Inflammatory Cytokines and cAMP Regulate Amelotin Gene Transcription

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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 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 (1ng/ml) and TNF-α (10 ng/ml) increased promoter activities of -211, -353, -501, -769 and -950AMTN LUC constructs in HGF. TNF-α (10ng/ml) induced LUC activities of these five AMTN constructs in Sa3 cells. FSK (1µM) increased AMTN mRNA levels at 12 and 24 h in Ca9-22 cells. FSK (1µM, 12h) 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.

Keywords: amelotin, cAMP, gene promoter, inflammatory cytokine, transcription

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 (1ng/ml) and TNF-α (10 ng/ml) increased promoter activities of -211, -353, -501, -769 and -950AMTN LUC constructs in HGF. TNF-α (10ng/ml) induced LUC activities of these five AMTN constructs in Sa3 cells. FSK (1µM) increased AMTN mRNA levels at 12 and 24 h in Ca9-22 cells. FSK (1µM, 12h) 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.

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similar characters of cells derived from JE, because they produce AMTN and FDC-SP (21, 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; Endogain®) 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.

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% CO2 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 including 10% FCS, then cultured for 12 h in α-MEM or DMEM without FCS, and stimulated with IL-1β (1ng/ml), TNF-α (10ng/ml) or forskolin (FSK; 1µM, Sigma-Aldrich, Tokyo Japan). Total RNA was purified from triplicate cultures at 0, 3, 6, 12 and 24 h following stimulation by FSK.

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'-GGTGAATGTACAACAGCAACTGCAC-3', AMTN Rev: 5'-TTCATCCTGGACATCTGGATTA-3', GAPDH For: 5'-GCACCGTCAAGGCTGAGAAC-3', GAPDH Rev: 5'-ATGTGGTGAGACGCAGCAG-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 (12.5µl), 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 2µg 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 (1mg/ml), TNF-α (10ng/ml) or FSK (1µM) for 12 h prior to harvest. The LUC activities were measured using a luminescence reader (AcuVuFlex Lumi 400; Aloka, Tokyo, Japan).

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

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 -950 AMTN) of the human AMTN gene (24, 25). Luciferase activities of -211, -353, -501, -769, and -950 AMTN 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 (API; 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).

**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 its expression is also found in internal basal lamina of JE. These characteristic distribution of AMTN suggest a role for cell adhesion and inflammatory blockade (9, 10). Previously, we 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 en-
abling 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 (28–30). In this study, FSK (1µM) significantly increased AMTN mRNA levels at 12 h and 24 h in Ca9-22 cells (Fig. 3). FSK (1µM) 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 (Figs. 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 (31). 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, 32) and initial periodontal therapy decreased

Fig. 2. TNF-α upregulates human AMTN gene promoter activities in Sa3 cells. The transcriptional activities of -211AMTN, -353 AMTN, -501 AMTN, -769 AMTN and -950 AMTN 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 (1µM) 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, -353 AMTN, -501 AMTN, -769 AMTN and -950 AMTN were increased by FSK (1µM, 12h) 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.
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 regulate various tissue functions and variety of cell differentiation (32). 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 (33). C/EBPα is expressed in T cells, and negatively regulates IFN-γ expression in T cells (34). YY1 plays a critical role in stimulating IL-6 expression in rheumatoid arthritis which contribute to the inflammation via promoting the differentiation of Th17 (35).

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 (1µM) at 12 and 24 h in Ca9-22 cells, and FSK (1µM) 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 requiring to analyze the role of cAMP on the expression and function of AMTN.

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Conflict of Interests

The authors have declared that no conflict of interest.

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