Effects of Inflammatory Cytokines on Follicular Dendritic Cell Secreted Protein Gene Transcription

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Abstract

Follicular dendritic cell secreted protein (FDC-SP) is similar in structure to statherin in saliva expressed by follicular dendritic cells, junctional epithelium and periodontal ligament. To determine the effects of inflammatory cytokines namely interleukin- 1β (IL- 1β) and tumor necrosis factor- α (TNF- α) on human FDC-SP gene transcription, we performed real-time PCR, transient transfection assays using luciferase (LUC) constructs containing varying length of human FDC-SP gene promoters and chromatin immunoprecipitation (ChIP) analyses in gingival epithelial Ca9-22 and Sa3 cells, adenocarcinoma HYS cells and immortalized human periodontal ligament (HPL-hTERT) cells. IL- 1β (1 ng/ml) increased FDC-SP mRNA expressions in Ca9-22 cells at 3 h, reached maximum at 6 and 12 h, at 24 h in Sa3 cells and at 6, 12 and 24 h in HSY cells. In transient transfection assays, IL- 1β (1 ng/ml) increased the LUC activities of -116, -210, -345 and -948 FDCSP constructs at 12 h including the promoter region of human FDC-SP gene in Ca9-22 cells. TNF- α (10 ng/ml) upregulated the transcription of LUC constructs between -116 and -948 FDCSP at 12 h in Sa3 and HSY cells. Results of ChIP analyses revealed that C/EBP α transcription factor bound to the C/EBP2 and C/EBP3 elements which were increased by IL- 1β in HPL-hTERT cells. These data indicate that IL- 1β and TNF- α stimulate human FDC-SP gene transcription.

Keywords :

follicular dendritic cell secreted protein, IL- 1β , inflammatory cytokine, junctional epithelium, TNF- α

Introduction

Three kinds of risk factors such as host, environmental and bacteria factors may induce periodontitis, and clinical conditions of the disease present swelling, redness of gum, tooth mobility and loss (1, 2). Immune response to the risk factors especially bacterial factor could induce inflammatory cytokines, prostanoide and matrix metalloproteinases. Interleukin- 1β (IL- 1β) and tumor necrosis factor- α (TNF- α) are typical inflammatory cytokines that cause inflammation in periodontal tissues and alveolar bone resorption (3, 4).

Follicular dendritic cell secreted protein (FDC-SP) is a low molecular weight secreted protein produced by follicular

dendritic cells in tonsils. FDC-SP gene exists on chromosome 4q13 and is located in a casein locus adjacent to odontogenic ameloblast-associated protein (ODAM) and casein kappa (CSN3). It is posttranslationally modified and can bind to the surface of B lymphoma, but not T lymphoma cells (5, 6). FDC-SP is also produced by the parotid gland, junctional epithelium (JE) and periodontal ligament. It has similar functions to saliva derived proteins, histatin and statherin, suggesting it might have an antimicrobial activity (7–10). Periodontal ligament cells transfected with FDC-SP could inhibit osteogenic differentiation, maintain character of fibroblast, and enhance osteoclastogenesis (11–14).

JE is a unique structure which contacts with both a tooth enamel and a basement membrane of the gingival connective tissue, and surrounds the tooth like a collar. Tooth enamel and internal basal lamina of JE are com-

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bined by hemidesmosome to each other(15). Body's defense against periodontal pathogen is crucial for the protection of periodontium(10, 16, 17). As an immune reaction to a bacterial infection, JE secretes several chemokines and cytokines. FDC-SP expression in the JE is tentative disappeared by gingival inflammation(10, 18). FDC-SP gene expression is upregulated in nifedipine-induced gingival overgrowth(19). These results suggest how to manage the capability of FDC-SP in the JE might provide fresh insight into defensive mechanisms in the periodontal tissues.

