a single administration of AGE and examined the change of the β -defensin 4 during 24 h. The data obtained by reverse transcription-quantitative PCR analysis indicated that AGE induced a transient and significant increase in the mRNA level of *Defb4* at 6 h (Fig. 2A). In addition, the protein level of β -defensin 4 was significantly elevated in the AGE group at 16 h compared to the control group (Fig. 2B).

Effect of single administration on the canonical Wnt signaling pathway in mouse gingiva. To explore the underlying mechanism of AGE-induced increase of β -defensin 4, we performed phosphoproteomics analysis using mouse gingival tissues treated with AGE for 6 h. Our phosphoproteomics analysis identified a total of 2,298 phosphopeptides, revealing 79 up-regulated and 52 down-regulated phosphopeptides in the AGE group compared to the control group (Fig. 3A). In addition, among the 131 phosphopeptides, the phosphorylated amino acid residues were distinctly defined in 47 up-regulated and 28 down-regulated phosphopeptides (Fig. 3A). We subsequently performed GO biological process enrichment analysis of proteins with differentially phosphorylated peptides in the AGE group using GeneCodis 4 (23). The top 5 significantly enriched GO terms in the biological process, based on the number of proteins with up-regulated phosphopeptides (adjusted P-value <0.05), were *axon extension*, *neurofilament bundle assembly*, *virion attachment to host cell*, *telomerase holoenzyme complex assembly*, and *intermediate filament bundle assembly* (Fig. 3B). Similarly, the top 5 significantly enriched GO terms for proteins with down-regulated phosphopeptides were *negative regulation of canonical Wnt signaling pathway*, *protein stabilization*, *ovulation from ovarian follicle*, *regulation of translational initiation*, and *response to cocaine* (Fig. 3B). The GO term 'negative regulation of canonical Wnt signaling pathway' included proteins that are involved in forkhead box protein O3 (S7), glycogen synthase kinase-3 alpha (GSK-3 α) (Y279), and catenin delta-1 (S252) (Table II). Non-targeted proteomics analysis revealed a decrease in axin-1 (adjusted P-value=0.047) among the 125 up-regulated and 73 down-regulated proteins (Fig. S2), indicating the involvement of the canonical Wnt pathway.

Effect of AGE on the β -defensin 4 production and Wnt/ β -catenin signaling pathway in mouse gingival epithelial

