Inflammatory cytokines and cAMP regulate amelotin gene expression and the effects of heated coral calcium mouthwash on plaque and periodontal tissue

（炎症性サイトカインと cAMP によるアメロチン遺伝子発現の調節と焼成サンゴカルシウム含有洗口剤のプラークおよび歯周組織に対する効果）

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Preface

This article is based on a main reference paper, “Inflammatory Cytokines and cAMP Regulate Amelotin Gene Transcription’’ in the International Journal of Oral-Medical Sciences, and a reference paper, “Effects of Heated Coral Calcium Mouthrinse on Plaque and Periodontal Tissue’’ in the Japanese Journal of Conservative Dentistry.

Abstract

Amelotin (AMTN) is an enamel protein secreted by ameloblasts at maturation stage and expressed in internal basal lamina of junctional epithelium (JE) which attaches to the tooth enamel. JE may prevent invasion of bacteria into the periodontal tissue. AMTN localization suggests that the function might be responsible for cell adhesion. The aim of this study was to elucidate the effect of interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α) or forskolin (FSK) on AMTN gene transcription in human gingival fibroblast (HGF), human gingival epithelial Sa3 or Ca9-22 cells. IL-1β (1 ng/ml) and TNF-α (10 ng/ml) increased promoter activities of -211, -353, -501, -769 and -950AMTN LUC constructs in HGF. TNF-α (10 ng/ml) induced LUC activities of these five AMTN constructs in Sa3 cells. FSK (1 μM) increased AMTN mRNA levels at 12 and 24 h in Ca9-22 cells. FSK (1 μM, 12 h) induced LUC activities of all six AMTN LUC constructs

in Ca9-22 cells. These results demonstrated that IL-1β and TNF-α increased AMTN gene transcription in HGF and Sa3 cells. FSK increased AMTN gene transcription mediated through AP1 site in the human AMTN gene promoter.

The purpose of the second study was to evaluate the antimicrobial effects and clinical efficacy of heated coral calcium mouthwash. Twenty male and female volunteers were assigned to two groups and used placebo (Placebo mouthwash group) or wash with heated coral calcium (Coral mouthwash group) for 1 month. In each patient, probing pocket depth (PPD), clinical attachment level (CAL), bleeding on probing (BOP), plaque Index (PlI) and gingival Index (GI) were measured before and after the mouthwash for 1 month. Gingival crevicular fluids were collected before and after the mouthwash and measure the numbers of total bacteria, *Aggregatibacter actinomycetemcomitans*, *Prevotella intermedia* and *Porphyromonas gingivalis* by PCR-invader method. PPD, CAL, BOP, PlI and GI did not change significantly after the mouthwash for 1 month in the both groups. However, there were improvement tendency in CAL, BOP, PlI and GI in the coral mouthwash group. Numbers of total bacteria, *A. actinomycetemcomitans*, *P. intermedia* and *P. gingivalis* did not change significantly after the mouthwash for 1 month in the both groups. Whereas, in the coral mouthwash group, there were reduction tendency in *P. intermedia* and *P. gingivalis* ratio % total bacteria after the coral mouthwash for 1 month.

Introduction

Periodontitis is an inflammatory disease coming from environmental, host and bacterial factors, one of the principal causes of tooth loss (1, 2). In the inflamed gingiva, immune responses were increase and inflammatory cytokines were extensively secreted (3). Tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) are typical inflammatory cytokines which induce inflammation of periodontium and alveolar bone resorption (4, 5).

Junctional epithelium (JE) is an epithelial tissue situated at the base of gingival sulcus and attached to the tooth enamel. JE might protect periodontal tissues against microbiological challenge, and it is nonkeratinized epithelium derived from the reduced enamel epithelium during tooth development (6-8).

Amelotin (AMTN) is enamel protein secreted by maturation stage of ameloblasts (9), which is also produced by internal basal lamina of JE and gingival fibroblasts (10-13). Targeted overexpression of AMTN revealed disorganized and thin tooth enamel compared to the enamel from the wild-type mice (14). Structural defects and enamel hypomineralization were detected in AMTN knockout mice (15). AMTN can advance hydroxyapatite mineralization (16, 17), and it is important for appropriate enamel

maturation (18). The task of AMTN in the JE remain to be clarified. It expresses in an internal basal lamina of JE, therefore AMTN could have function of adhesion between JE and tooth enamel (19, 20). Odontogenic ameloblast-associated protein (ODAM) and follicular dendritic cell secreted protein (FDC-SP) are also expressed in the JE, whereas localization of these three proteins in the JE are a bit different (21, 22). AMTN and FDC-SP express only in the internal basal lamina, whereas ODAM is detected in the entire JE (23). ODAM became detectable at edge of the wound first and then entire of the JE after gingivectomy. AMTN expressed later and detected only at the interface between cell and tooth (23). Ca9-22 gingival epithelial cells were used in this study, which have similar characters of cells derived from JE, because they produce AMTN and FDC-SP (22, 24, 25). We have reported previously, AMTN gene expression was upregulated in inflamed gingiva from periodontitis patients (12, 13), and TNF-α and IL-1β increased AMTN gene transcription in Ca9-22 cells (24, 25). Enamel matrix derivative (EMD; Emdogain®) is used for periodontal regeneration therapy and employed to restore functional periodontal ligament, cementum and alveolar bone. Epithelial cells increased intracellular cyclic AMP (cAMP) and platelet-derived growth factor AB secretion when EMD was present, but proliferation was inhibited (26, 27). Therefore, we focused on the TNF-α and IL-1β effects on AMTN gene expression in human gingival fibroblasts (HGF) and human

gingival epithelial Sa3 cells by luciferase (LUC) assays, and cAMP effect on AMTN gene expression by real-time PCR and LUC analyses.

