The comparison of IL-6, elastase and α1-PI expressions in human chronic periodontitis with type 2 diabetes mellitus

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I. INTRODUCTION

Diabetes mellitus is an important risk factor of periodontal diseases. It is one of the main contributing factors for periodontal disease and also limiting factor for periodontal treatments such as implant therapy. Although diabetes itself does not cause periodontitis, periodontal disease progresses more rapidly and leads to more tooth losses in patients with poorly controlled blood glucose1-5. Severe periodontitis has been associated with an increased risk of poor glycemic control and, in turn untreated advanced periodontal disease can deteriorate the metabolic control of diabetes6. Various pathogenetic factors have been suggested to explain the increased prevalence and severity of periodontitis in diabetes7,8.

Reduced polymorphonuclear leukocyte (PMN) function has been found in patients with diabetes. This impairment of function was noted in assays of PMN chemotaxis, adherence and phagocytosis9-11. Studies of PMN defects suggest that this dysfunction could lead to impaired host resistance to infection12. Gingival fibroblasts from diabetic patients synthesize less collagen compared to non-diabetic subjects13. In addition to decreased collagen production, crevicular fluid collagenolytic activity also was increased in diabetic patients14. This increased crevicular fluid collagenase activity appears to be primarily of neutrophil origin. Non-enzymatic glycosylation process results in increased cross-linking between collagen molecules. This cross-linking of collagen significantly contributes to reduced solubility and de-
CREASES TURNOVER RATE\textsuperscript{15-17}. Vascular changes are common in patients with diabetes. Basement membrane proteins become glycosylated in a hyperglycemic environment, with thickening and changes in the physical properties\textsuperscript{1,8,19}.

Chronic periodontitis is an inflammatory disease initiated and maintained by bacterial plaque and its metabolic products that trigger the local infiltration of inflammatory cells associated with the breakdown of collagenous extracellular matrices (ECM)\textsuperscript{20}. The degradation of gingival connective tissue during periodontitis could be a disturbance of cell-cell and cell-matrix interactions involving the production of enzymes, activators, inhibitors, and regulatory molecules such as cytokines and growth factors\textsuperscript{21,22}.

Interleukin-6(IL-6) is recognized factors for progression of gingivitis to periodontitis.

It has been suggested that IL-6 accumulation within inflamed gingiva is also a significant factor in progression of periodontal disease. IL-6 concentrations become elevated later in the pathogenesis of periodontal disease\textsuperscript{23,24} or at refractory sites\textsuperscript{25,26}. IL-6 increase the potential for periodontitis and alveolar bone loss, as this cytokine has been implicated in the tissue destruction characteristic of this disease\textsuperscript{27,28}.

Elastase is secreted upon release of azurophilic granules during neutrophil phagocytosis, stimulation, and cell lysis. Parts of these proteinase is bound to the external surface of the cell membrane after their transport from the granules. Membrane-bound leukocyte elastase has a strong activity against fibronectin and type IV(basement membrane) collagen\textsuperscript{29}. Consequently elastase may be an important enzyme facilitating neutrophil transmigration through subendothelial and subepithelial basement membranes. The secreted elastase has potential for extensive degradation of extracellular matrix\textsuperscript{30,31}. Elastase activity in gingival crevicular fluid(GCF) of humans is elevated during adult periodontitis and this analyte has been used as an indicator to predict gingival attachment loss in longitudinal studies on humans with periodontal disease\textsuperscript{32,33}.

\(\alpha_1\)-Proteinase Inhibitor(\(\alpha_1\)-PI), has been implicated in the modulation of periodontal inflammation and destruction\textsuperscript{34}. This plasma protein, produced by liver cells and monocytes, is an acute phase reactant and elevated during inflammation and other disease conditions\textsuperscript{35}. It was reported that the \(\alpha_1\)-PI rapidly forms a 1:1 inactive complex with the enzyme and the \(\alpha_1\)-PI/elastase complex is also increased during gingival inflammation in regulating elastase activity\textsuperscript{36}. Conversely, deficiency of \(\alpha_1\)-PI levels in serum can result in several disease processes associated with excess degradation of elastase substrates(e.g. elastic fibers, fibronectin, proteoglycan)\textsuperscript{37}.

