

Original

Proinflammatory cytokines induce amelotin transcription in human gingival fibroblasts

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Abstract: Amelotin (AMTN) is a secreted protein transcribed predominantly during the maturation stage of enamel formation and localized in the junctional epithelium. We investigated differences in the levels of AMTN gene expression between non-inflamed gingiva and inflamed gingiva from patients with chronic periodontitis. Total RNAs were isolated from these tissues and their gene expression profiles were monitored by DNA microarray. The observed induction of AMTN mRNA in inflamed gingiva and cultured human gingival fibroblasts (HGF) was confirmed by real-time PCR. Transient transfection assays were performed using chimeric constructs of mouse AMTN gene promoter fragments linked to a luciferase reporter gene. Immunohistochemical localization of AMTN in inflamed and non-inflamed gingiva was assessed by immunohistochemistry. Among many differentially expressed genes, the level of AMTN mRNA was significantly increased in inflamed gingiva. Treatment of HGF with interleukin-1 β (IL-1 β), IL-6 and tumor necrosis factor- α (TNF- α) induced the expression of AMTN mRNA, and increased the luciferase activities of the AMTN promoter constructs. AMTN protein was detected in inflamed gingival connective tissue and

junctional epithelium. These findings demonstrate that proinflammatory cytokines induce AMTN gene expression in human gingival fibroblasts and suggest a role for AMTN in gingival inflammation.

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Keywords: amelotin; gingival fibroblast; inflammation; cytokines.

Introduction

Chronic periodontitis is an infectious disease caused by periodontopathic bacteria, and both host and environmental factors are crucial for its onset and progression (1-3). It is well established that the immune response to bacterial products, such as lipopolysaccharide, fimbriae and proteolytic enzymes, and subsequent production of proinflammatory cytokines and other inflammatory mediators, play a particularly important role in periodontal tissue destruction (4,5).

Microarray-based methodologies have greatly facilitated the analysis of differential gene expression in large numbers of specimens because – unlike more traditional experimental methods – they allow simultaneously monitoring of the relative expression levels of a large number of genes (6,7). In the present study, inflamed gingiva from patients with chronic periodontitis and non-inflamed gingiva from edentulous residual ridges during dental implant surgery were examined by a gene microarray technique in order to identify differentially expressed genes that might play a role in gingival inflammation.

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Amelotin (AMTN) is a recently discovered secreted enamel protein with very limited sequence similarity to the major enamel matrix proteins (8). The AMTN protein is rich in proline, leucine, glutamine and threonine, and is localized in the basal lamina of maturation-stage ameloblasts of incisors and unerupted molars, and the internal basal lamina of the junctional epithelium (JE) in molars (9-11). This specific protein localization suggests that AMTN may be involved in dentogingival attachment, and thus the maintenance of periodontal integrity. To understand the molecular mechanisms involved in the cell-type-specific expression and transcription of AMTN in the inflamed gingiva, we analyzed the effects of proinflammatory cytokines on AMTN transcription in human gingival fibroblasts (HGF).

Materials and Methods

Reagents

Dulbecco's modified Eagle medium (DMEM) was purchased from Wako (Tokyo, Japan). Fetal calf serum (FCS), penicillin, streptomycin, and TrypLE Express were obtained from Invitrogen (Carlsbad, CA, USA). PGL3-promoter, pSV- β -galactosidase (β -Gal) control vector, and MultiFectam were purchased from Promega Co., (Madison, WI, USA). Interleukin-1 β (IL-1 β), IL-6, and tumor necrosis factor- α (TNF- α) were purchased from Genzyme, Techne (Minneapolis, MN, USA). Isogen II was purchased from Nippongene (Tokyo, Japan). The miRNeasy Mini Kit was purchased from Qiagen (Valencia, CA, USA). The EXScript RT reagent Kit and SYBR Premix Ex Taq were purchased from TaKaRa (Tokyo, Japan).