Routine plaque control as a cause removal is very important for the prevention and treatment of periodontal disease. There are two methods of plaque control, mechanical plaque control and chemical plaque control. Mechanical plaque controls such as brushing is the most effective means, but due to the limitation of reachability of hair tips, chemical plaque control methods such as antibacterial agents and enzymes are used as an auxiliary method (28). In addition, the role of chemical plaque control may become more important when mechanical plaque control is difficult for the elderly, disabled people, and even infants. Since chlorhexidine gluconate has a plaque-suppressing effect, 0.12% chlorhexidine gluconate is used in Europe and the United States. In Japan, products with a stock solution concentration of 0.05% are currently used as mouthwashes after dilution because of reports of side effects such as anaphylactic shock when used on mucous membranes (29). Therefore, it is desired to develop a chemical plaque control agent with few side effects. We aimed to search for the usefulness of an alkaline non-alcohol type mouthwash using heated coral calcium as a chemical plaque control agent. We analyzed the improvement of clinical findings of the periodontitis and changes in the number of bacteria in the gingival crevicular fluid before and after use of the mouthwash.

Materials and Methods

Cell culture

Human gingival epithelial-like Ca9-22 cells, or Sa3 cells (21, 24) and HGF (12) were cultured in alpha-minimum essential medium (α-MEM) or Dulbecco's Modified Eagle Medium (DMEM) at 37°C in 5% CO_2 and 95% air containing 10% fetal calf serum (FCS) (Wako, Tokyo Japan). The cells were grown to confluence in 35 mm or 60 mm culture dishes in α-MEM or DMEM including 10% FCS, then cultured for 12 h in α-MEM or DMEM without FCS, and stimulated with IL-1β (1 ng/ml, Wako, Tokyo Japan), TNF-α (10 ng/ml, R&D systems, Minneapolis, USA) or forskolin (FSK; 1 μM, Sigma-Aldrich, Tokyo Japan). Total RNA was purified from triplicate cultures at 0, 3, 6, 12 and 24 h following stimulation by FSK. Cell culture of HGF was carried out with the approval of the Ethics Review Committee of the Faculty of Dentistry, Nihon University School of Dentistry at Matsudo (EC03-041).

Real-time PCR

Total RNAs were isolated using Isogen II (Wako, Tokyo, Japan) from Ca9-22 cells and used as a template for cDNA synthesis. cDNA was prepared using the PrimeScript RT

reagent kit (Takara-Bio, Tokyo Japan). Quantitative real-time PCR (qPCR) was performed using AMTN For; 5'-GTTGAATGTACAACAGCAACTGCAC-3', AMTN Rev; 5'-TTCCATCCTGGACATCTGGATTA-3', GAPDH For; 5'-GCACCGTCAAGGCTGAGAAC-3'; GAPDH Rev; 5'-ATGGTGGTGAAGACGCCAGT-3', using the SYBR Premix Ex Taq II in a TP800 thermal cycler dice real-time system (Takara-Bio, Tokyo, Japan). The amplification reactions were performed in a total volume of 25 μl 2x SYBR Premix Ex Taq II (12.5 μl), 10 μM forward and reverse primers and 50 ng cDNA for AMTN and 10 ng for GAPDH. To reduce variability between replicates, PCR premixes containing all reagents except for cDNA were prepared and aliquoted into 0.2 ml PCR tubes. The conditions for thermal cycling were 10 s at 95°C, 40 cycles of 5 s at 95°C and 30 s at 60°C. Post-PCR melting curves confirmed the specificity of single-target amplification, and the expression of AMTN relative to GAPDH was determined in triplicate.

LUC assays

To elucidate the effects of IL-1β, TNF-α or FSK on human AMTN gene transcription, we prepared chimeric constructs by ligating various length of human AMTN gene promoters into LUC reporter plasmid (-100AMTN; -100~+60, -211AMTN; -211~+60, -353AMTN;

-353~+60, -501AMTN; -501~+60, -769AMTN; -769~+60, -950AMTN; -950~+60) (24, 25). Exponentially growing HGF, Sa3 and Ca9-22 cells were used for LUC assays. Twenty-four hours after plating, cells at 60-70% confluence were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The transfection mixture included 1 μg of a LUC plasmid and 2 μg of β-galactosidase (β-Gal) plasmid (Promega, Madison, WI, USA) as an internal transfection control. β-Gal activities were determined separately to normalize the LUC activities. Two days after transfection, the cells were cultured in α-MEM without FCS for 12 h, and then stimulated with IL-1β (1 ng/ml), TNF-α (10 ng/ml) or FSK (1 μM) for 12 h prior to harvest. The LUC activities were measured using a luminescence reader (AcuuFlex Lumi 400; Aloka, Tokyo, Japan).

Target patients and usage of mouthwash

Twenty men and women aged 39 to 86 (10 men, 10 women, average age 68.6 ± 11.5 years) visiting for supportive periodontal therapy at the Nihon University Hospital at Matsudo) (Table 1). The subjects were those who had no systemic disease and had not received antibacterial drugs in the past 3 months. This study was carried out with the approval of the Ethics Review Committee of the Faculty of Dentistry, Nihon University School of Dentistry at Matsudo (EC14-024), and the coral mouthwash group and placebo

mouthwash group were randomly assigned.

