Effects of inflammatory cytokines on follicular dendritic cell secreted protein gene expression and comparison of the amount of aspartate aminotransferase and clinical parameters pre- and post-periodontal regeneration therapy

（炎症性サイトカインの濾胞性樹状細胞分泌タンパク質の遺伝子発現に対する効果と歯周組織再生治療前後における臨床パラメーターとアスパラギン酸アミノトランスフェラーゼ量の比較検討）

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Preface

This article is based on a main reference paper, “Effects of Inflammatory Cytokines on Follicular Dendritic Cell Secreted Protein Gene Transcription’’ in the International Journal of Oral-Medical Sciences, and a reference paper, “Comparison of amount of aspartate aminotransferase and clinical parameters pre- and post-initial therapy and periodontal surgery’’ in the Journal of Japanese Society of Periodontology.

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 HSY 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 -948FDCSP 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 -948FDCSP at 12 h in Sa3 and HSY cells. Results of ChIP analyses revealed that C/EBPα 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.

Aspartate aminotransferase (AST) is an enzyme used clinically as an indicator of cell injury in pathological conditions in which cytoplasmic enzymes are liberated into the extracellular fluid. Elevated gingival crevicular fluid (GCF) levels of AST provide useful indicator of disease activity of periodontitis. The objective of the second study is to measure the AST levels in GCF from periodontitis patients before and after periodontal regeneration therapy by Emdogain®gel using Periodontal Tissue Monitor (PTM) kit and investigate the relationship between AST levels (PTM values) and probing pocket depth (PPD), clinical attachment level (CAL) and bleeding on probing (BOP). After periodontal regeneration therapy, PTM values were improved at 42 out of 56 treatment sites. In the 42 sites, 42 PPD, 38 CAL, and 41 BOP were either improved or no change. When we explored the relationship between PTM values and mean PPD and CAL after periodontal

regeneration therapy. In the improved 42 sites, mean PPD and CAL was decreased significantly. However, in the 14 no change and deteriorated sites, only the mean PPD was decreased significantly. The results suggest that sites with improved PTM values after periodontal regeneration therapy might have not only shallow PPD but also CAL gain. These studies suggest that the application of AST levels in GCF for clinical evaluation is useful as a prognostic indicator of periodontal regeneration therapy.

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, prostanoid 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 post translationally 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 combined 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 tentatively 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-1β and TNF-α on FDC-SP gene

expression in gingival epithelium cells, parotid salivary ductal cells and periodontal ligament cells.

Several diagnostic methods for chronic periodontitis (CP) have recently established and utilized for the causal factors for CP. It is generally accepted that periodontitis is a multifactorial disease principally caused by periodontopathic bacteria, environment and host factors, and has both active and quiescence phases (20-24). The assessments of destructed periodontal tissues due to periodontitis are important for an accurate diagnosis of periodontitis using clinical periodontal parameters, such as probing pocket depth (PPD), clinical attachment level (CAL) and bleeding on probing (BOP). They are usually utilized in making a diagnosis of periodontal disease. Aspartate aminotransferase (AST) levels in gingival crevicular fluid (GCF) reflect cellular damage arising from active periodontal disease (25), and they used for a clinical predictor of periodontitis and prognosis after periodontal therapy (25-30). Positive correlation between AST levels in GCF, and PPD and CAL at untreated sites in patients using PocketWatch™ (Pacific Pharmaceuticals Inc; San Diego, CA) was reported by cross-sectional study (31). AST levels were supportively utilized in periodontal initiation therapy (32), including scaling and root planing (SRP), and periodontal regeneration therapies (33-36). Therefore, the clinical usefulness of AST levels was widely accepted in periodontal therapies. In contrast, it has demonstrated that

elevated AST levels did not subsequently exhibit the progression of periodontal disease after SRP and prophylaxis over a 12-month period (37).

Periodontal Tissue Monitor (PTM) kit (Shofu; Kyoto, Japan) is semi quantitative measuring kit to measure the AST activities (PTM values). There are few studies have reported correlation between change in the amount of PTM values and periodontal parameters. Moreover, the clinical usefulness of AST levels as a predictor of periodontal regeneration therapy has not yet been evaluated. Therefore, the aim of this study was to evaluate the correlation between PTM values and periodontal parameters at pre- and post-periodontal regeneration therapy by enamel matrix derivative (EMD; Emdogain® gel, Straumann Japan; Japan), and demonstrate the clinical usefulness of the PTM values as a pre-operative predictor of prognosis after regeneration therapies.

Materials and Methods

Cell culture

Human gingival carcinoma epithelial-like Ca9-22 cells, human parotid salivary ductal cell line HSY cells (38) and immortalized human periodontal ligament (HPL-hTERT) cells (39-41) or human gingival squamous cell carcinoma Sa3 cells (38) 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) 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-1β.