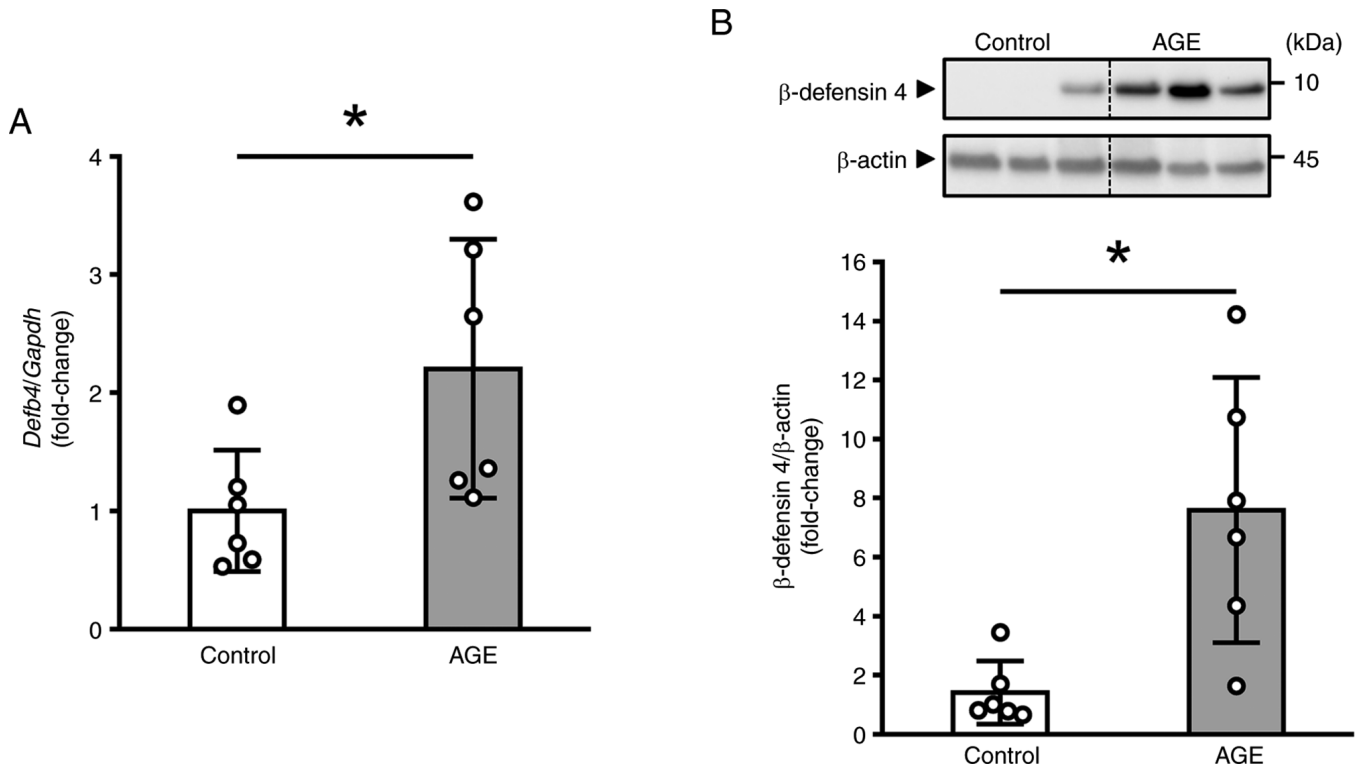


Figure 1. Effect of daily administration of AGE on the mRNA and protein levels of β -defensin 4 in mouse gingiva. ddY mice were orally administered deionized water (control) or AGE (2 g/kg/day) for 2 weeks. The levels of β -defensin 4 in gingival tissues were analyzed by reverse transcription-quantitative PCR and western blotting. (A) Graph showing the mRNA levels of *Defb4* normalized to those of *Gapdh*. (B) Representative images of western blotting for β -defensin 4 with β -actin as an internal control, using the samples of control and AGE-treated mice. Graph showing the protein levels of β -defensin 4 normalized to those of β -actin. Data are presented as the mean \pm standard deviation (n=6). *P<0.05 (Welch's t-test). AGE, aged garlic extract.