The coral mouthwash is an alkaline non-alcohol type mouthwash (pH 12) containing heated coral calcium compatible with existing food additives, and contains heated coral calcium, water, glycerin, xylitol, and peppermint (Magnacaps Dent Safe®, Asada Chemical Co., Ltd.). The components of heated coral calcium powder are H_2O; 0.06%, Na; 3.63 mg/100 g, P; 17.8 mg/100 g, Fe; 35.2 mg/100 g, Ca; 63.2 g/100 g, Mg; 568 mg/100 g, S; 0.2 g/100 g, and the calcium oxide (CaO) content is 96.9%. As a placebo mouthwash, we used a mouthwash (pH 7) that did not contain heated coral calcium and had the same other components and containers. Coral and placebo mouthwashes were used 3 times a day (about 10 ml each time, 1 minute mouthwash), and the mouthwash was performed for 1 month.

Clinical evaluation

Clinical evaluation of periodontitis includes probing pocket depth (PPD) 30), clinical attachment level (CAL), and bleeding on probing (BOP) of the test tooth. Plaque Index (PlI) (31) and Gingival Index (GI) (32) were measured before and after the use of mouthwash. A periodontal probe (CP11, Hu-Friedy, USA) was used to measure PPD and CAL. The probing pressure was 20 to 25 g, and the CAL was measured as the distance

from the cement-enamel junction or the edge of the prosthetic device to the tip of the probe. The deepest part of the PPD measured by the 6-point method was adopted as the PPD value.

Bacterial test

Gingival crevicular fluid (GCF) is simply moisturized with a cotton roll after removing the supragingival plaque with a cotton ball, collected from the deepest PPD site three times for 30 seconds at a sterile paper point, and the number of periodontopathic bacteria were measured by the PCR invader method. We requested BML Co., Ltd. (Tokyo, Japan) to measure the three types of pathogenic bacteria (*Actinobacillus actinomycetemcomitans*, *Prevotella intermedia*, *Porphyromonas gingivalis*). GCF was collected before and after the use of mouthwash.

Statistical analysis

Triplicate samples were analyzed for each experiment, and experiments were replicated to ensure the consistency of the responses to drugs. Significant differences between the control and treatment groups were determined using the one-way ANOVA.

Results

1. Regulation of AMTN gene expression by IL-1β, TNF-α and FSK

Previously, we have reported that the differences in the gene expression profiles between non-inflamed gingiva collected from alveolar ridges at dental implant surgery and inflamed gingiva from periodontitis patients during periodontal flap surgery using DNA microarray. A scatter plot of the results of microarray showed that AMTN, ODAM, IL-1β, IL-1α and IL-6 gene expressions were increased in inflamed gingiva (12). To elucidate the effects of inflammatory cytokines (IL-1β and/or TNF-α) on AMTN gene transcription in HGF and Sa3 by LUC assays, we used human AMTN LUC constructs containing different promoter regions (-100, -211, -353, -501, -769 and -950AMTN) of the human AMTN gene (24, 25). Luciferase activities of -211, -353, -501, -769 and -950AMTN LUC constructs were increased by IL-1β (1 ng/ml) and TNF-α (10 ng/ml) in HGF (Fig. 1). TNF-α (10 ng/ml) induced LUC activities of five AMTN constructs in Sa3 cells (Fig. 2). In the human AMTN proximal gene promoter, there are several response elements for transcription factors such as activator protein 1 (AP1; nts -84 to -94), two kinds of C/EBP elements (C/EBP1; nts -105 to -118), C/EBP2 (nts -150 to -163), and Yin Yang 1 (YY1; nts -212 to -228) (Fig. 5) (24, 25).

To study the effect of FSK on human AMTN gene expression, we performed qPCR

and LUC assays. Total RNA was extracted from gingival epithelial Ca9-22 cells after stimulation by FSK (1 μM) for 3, 6, 12 and 24 h. AMTN mRNA levels were increased at 12 h, reached peak at 24 h in Ca9-22 cells (Fig. 3). FSK (1 μM, 12 h) induced LUC activities of all AMTN LUC constructs in Ca9-22 cells (Fig. 4).

2. Effects of heated coral mouthwash on plaque and periodontal tissue

No adverse events were observed during the study period in either coral or placebo mouthwash group, and no pathological changes in the oral mucosa were observed. Mean PPD, CAL, BOP, GI and PlI of all target patients before mouthwash (baseline) were 6.3 ± 0.66 mm, 8.1 ± 1.68 mm, 55%, 1.6 ± 0.5 and 0.95 ± 0.76 (Table 1). Before using the coral mouthwash, the average PPD, CAL, BOP, GI and PlI at base line were 6.3 ± 0.67 mm, 8.3 ± 1.7 mm, 60%, 1.61 ± 0.52 and 0.9 ± 0.74. After 1 month of coral mouthwash, PPD, CAL, BOP, GI and PlI were changed to 6.3 ± 0.95 mm, 8.2 ± 2.1 mm, 50%, 1.5 ± 0.53 and 0.8 ± 0.92 (Table 2A). The average PPD, CAL, BOP, GI and PlI of the placebo mouthwash group were 6.3 ± 0.67 mm, 7.9 ± 1.73 mm, 50%, 1.6 ± 0.52, and 1.0 ± 0.82 at base line. After 1 month of placebo mouthwash, PPD, CAL, BOP, GI and PlI were changed to 6.1 ± 1.45 mm, 7.7 ± 2.31 mm, 60%, 1.6 ± 0.52, and 1.1 ± 0.74 (Table 2B). In the both groups, there was no significant improvement in PPD, CAL, BOP, GI and PlI

after 1 month compared to the base line. In the coral mouthwash group, there was no significant difference, but a decreasing tendency was observed in CAL, BOP, GI and PlI after 1 month (Table 2).