To determine how inflammatory cytokines control human FDC-SP gene transcription, we investigated the effect of IL-1 β and TNF- α on FDC-SP gene expression in gingival epithelium cells, parotid salivary ductal cells and periodontal ligament cells.

Materials and Methods

Cell culture

Human gingival carcinoma epithelial-like Ca9-22 cells, human parotid salivary ductal cell line HSY cells(20) and immortalized human periodontal ligament(HPL-hTERT) cells(21-23) or human gingival squamous cell carcinoma Sa3 cells(20) were cultured in α -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 without FCS, and stimulated with IL-1 β (1 ng/ml) or TNF- α (10 ng/ml) (Wako, Tokyo Japan). Total RNA was purified from triplicate cultures at 0, 3, 6, 12 and 24 h following stimulation by IL-1 β .

Real-time PCR

Total RNAs were isolated using Isogen II(Wako, Tokyo, Japan) from Ca9-22, HSY and Sa3 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 FDC-SP For; 5'-GCCAGTCACTTGCCATTTCT-3', FDC-SP Rev; 5'-GGGCAGATTTCAGGTATTGGA-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 μ l 2x SYBR Premix Ex Taq II(12.5 μ l), 10 μ M forward and reverse primers and 50 ng cDNA for FDC-SP 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 FDC-SP relative to GAPDH was determined in triplicate.

Luciferase(LUC) assays

To elucidate the effect of TNF- α on human FDC-SP gene transcription, we prepared chimeric constructs by ligating various length of human FDC-SP gene promoters into LUC reporter plasmid(-116FDCSP; -116~+60, -210FDCSP; -210~+60, -345FDCSP; -345~+60, -501FDCSP; -501~+60, -717FDCSP; -717~+60, -948FDCSP; -948~+60) (19, 22). Exponentially growing Ca9-22, Sa3 and HSY 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 μ g of a LUC plasmid and 2 μ g 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-1 β (1 ng/ml) or TNF- α (10 ng/ml) for 12 h prior to harvest. The LUC activities were measured using a luminescence reader(AcuuFlex Lumi 400; Aloka, Tokyo, Japan).

Chromatin immunoprecipitation(ChIP) assays

To identify interaction between C/EBP α and human FDC-SP gene promoter *in vivo*, ChIP assays were carried out using HPL-hTERT cells. Confluent HPL-hTERT cells in 100 mm culture dishes were stimulated by IL-1 β (1 ng/ml) for 0, 3, 6, 12 and 24 h, and then the cells were fixed with 100 μ l formaldehyde for 10 min to crosslink the protein-DNA complexes according to the previously(20). For the immunoprecipitation of protein-DNA complexes, 2 μ g of rabbit polyclonal anti-C/EBP α (SAB4500111, Sigma-Aldrich) and the appropriate normal rabbit IgG(sc-

Fig. 1. Effects of IL-1 β on FDC-SP mRNA levels in Ca9-22 cells. (A) Dose-response effects of IL-1 β on FDC-SP mRNA levels in Ca9-22 cells treated for 12 h. (B) Ca9-22 cells were treated with or without IL-1 β (1 ng/ml) for 3, 6, 12, and 24 h. FDC-SP 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; * $P < 0.05$ and ** $P < 0.01$.