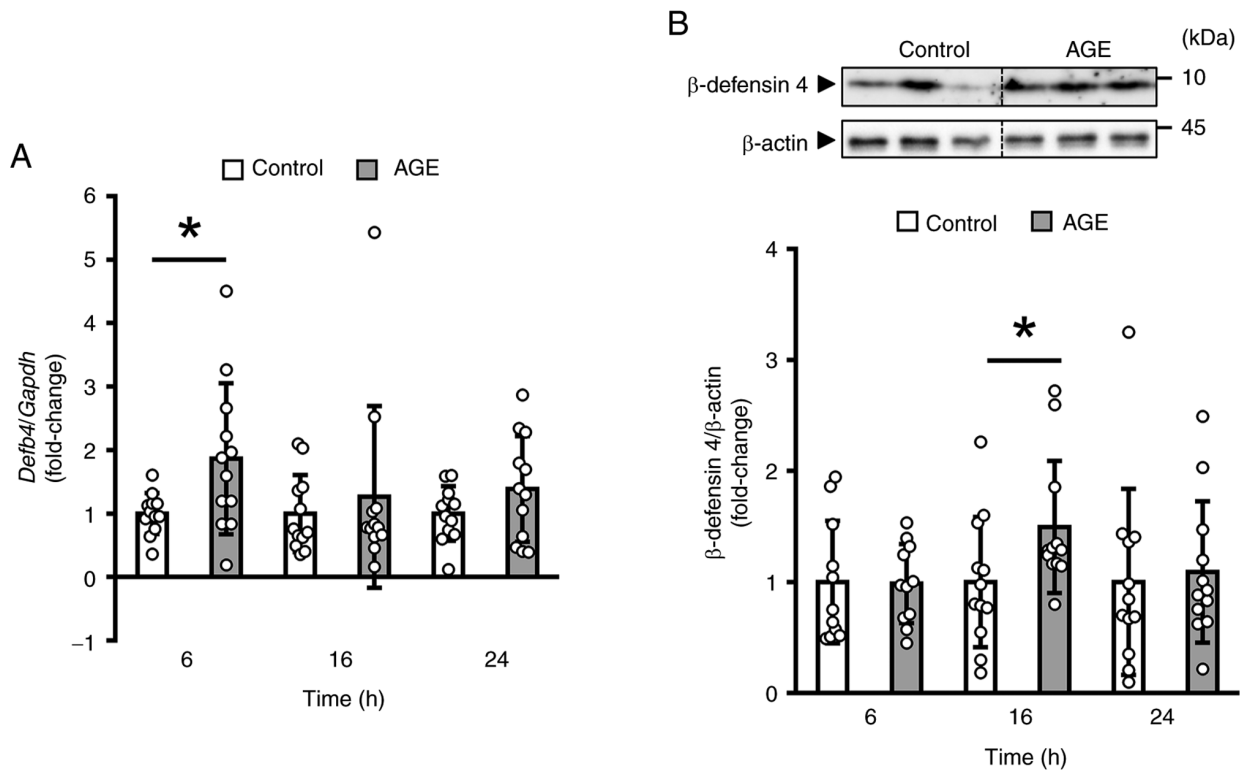


Figure 2. Effect of single administration of AGE on the levels of β -defensin 4 in mouse gingiva. ddY mice were orally administered deionized water (control) or AGE (2 g/kg). After 6, 16 and 24 h, the mRNA and protein levels of β -defensin 4 in gingival tissues were analyzed by reverse transcription-quantitative PCR and western blotting. (A) Graph showing the mRNA levels of *Defb4* normalized to those of *Gapdh*. (B) Representative images of western blotting for β -defensin 4 with β -actin as an internal control, using the samples of control and AGE-treated mice. Graph shows the protein levels of β -defensin 4 normalized to those of β -actin. Data are presented as the mean \pm standard deviation (n=11-12). *P<0.05 (Welch's t-test). AGE, aged garlic extract.