Number of total bacteria of all target patients was 4.43 ± 0.8 (log copies), average numbers of *A. actinomycetemcomitans*, *P. intermedia* and *P. gingivalis* were 0.18 ± 0.54, 1.81 ± 0.96, and 2.47 ± 1.35 (log copies) at baseline (Table 1).

Number of total bacteria in coral mouthwash group was 4.40 ± 0.84 (log copies), and average numbers of *A. actinomycetemcomitans*, *P. intermedia* and *P. gingivalis* were 0.18 ± 0.57, 2.11 ± 1.06, and 2.71 ± 1.31 (log copies) at baseline. Number of total bacteria using coral mouthwash was changed to 4.29 ± 0.88 (log copies), and average numbers of *A. actinomycetemcomitans*, *P. intermedia* and *P. gingivalis* were changed to 0.16 ± 0.51, 1.89 ± 0.89, and 2.69 ± 1.39 (log copies) after 1 month. The ratio of *A. actinomycetemcomitans*, *P. intermedia* and *P. gingivalis* to the number of total bacteria in coral mouthwash group were 0.02 ± 0.07%, 1.98 ± 2.77%, and 7.14 ± 7.53% at base line. They improved to 0.01 ± 0.04%, 1.22 ± 1.57%, and 6.90 ± 7.47% after 1 month (Table 3A).

Number of total bacteria using placebo mouthwash was 4.45 ± 0.80 (log copies), and average numbers of *A. actinomycetemcomitans*, *P. intermedia* and *P. gingivalis* were 0.17

± 0.54, 1.51 ± 0.80, and 2.58 ± 1.58 (log copies) at baseline. Number of total bacteria using placebo mouthwash was changed to 4.23 ± 0.88 (log copies), and average numbers of *A. actinomycetemcomitans*, *P. intermedia* and *P. gingivalis* were changed to 0, 1.45 ± 0.91, and 2.58 ± 1.53 (log copies) after 1 month. The ratio of *A. actinomycetemcomitans*, *P. intermedia* and *P. gingivalis* to the number of total bacteria in placebo mouthwash group were 0.12 ± 0.36%, 0.52 ± 0.76%, and 6.25 ±8.03% at base line. They changed to 0%, 0.88 ± 1.26%, and 14.42 ± 21.85% after 1 month (Table 3B).

Discussion

In the present study, we have shown that the AMTN gene expression was upregulated by IL-1β and TNF-α in HGF (Fig. 1), and induced by TNF-α in gingival epithelial-like Sa3 cells (Fig. 2). AMTN is secreted by maturation stage ameloblasts, however it's expressions also found in internal basal lamina of JE. This characteristic distribution of AMTN suggest a role for cell adhesion and infectious blockade (9, 10). Previously, we have reported IL-1β, IL-6 and TNF-α induced AMTN mRNA levels in HGF after 24 h (12). If we used inflamed gingiva for immunostaining using anti-AMTN antibody, positive staining of AMTN was observed in the gingival connective tissues and JE. However, we could not detect any immunostaining of AMTN in the non-inflamed gingiva

collected from alveolar ridges (12). These results suggest that some function of the AMTN in the gingival epithelium and connective tissues could be related to inflammation. FSK is a labdane diterpene which is produced by the Indian Coleus plant and commonly used to increase intercellular cAMP levels by stimulation of adenylate cyclase. cAMP is a second messenger synthesized from adenosine triphosphate (ATP) by adenylate cyclase, binds to protein kinase A (PKA), and causes dissociation between the regulatory and catalytic units of PKA, thus enabling those catalytic units to phosphorylate serine or threonine residues of substrate proteins. The phosphorylated proteins may act as an ion channels directly or activate enzymes. PKA can also phosphorylate cAMP response element (CRE) binding protein that bind to CRE (5'-TGACGTCA) in the gene promoter, causing increases or decreasing in gene transcription (33-35). In this study, FSK (1 μM) significantly increased AMTN mRNA levels at 12 h and 24 h in Ca9-22 cells (Fig. 3). FSK (1 μM) induced transcriptional activities of all AMTN LUC constructs at 12 h in Ca9-22 cells (Fig. 4), whereas IL-1β and TNF-α could not induce luciferase activity of -100AMTN construct (Fig. 1 and 2). The results suggest that CRE may exist between -100 ~ -1 from the transcription start site in the AMTN gene promoter. There is an AP1 site (AATGATTTAAC) between -94 ~ -84 and sequence of the AP1 is similar to CRE (Fig. 5). Therefore, AP1 site may be response element for the effect of FSK. Single

nucleotide mutation (p.S34N mutation) of cAMP gene could be contributing factor for generalized aggressive periodontitis (36). The result suggests the association of cAMP in periodontitis.

Concentrations of IL-1β and TNF-α in gingival crevicular fluid (GCF) were increased in the periodontitis patients (4, 37) and initial periodontal therapy decreased IL-1β levels in the GCF (4). IL-1β and TNF-α upregulated LUC activities of the AMTN constructs containing promoter sequence between -101 to -950 base pair upstream from the transcriptional start site of the human AMTN gene in HGF (Fig. 1), and TNF-α induced LUC activities of the AMTN constructs (-101 to -950) in Sa3 cells (Fig. 2). In the previous report, we have reported that IL-1β and TNF-α increased human AMTN gene transcription in Ca9-22 cells via C/EBP1, C/EBP/2 and YY1 in the human AMTN gene promoter (24, 25). These 3 response elements exist between -101 to -950 of the human AMTN gene promoter. Therefore, C/EBP1, C/EBP/2 and YY1 could be response elements for the effects of IL-1β and TNF-α in HGF.