Cell culture

Primary HGF were established from patients' gingival connective tissue explants as described previously (12). The cells were cultured at 37°C in 5% CO₂/95% air in DMEM containing 10% FCS. They were grown to confluence in 100-mm tissue culture dishes, and the medium was then changed to DMEM containing 1% FCS for 12 h, followed by incubation in this medium with or without IL-1 β (1 ng/mL), IL-6 (1 ng/mL), and TNF- α (10 ng/mL) for 24 h as described previously (13-19). Total RNA was isolated from quadruplicate cultures and analyzed for the expression of AMTN and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA by real-time PCR as described below.

RNA preparations and DNA microarray

Gingival tissues (containing both epithelial and connective tissues), obtained during periodontal flap surgery (inflamed gingiva from four patients with chronic periodontitis; four females, mean \pm SD age, 53.8 \pm 8.5 years, no systemic disease) or dental implant surgery (non-inflamed gingiva from four edentulous residual ridges; one male and three females, 59.0 \pm 2.2 years old, no systemic disease) were used in this study. The Institutional Internal Review and Ethics Board at the Nihon University School of Dentistry at Matsudo approved the study (EC03-016, EC03-041, EC10-040). Written informed consent was obtained from each study subject after all procedures had been fully explained. Periodontal status was assessed on the basis of probing depth (PD), clinical attachment level (CAL) and bleeding on probing (BOP). PD and CAL were measured with a PCP11 probe (Hu-Friedy, Chicago, IL, USA). Patients were judged to have chronic periodontitis if they had at least two sites with PD \geq 6 mm and attachment loss of more than 6 mm. The average PD and CAL ($n = 4$) in these patients were 7.25 \pm 1.09 mm and 9.0 \pm 1.15 mm, respectively. BOP was detected in all four sites.

Total RNA was isolated using the miRNeasy Mini Kit. The RNA quality was assessed using an Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Gene expression profiling was performed for RNA samples using a SurePrint G3 Human GE microarray 8 \times 60k (Agilent Technologies). RNA preparation and hybridization were performed according to the Agilent user manual, and the data were imported into GeneSpring GX software (Agilent Technologies) for selection of induced and repressed genes.

Real-time PCR

Total RNA was used as a template for cDNA synthesis with an ExScript RT reagent kit (TaKaRa). Quantitative real-time PCR was performed using the following primer sets: Human AMTN forward, 5'-GTTGAATGTACAACAGCAACTGCAC-3'; Human AMTN reverse, 5'-TTCCATCCTGGACATCTGGATTAG-3'; Human GAPDH Forward, 5'-GCACCGTCAAGGCTGAGAC-3'; Human GAPDH Reverse, 5'-ATGGTGGTGAAGACGCCAGT-3'; using SYBR Premix Ex Taq in a TP800 thermal cycler dice real time system (TaKaRa). The amplification reactions were performed in 25 μ L of a final reaction mixture containing: 2 \times SYBR Advantage qPCR Premix or SYBR Premix EX Taq (12.5 μ L); 10 μ M forward and reverse primers (final concentration was 0.2 μ M); 25 ng (2.5 μ L) cDNA for AMTN and 10 ng (1.0 μ L) cDNA for GAPDH. To reduce variability

between replicates, PCR premixes which contained all reagents except for cDNA were prepared and aliquoted into 0.2 mL Hi-8-tubes (TaKaRa). The thermal cycling conditions were 10 s at 95°C and 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. The initial amount of RNA was quantified using a standard curve, and fold expressions of AMTN relative to GAPDH were determined in quadruplicate.