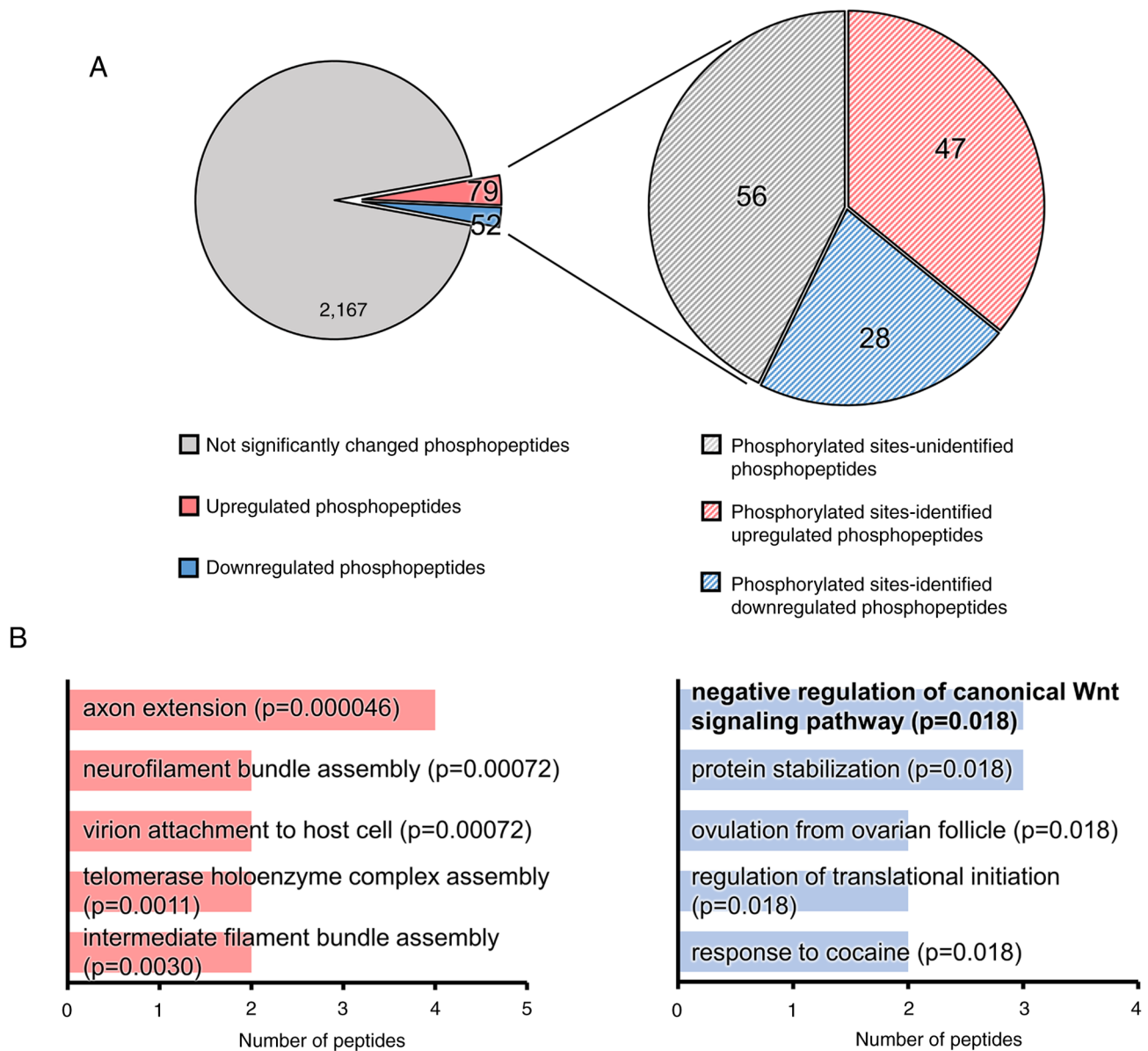


Figure 3. Enrichment analysis of the differentially phosphorylated peptides during AGE treatment in gingiva. ddY mice were orally administrated deionized water (control) or AGE (2 g/kg). After 6 h, gingival tissues were analyzed using phosphoproteomics. (A) Pie charts showing the number of differentially phosphorylated (left) and modified peptides with identified phosphorylation sites (right) in the AGE group compared with the control group. The adjusted P-value threshold was set to <0.05. (B) Graphs showing the top 5 enriched Gene Ontology biological process terms based on the number of proteins with phosphorylated sites identified to be upregulated (left) and downregulated (right) phosphopeptides. The adjusted P-value threshold was set to <0.05. AGE, aged garlic extract.

GE1 cells. The proteomics analysis on mouse gingiva suggested the possible involvement of the canonical Wnt signaling pathway in AGE-induced β -defensin 4 production. This hypothesis was supported by two key observations: (1) the suppressed phosphorylation level of GSK-3 α , a negative regulator of the Wnt/ β -catenin pathway (Fig. 3B; Table II), and (2) the decreased protein level of axin-1 (adjusted P-value=0.047), a component of the β -catenin destruction complex along with GSK-3 α . Since β -catenin functions as a transcription factor downstream of this pathway and its localization to the nucleus is essential for exerting its transcriptional effects (24), we investigated the mechanism by which AGE induces β -defensin 4 in gingiva, using mouse gingival epithelial GE1 cells in culture.