C/EBPs are leucine zipper transcription factor family member that regulate a various tissue functions and variety of cell differentiation (37). C/EBPβ binds to IL-6 response elements in the G-CSF, IL-8 and TNF-α gene promoters and mediates IL-6 signaling pathway, and nuclear localization of C/EBPβ is regulated by inflammation (38). C/EBPα

is expressed in T cells, and negatively regulates IFN-γ expression in T cells (39). YY1 plays a critical role in stimulating IL-6 expression in rheumatoid arthritis which contribute to the inflammation via promoting the differentiation of Th17 (40). In conclusion, we demonstrate that IL-1β and TNF-α induced LUC activities of AMTN promoter constructs (-211, -353, -501, -769 and -950AMTN) in HGF and TNF-α upregulated -211, -353, -501, -769 and -950AMTN gene promoter constructs in Sa3 cells. AMTN mRNA levels were increased significantly by FSK (1 μM) at 12 and 24 h in Ca9-22 cells, and FSK (1 μM) induced transcriptional activities of all AMTN LUC constructs at 12 h in Ca9-22 cells. These results suggest that FSK induced AMTN gene transcription via AP1 between -94 ~ -84 in the human AMTN gene promoter. Further research is required to analyze the role of cAMP on the expression and function of AMTN.

In the 2nd study, we have performed with a mouthwash containing heated coral calcium as a chemical plaque control agent, and after 1 month of coral and placebo mouthwash, the degree of improvement in clinical parameters and the therapeutic effect due to changes in the bacterial flora in the periodontal pocket were evaluated. In the previous studies, a 6-month mouthwash with 0.12% chlorhexidine gluconate or a 9-month mouthwash with Listerine was found to reduce plaque volume and gingival inflammation (29, 41). In this study, CAL, BOP, GI and PlI values tended to decrease with the use of coral mouthwash

for 1 month, however there were no significant differences. These results could be due to the fact that the mouthwash period of chlorhexidine and Listerine were 6 to 9 months, whereas in this study the period of coral mouthwash was as short as 1 month. For the patients with periodontitis with PPD of 6-7 mm on 2 or more teeth, use dentifrice Gelcoat F containing 0.05% chlorhexidine hydrochloride, β-glycyrrhetinic acid, sodium fluoride and sodium polyphosphate was applied for 1 month, and a dentifrice was applied daily for 10 minutes in a retainer before going to bed. Significant improvements in PlI and GI were observed compared to the control group using dentifrices excluding chlorhexidine hydrochloride and β-glycyrrhetinic acid (42). In a one-month study using a mouthwash containing 0.01% chlorhexidine hydrochloride and 0.01% cetylpyridinium chloride (CPC), significant reductions in saliva total bacterial count, *S. mutans* count, tongue coating total bacterial count, and total *streptococcal* count in marginal plaque were reported. (43). When rinsing with placebo and mouthwash (chlorhexidine, Listerine or Meridol) twice daily during the 3-week brushing discontinuation period, mouthwashing with chlorhexidine solution showed the most inhibitory effect on marginal plaque (44). Although CPC is a cationic detergent (45), mouthwashes containing CPC have a bactericidal effect against gram-negative bacteria, and show an effect of suppressing supragingival plaque and suppressing gingival inflammation caused by plaque (46). As a

result of mouthwashing with tea catechin or distilled water during the 10-day brushing discontinuation period, it has been reported that plaque growth suppression, gingival inflammation and halitosis suppression tendencies were observed in the catechin group (47).

Since the mean PPD, GI, and PlI before mouthwash were almost same between the coral mouthwash group and the placebo mouthwash group, there was almost no difference between the groups in the clinical parameters at baseline (Table 2). In the both groups, there were no significant improvements in PPD, CAL, BOP, GI and PlI after 1 month of the mouthwash compared to the baseline, but the improvement tendencies were observed in CAL, BOP, GI and PlI after 1 month of use of the coral mouthwash (Table 2A). Heated coral calcium has a bactericidal action, but it is doubtful whether the bactericidal effect affects the subgingival flora using mouthwash. However, since it has been reported that toothbrush instruction on the supragingival plaque has the effect of suppressing subgingival plaque (48, 49). If mouthwash with heated coral calcium having a bactericidal action is performed for a longer period, it may lead to further improvements in clinical parameters and bacterial flora in the periodontal pocket. The number of bacteria detected by *P. intermedia* showed a decreasing tendency after mouthwash in both groups, but there was no significant difference, and the number of bacteria detected by *P. gingivalis* was

almost unchanged before and after mouthwash in both groups (Table 3). However, the ratio of *P. intermedia* and *P. gingivalis* to the total number of bacteria after 1 month of use of placebo mouthwash tends to increase. While the ratio of *P. intermedia* and *P. gingivalis* to the total number of bacteria after 1 month of use of coral mouthwash show decreasing tendancy, it is considered that a longer-term comparative study is necessary.

It has been reported that *P. intermedia* is highly detected in periodontal pockets of patients with periodontitis, whose growth is promoted by female hormones estrogen and estradiol, and is related to pregnancy gingivitis. (50). In addition to *P. gingivalis*, observing changes in the number of *P. intermedia* in the periodontal pocket associated with mouthwash was considered to be an important index to test the effect. From the above results, 1 month mouthwash with heated coral calcium had no significant effect on the subgingival periodontopathic bacteria in the periodontal pocket and clinical parameters of periodontal disease. However, there were improvement tendency in CAL, BOP, PlI and GI in the coral mouthwash group. Therefore, it will be necessary to examine the effects of long-term use of the mouthwash, or of additive or synergistic effects with other medicinal ingredients.