2027, Santa Cruz Biotechnology) were used. The purified DNA was subjected to PCR amplification (1 cycle, 95°C for 3 min; amplification was performed for 35 cycles, denature at 95°C for 15 s, anneal at 59 or 60°C for 15 s, and extend at 72°C for 1 min; final extension was at 72°C for 1 min) mainly for the CCAAT-enhancer-binding protein 2 (C/EBP2) and C/EBP3 sites within the human FDC-SP gene promoter using primer sets. In addition, protein kinase A inhibitor KT5720 (100 nM), tyrosine kinase inhibitor herbimycin A (HA; 1 μ M), mitogen-activated protein kinase (MEK1/2) inhibitor U0126 (5 μ M) and phosphatidylinositol 3-kinase (PI3-K) inhibitor LY249002 (10 μ M) were used for protein kinases inhibition and then stimulated with IL-1 β (1 ng/ml) for 12 h to extract protein-DNA complexes. KAPA Taq Extra HotStart was utilized for the PCR procedure and the PCR products were separated on 2 % agarose gels and visualized with ultraviolet light. Human FDC-SP promoter using C/EBP2 ChIP For; 5'-CCAGTAAATGCTTAGAGGT-3', C/EBP2 ChIP Rev; 5'-CTCCAAATTTTGTGTCTTGT-3', C/EBP3 ChIP For; 5'-CTTAAGATTCCAGCACTATC-3', C/EBP3 ChIP Rev; 5'-CTCACAATTTTTCCTTTAC-3' primers.

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

To study the effect of IL-1 β on FDC-SP gene expression, we performed qPCR. Total RNA extracted from Ca9-22 and Sa3 gingival epithelial cells, and adenocarcinoma-derived HSY parotid gland epithelial cells. Firstly, effects of different concentrations of IL-1 β on the FDC-SP mRNA levels for 12 h in Ca9-22 cells were investigated. IL-1 β upregulated FDC-SP mRNA levels significantly at 1 and 10 ng/ml (Fig. 1A). IL-1 β (1 ng/ml) induced FDC-SP mRNA expressions at 3 h, achieved peaks at 6 and 12 h in Ca9-22 cells (Fig. 1B). IL-1 β (1 ng/ml) increased FDC-SP mRNA expressions at 3 h, and reached maximally at 24 h in Sa3 cells (Fig. 2A). Treatment of HSY cells with IL-1 β (1 ng/ml) up-regulated FDC-SP mRNA expressions at 3 h, reached peaks at 6, 12 and 24 h (Fig. 2B).

Since the impacts of IL-1 β or TNF- α likely involved in

Fig. 2. Effects of IL-1 β on FDC-SP mRNA levels in Sa3 and HSY cells. Sa3(A) and HSY(B) cells were treated with or without IL-1 β (1 ng/ml) for 3, 6, 12, and 24 h. FDC-SP 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 SD. Significant differences between the control and IL-1 β treatment groups were determined using the one-way ANOVA; *P<0.05 and **P<0.01.

Fig. 3. IL-1 β upregulates human FDC-SP gene promoter activities. The transcriptional activities of -116FDCSP(-116~+60), -210FDCSP(-210~+60), -345FDCSP(-345~+60) and -948FDCSP(-948~+60) were increased by IL-1 β (1 ng/ml, 12 h) in Ca9-22 cells. Results of transcriptional activities obtained from three separate transfections with constructs, pGL3basic and -116FDCSP to -948FDCSP were combined and values expressed with SD. Significant differences between the control and IL-1 β treatment in each construct were determined using the one-way ANOVA; *P<0.05 and **P<0.01.

the interaction between transcription factors and response elements in the gene promoter of human FDC-SP, following LUC assays were performed. Transient transfection of LUC constructs containing different promoter regions of the human FDC-SP gene(-116, -210, -345, -501, -717 and -948FDCSP) was performed in Ca9-22,

Sa3 and HSY cells. IL-1 β (1 ng/ml) induced LUC activities of -116, -210, -345 and -948FDCSP LUC constructs in Ca9-22 cells (Fig. 3). TNF- α (10 ng/ml) increased LUC activities of the six constructs in Sa3 and HSY cells (Figs. 4 and 5). Human FDC-SP gene promoter has several transcription factor binding sites such as Yin Yang 1

Fig. 4. TNF- α upregulates human FDC-SP gene promoter activities. The transcriptional activities of -116FDCSP, -210FDCSP, -345FDCSP, -501FDCSP, -717FDCSP and -948FDCSP 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 -116FDCSP to -948FDCSP were combined and values expressed with SD. **P<0.01