Transient transfection assays

Exponentially growing HGF was used for transfection assays. Twenty four hours after plating, cells at 50-70% confluence were transfected using MultiFectam reagent. The transfection mixture included 1 µg of the respective luciferase (LUC) construct (-878 AMTN, -878~+65 mouse AMTN gene promoter; -1651AMTN, -1651~+65; -2200AMTN, -2200~+65) and 2 µg β-Gal vector as an internal control. β-Gal activity was determined separately to normalize values. Two days post-transfection, cells were deprived of FCS for 12 h, then IL-1β (500 pg/mL), IL-6 (1 ng/mL) or TNF-α (10 ng/mL) was added for a further 12 h prior to harvesting. The luciferase activity measurements were performed according to the supplier's protocol (PicaGene, Toyo Ink, Tokyo, Japan) using a luminescence reader (Acuu FLEX Lumi 400; Aloka, Tokyo, Japan).

Primer extension

A 20-mer ³²P-end-labeled oligonucleotide, 5'-AGCAG-TAGAATCATGGTCTT-3', complementary to nucleotides immediately downstream of the ATG start codon, was hybridized overnight at 30°C to total RNA isolated from mouse mandibular incisor ameloblasts. Nascent cDNA was synthesized by avian myeloblastosis virus (AMV) reverse transcriptase and the ³²P-labeled cDNA generated was analyzed on an 8% polyacrylamide/8 M urea gel. A plasmid containing a corresponding genomic DNA fragment was used in a Sanger dideoxy sequencing reaction to determine the relative position of the primer extension product.

Immunohistochemistry

Human gingival tissues were obtained during periodontal flap surgery (inflamed gingiva) or dental implant surgery (non-inflamed gingiva from edentulous residual ridges). We used the same gingival tissues that were analyzed by hematoxylin/eosin (HE) and immunohistochemical staining. Appropriate sections for immunohistochemistry were selected based on the analysis of serial sections stained with HE. After they had been deparaffinized

and rehydrated, antigen retrieval was performed by immersing the tissues in hot (80°C) citrate buffer (10 mM citric acid, 0.05% Tween 20, pH 6.0) for 20 min. The tissues were washed with Tris-buffered saline (pH 7.4), blocked with peroxidase block (DAKO) for 5 min, incubated with the primary antibody (rabbit polyclonal anti-AMTN antibody; ab122312, Abcam, dilution 1:50) for 1 h, washed and incubated with a secondary antibody for 30 min (EnVision + System HRP Labelled Polymer Anti-Rabbit, K4003, DAKO). Following color development with diaminobenzidine, the sections were counterstained with methyl green (S1962, DAKO).

Statistical analysis

Quadruplicate samples were analyzed for each experiment, and experiments were replicated to ensure the consistency of the responses to IL-1β, IL-6 and TNF-α. The significance of differences between the control and treatments was determined using one-way analysis of variance (ANOVA).

Results

Analysis of differences in gene expression in non-inflamed and inflamed gingiva

Flag analysis of raw signal intensity using the DNA microarray operating system showed that a flag was present (P) for the level of AMTN mRNA in inflamed gingival samples (data not shown). A scatter plot of gene expression in non-inflamed gingiva (control) vs. inflamed gingiva is shown in Fig. 1A. Interestingly, higher levels of gene expression for AMTN, odontogenic ameloblast-associated/amyloid in Pindborg tumors (ODAM/APIN), IL-1β, IL-1α, and IL-6 were found in inflamed gingiva from patients with chronic periodontitis than was the case in samples of non-inflamed gingiva. The relative change in the expression of AMTN (raw data) demonstrated by the DNA microarray is shown in Fig. 1B. On the other hand, lower levels of expression of the asporin (ASPN) and aggrecan (ACAN) genes were found in inflamed gingiva than in non-inflamed gingiva (Fig. 1A).