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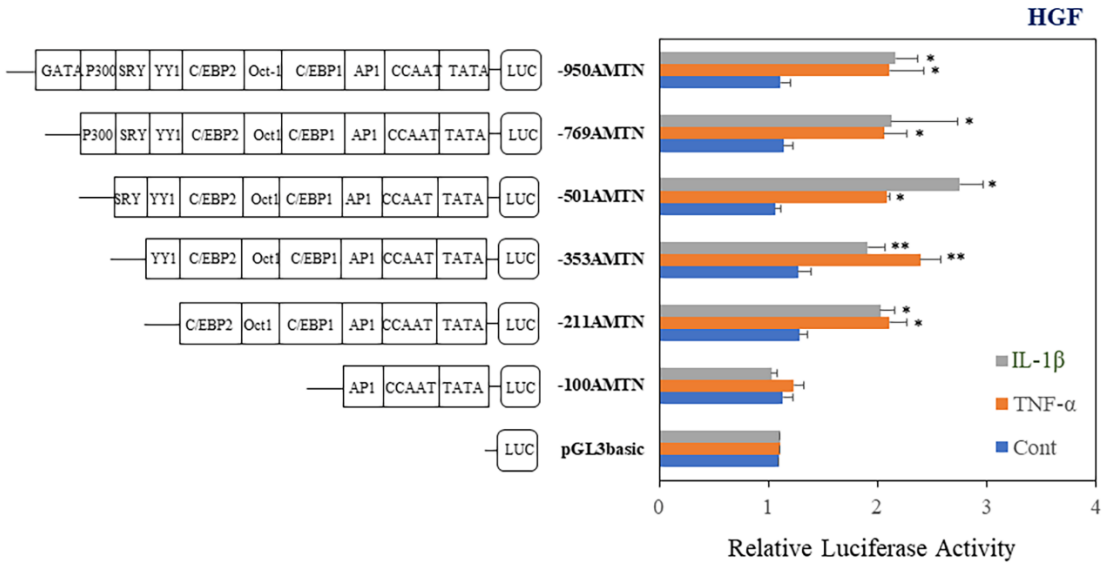

Fig. 1 IL-1β and TNF-α upregulates human AMTN gene promoter activities in HGF. The transcriptional activities of -211AMTN (-211~+60), -353AMTN (-353~+60), -501AMTN (-501~+60), -769AMTN (-769~+60) and -950AMTN (-950~+60) were increased by IL-1β (1 ng/ml) and TNF-α (10 ng/ml) at 12 h in HGF. Results of transcriptional activities obtained from three separate transfections with constructs, pGL3basic and -211AMTN to -950AMTN were combined and values expressed with standard error (SE). *P<0.05 and **P<0.01.

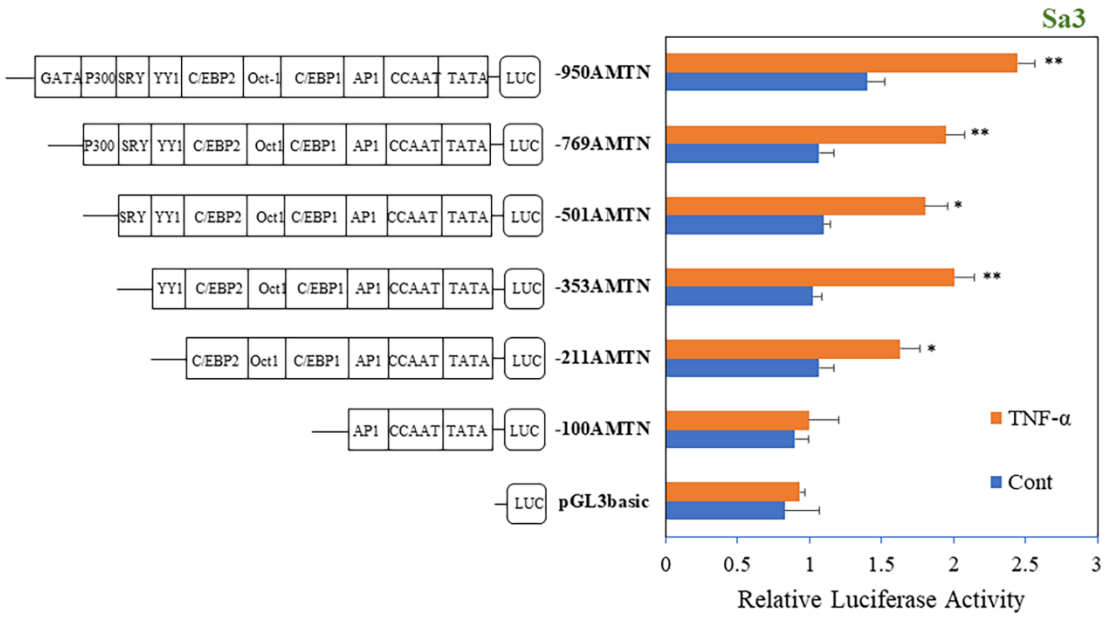

Fig. 2 TNF-α upregulates human AMTN gene promoter activities in Sa3 cells. The transcriptional activities of -211AMTN, -353AMTN, -501AMTN, -769AMTN and -950AMTN were increased by TNF-α (10 ng/ml, 12 h) in Sa3 cells. Results of transcriptional activities obtained from three separate transfections with constructs, pGL3basic and -211AMTN to -950AMTN were combined and values expressed with SE. *P<0.05 and **P<0.01