Fig. 5. TNF- α upregulates human FDC-SP gene promoter activities. The transcriptional activities of -116FDCSP, -210FDCSP, -345FDCSP, -501FDCSP, -717FDCSP and -948FDCSP were increased by TNF- α (10 ng/ml, 12 h) in HSY cells. Results of transcriptional activities obtained from three separate transfections with constructs, pGL3basic and -116FDCSP to -948FDCSP were combined and values expressed with SD. **P<0.01

(YY1; nts -69 to -53), GATA (nts -122 to -106) and three kinds of C/EBP elements (C/EBP1; nts -143 to -133), C/EBP2 (nts -194 to -181), and C/EBP3 (nts -282 to -269) (20, 23).

To clarify whether C/EBP α transcription factor is able to interact with C/EBP2 and C/EBP3 in the gene promoter of human FDC-SP *in vivo* after IL-1 β stimulation. ChIP analyses were conducted by HPL-hTERT cells.

IL-1 β (1 ng/ml) induced C/EBP α binding to the C/EBP2 and C/EBP3 elements in a time dependent profile (Fig. 6A). Next, we used several kinase inhibitors to elucidate which kinases can control C/EBP α bindings to C/EBP2 and C/EBP3 after IL-1 β stimulation. When HPL-hTERT cells were stimulated by IL-1 β for 12 h, C/EBP α binding to C/EBP2 and C/EBP3 were almost completely inhibited by protein kinase A inhibitor KT5720, tyrosine kinase

Fig. 6. ChIP analyses of C/EBP α binding to C/EBP2 and C/EBP3 in the human FDC-SP gene promoter in HPL-hTERT cells. (A) PCR bands amplified and corresponding to DNA-protein complexes immunoprecipitated with anti-C/EBP α antibody showed that C/EBP α interacted with a chromatin fragment containing the C/EBP2 and C/EBP3, which were increased in HPL-hTERT cells following stimulation with IL-1 β (1 ng/ml). Input DNA was also used as a control in PCR analysis. (B) ChIP analyses of C/EBP α binding to C/EBP2 and C/EBP3 under the treatment by IL-1 β (1 ng/ml, 12 h) with kinase inhibitors. Treatments of KT5720, HA, U0126 and LY294002 almost completely abolished the induction of C/EBP α bindings to C/EBP2 and C/EBP3 by IL-1 β .

inhibitor HA, MEK1/2 inhibitor U0126 and PI3K inhibitor LY2940024 (Fig. 6B).

Discussion

Periodontitis is an inflammatory disorder induced by periodontal pathogen in the gingival sulcus, and periodontitis might elicit the destruction of periodontal tissue, alveolar bone resorption and tooth loss (4, 24). IL-1 β and TNF- α are inflammatory cytokines need for activate innate immune reaction, mediating the recruitment, activation and adherence of circulating neutrophils and macrophages. IL-1 β and TNF- α -induced changes in gene transcription are mediated by similar transcription factors, whereas TNF- α and IL-1 receptor knockout mice show different sensitivities to an initiator of the innate immune reaction (25). IL-1 β concentration in gingival crevicular fluid (GCF) was increased as a characteristic of periodontitis (26). Initial periodontal therapy decreased IL-1 β levels in GCF from periodontitis patients (27). Gene cluster polymorphisms of IL-1 is connected to periodontitis in type 2 diabetes (28). A positive correlation was detected between severity of periodontitis and the

concentrations of TNF- α in the GCF (29). Adipose tissues express high concentrations of TNF- α (30) and serum levels of TNF- α are increased in type 2 diabetes, obese patients (31, 32). Antimicrobial periodontal therapy decreased serum levels of TNF- α , C-reactive protein and glycated hemoglobin levels in chronic periodontitis patients (33, 34). These data suggest that IL-1 β and TNF- α could be deeply involved in the onset and progression of periodontitis.