Real-time PCR analyses

To confirm the induction of AMTN gene expression using the DNA microarray, real-time PCR analyses were performed on samples of inflamed and non-inflamed gingiva. The level of AMTN mRNA in inflamed gingiva was significantly higher than that in non-inflamed gingiva (Fig. 2A). Next, we cultured HGF from two patients to study the effects of selected pro-inflammatory cytokines on AMTN mRNA levels. Treatment with IL-1β (1 ng/mL), IL-6 (1 ng/mL), or TNF-α (10 ng/mL) led to a

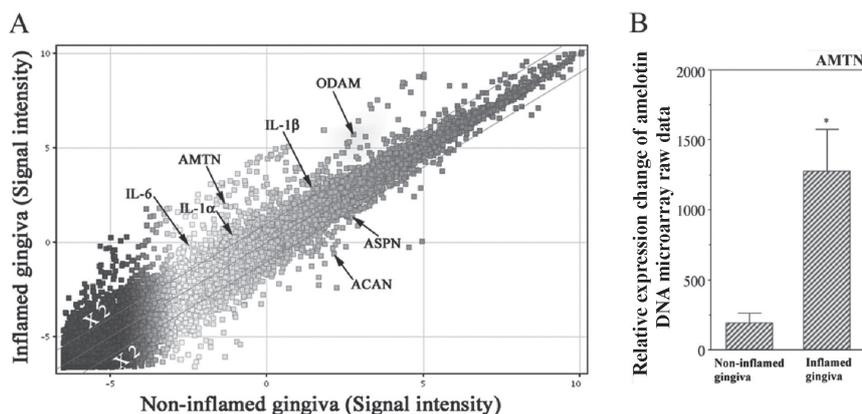


Fig. 1 A scatter plot of hybridization signals in non-inflamed and inflamed gingiva. **A:** Levels of expression of the AMTN, ODAM, IL-1 β , IL-1 α and IL-6 genes were higher in inflamed gingiva from patients with chronic periodontitis than in samples of non-inflamed gingiva. Levels of expression of the asporin (ASPN) and aggrecan (ACAN) genes were lower in inflamed gingiva. **B:** Relative change in expression of AMTN (raw data from DNA microarray) in inflamed gingiva from patients with chronic periodontitis compared with samples of non-inflamed gingiva. Quantitative analyses of the data sets are shown with standard errors. Significant differences from control. * $P < 0.05$.

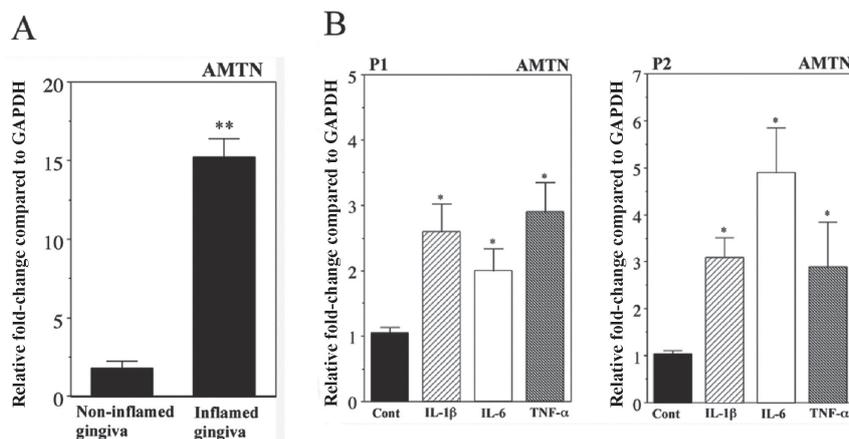


Fig. 2 Levels of AMTN mRNA in non-inflamed gingiva, inflamed gingiva and HGF. **A:** The expression of AMTN and GAPDH mRNA in four samples of non-inflamed and inflamed gingiva was measured by real-time PCR. Levels of AMTN mRNA in inflamed gingiva were significantly higher than in non-inflamed gingiva. **B:** The levels of expression of AMTN and GAPDH mRNA in HGF from two patients (patient 1; P1 and patient 2; P2) were measured by real-time PCR. IL-1 β (1 ng/mL), IL-6 (1 ng/mL), and TNF- α (10 ng/mL) increased the level of AMTN mRNA at 24 h. The experiments were performed in quadruplicate. Quantitative analyses of the data sets are shown with standard errors. Significant differences from control. * $P < 0.05$, ** $P < 0.01$.

