Expression of Matrix metalloproteinase-1 between Simple Chronic Periodontitis and Type 2 Diabetes associated Chronic Periodontitis on Protein level

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Ⅰ. Introduction

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(ECMs)[1]. 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[2,3].

In periodontitis, although some tissue-destructive enzyme activities may derive from specific bacteria, it is more likely that plaque bacteria inaddition to their other pro-inflammatory effects, generate proteinases that activate latent forms of mammalian collagenase, or even stimulate the release of collagenase and other matrix metalloproteinases(MMPs) from host cells[4]. MMPs are a family of zinc-dependent endopeptidases that collectively degrade all extracellular matrix proteins and basement membrane at neutral pH and are essential for cellular migration and tissue remodeling under physiological and pathological conditions[5,6]. Most of them are secreted as inactive form(proenzyme) and are activated in the extracellular compartment or in the vicinity of the cell membranes by other MMPs or serine proteinases[7]. Interstitial collagenase, including matrix metalloproteinase-1(MMP-1) and MMP-8, serve as ini-

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tiators of extracellular matrix destruction in periodontal disease. Fibroblast-type interstitial collagenase(MMP-1) is distributed widely in tissues and expressed by fibroblasts, keratinocytes, endothelial cells, osteoblasts, chondrocytes and monocytes/macrophages[4,8]. MMP-1 is a regulator of connective tissue remodeling and is present in high concentrations especially in inflamed gingival regions, including in periodontal disease[8]. Enhanced protein levels and m-RNA expression of MMP-1 have been demonstrated in inflammatory disease, including periodontitis[9-11]. The increased levels of MMP-1 observed in periodontitis may be a result of an alteration in the regulation of MMP-1 by gingival fibroblast, which is the most predominant cell in the gingivae. MMP-1 production in human gingival fibroblasts is stimulated by cytokines such as interleukin-1β and tumor necrosis factor-α[12].

Periodontal disease is frequently mentioned among the oral problems encountered in patients with diabetes. Although diabetes in itself does not cause periodontitis, periodontal disease progresses more rapidly and leads to more tooth loss in patients whose diabetes is poorly controlled[13-17]. 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 diabetes[18-22]. Collagen undergoes non-enzymatic glycosylation when subjected to a hyperglycemic environment and the glucose-derived cross-links between the molecules contribute to reduced collagen solubility and turn-over rate[23]. Advanced glycosylation end-products(AGEs) play a central role in diabetic complications. Several studies have linked an increased development of periodontal disease in diabetes to an decreased collagen production and exaggerated collagenase activities in periodontium. In diabetic patients, collagenolytic activity of gingival crevicular fluid is increased and gingival fibroblasts from diabetic patients synthesize less collagen than those form non-diabetic subjects[24,25].

It is well known that MMP-1 regulates ECM turnover, however, whether diabetes affects the expression and activity of the MMP-1 in patients with diabetes remains unknown. Therefore, the aim of this study is to investigate the MMP-1 levels in human gingival tissue and to compare MMP-1 levels of diabetic and nondiabetic periodontal patients, using systemically healthy individuals as the control group.

Ⅱ. Materials and Methods

1. Study population and Tissue sampling

Study population comprised 8 patients with type 2 diabetes and chronic periodontitis, 8 patients with chronic periodontitis, and 8 healthy individuals. Marginal gingival tissue samples were obtained during periodontal surgery(including surgical crown lengthening) or tooth extraction. Before surgery, patient's systemic condition(age, sex, blood glucose level, smoking, obesity) and clinical criteria of gingiva were recorded.

According to the patient's systemic condition & clinical criteria of gingiva(gingival

color, gingival bleeding, probing depths, and radiographic evidence of bone resorption), each gingival sample was devided into the three group. Group 1(control, n=8, aged 38.1 [20-48], 5 males and 3 females) is clinically healthy gingiva without bleeding and no evidence of bone resorption or periodontal pockets, obtained from systemically healthy 8 patients. Group 2(periodontitis, n=8, aged 42.8 [38-48], 5 males and 3 females) 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 diseases and conditions[26]. All patient in group 2 was systemically healthy and had more than one pocket ≥5 mm and at least on pocket with ≥4 mm loss of attachment. All gingival samples were obtained from teeth with probing depth ≥5 mm, swelling of the marginal gingiva, and bleeding corresponding to sulcus bleeding index 3 according to Muhlemann and Son[27]. Group 3 (periodontitis & type 2 DM, n=8, aged 58.3 [44-71], 6 males and 2 females) is inflamed gingiva from patients with chronic periodontitis and type 2 diabetes. Patients in group 2 & 3 has similar periodontal condition, but systemically, patients in group 2 was healthy and patients in group 3 was type 2 diabetics. Mean fasting glucose level in group 3 was 142.88 mg/dl and mean 2 hour postprandial glucose level was 212.5 mg/dl. Diabetic control was performed by insulin medication in all 8 patients and, additionally, diet control was also performed in 4 of them. Gingival samples were obtained by similar way described above.