Several groups have reported about relation of elastase-complex with \(\alpha_1\)-PI in GCF\textsuperscript{33,38}.

In inflammatory response, the roles and interactions of IL-6, elastase and \(\alpha_1\)-PI are not clear. The relative contribution of IL-6, elastase and \(\alpha_1\)-PI in the pathogenesis of periodontitis is still not entirely established. Moreover none of the in vivo studies simul-
simultaneously analysed each IL-6, elstase and α-PI and it’s interrelationship for the diabetic and nondiabetic patients with chronic periodontitis. The purposes of this study were to compare and quantify the expression of IL-6, elstase, α-PI and α-PI/elstase relation in the gingival tissues of patients with type 2 diabetes mellitus and healthy adults with chronic periodontitis.

II. MATERIALS AND METHODS

1. Study population and Tissue sampling

Study population consisted of 8 patients with type 2 diabetes and chronic periodontitis, 8 patients with chronic periodontitis, and 8 healthy individuals. Marginal gingival tissue samples were obtained by internal bevel incision at the time of periodontal surgery (including surgical crown lengthening) or tooth extraction and informed consent was obtained from all of the participants before the surgery.

Clinical criteria of gingiva (Sulcus bleeding index value, probing depths) and radiographic evidences of bone resorption, each gingival sample was divided into the three groups. Group 1 (normal, n=8) is clinically healthy gingiva without bleeding and no evidence of bone resorption or periodontal pockets, obtained from systemically healthy 8 patients. Group 2 (chronic periodontitis, n=8) is inflamed gingiva from patients with chronic periodontitis. The diagnosis of chronic periodontitis was established on the basis of clinical and radiographic criteria (bone resorption) according to the classification system for periodontal disease and condition. All patients of group 2 were systemically healthy and had more than one periodontal pockets ≥5 mm and at least one pocket with ≥4 mm loss of attachment. All gingival samples were obtained from the teeth with probing depth ≥5 mm, swelling of the marginal gingiva, and bleeding corresponding to gingival sulcus bleeding indexes 3 according to Mühlman and Son. Group 3 (chronic periodontitis & type 2 DM, n=8) is inflamed gingiva from patients with chronic periodontitis associated with type 2 diabetes. Patients in group 3 were diagnosed type 2 diabetes mellitus since 6 months and showed above 200 mg/dl blood glucose level in postprandial 2 hours. Patients in group 2 & 3 have similar periodontal condition, but systemically patients in group 2 were healthy and patients in group 3 had type 2 diabetes with treatment. Gingival sample were obtained by similar way described above.

Following surgery, excised tissue specimens were immediately placed on liquid nitrogen and subsequently frozen (-70°C).

2. Protein Isolation and Western blotting

For Western blotting, as previously described technique by Kim et al40) frozen tissues were homogenized in RIPA lysis buffer (10 mM EDTA, 0.15M NaCl) with
1:30 diluted protease inhibitor cocktail (Roche, Germany). The lysates were sonicated 3 times for 10 seconds and centrifuged at 12,000g for 15 minutes. Protein concentrations of supernant were routinely determined by a Bradford protein assay (Quick Start, BIO-RAD, USA) using BSA as standard.

Lysates were boiled in SDS samples buffer (1M Tris-Cl (pH6.8), 40% glycerol, 8% SDS, 2% mercapto-ethanol, 0.002% Bromophenole blue). Prepared samples were separated by 15% sodium dodecyl sulfate (SDS)- polyacrylamide gels and transferred to a polyvinylidene difluoride membrane.

The membranes were subsequently blocked in Tris-buffered saline (TBS) containing 5% powdered milk and 1% BSA for 1 hour, and then incubated with polyclonal anti-IL-6, anti-elastase, and anti-α-PI (Santa Cruz Biotechnology, Inc. USA) antibody for 1.5 hours at room temperature.

The membranes were washed (five times for 5 minutes with Tween 20) and incubated with a horseradish peroxidase (HRP)- conjugated goat anti-rabbit secondary antibody for anti-IL-6 antibody and donkey anti-goat secondary antibody for anti-elastase, anti-α-PI antibody (diluted 1: 2000 in TBS) for 1 hour at room temperature. After additional washing (five times for 5 minutes with Tween 20) the Western blot procedure was completed with an ECL Plus development kit (Amsterdam, Beckinghamshire, U.K.)