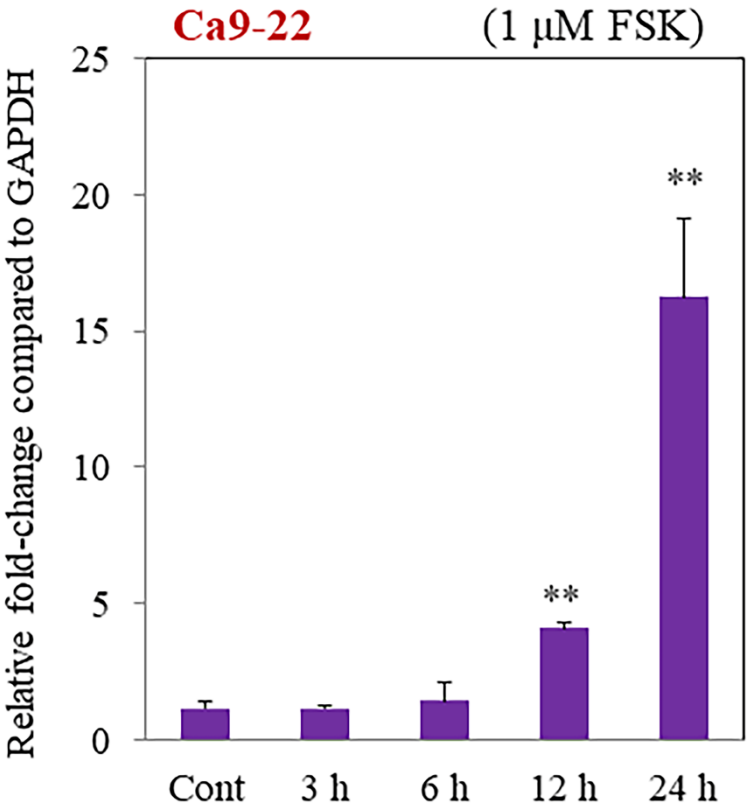

Fig. 3 Effects of FSK on AMTN mRNA levels in Ca9-22 cells. Ca9-22 cells were treated with or without FSK (1 μM) for 3, 6, 12, and 24 h. AMTN and GAPDH mRNA levels were measured by real-time PCR. The experiments were performed in triplicate for each data point. Quantitative analyses of the data sets are shown with standard deviation (SD). Significantly different from control; **P<0.01.

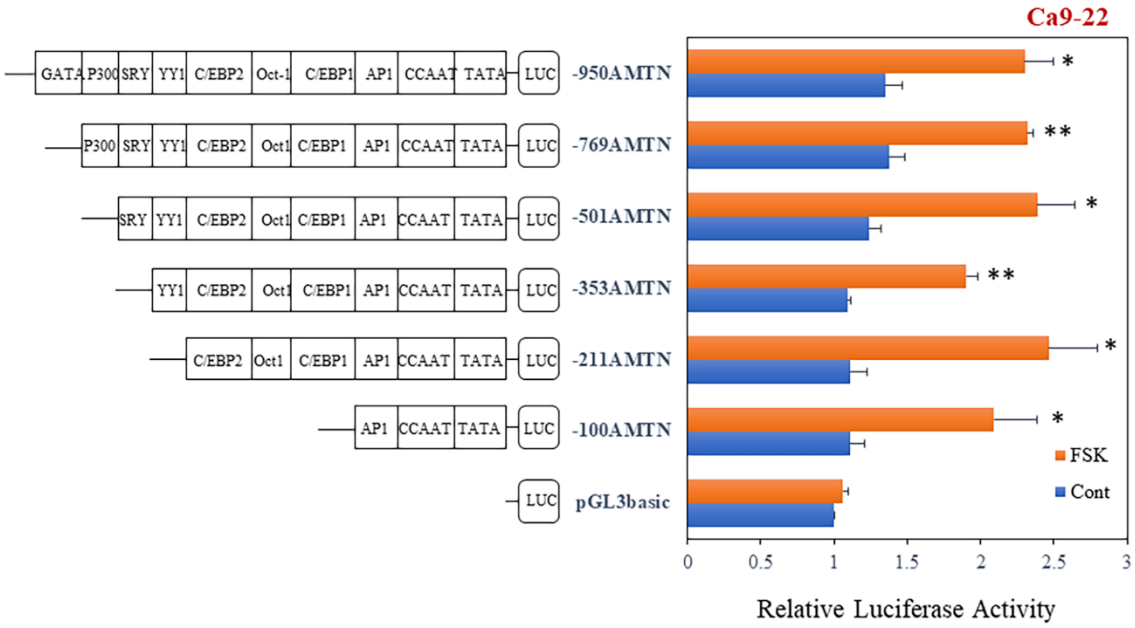

Fig. 4 FSK upregulates human FDC-SP gene promoter activities in Ca9-22 cells. The transcriptional activities of -100AMTN, -211AMTN, -353AMTN, -501AMTN, -769AMTN and -950AMTN were increased by FSK (1 μM, 12 h) in Ca9-22 cells. Results of transcriptional activities obtained from three separate transfections with constructs, pGL3basic and -100AMTN to -950AMTN were combined and values expressed with SE. **P<0.01