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'-GGGCAGATTCAGGTATTGGA-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 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, -948 FDCSP; -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 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) 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/EBPα 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-1β (1 ng/ml) for 0, 3, 6, 12 and 24 h, and then the cells were fixed with 100 μl formaldehyde for 10 min to crosslink the protein-DNA complexes according to the previously (38). For the immunoprecipitation of protein-DNA complexes, 2 μg of rabbit polyclonal anti-C/EBPα (SAB4500111, Sigma-Aldrich) and the appropriate normal rabbit IgG (sc-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 μM), mitogen-activated protein kinase kinase (MEK1/2) inhibitor U0126 (5 μM) and phosphatidylinositol 3-kinase (PI3-

K) inhibitor LY249002 (10 μM) were used for protein kinases inhibition and then stimulated with IL-1β (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'-CCAGTAAAATGCTTAGAGGT-3', C/EBP2 ChIP Rev; 5'-CTCCAAATTTTGTGTCTTGT-3', C/EBP3 ChIP For; 5'-CTTAAGATTCCAGCACTATC-3', C/EBP3 ChIP Rev; 5'-CTCACAATTTTTTCCTTTAC-3' primers.

Participants

Patients with CP who visited Nihon University Hospital at Matsudo, Chiba, Japan from September 2011 to May 2014, were screened according to the following inclusion and exclusion criteria. Inclusion criteria consisted of**:** 1) having one or more interproximal sites; 2) showing a PPD of 5 to 8 mm with attachment loss; and 3) radiographic evidence of alveolar bone loss. Exclusion criteria consisted of**:** 1) with systemic diseases; and 2) who are smokers were excluded. A total of 27 patients (12 males and 15 females, mean age: 54.3 ± 12.73 years) and 56 surgical sites for the periodontal regeneration therapy by EMD were enrolled in this study.

Clinical examination and protocol

This study received ethical approval from the ethics committee of Nihon University School of Dentistry at Matsudo, Chiba, Japan [EC12-23]. Periodontal examinations were carried out using a periodontal probe (PCP11, Hu-Friedy; Chicago, IL). The following clinical parameters were used: 1) PPD (42); 2) CAL; and 3) BOP. PPD was measured from the top of the marginal gingiva to the apical extent of the periodontal probe penetration at six sites for each tooth. CAL was measured from the cement-enamel junction to the apical extent of periodontal probe penetration. BOP was recorded as presence or absence of BOP after measurement of PPD, and BOP rate for all measurement sites were calculated in percentage. A total of 27 patients received periodontal examinations, initial periodontal therapy, such as standard oral hygiene instruction, scaling and root planing (SRP) and professional mechanical tooth cleaning. After initial periodontal therapy, periodontal examination for re-evaluation was performed, the participants were selected according to the selection criteria mentioned above, candidates were asked to participate in this study, and written informed consents were obtained from all patients after the detail of investigative approach had been explained to them. PTM values were evaluated by a PTM kit for one to three sites with a PPD of 5 to 8 mm in one

participant. The day of periodontal regeneration therapy was set as a baseline (BL). After six months of EMD procedures, periodontal examinations for re-evaluation (RE) were performed. Periodontal examinations were performed concurrently with PTM values at BL and RE.

Sampling method and detection of AST by PTM kit

PTM values were measured following the instructions. PTM kits are based on a colorimetric reaction in which an examiner compares three positive control wells of known AST levels (800 μIU, 1,200 μIU and 1,800 μIU) to test wells, which contain the PerioPaper (Oraflow Inc; Hewlett, NY.) impregnated with GCF from the sites under investigation. The test was scored as follows: score 0 (– –); < 800 μIU, score 1 (–); < 1,200 ≥ 800 μIU, score 2 (+); < 1800 ≥ 1200 μIU and score 3 (++); ≥ 1800 μIU [17]. Just 15 min prior to GCF sampling, dried reagent wells were prepared by three drops of reagent liquid solution. After supragingival plaque was removed by sterilized cotton pellets, GCF was collected by PerioPaper put onto the periodontal pocket for 30 sec. Each PerioPaper was immediately immersed into the dissolved reagent wells, and a drop of start solution was put into the test wells and three control wells, respectively. The decision of representing the intensity of pink coloring was carried out at 4, 6 and 10 mins at room

temperature to evaluate four grades based on the levels of AST, namely – –, –, + and ++. The results of – – and – were estimated negative, meanwhile those of + and ++ were positive by comparison with controls in the description of the PTM kit.

Periodontal regeneration therapies

All surgical interventions were performed by trained periodontists according to general methods. Simplified papilla preservation technique was used for the incision to access the intrabony defects. After elevation of the mucoperiosteal flap, the flap was extended one or two teeth mesially and distally of the intrabony defects. In case they were needed, vertical releasing incisions were performed, especially at palatal sites. Then, the granulation tissue was removed from intrabony defects. After elucidation of vertical bone loss with one-, two- and three-wall components, SRP was carried out by Gracey curettes (SG11/12R, SG13/14R, Hu-Friedy; Chicago, IL) and ultrasonic scalars. Before the EMD treatment, 10% citric acid etching was performed on root surfaces facing to intrabony defects. After rinsing with saline solution, EMD was applied to the root surface and the bottom of intrabony defects. If an autogenous bone followed by osseous resection was gained, an autogenous graft was utilized regardless of the classification of bone walls. The flaps were repositioned and sutured using nylon or a Teflon-coated suture. For the

infection control at post-surgery, 500 mg of azithromycin per day was prescribed for 3 days. Sixty mg of loxoprofen sodium hydrate was also prescribed as painkiller when necessary. For post-surgical instructions, the prohibition of brushing and the use of 0.2% benzethonium chloride for operative sites and patients were instructed to rinse after each meal for the first two weeks until the sutures were removed. After about six months, follow-up periodontal examinations and evaluations with a PTM kit were performed.