The quantification analysis of IL-6, elastase, α-PI expression was performed using a densitometer (Image Gauge V 3.46, Koshin Graphic Systems, FUJI PHOTO FILM CO, Japan). After normalization to β-actin (Abcam® U.K.) in each sample, level of IL-6, elastase and α-PI were expressed as a ratio of IL-6 or elastase or α-PI/β-actin and the differences of density between 3 groups were determined. α-PI/elastase ratio was also compared.

3. Statistical Analysis of the Western blot results

All data were presented as means ± standard deviation and results were statistically analyzed. The IL-6, elastase, α-PI levels and α-PI/elastase ratio among each 3 groups were compared using one way ANOVA followed by Tukey test. P value < 0.05 was considered to statistically significant.

III. RESULTS

Both chronic periodontitis group & chronic periodontitis with type 2 DM group showed the expression of IL-6, elastase and α-PI in all samples. To compare IL-6 expression levels in human gingiva with chronic periodontitis with or without associated to Type 2 diabetes mellitus, IL-6 specific antibodies were used to detect the cytokine in the tissues (Figure 1A, B). Representative Western blot data were presented in Figure 1A. The expression levels of β-actin were also measured by anti-β-actin specific western blot analysis. In order to quantify the level of IL-6 expression in the groups, the
**Figure 1A.** IL-6 Western analysis showing 4 representative samples in each group. IL-6 corresponding to molecular weight 21kDa was shown to be expressed in all samples including healthy gingiva, and the expression levels of IL-6 were increased in order of group 1, group 2, group 3. In order to quantify the IL-6 levels, β-actin levels were also performed.

Group 1: healthy gingiva from systemically healthy person  
Group 2: inflamed gingiva from patient with chronic periodontitis  
Group 3: inflamed gingiva from patient with chronic periodontitis and type 2 DM

**Figure 1B.** Graphics showing the average amounts (Ratio of IL-6/β-actin) and standard deviation of IL-6 in group 1, 2 and 3. In the inflamed gingiva with diabetes (group 3), IL-6 was highest in to group 3.

Group 1: healthy gingiva from systemically healthy person  
Group 2: inflamed gingiva from patient with chronic periodontitis  
Group 3: inflamed gingiva from patient with chronic periodontitis and type 2 DM

+significant difference between group 1 and group 2 (P<0.05)  
* significant difference between group 2 and group 3 (P<0.05)  
** significant difference between group 1 and group 3 (P<0.05)

The mean amount of IL-6 expression (ratio of IL-6/β-actin) were 0.793±0.135 in group 1, 0.962±0.113 in group 2, 1.148±0.142 in group 3. The expression levels of IL-6 among group 1, group 2 and group 3 were significantly different and were highest in group 3. (P<0.05)

The comparison of elastase expression levels were also studied by Western blot analysis using elastase specific antibody which
Figure 2A. Elastase Western analysis showing 4 representative samples in each group. Elastase corresponding to molecular weight 30kDa was shown to be expressed in all samples including healthy gingiva. The expression levels of elastase were increased in order of group 1, group 2, group 3. In order to quantify detected elastase, β-actin levels were also measured.

Group 1: healthy gingiva from systemically healthy person
Group 2: inflamed gingiva from patient with chronic periodontitis
Group 3: inflamed gingiva from patient with chronic periodontitis and type 2 DM

Figure 2B. Graphics showing the average amounts (Ratio of elastase/β-actin) and standard deviation of elastase in group 1, 2 and 3. In the inflamed gingiva with diabetes (group 3), elastase seemed to be increased compared to group 1 and group 2.

Group 1: healthy gingiva from systemically healthy person
Group 2: inflamed gingiva from patient with chronic periodontitis
Group 3: inflamed gingiva from patient with chronic periodontitis and type 2 DM

+ significant difference between group 1 and group 3 (P<0.05)
* significant difference between group 2 and group 3 (P<0.05)

detected about 30kDa molecular weight of elastase in all three groups (Figure 2A). The levels of elastase expression were also quantified with β-actin normalization (Figure 2B). The mean amounts of elastase expression (ratio of elastase/β-actin) were 0.818 ± 0.112 in group 1, 0.977 ± 0.174 in group 2 and 1.500 ± 0.231 in group 3. There was no significant difference between group 1 and group 2, but the differences between group 1 and group 3 and between group 2 and group 3 were statistically significant (P<0.05).