significant increase in the level of AMTN mRNA in HGF after 24 h, relative to untreated controls (Fig. 2B). The threshold cycle (Ct) values of AMTN expression in HGF were about 25 cycles (with cytokines) and 29 cycles (without cytokine), being higher than the corresponding value for GAPDH (18 cycles). Therefore the level of AMTN gene expression in HGF was considered to be very low. The amplified product of the AMTN real time PCR reaction was visible as a single, sharp, 191-bp band when analyzed by electrophoresis on 2% agarose gel (data not shown).

Transient transfection analyses of mouse AMTN promoter constructs

To delineate the portions in the promoter region of the AMTN gene that mediate transcriptional regulation in response to IL-1 β , IL-6, and TNF- α , subsequent studies were performed utilizing chimeric constructs of various lengths prepared from the mouse AMTN gene promoter. Transient transfection of these constructs encompassing different regions of the mouse AMTN gene promoter ligated to a luciferase reporter gene (-878AMTN, -1651AMTN, -2200AMTN) were performed in HGF. The results of these luciferase assays indicated an

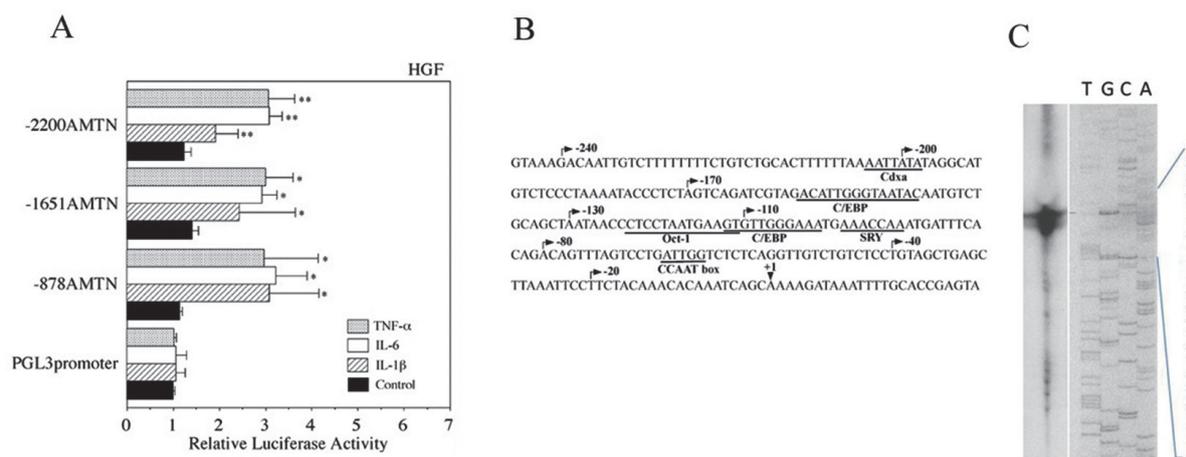


Fig. 3 A: IL-1 β , IL-6, and TNF- α up-regulate activity of the mouse AMTN promoter. The transcriptional activities of -878AMTN (-878 to +65), -1651AMTN (-1651 to +65), and -2200AMTN (-2200 to +65) were increased after 12 h of treatment with IL-1 β (500 pg/mL), IL-6 (1 ng/mL), and TNF- α (10 ng/mL) in HGF (P2). The transcriptional activities obtained from four separate transfections with constructs, the PGL3 promoter and -878AMTN, -1651AMTN and -2200AMTN, have been combined, and the values expressed with standard deviation. * $P < 0.05$; ** $P < 0.01$. B: Regulatory elements in the proximal promoter of the mouse AMTN gene. The nucleotide sequence of the mouse AMTN gene proximal promoter is shown from -245 to +24. An inverted CCAAT box, the sex-determining region Y (SRY) site, two CCAAT enhancer binding protein (C/EBP) motifs, the POU homeodomain protein Oct-1 site and the chicken homeodomain protein CdxA motif are present. C: Primer extension analysis to determine the AMTN transcription start site. A standard primer extension protocol, using the primer AGCAGTAGAATCATGGTCTT, was applied to total RNA isolated from mouse mandibular incisor ameloblasts. The position of the major labeled product is shown next to a sequencing reaction of a corresponding genomic DNA fragment.