The sample cohort consisted of 8 clinically healthy, 8 inflamed and 8 diabetic patients' inflamed samples from total of 24 subjects. Following surgery, excised tissue specimens were immediately placed on liquid nitrogen and subsequently frozen(-70℃).

2. Protein Isolation and Western blotting

The visualization of Protein levels of MMP-1 in tissue samples was performed by immunoblotting using antibody specific for the species.

For Western blotting, as previously described technique by Cho[28], frozen tissues were homogenized in RIPA lysis buffer(10 mM phosphate buffer, pH 7.4, 10% glycerol, 1% NP-40, 0.1% SDS, 4 mM EDTA, 0.15 M NaCl) with 1:30 diluted protease inhibitor cocktail(Roche). The lysates were sonicated 3 times for 10 seconds and centrifuged at 12,000g for 15 minutes. Protein concentration of supernatant were routinely determined by a Bradford protein assay(Bio-Rad) using BSA as a standard.

Lysates were boiled in SDS sample buffer (1M Tris-Cl (pH6.8), 40% glycerol, 8% SDS, 2%-mercaptoethanol, 0.002% Bromophenole blue). Prepared sample were separated by 10% sodium dodecyl sulfate(SDS)-polyacrylamide gels and transferred to a polyvinylidene difluride membrane.

The membranes were subsequently blocked in Tris-buffered saline(TBS) containing 5% powdered milk and 1% BSA for 1 hours, and then incubated with anti-MMP-1 anti-

body(Sigma, ST. Luis, U. S. A., prepared in rabbits, diluted 1:1000 in TBS/1% BSA) for 1.5 hours at room temperature. The membrane was washed(five times for 5 minutes with 0.2% Tween 20) and incubated with a horseradish peroxidase(HRP)-coupled goat anti-rabbit secondary 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(Amersham, Beckinghamshire, U. K.)

The quantitative analysis(the ratio of MMP-1/β-actin) of MMP-1 was performed using a densitometer(Image Gauge V 3.46, Altura Software, Koshin Graphic Systems, FUJI PHOTO FILM CO). After normalization to β-actin(Abcam® Cambridge Science Park, U. K.) in each sample, level of MMP -1 was expressed as a ratio and the differ- ence of density among 3 group was determined.

3. Statistical Analysis of the Western blot results

All data were presented as means and standard deviation and results were statistically analyzed. The MMP-1 levels among each 3 groups were compared using one way ANOVA followed by Scheffe test.

It was considered to be statistically significant when $P<0.05$. Statistical analysis in this study was performed using appropriate SPSS 12.0 K software(SPSS, Korea).

Ⅲ. Results

The purpose of this study was to compare the relative amounts of MMP-1 in healthy versus diseased gingiva with or without type 2 diabetes mellitus. Antibodies to MMP-1 cross-reacted with 53 kDa of MMP-1 in all 3 groups were observed(Figure 1). In order to quantify detected MMP-1, normalization to β -actin was performed.

Using a densitometer, the quantitative analysis(the ratio of MMP-1/β-actin) of MMP-1 of each sample was performed. The identified MMP-1 levels of gingival tissue samples are presented in Table 1. Average amounts of MMP-1 between 3 groups were compared(Figure 2).

MMP-1 levels of gingival tissue samples were varied within the same group(Table 1). The mean amount of MMP-1 in group 1, 2 and group 3 was 1.009, 1.209 and 1.104, respectively. But, the differences among 3 groups were not statistically significant ($P>0.05$). It is because sample size was too small and various factor of each individuals was not excluded.

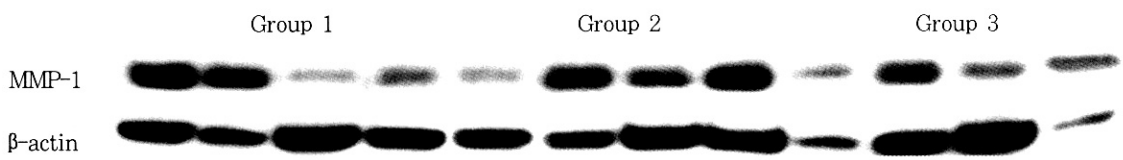

Figure 1. Western analysis of samples for MMP-1. MMP-1 corresponding to molecular weight 53 kDa was expressed in all samples including healthy gingiva, and expression of MMP-1 was increased in Group 2 than Group 1, 3. In order to quantify detected MMP-1, normalization to β -actin was performed.