Statistical analysis

Triplicate samples were analyzed for each experiment, and experiments were replicated to ensure the consistency of the responses to IL-1β or TNF-α. Significant differences between the control and IL-1β or TNF-α treatment groups, and change and variation of the PTM values at sites with periodontitis at BL and RE were determined using the one-way ANOVA. It was also used to compare the differences of measurement values of PPD, CAL and BOP at BL and RE in each group, respectively.

Results

To study the effect of IL-1β 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-1β on the FDC-SP mRNA levels for 12 h in Ca9-22 cells were investigated. IL-1β upregulated FDC-SP mRNA levels significantly at 1 and 10 ng/ml (Fig. 1A). IL-1β (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-1β (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-1β (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-1β or TNF-α likely involved in 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-1β (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 (Fig. 4 and 5). Human FDC-SP gene promoter has several transcription factors binding sites such as Yin Yang 1 (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) (38).

To clarify whether C/EBPα transcription factor is able to interact with C/EBP2 and C/EBP3 in the gene promoter of human FDC-SP *in vivo* after IL-1β stimulation. ChIP analyses were conducted by HPL-hTERT cells. IL-1β (1 ng/ml) induced C/EBPα 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/EBPα bindings to C/EBP2 and C/EBP3 after IL-1β stimulation. When HPL-hTERT cells were stimulated by IL-1β for 12 h, C/EBPα binding to C/EBP2 and C/EBP3 were almost completely inhibited by protein kinase A inhibitor KT5720, tyrosine kinase inhibitor HA, MEK1/2 inhibitor U0126 and PI3K inhibitor LY2940024 (Fig. 6B).

Table 1 describes clinical parameters of 56 surgical sites (27 patients) for the periodontal regeneration therapy by EMD at BL and RE. Mean PPD (5.82 ± 1.44 mm), CAL (7.34 ± 2.17 mm) and BOP (58.9%) at BL significantly improved to mean PPD (3.45 ± 0.99 mm), CAL (5.59 ± 1.79 mm) and BOP (19.6 %) at RE. After periodontal regeneration therapies, PTM values were improved at 42 out of 56 treatment sites. In the 42 sites, 42 PPD, 38 CAL, and 41 BOP were either improved or no change (Fig. 7). Next, we explored the relationship between PTM values and mean PPD and CAL after periodontal regeneration therapies. In the improved 42 sites, mean PPD and CAL were decreased significantly (Fig. 8A). On the other hand, in the 14 no change and deteriorated

sites, only the mean PPD was decreased significantly (Fig. 8B). The results suggest that sites with improved PTM values after periodontal regeneration therapies might have not only shallow PPD but also CAL gain. These studies suggest that the application of AST levels in GCF for clinical evaluation is useful as a prognostic indicator of periodontal disease.

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, 43). IL-1β and TNF-α are inflammatory cytokines required to activate innate immune reaction, mediating the recruitment, activation and adherence of circulating neutrophils and macrophages. IL-1β 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 (lipopolysaccharide) of the innate immune reaction (44). IL-1β concentration in gingival crevicular fluid (GCF) was increased as a characteristic of periodontitis (45). Initial periodontal therapy decreased IL-1β levels in GCF from periodontitis patients (46). Gene cluster polymorphisms of IL-1 is connected to periodontitis in type 2 diabetes (47). A positive

correlation was detected between severity of periodontitis and the concentrations of TNF-α in the GCF (48). Adipose tissues express high concentrations of TNF-α (49) and serum levels of TNF-α are increased in type 2 diabetes, obese patients (50, 51). Antimicrobial periodontal therapy decreased serum levels of TNF-α, C-reactive protein and glycated hemoglobin levels in chronic periodontitis patients (52, 53). These data suggest that IL-1β and TNF-α could be deeply involved in the onset and progression of periodontitis.