In the study of α1-PI expression levels using Western blot analysis, molecular weight of α1-PI was identified as 42kDa size (Figure 3A). The mean amounts of α1-PI expression (ratio of α1-PI/β-actin) were 0.892 ± 0.053 in group 1, 0.989 ± 0.054 in group 2 and 1.107 ±
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Figure 3A. α1-PI Western analysis showing 4 representative samples in each group. α1-PI corresponding to molecular weight 42kDa was shown to be expressed in all samples including healthy gingiva and the expression level of α1-PI was increased in patients with type 2 diabetes mellitus than in control healthy subjects. In order to quantify detected elastase, β-actin levels were also measured.

Group 1: healthy gingiva from systemically healthy person
Group 2: inflamed gingiva from patient with chronic periodontitis
Group 3: inflamed gingiva from patient with chronic periodontitis and type 2 DM

Figure 3B. Graphics showing the average amounts (Ratio of α1-PI/β-actin) and standard deviation of α1-PI in group 1, 2 and 3. In the inflamed gingiva (with or without diabetes, group 2 & 3), the levels of α1-PI was higher compared to healthy gingiva.

Group 1: healthy gingiva from systemically healthy person
Group 2: inflamed gingiva from patient with chronic periodontitis
Group 3: inflamed gingiva from patient with chronic periodontitis and type 2 DM

* Significant difference between group 1 and group 3 (P<0.05)

0.226 in group 3. The significant difference was observed only in between group 1 and group 3 (P<0.05)

The ratios of α1-PI/elastase were also calculated, and compared between groups. The mean amounts of α1-PI/elastase ratio were 1.113±0.192 in group 1, 1.038±0.177 in group 2 and 0.742±0.114 in group 3. The ratio was decreased in the order of group 1, 2, and 3. The differences between group 1 and group 2 and between group 1 and group 3 were statistically significant (P<0.05)

In the interrelationship of IL-6, elastase and α1-PI expressions, as expression of IL-6 was increased, elastase expressions showed increasing tendency in chronic periodontitis associated to type 2 DM. Although α1-PI levels were increased in DM according to increase of IL-6 and elastase, α1-PI/elastase ratios were decreased in chronic periodontitis with type 2 DM.
Figure 4. Graphics showing the average amounts and standard deviation of α₁-PI/elastase ratio. The inflammed gingiva with DM is lower than group 2 and 3.

Group 1: healthy gingiva from systemically healthy person
Group 2: inflammed gingiva from patient with chronic periodontitis
Group 3: inflammed gingiva from patient with chronic periodontitis and type 2 DM

+ significant difference between group 1 and group 3 (P<0.05)
* significant difference between group 2 and group 3 (P<0.05)

IV. DISCUSSION

Multiple studies have demonstrated the link between diabetes and periodontal disease in human subjects. Although diabetes itself does not cause periodontitis, periodontal disease progresses more rapidly and leads to more tooth loss in poorly controlled patients. Various pathogenetic factors have been suggested to explain the increased prevalence and severity of periodontitis in diabetes.

The purpose of this study was to quantify and compare the expression of IL-6, elastase and α₁-PI in the gingival tissues of the patients with chronic periodontitis associated to type 2 DM, in order to understand the contribution of these proteins to periodontal destruction in type 2 diabetic patients, especially elastase-mediated host response in type 2 diabetic patients.

The amount of IL-6 expression was higher in inflammed gingiva with chronic periodontitis associated to type 2 DM than in healthy gingiva from systemically healthy person and inflammed gingiva from patients with chronic periodontitis. The differences among three groups were statistically significant between group 1, 2 and group 3. (P<0.05) (Figure 1B)

When the expression levels of IL-6 between chronic periodontitis without type 2 DM and chronic periodontitis with type 2 DM were compared, chronic periodontitis with type 2 DM showed higher level than chronic periodontitis without DM. These results were similar with previous reports seen in inflammatory conditions. It was considered that IL-6 levels were overexpressed in inflammed gingiva with or without type 2 DM and chronic periodontitis patient with type 2 DM expressed higher cytokine activity and inflammatory response.