As shown in Fig. 4A, treatment with AGE at 2 mg/ml for 24 h significantly increased the amount of β -defensin 4 in culture medium. We next examined β -catenin localization to assess the involvement of the Wnt/ β -catenin pathway. We found that β -catenin protein accumulated within the nucleus of cells treated with AGE (2 mg/ml) for 3 h (Fig. 4B). To further examine the involvement of the Wnt/ β -catenin pathway, we used two specific inhibitors of this pathway, LF3 and BIO. Simultaneous treatment of GE1 cells for 24 h with LF3 (30 μ M), a specific inhibitor of β -catenin on canonical Wnt signaling, significantly suppressed the AGE-induced increase in the β -defensin 4 protein production (Fig. 4C). Moreover, treatment with a GSK-3 specific inhibitor BIO (0.1 and 1 μ M) alone resulted in a statistically significant

Table II. Changes in the phosphorylation levels of proteins related to the canonical Wnt pathway in mouse gingiva treated with AGE for 6 h.

Description	Phosphorylated site	Log ₂ ratio (AGE/DW)	Adjusted P-value
Forkhead box protein O3	1xPhospho [S7]	-6.6439	2.38x10 ⁻¹⁶
Glycogen synthase kinase-3 α	1xPhospho [Y279]	-0.7442	7.76x10 ⁻³
Catenin δ 1	1xPhospho [S252]	-0.4170	4.66x10 ⁻²

AGE, aged garlic extract; DW, deionized water.