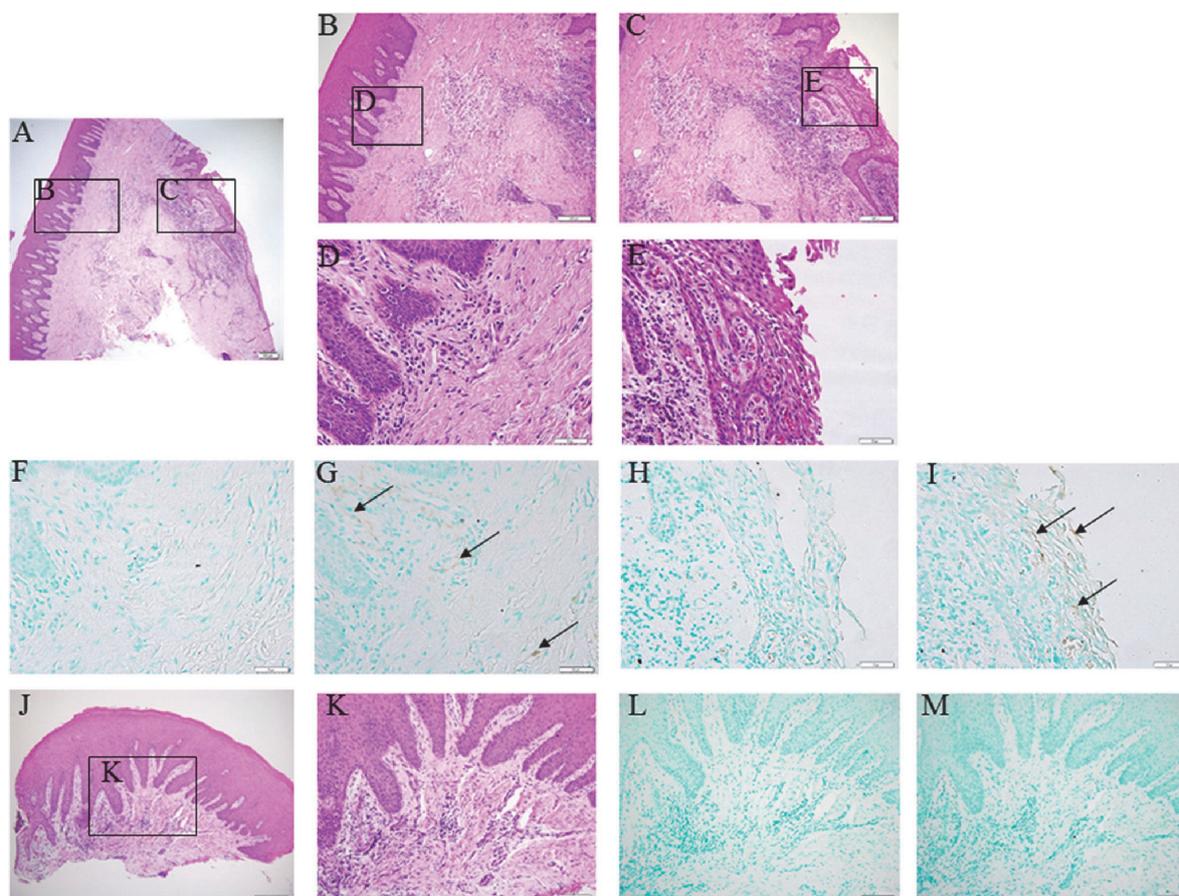


Fig. 4 HE and immunohistochemical staining of AMTN expression in inflamed (A; $\times 40$, B and C; $\times 100$, D-I; $\times 400$) and non-inflamed gingiva (J; $\times 100$, K-M; $\times 200$). HE staining for inflamed (A-E) and non-inflamed gingiva (J and K). Control staining without the first antibody for inflamed (F and H) and non-inflamed gingiva (L). Immunolocalization (brown staining) of AMTN in the inflamed gingival connective tissue (G; arrowheads), junctional epithelium (I; arrowheads) and non-inflamed gingiva (M) is shown.

increase in transcription after 12 h of treatment with IL-1 β , IL-6, and TNF- α using the three constructs (Fig. 3A). The sequence of -245 nucleotides comprising the 5'-flanking sequence of the mouse AMTN gene is shown in Fig. 3B to demonstrate the presence of predicted regulatory elements in the promoter region. An inverted CCAAT box (ATTGG) is located -66 to -62 nts from the putative transcription initiation site. In addition, a binding site for the Sex-determining region Y (SRY) transcription factor (AAACCAA) is located at nts -99 to -93, a CCAAT enhancer binding protein (C/EBP) motif (GTGTTGGGAAA) is found at -112 to -102, and a second C/EBP motif (ACATTGGGTAATA) is present at nts -157 to -144. A predicted binding site for the POU homeodomain protein Oct-1 (CTCCTAATGAAGT) is found at -123 to -111, and chicken homeodomain protein Cdx motif (AATTATA) is present at -204 to -198. Further study will be necessary to find functional response