It was reported the impaired PMN func-
tion in diabetes regarding to chemotaxis, chemokinesis and degranulation\textsuperscript{41,42}. This might explain the increased susceptibility of diabetic patients to various infectious diseases including periodontitis. These results make it possible to consider that PMN dysfunction may be reflected in the gingival neutrophil degranulation product, which is released from neutrophils recruited to inflammatory gingiva.

The amounts of elastase expression were higher in chronic periodontitis with type 2 DM compared to chronic periodontitis group of systemic healthy person and healthy gingiva from systemically healthy person.

The levels in inflamed gingiva with type 2 DM were higher than chronic periodontitis in systemic healthy person and there was statistically significant difference between inflamed gingiva from patients with chronic periodontitis and inflamed gingiva with chronic periodontitis associated to type 2 DM. These results were similar with the results reported in GCF of chronic periodontitis condition of various report\textsuperscript{43-45}. It is assumed that IL-6 stimulated elastase activity in chronic periodontitis associated with type 2 DM. But, it is also considered that other cytokines or other tissue degradation enzymes are involved complexly in elastase expression.

\(\alpha\)-PI has been studied in this study. The level of \(\alpha\)-PI was increased in inflamed gingiva. In inflammatory response, as the level of elastase increased, \(\alpha\)-PI level was increased, but in the case of inflamed gingiva with type 2 DM, the elastase value was not proportional to \(\alpha\)-PI. There was significant difference between group 1 and group 3.

The comparison of \(\alpha\)-PI/elastase ratio was also studied in some previous reports as an important factor in inflammatory condition. Some studies had investigated balance between \(\alpha\)-PI and elastase in GCF\textsuperscript{43,46} and a few reports in whole mouth saliva\textsuperscript{47}. It was considered that the ratio was decreased in inflamed gingiva from patients with chronic periodontitis and inflamed gingiva with chronic periodontitis associated to type 2 DM. There was significant difference between group 1, 2 and 3. It was considered that \(\alpha\)-PI activity was suppressed because the relative amount of \(\alpha\)-PI was decreased due to the relative increase of elastase in chronic periodontitis group without DM. The ratio in DM group was further decreased. Neutrophils and monocytes migrating into the gingival crevice were probably the main source of elastase while \(\alpha\)-PI would have derived from extravasated serum, possibly from some local production by macrophage\textsuperscript{48,49}. It was considered that \(\alpha\)-PI activity of playing a role in controlling elastase activity was decreased in DM and it is suggested that defensive factors are associated with immune response related to neutrophils, monocytes, and macrophage were further decreased in DM group, but relationship with other immune factors should also be considered.

In interrelationship between IL-6, elastase
and α-PI, α-PI level of Group 3 showed increasing pattern according to increasing tendency of IL-6 and elastase expressions in chronic periodontitis associated to type 2 DM. It was shown positive relation between IL-6 and elastase expression, although they were shown reverse effect to α-PI ratio in inflammed tissue with DM.

In inflammed gingiva from patient with chronic periodontitis and type 2 DM, despite of increasing IL-6 and elastase, α-PI values were not significantly different from the gingiva with chronic periodontitis.

In conclusion, this study demonstrated that the expression levels of IL-6 and elastase had increasing tendency and positive relation in inflammed tissue and inflammed tissue associated with DM. It is suggested that IL-6 and elastase may be partly involved in the progression of periodontal inflammation associated with type 2 DM. The α-PI/elastase ratio also was further decreased in DM group. It is considered that as the quantity and activity of defensive factors associated with immune response is depressed in DM group, the inflammatory response in DM is higher, so the IL-6 and elastase levels are higher.

This ratio of α-PI/elastase also may be important measuring inflammatory factors in the progression of periodontal inflammation associated to type 2 DM.

Finally, it seemed that more studies are needed to investigate the effect and inter-relationship between inflammatory enzymes and other immune factors that affect the progression of periodontal disease in inflammaotory tissue with DM.