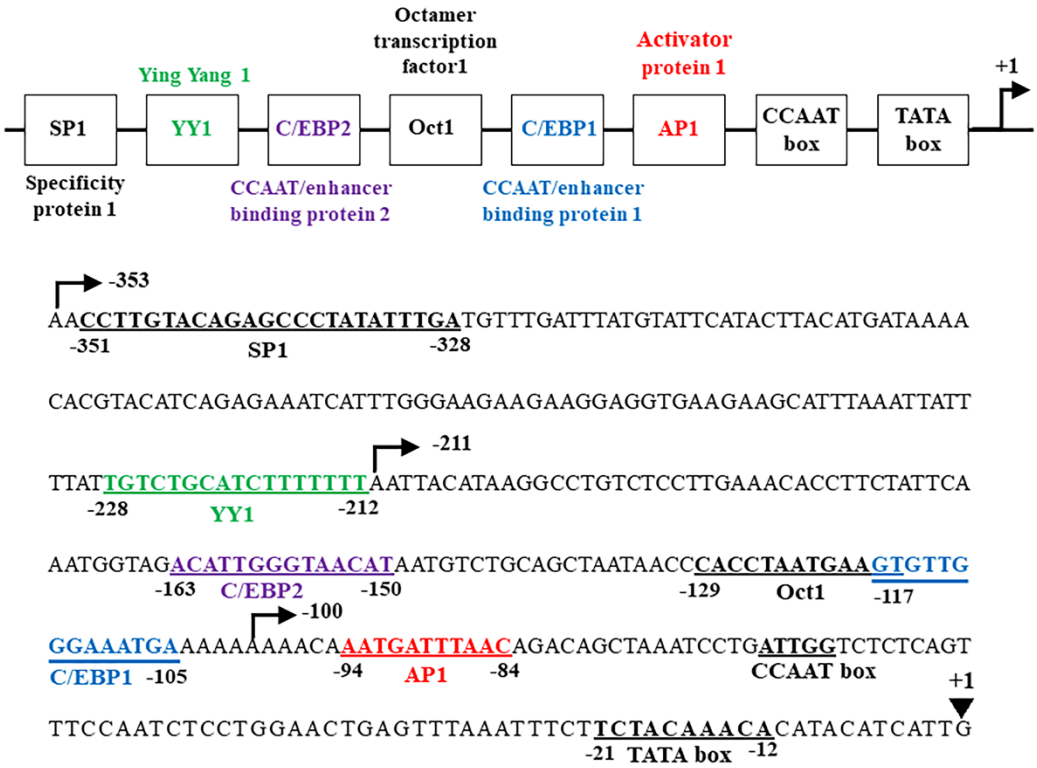

Fig. 5 Regulatory elements in the proximal promoter of the human AMTN gene. **Upper panel:** The schematic diagram of human AMTN gene proximal promoter. **Lower panel:** The nucleotide sequence of the human AMTN gene proximal promoter from -353 to transcription start point. An AP1, C/EBP1, C/EBP2 and YY1 are present.

Table 1 Study patients

Base line (20 patients with chronic periodontitis)	
Age	68.6 ± 11.5
Male	10 (50%)
Female	10 (50%)
PPD	6.3 ± 0.66
CAL	8.1 ± 1.68
BOP	11 (55%)
GI	1.6 ± 0.5
PlI	0.95 ± 0.76
Number of total bacteria (log copies)	4.43 ± 0.8
A.actinomycetemcomitans	0.18 ± 0.54
P. intermedia	1.81 ± 0.96
P. gingivalis	2.47 ± 1.35

Table 2A Clinical parameters at base line and after 1 month of coral mouthwash

Coral mouthwash group	Base line	After 1 month
PPD (mm)	6.3 ± 0.67	6.3 ± 0.95
CAL (mm)	8.3 ± 1.7	8.2 ± 2.1
BOP (%)	60	50
GI	1.61 ± 0.52	1.5 ± 0.53
PlI	0.9 ± 0.74	0.8 ± 0.92

Table 2B Clinical parameters at base line and after 1 month of placebo mouthwash

Placebo mouthwash group	Base line	After 1 month
PPD (mm)	6.3 ± 0.67	6.1 ± 1.45
CAL (mm)	7.9 ± 1.73	7.7 ± 2.31
BOP (%)	50	60
GI	1.6 ± 0.52	1.6 ± 0.52
PlI	1.0 ± 0.82	1.1 ± 0.74

Table 3A Bacterial test at base line and after 1 month of coral mouthwash

Coral mouthwash group	Base line	After 1 month
Total bacteria (log copies)	4.40 ± 0.84	4.29 ± 0.88
A. actinomycetemcomitans (log copies)	0.18 ± 0.57	0.16 ± 0.51
A.actinomycetemcomitans ratio (%)	0.02 ± 0.07	0.01 ± 0.04
P. intermedia (log copies)	2.11 ± 1.06	1.89 ± 0.89
P. intermedia ratio (%)	1.98 ± 2.77	1.22 ± 1.57
P. gingivalis (log copies)	2.71 ± 1.31	2.69 ± 1.39
P. gingivalis ratio (%)	7.14 ± 7.53	6.90 ± 7.47

Placebo mouthwash group	Base line	After 1 month
Total bacteria (log copies)	4.45 ± 0.80	4.23 ± 0.88
A. actinomycetemcomitans (log copies)	0.17 ± 0.54	0
A.actinomycetemcomitans ratio (%)	0.12 ± 0.36	0
P. intermedia (log copies)	1.51 ± 0.80	1.45 ± 0.91
P. intermedia ratio (%)	0.52 ± 0.76	0.88 ± 1.26
P. gingivalis (log copies)	2.58 ± 1.58	2.58 ± 1.55
P. gingivalis ratio (%)	6.25 ± 8.03	14.42 ± 21.85