elements for inflammatory cytokines in the AMTN gene promoter. Figure 3C shows the results of primer extension analysis to determine the AMTN transcription start site. The position of the major labeled product, run in parallel with a sequencing reaction of a corresponding genomic DNA fragment, indicates the transcription start site at position -91 to -93 relative to the translation start codon ATG.

Immunohistochemistry

Results of HE staining for inflamed (Fig. 4A-E, B and C; boxed area in Fig. 4A, D and E; boxed area in Fig. 4B and C) and non-inflamed gingiva (Fig. 4J and K; boxed area in Fig. 4J) are shown. When a primary anti-AMTN antibody was used, staining for AMTN was observed in the inflamed gingival connective tissue (Fig. 4G; arrowheads) and junctional epithelium (Fig. 4I; arrowheads). However, no staining for AMTN was observed in non-inflamed gingiva from edentulous residual ridges (Fig. 4M), and without the first antibody (Fig. 4F, H and L; Control).

Discussion

In this study, we demonstrated that AMTN and ODAM/APIN, which are normally found in maturation-stage ameloblasts and the JE (8-10), are expressed at increased levels in inflamed gingiva from patients with chronic periodontitis, together with inflammatory cytokines such as IL-1 β , IL-1 α , and IL-6. It is of particular interest that AMTN and ODAM were upregulated to a similar extent; a recent study (20) showed that AMTN and ODAM are re-expressed during gingival regeneration, and both proteins have been shown to interact (21). Therefore it is