V. SUMMARY

The purposes of this study were to compare and quantify the expression of IL-6, elastase and α-PI in the gingival tissues of patients with type 2 diabetes mellitus and healthy adults with chronic periodontitis. Gingival tissue samples were obtained during periodontal surgery or tooth extraction. According to the patient’s systemic condition & clinical criteria of gingiva, each gingival sample was devided into three groups. Group 1 (n=8) is clinically healthy gingiva without bleeding and no evidence of bone resorption or periodontal pockets, obtained from systemically healthy 8 patients. Group 2 (n=8) is inflammed gingiva from patients with chronic periodontitis. Group 3 (n=8) is inflammed gingiva from patients with chronic periodontitis associated with type 2 diabetes. Tissue samples were prepared and analyzed by Western blotting. The quantification of IL-6, elastase and α-PI were performed using a densitometer and statistically analyzed by one-way ANOVA followed by Tukey test.

1. The expression levels of IL-6 showed increasing tendency in group 2 and 3, and it was highest in group 3.
2. The expression of elastase showed increasing tendency in group 2 and 3, and it was highest in group 3.
3. The expression of α-PI showed increas-
ing tendency in group 3 compared to group 1.

4. The α1-PI/elastase ratio was decreased in group 2 and 3 compared to group 1, especially most decreased in group 3.

5. As IL-6 levels were increasing, elastase showed increasing tendency in group 3, and although IL-6 and elastase levels were increasing, α1-PI level in group 3 showed slightly increasing pattern comparing to group 1.

In conclusion, this study demonstrated that the expression levels of IL-6 and elastase will be inflammatory markers of periodontal inflammed tissue and DM. The α1-PI/elastase ratio also may be important measuring inflammatory factors in the progression of periodontal inflammation associated to type 2 DM.

VI. REFERENCES


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단순 만성 치주염 환자 및 2형 당뇨병환자의 만성 치주염 치은조직에서 IL-6, elastase 및 α₁-PI의 발현 양상 비교

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본 실험이는 제2형 당뇨병 환자와 비당뇨 환자들에서 만성 치주염 부위의 치은 및 건강한 치은에서 염증 매개체 중 하나인 IL-6, elastase 및 α₁-PI의 발현에 대해 상호 비교 분석함으로서 염증, 혈당이 미치는 영향을 밝히고 제 2형 당뇨병 환자에서 심한 치주조직 파괴의 기전을 연구하고자 하였다.

경북대학교병원 치주과 내원환자 중 제2형 당뇨병 환자와 비당뇨 환자들 및 치주질환이 없는 건강인 대조군을 대상으로 여러 가지 환자요소, 임상 치주상태를 기록하고, 전신적으로 건강한 환자의 건강한 부위 (n=8, Group 1), 전신적으로 건강한 환자의 만성 치주염 부위 (n=8, Group 2), 제2형 당뇨병 환자의 만성 치주염 부위 (n=8, Group 3)에서 각각 변연치은을 체득하고 액화질소에 급속 동결하였다. Western blotting을 이용하여 각 조직 내 IL-6, elastase 및 α₁-PI의 발현을 관찰, densitometer를 이용하여 상대적 발현을 정량, 각 조직의 β-actin을 이용하여 표준화하여 실험군과 대조군들의 평균치를 비교하여 다음과 같은 결과를 얻었다.

1. IL-6의 발현은 2군과 3군에서 증가되는 경향을 보였으며 3군에서 가장 높게 나타났다.
2. Elastase의 발현도 2군과 3군에서 증가장을 보였으며 3군에서 가장 높게 나타났다.
3. α₁-PI의 발현경도는 1군에 비해 3군에서 증가되는 경향을 보였다.
4. α₁-PI/elastase 비율은 1군에 비해 2군과 3군에서 감소되었으며, 특히 3군에서 가장 감소되었다.
5. 3군에서 IL-6 발현이 증가됨에 따라 Elastase 발현이 증가되는 경향을 보였으나, IL-6와 Elastase 수치가 증가함에도 불구하고 α₁-PI 수치는 1군에 비해 약간 증가되는 경향을 보였다.

결론적으로 IL-6와 elastase는 만성 치주염과 제2형 당뇨병 환자의 만성 치주염 부위에서 염증상태에 따른 지표로 응용할 수 있으리라 생각되며, α₁-PI/elastase 비율 또한 2형 당뇨환자의 만성치주염 부위에서 중요한 측정인자가 될 수 있으리라 생각된다.

Key words: inflammation, inflammatory mediator, chronic periodontitis, diabetes mellitus