In the present study, we showed that IL-1β upregulated human 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-1β 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 (38). 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 (Fig. 4 and 5), whereas IL-1β 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-1β 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, 54). 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-1β 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 (55-60). YY1 plays an important function in stimulating IL-6 expression in rheumatoid arthritis which contribute to the inflammation via promoting the differentiation of Th17 (55). 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 (56). GATA-6 and co-factor friend of GATA (FOG) induced hepcidin gene transcription in hepatocytes during inflammation (57). C/EBPs are member of leucine zipper transcription factor family regulate a variety of cell differentiation and various

aspects of tissue functions (58). C/EBPβ 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/EBPβ is regulated by inflammatory stress (59). C/EBPα is expressed high levels in T follicular helper cells, and restricts IFN-γ expression in T cells to allow B cells class switching (60). Previously, we have reported C/EBPβ play crucial roles in human FDC-SP gene transcription after stimulation by IL-1β in HPL-hTERT cells (41). However, we did not analyze the function of C/EBPα in FDC-SP gene transcription. IL-1β increased human FDC-SP gene transcription mediated through C/EBPα targeting C/EBP2 and C/EBP3 in the human FDC-SP gene promoter (Fig. 6). In the first study, we demonstrate that IL-1β upregulated human FDC-SP mRNA expressions in Ca9-22, Sa3 and HSY cells. IL-1β 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-1β induced C/EBPα 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-1β and TNF-α on the expression and function of FDC-SP.

In the second study, we demonstrated that PTM values (AST levels) can be critically useful in prognosis after periodontal regeneration therapies. Shimada *et al*. reported that the correlation coefficients between AST levels (measured by PocketWatch™) and the

PPD, and CAL were 0.436 and 0.266 respectively, and were statistically significant (31). AST positive sites showed significantly higher occurrence of co-infection of periodontopathic bacteria than AST negative sites (61). Periodontal regeneration therapies improved severe periodontal conditions as effective as previous studies (62). There were limitations of uniformity for the procedure of periodontal surgery and surgical techniques such as papilla preservation technique (63-65) with or without autologous bone graft (66-68), because the efficacy the periodontal regeneration therapies are substantially affected by the shape of intrabony defects (69, 70). Therefore, the variation of periodontal parameters after regeneration therapy by EMD might arise, if the PTM values were improved at RE (Fig.7). Next, we compared relationship between PTM values and PPD or CAL after periodontal regeneration therapies. In the improved sites of PTM values (42 sites), PPD and CAL were decreased significantly (Fig. 8A). On the other hand, in the 14 no change and deteriorated sites, only the PPD was decreased significantly (Fig. 8B). The results suggest that sites with improved PTM values after periodontal regeneration therapies might have not only shallow PPD but also CAL gain. The application of AST levels in GCF for clinical evaluation is useful as a prognostic indicator of periodontal disease. Moreover, the premature healing of periodontal tissues could influence the results, although we evaluated periodontal parameters and PTM values six

months after regeneration therapies, EMD is capable of enhancing early wound healing by early angiogenesis and resolution of inflammation (71, 72). These findings support that EMD might improve AST activities due to early wound healing after regeneration therapies.

Taken together, PTM values might support the clinical improvements by the assessment of periodontal parameters after periodontal regeneration therapies. Moreover, these results raise the possibility that PTM values were utilized not only for the efficacious evaluation of postoperative restoration, but also as preoperative predictors for prognosis after periodontal regeneration therapies.

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Table 1 Clinical parameter before and after periodontal regeneration therapy

56 sites	BL	RE
PPD (mm)	5.82±1.44	3.45±0.99*
CAL (mm)	7.34±2.17	5.59±1.79*
BOP (%)	58.9	19.6 *

BL, baseline; RE, re-evaluation; *P＜0.01

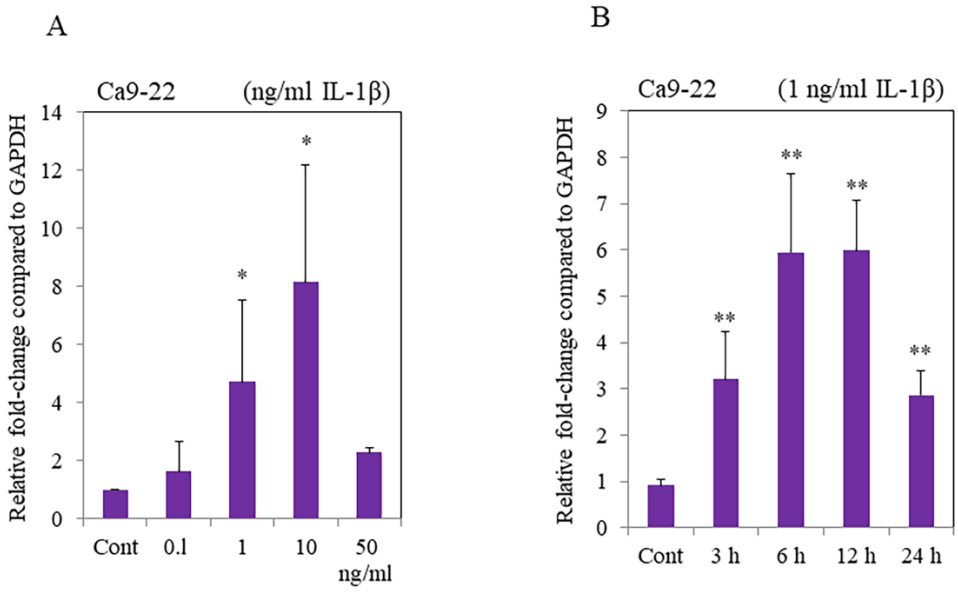


Fig. 1 Effects of IL-1β on FDC-SP mRNA levels in Ca9-22 cells.