In the present study, we showed that IL-1 β upregulated FDC-SP mRNA levels in Ca9-22 (Fig. 1A, B) and Sa3 (Fig. 2A) human gingival epithelial cells, and HSY (Fig. 2B) human parotid salivary ductal cells. In the LUC assay, IL-1 β increased transcription of LUC constructs containing various length of human FDC-SP gene promoter in Ca9-22 cells (Fig. 3). Previously, we have shown that TNF- α induced human FDC-SP mRNA expressions in Ca9-22, Sa3 and HSY cells (20). However, we could not report the TNF- α effects on LUC activities of the FDC-SP gene promoter constructs in Sa3 and HSY cells. In this study, TNF- α induced LUC activities of the all constructs in Sa3 and HSY cells (Figs. 4 and 5), whereas

IL-1 β could not induce LUC activities of -501 and -717FDCSP LUC constructs in Ca9-22 cells (Fig. 3). There may be a suppressor element for IL-1 β between -346 and -717 in the FDC-SP gene promoter. Additional study could be necessary to clarify these discrepancies.

FDC-SP expresses not only in the JE and periodontal ligament, but also in the tonsil, prostate gland and trachea. The latter three organs are involved in the innate immunity. Therefore, FDC-SP might have function with the host defense against bacteria and infection in the JE (9, 19, 35). FDC-SP could not be detected in the JE 3 h after topical application of *E. coli* lipopolysaccharide to the gingival sulcus, and then it was detected again after 3 days (10). FDC-SP expression was disappeared concurrently with the increase of interleukin-17 at 5 and 7 days after initiation of experimental periodontitis, and FDC-SP expression recovered to the normal levels at 2 and 4 weeks (18). In our previous studies, using LUC assays, we identified several IL-1 β and TNF- α response elements in the proximal region of the human FDC-SP gene promoter (-345 to +60), which encompasses YY1, GATA, C/EBP2 and C/EBP3 elements (20, 23). YY1 plays an important function in stimulating IL-6 expression in rheumatoid arthritis which contribute to the inflammation via promoting the differentiation of Th17 (36). GATA (GATA-1~6) transcription factors cognize (A/T)GATA (A/G), and hematopoietic cells expressed GATA-1/2/3 isoforms. Endoderm-derived tissues and cardiovascular system expressed GATA-4/5/6 (37). GATA-6 and co-factor friend of GATA (FOG) induced hepcidin gene transcription in hepatocytes during inflammation (38). C/EBPs are member of leucine zipper transcription factor family regulate a variety of cell differentiation and various aspects of tissue functions (39). C/EBP β binds to IL-6 response elements in the TNF- α , IL-8 and G-CSF gene promoters and mediates IL-6 signaling, and nuclear localization of C/EBP β is regulated by inflammatory stress (40). C/EBP α is expressed high levels in T follicular helper cells, and restricts IFN- γ expression in T cells to allow B cells class switching (41). Previously, we have reported C/EBP β play crucial roles in human FDC-SP gene transcription after stimulation by IL-1 β in HPL-hTERT cells (23). However, we did not analyze the function of C/EBP α in FDC-SP gene transcription. IL-1 β increased human FDC-SP gene transcription mediated through C/EBP α targeting C/EBP2 and C/EBP3 in the human

FDC-SP gene promoter (Fig. 6).

In conclusion, we demonstrate that IL-1 β upregulated human FDC-SP mRNA expressions in Ca9-22, Sa3 and HSY cells. IL-1 β induced LUC activities of FDC-SP promoter constructs in Ca9-22 and TNF- α upregulated LUC activities of FDC-SP promoter constructs in Sa3 and HSY cells. IL-1 β induced C/EBP α binding to the C/EBP2 and C/EBP3 elements via PKA, tyrosine kinase, MEK1/2 and PI3 kinase pathways. Further research is requiring to analyze the differential effects of IL-1 β and TNF- α on the expression and function of FDC-SP.

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Conflict of Interests

The authors have declared that no conflict of interest.

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