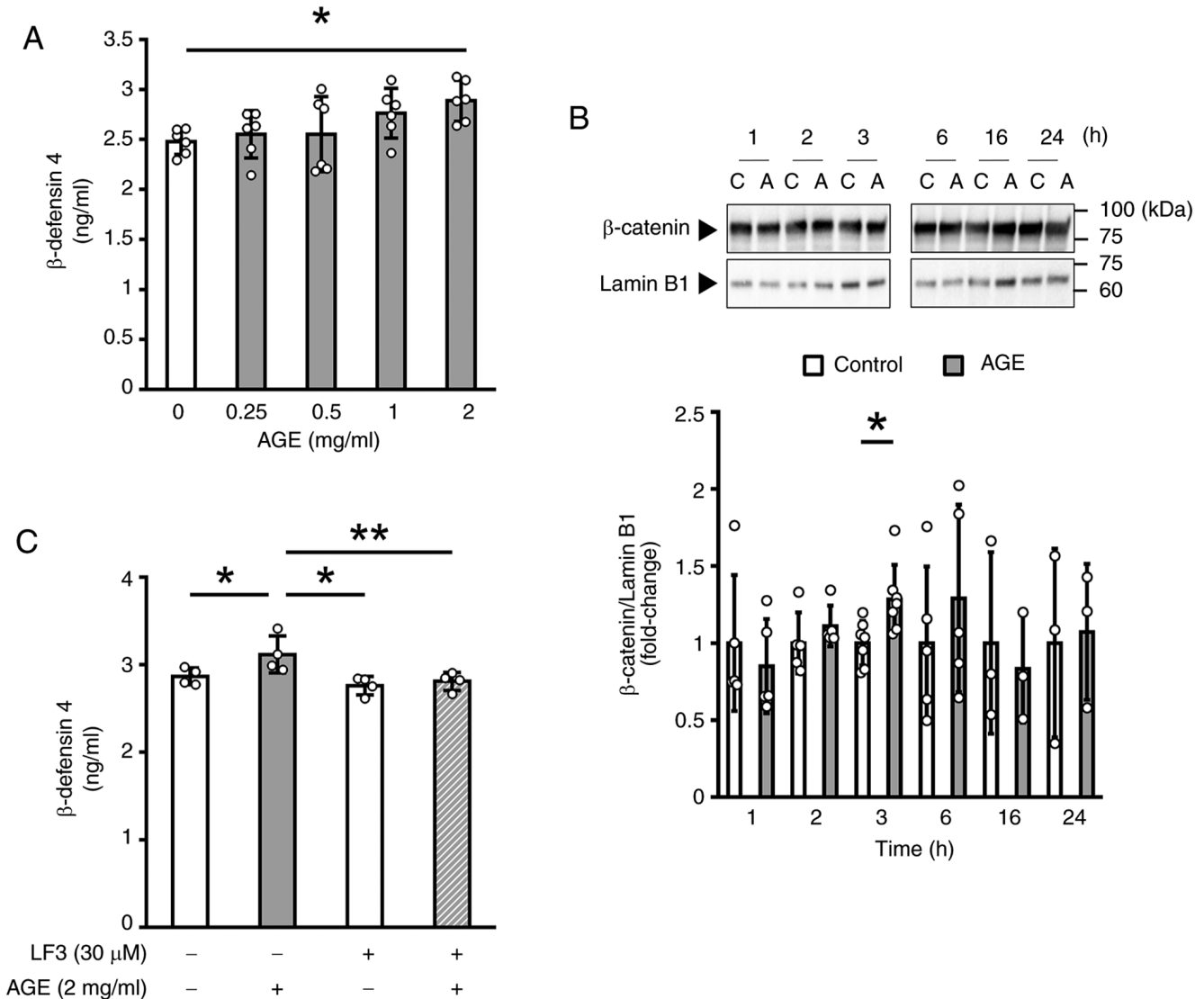


Figure 4. Effect of AGE on the β -defensin 4 production and canonical Wnt pathway in GE1 mouse gingival epithelial cells. (A) Cells were treated with AGE at the indicated concentrations (0.25–2 mg/ml) for 24 h. The amount of β -defensin 4 protein secreted into the culture medium was determined using an ELISA. The graph shows the concentration of β -defensin 4 in the medium. Data are presented as the mean \pm standard deviation ($n=6$). * $P<0.05$ (Dunnett's test). (B) Cells were treated with AGE (2 mg/ml) for the indicated duration (1–24 h). The protein level of β -catenin in the nucleus was analyzed by western blotting. Images show representative data of western blotting for β -catenin with lamin B1 as the internal control. The graph shows the level of β -catenin protein normalized to that of lamin B1. Data are presented as the mean \pm standard deviation ($n=3-7$). * $P<0.05$ (Mann-Whitney U test). (C) Cells were treated with AGE for 24 h in the presence or absence of LF3 (30 μ M; 30-min pretreatment), an inhibitor of β -catenin in the canonical Wnt pathway. The amount of β -defensin 4 protein secreted into the culture medium was determined using an ELISA. The graph shows the concentration of β -defensin 4 in the medium. Data are presented as the mean \pm standard deviation ($n=4$). * $P<0.05$, ** $P<0.01$ (Holm-Bonferroni test). A, AGE; AGE, aged garlic extract; C, control.

increase of the β -defensin 4 production (Fig. S3). These results suggested the involvement of the Wnt/ β -catenin pathway in the

β -defensin 4 production induced by AGE in mouse gingival epithelial cells.

Discussion

Gingival epithelium serves as a mechanical barrier, protecting the soft and hard tissues of the periodontal structures. In addition, the gingival epithelium secretes antimicrobial peptides, mainly β -defensin family that play a significant role in the innate immune system of periodontal tissues (25). In human, the most well-characterized β -defensins are β -defensin 1, 2, and 3 (26). The production of β -defensin 1 is essentially constitutive, whereas β -defensin 2 and 3 are inducible in response to inflammatory stimuli in human gingival keratinocytes (27). Several functional foods, such as human milk oligosaccharides (28), *Lactobacillus helveticus* SBT2171 (29) and green tea extracts (30), have been shown to up-regulate inducible human β -defensin 2 and/or 3 in human gingival epithelial cells.