(A) Dose-response effects of IL-1β on FDC-SP mRNA levels in Ca9-22 cells treated for 12 h. **(B)** Ca9-22 cells were treated with or without IL-1β (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). Significant differences between the control and IL-1β treatment groups were determined using the one-way ANOVA; *P<0.05 and **P<0.01.

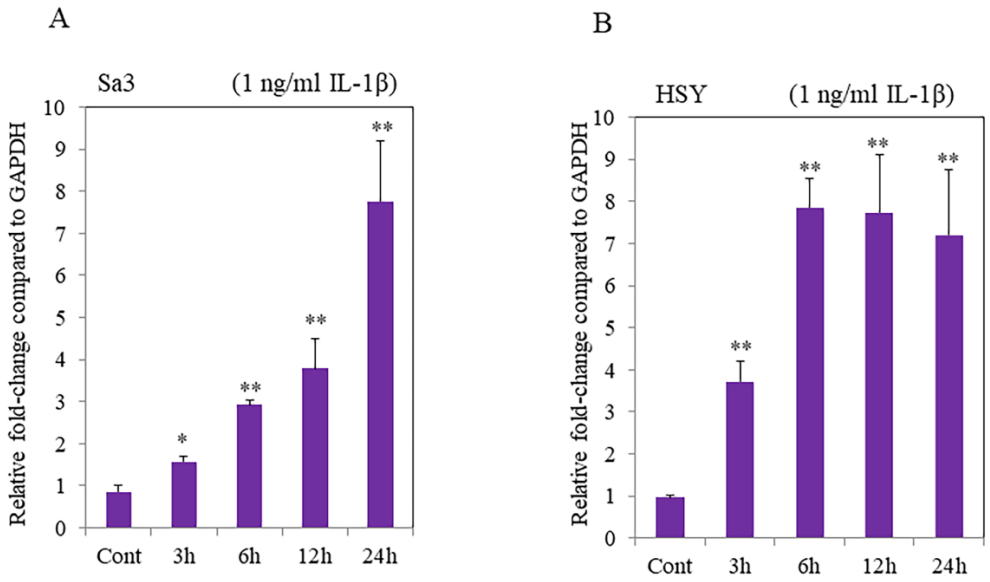


Fig. 2 Effects of IL-1β on FDC-SP mRNA levels in Sa3 and HSY cells.

Sa3 **(A)** and HSY **(B)** cells were treated with or without IL-1β (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-1β treatment groups were determined using the one-way ANOVA; *P<0.05 and **P<0.01.

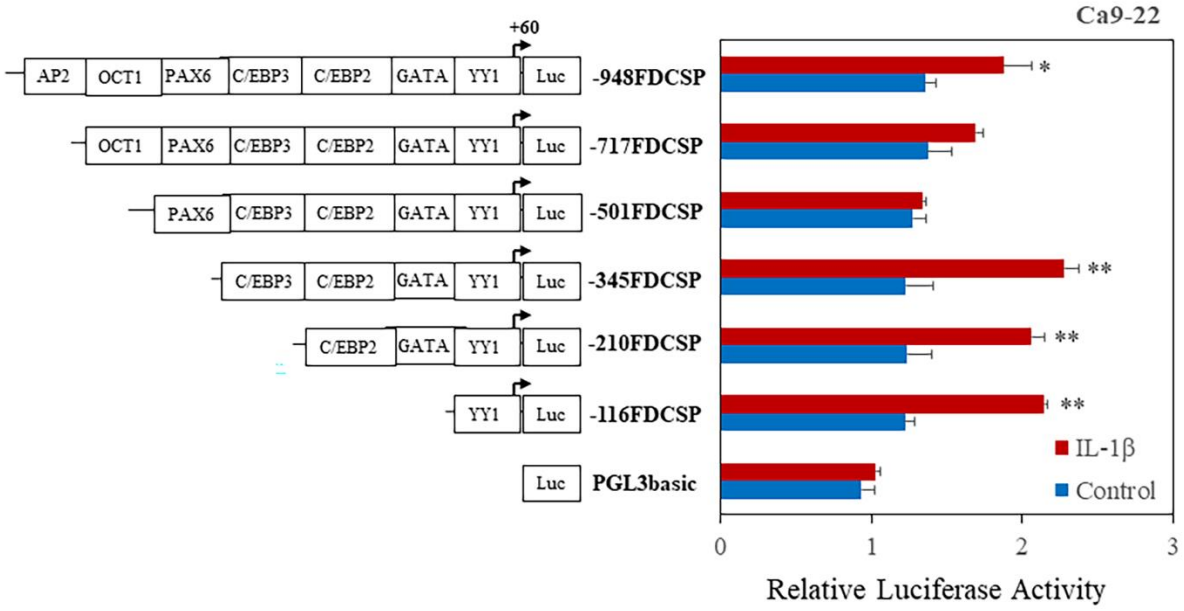


Fig. 3 IL-1β upregulates human FDC-SP gene promoter activities. The transcriptional activities of -116FDCSP (-116~+60), -210FDCSP (-210~+60), -345FDCSP (-345~+60) and -948FDCSP (-948~+60) were increased by IL-1β (1 ng/ml, 12 h) in Ca9-22 cells. Results of transcriptional activities obtained from three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