conceivable that the regulation of AMTN and ODAM in gingival cells is not independent. In contrast, decreased levels of ASPN and ACAN gene expression were found in inflamed gingiva relative to non-inflamed gingiva (Fig. 1A). The JE adheres to the tooth surface, and seals off periodontal tissues from the oral environment. It is formed initially by fusion of the reduced enamel epithelium with the oral epithelium (20), and is connected to the enamel via an inner basal lamina and hemidesmosomes (22). Two proteins, AMTN and ODAM/APIN, have been identified in the JE (9-11). Immunolabeling has shown that both AMTN and ODAM/APIN are localized to the basal lamina of ameloblasts. At the beginning of maturation, there ODAM/APIN is concentrated on the cell side of the basal lamina, while AMTN appears more concentrated on the enamel side (23). Our specimens of inflamed gingiva obtained by periodontal flap surgery contained the JE, whereas our specimens of non-inflamed gingiva from edentulous residual ridges did not. This may explain the higher expression of AMTN in inflamed gingiva. Gingival fibroblasts might support the growth and patterns of JE cell differentiation (24), and we showed here that proinflammatory cytokines (IL-1 β , IL-6, and TNF- α) induced expression of the AMTN gene in HGF. Therefore, the induction of AMTN gene expression could be part of the host defense response to inflammation in gingival fibroblasts. The fact that AMTN staining was observed in the inflamed gingival connective tissue and junctional epithelium (Fig. 4G and I) supports this possibility, and confirms the microarray data. It remains to be demonstrated whether AMTN is transcribed and secreted by gingival connective tissue cells or by cells of the gingival epithelium. In any event, the transcription level of AMTN has been found to be too low for detection by *in situ* hybridization (8,25), and therefore this issue remains beyond the scope of the present study.

ASPN belongs to the class I small-rich proteoglycan family and ACAN is a predominant component of cartilage proteoglycan (26,27). ACAN is composed of two types of glycosaminoglycan side chains, chondroitin sulfate and keratan sulfate. Keratan sulfate may become a therapeutic agent for inflammatory diseases such as rheumatoid arthritis (27).

Beyond demonstrating a potential role for AMTN in the response of gingival tissue to inflammation, our study is the first characterization of the mouse AMTN gene promoter in the context of gingival inflammation. Luciferase assays (Fig. 3A) showed that regulatory elements mediating the induction of AMTN expression by inflammatory cytokines are located within -878

base pairs or less of the AMTN gene transcription start site. An examination of the 5'-flanking sequences of the mouse proximal AMTN gene promoter revealed a number of sequences resembling consensus eukaryotic *cis*-acting elements (Fig. 3B). While we were unable to find an inverted TATA box (TTTATA) sequence, which is generally present in the gene promoter of small integrin-binding ligand N-linked glycoprotein (SIBLING) family members together with the inverted CCAAT box (28-30), an A/T-rich sequence similar to that of a TATA box (TTAAATT) is present at nucleotides -29 to -23.

To test the promoter activity, we used HGF for transient transfection. Luciferase activity of the shortest luciferase construct (-878 to +65) was almost the same (1.14-fold) as that of the empty plasmid (PGL3 promoter). However, after stimulation with IL-1 β , IL-6, and TNF- α for 12 h, the luciferase activities of the -878AMTN construct were increased 3.09-, 3.23-, and 2.97-fold, respectively. While all our experiments indicate an inductive effect of the pro-inflammatory cytokines IL-1 β , IL-6, and TNF- α on AMTN expression, quantitative differences were evident among the results obtained by real-time PCR and luciferase assays. The higher induction of AMTN expression particularly by IL-1 β and TNF- α in Patient 1, and by IL-6 in Patient 2 (Fig. 2B), compared to the results in Fig. 3A, indicated that several responsive regulatory elements may exist upstream of the longest promoter fragment that was investigated by luciferase assays. It may also indicate quantitative differences in regulation between the human and mouse AMTN promoter sequences, although qualitatively, both sequences produced a clearly inductive response. The individual contributions of these potential regulatory elements to the increase of AMTN gene transcription mediated by IL-1 β , IL-6, and TNF- α remain to be analyzed in detail. Nevertheless, the regulation of AMTN expression at the mRNA level and activation of the AMTN promoter by proinflammatory cytokines provide substantial new insight into the molecular mechanisms of gingival inflammation.

In summary, we have demonstrated that inflamed gingiva from patients with chronic periodontitis expresses AMTN, and that proinflammatory cytokines (IL-1 β , IL-6, and TNF- α) induce expression of the AMTN gene in HGF. Upstream elements, including a conserved NF- κ B and other inflammation-related elements, may be involved in expression of the AMTN gene in gingival fibroblasts.

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