In this study, we used the ddY strain of mice for several reasons. The first one is related to its genetic diversity. It is not as extensive as in humans but is substantially greater than that of inbred strains such as C57BL/6. Thus, the finding obtained by this study may be more applicable to humans when we consider the possible use of AGE for our oral health. The second one is that in our preliminary studies, ddY strain gave the best response of β -defensin to AGE treatment among a few strains tested. Thus this strain serves as a good experimental model to assess the effect of AGE on β -defensin production.

AGE is reported to be beneficial for patients with hypertension (31), atherosclerosis (32), and metabolic syndrome (33). Furthermore, recent findings have indicated that AGE suppresses inflammation and subsequent tissue destruction in the gingiva, thereby preventing the progression of periodontal disease (11-15). However, the effects of AGE on the innate immune function of periodontal tissues remained unclear. The present study demonstrated that AGE increased the production of mouse β -defensin 4, which is an ortholog to human β -defensin 2 (34), in both mouse gingival tissue and epithelial GE1 cells, suggesting that AGE is capable of bolstering antimicrobial efficacy in gingival epithelium.

The canonical Wnt pathway, also termed Wnt/ β -catenin signaling, is well-known to contribute to cell fate determination during developmental processes and tissue homeostasis (24). Recently it was reported that this pathway is involved in the maintenance of the periodontium and the progression of periodontal disease (35). Our proteomics analysis showed that AGE suppressed the phosphorylation of GSK-3 α (Y279) as well as the protein level of axin-1, a well-known component of the destruction complex of β -catenin (36). Furthermore, AGE reduced the phosphorylation of forkhead box protein O3 and catenin delta-1 that also participate in the degradation of β -catenin (37,38). Moreover, the present study revealed that AGE increased the protein level of nuclear β -catenin in GE1 cells. These results suggested that AGE activates the canonical Wnt pathway in gingival tissue by inhibiting the degradation of β -catenin.

Wnt3a, an endogenous Wnt agonist, is reported to elevate the mRNA level of mouse β -defensin 1 in mouse macrophage-like RAW264.7 cells (39). Moreover, Wang *et al* (40) have shown that DEAD-box Helicase 15 induces α -defensins in Paneth cells through the Wnt/ β -catenin signaling. These findings suggested that activation of the Wnt pathway is involved in the production of various defensins. Recently, Chen Y. and

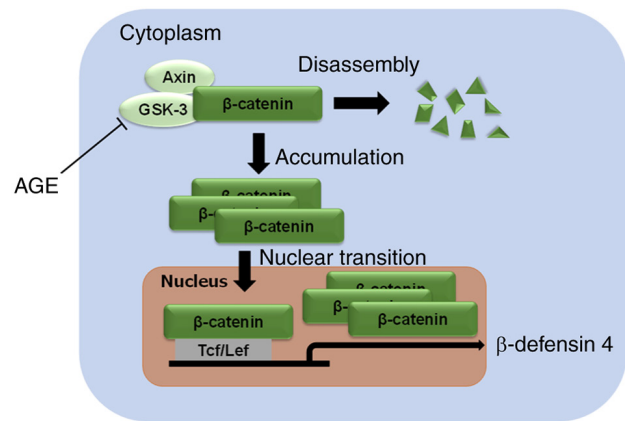


Figure 5. Putative mechanism by which AGE promotes the production of β -defensin 4 in mouse gingiva via the canonical Wnt signaling pathway. At the steady state, GSK-3 degrades β -catenin by forming a destruction complex with axin-1, suppressing the canonical Wnt pathway. When AGE inhibits GSK-3, β -catenin is not degraded, resulting in the accumulation of β -catenin in the cytoplasm. The accumulated β -catenin is then transferred into the nucleus and interacts with the transcription factor Tcf/Lef to promote the transcription of the β -defensin 4 gene. AGE, aged garlic extract; GSK-3, glycogen synthase kinase-3; Tcf/Lef, T cell factor/lymphoid enhancer factor.