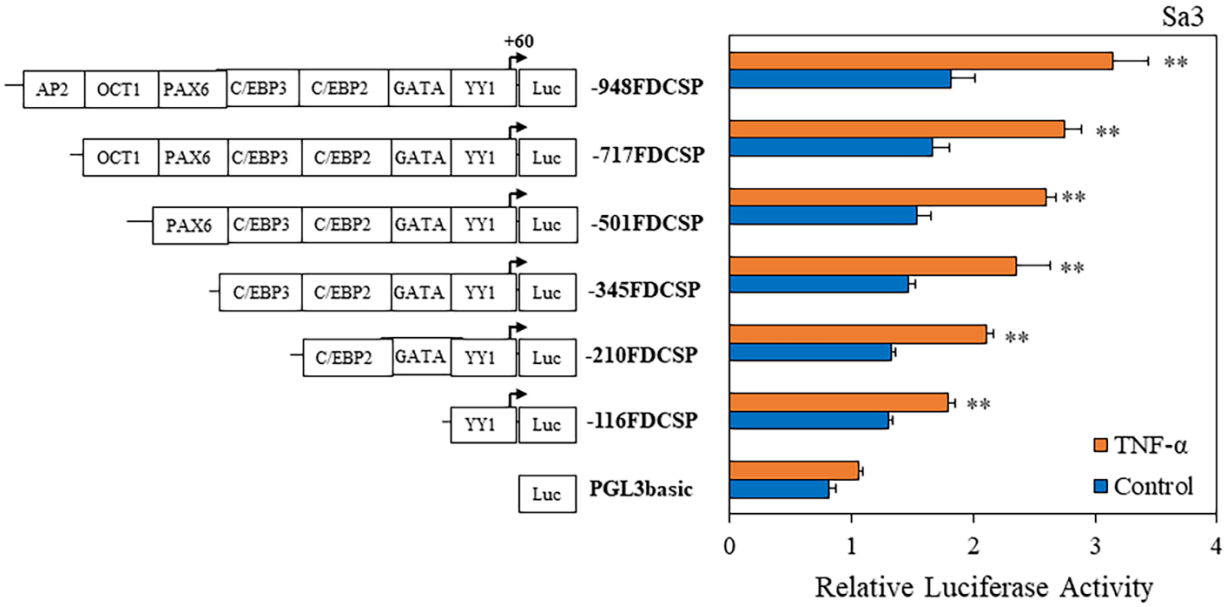


Fig. 4 TNF-α upregulates human FDC-SP gene promoter activities. The transcriptional activities of -116FDCSP, -210FDCSP, -345FDCSP, -501FDCSP, -717FDCSP and -948FDCSP were increased by TNF-α (10 ng/ml, 12 h) in Sa3 cells. Results of transcriptional activities obtained from three separate transfections with constructs, pGL3basic and -116FDCSP to -948FDCSP were combined and values expressed with SD. Significant differences between the control and TNF-α treatment in each construct were determined using the one-way ANOVA; **P<0.01

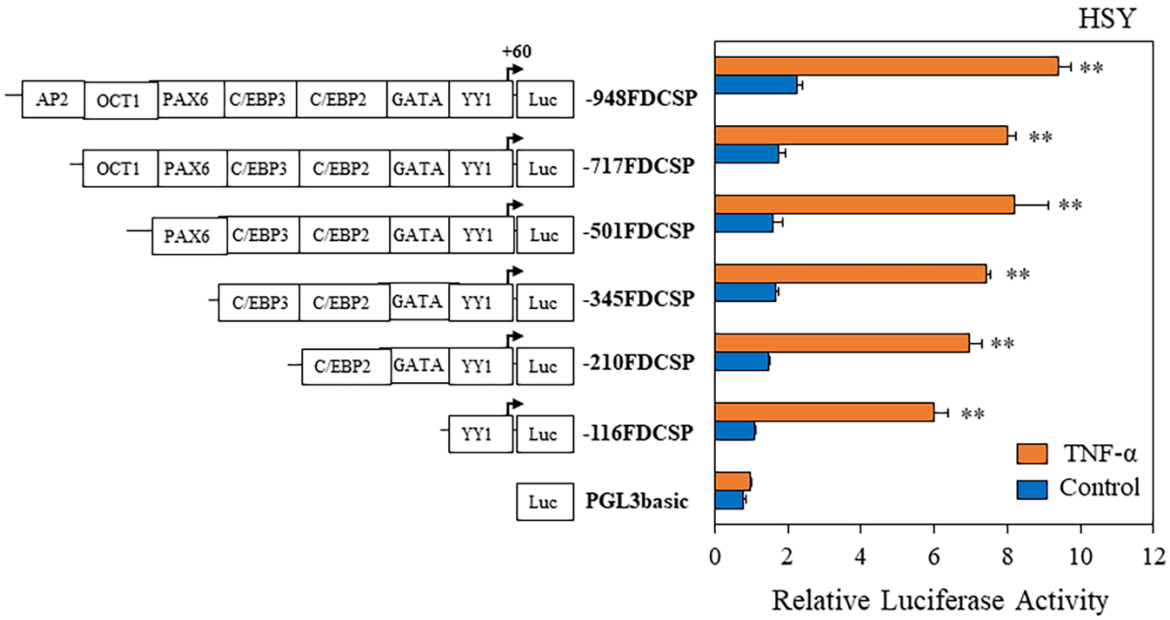


Fig. 5 TNF-α upregulates human FDC-SP gene promoter activities. The transcriptional activities of -116FDCSP, -210FDCSP, -345FDCSP, -501FDCSP, -717FDCSP and -948FDCSP were increased by TNF-α (10 ng/ml, 12 h) in HSY cells. Results of transcriptional activities obtained from three separate transfections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

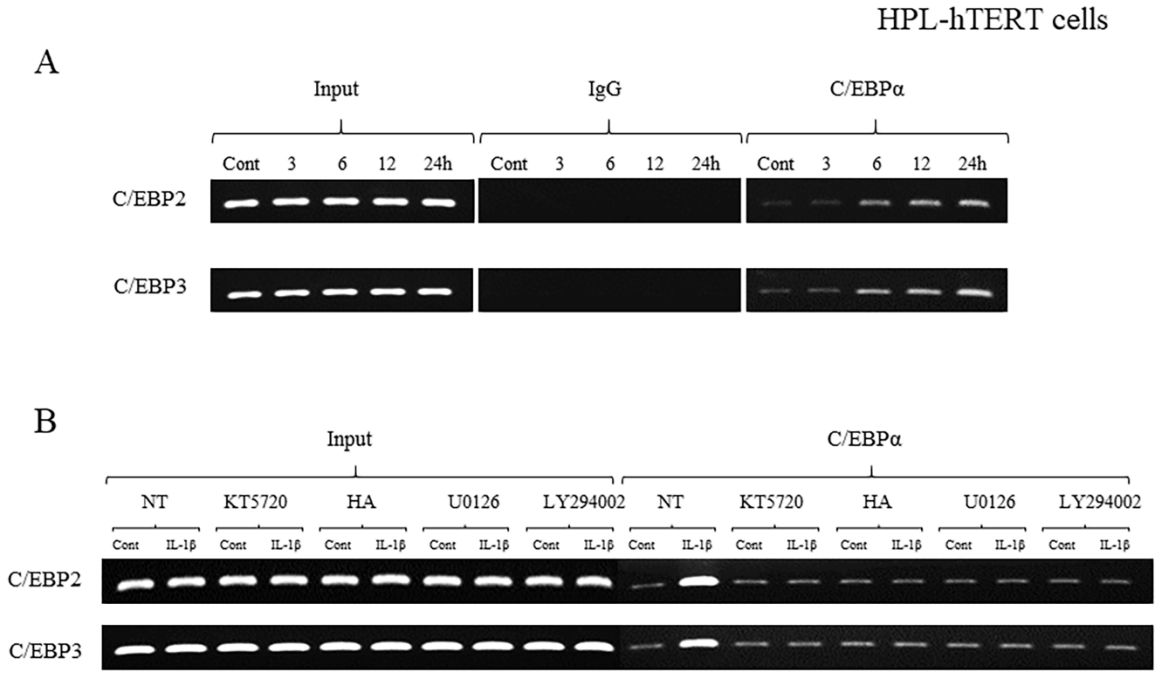


Fig. 6 ChIP analyses of C/EBPα binding to C/EBP2 and C/EBP3 in the human FDC-SP gene promoter in HPL-hTERT cells. **(A)** PCR bands amplified and corresponding to DNA-protein complexes immunoprecipitated with anti-C/EBPα antibody showed that C/EBPα interacted with a chromatin fragment containing the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