Hu Y. have reported that the level of activated β -catenin is increased in gingiva of *Porphyromonas gingivalis*-associated ligature-induced periodontitis model mice, and that Wnt3a induced the production of tumor necrosis factor- α (TNF- α) in Raw264.7 cells (41). In the present study, LF3, a specific inhibitor of β -catenin in the canonical Wnt signaling, suppressed AGE-induced production of β -defensin 4 in GE1 cells. To elucidate the relationship between the AGE-induced decrease in the phosphorylation level of GSK-3 α (Y279) and β -defensin 4 production, we examined the effect of BIO, a GSK-3 specific inhibitor, on the β -defensin 4 production in GE1 cells, and found that this inhibitor increased the β -defensin 4 production. These findings suggested that AGE regulates the production of β -defensin 4 in mouse gingiva through the activation of the canonical Wnt pathway. We plan to investigate the involvement of GSK and/or its phosphorylation and other key molecules by conducting intervention and other experiments both *in vivo* and *in vitro* to elucidate the mechanism action of AGE.

Periodontal health is linked to the balance of the oral microbiome, with dysbiosis being a key factor in the onset and progression of periodontal disease (1,2). Bee pollen, which is a pollen ball or pellet that is carried by the honey bee, has been reported to elevate the mRNA level of β -defensin-2 and -3 and alter the oral microbiota in the oral cavities of mice (42). It is possible that AGE helps to maintain the oral microbiome in a healthy state by increasing the production of antimicrobial peptides, and thus foster an oral environment less susceptible to periodontal disease. Further investigation is needed to clarify whether AGE affects the oral microbiota in periodontal disease patients and model mice.

In conclusion, our findings showed that AGE can up-regulate antimicrobial defense potential by promoting the production of β -defensin 4 via the canonical Wnt signal transduction pathway in gingiva (Fig. 5). Although more studies *in vivo* are required to clarify the role of β -defensin 4 induced by AGE, the present study suggests that AGE serves as a

potential oral supplement for preventing onset of periodontal disease.

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Availability of data and materials

The mass spectrometry immunopeptidomics and proteomics data generated in the present study may be found in the ProteomeXchange Consortium (43) via the jPOSTrepo partner repository (44) under the accession numbers PXD053155 and PXD053156 for ProteomeXchange, and JPST002964 and JSPT002963 for jPOSTrepo or at the following URLs: <https://proteomecentral.proteomexchange.org/cgi/GetDataset?ID=PXD053155>, <https://proteomecentral.proteomexchange.org/cgi/GetDataset?ID=PXD053156>, <https://repository.jpostdb.org/entry/JPST002963.0> and <https://repository.jpostdb.org/entry/JPST002964.0>. All other data generated in the present study may be requested from the corresponding author.

Authors' contributions

HN and MO designed the experimental procedures. HN performed animal experiments. DF and HN performed cell experiments, reverse transcription-quantitative PCR and western blot analysis. HN performed the proteomics analysis and ELISA. DF and HN performed data analysis. DF, HN and MO confirm the authenticity of all the raw data. MO validated the results to ensure accuracy. DF and HN created graphical representations of the data. HN wrote the original draft. DF and MO reviewed and edited the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Animal care and experiments were performed in accordance with the guidelines for the care and use of laboratory animals of the Wakunaga Pharmaceutical Co., Ltd., and animal experiments were reviewed and approved by the Wakunaga Pharmaceutical Company Institutional Animal Care and Use Committee (approval no. 360; Akitakata, Japan).

Patient consent for publication

Not applicable.

Competing interests

All the authors are employees of Wakunaga Pharmaceutical Co., Ltd., who provided funding for this study.

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