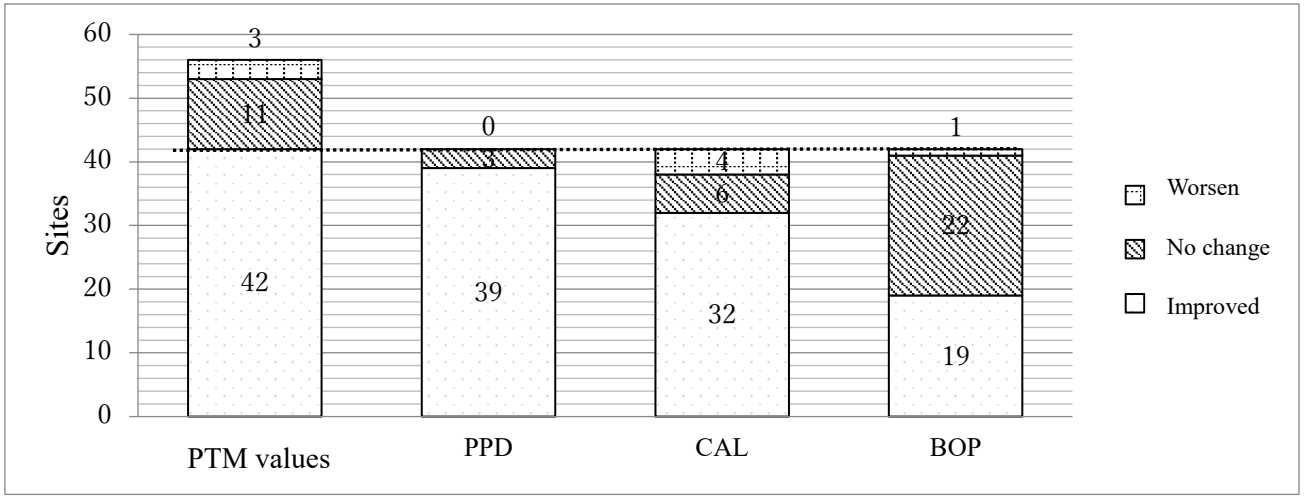
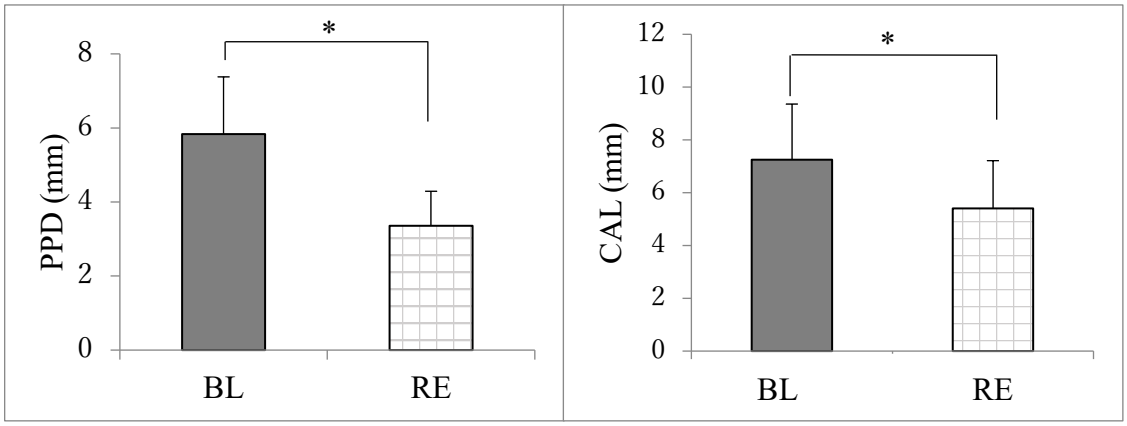


Fig. 7 Changes in PTM values and clinical parameters after periodontal regeneration therapies at 56 treatment sites.

A



B

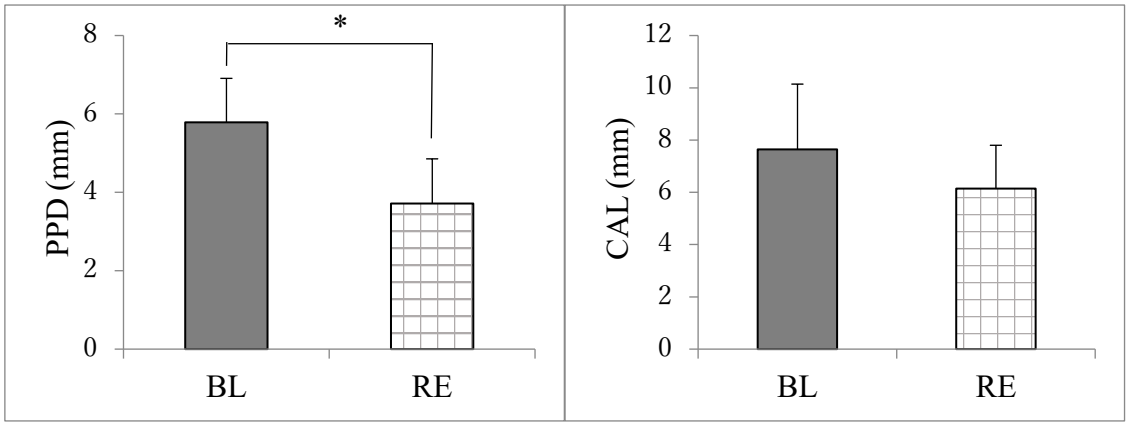


Fig. 8 Relationship between PTM values and mean PPD and CAL after periodontal regeneration therapies. **(A)** Changes in PPD and CAL before (BL) and after surgery (RE) at PTM values have improved (n=42, *P＜0.01). **(B)** Changes in PPD and CAL before (BL) and after surgery (RE) at PTM values unchanged and at the sites of deterioration. (n=14, *P＜0.01)