Table 1. The quantitative analysis(the ratio of MMP-1/β -actin) of MMP-1 of each sample was performed using a densitometer and mean amount of MMP-1 in 3 groups were identified

Sample	Group 1	Group 2	Group 3
1	1.213	1.619	0.947
2	1.213	1.263	1.069
3	0.564	0.770	0.673
4	0.725	1.099	1.240
5	1.264	1.335	1.014
6	1.146	1.408	0.794
7	1.087	1.322	1.601
8	0.860	1.553	1.495
Mean±SD	1.009±0.260	1.210±0.268	1.104±0.324

Group 1: healthy gingiva from systemically healthy patient
Group 2: inflamed gingiva from systemically healthy patient with chronic periodontitis
Group 3: inflamed gingiva from patients with diabetic patient with chronic periodontitis

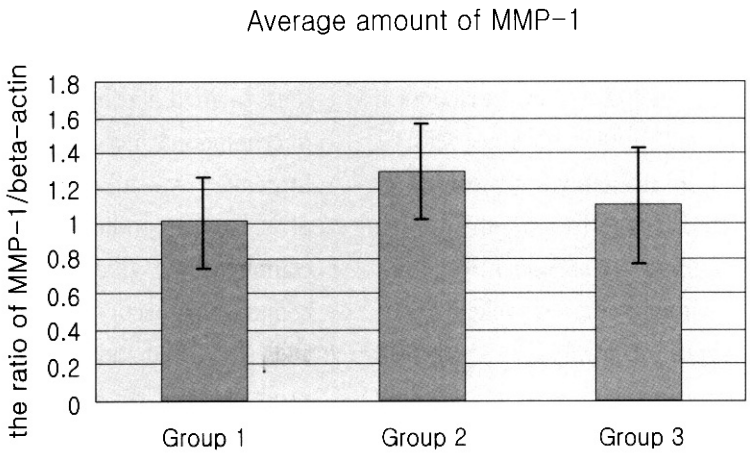

Figure 2. Mean amount(the ratio of MMP-1/β -actin) and standard deviation of MMP-1 in group 1, 2, and 3. In group 2, MMP-1 increased compared to group 1. But the difference was not statistically significant(P>0.05).

Ⅳ. Discussion

Diabetic subjects are 2.3 times more likely to afflicted with periodontal disease than non-diabetic subjects[30] and several cross-sectional studies suggested that the bidirectional relationship between DM and periodontal diseases[14,21,29]. Considering the increasing number of diabetics in the aging population, assessment of MMP system in periodontal tissue and diabetic patients may help to provide appropriate health/oral care to this population.

In present study, MMP-1 levels of in-

flamed gingiva of systemically healthy patient with chronic periodontitis were higher than normal gingiva of healthy patient. Periodontal disease is characterized by loss of collagen fiber and other extracellular matrix constituents in periodontal tissues. Most likely, periodontal tissue destruction is mediated to a significant extent by the host cell-derived MMPs. The neutrophil-type collagenase(MMP-8) secreted by PMN leukocytes may have a more important role than the fibroblast-type collagenase(MMP-1) in the pathological destruction of periodontal connective tissues[24,35]. However, MMP-1 is an important regulator of CT remodelling and is present in high concentration in inflamed regions, including in periodontal disease. Aiba et al.[8] indicated that MMP-1 mRNA increased in inflammatory lesions of adult periodontitis. It was in agreement with the findings obtained by Nomura et al[36]. Meikle et al.[37] immunohistochemically detected the presence of MMP-1, -2, and -3 and TIMP-1 in inflamed gingiva of patients with chronic inflammatory periodontal disease. Moreover the protein amounts and activities of MMP-1 in gingival crevicular fluid (GCF) and gingival tissue were reported to be higher in periodontitis affected sites than in healthy site[11]. Thus, MMP-1 may participate in collagen degradation in advanced periodontal disease. These are consistent with this result.

In this study, although the severity of gingival inflammation in group 2 and 3 are similar, MMP-1 expression is decreased in diabetic patients than systemically healthy periodontal patients. It is assumed that AGE formation of collagen might reduced MMP-1 production and decreased remodelling capacity of fibroblast. Uncontrolled diabetes with peridontal disease frequently exhibit an altered inflammatory cell functions and impaired neutrophil and monocyte /macrophage functions[23,31-33]. Increasing glucose concentrations can reduce the synthesis of collagens and glycosaminoglycans[23]. Both the function of proteins and cells involved in the host defence can be modified by non-enzymatic glycosylation[23,32]. And prolonged exposure to hyperglycemia may result in vascular dysfunction and cellular changes[33]. Most histological studies have demonstrated that small blood vessels of the gingiva in long-term diabetic patients frequently show microangiopathic changes with occlusion and increased vascular thickness. Chappey et al.[32] have indicated that microvascular complications due to long term hyperglycemia may occur due to modified proteins, the so called advanced glycosylation end-products(AGEs). AGEs can induce diabetic collagen cross-links and expansion of ECM, such as hardening of arteries and narrowing of vascular lumina. Potentially AGEs may also induce oxidant stress in the gingiva, resulting in accelerated periodontal tissue destruction[34].

There is little data on MMP expression in human type 2 diabetes, despite of the great interest in the MMP system and periodontal disease. Death et al.[38] showed that high glucose exposure could promote increased MMP-1 expression from two key vascular cell, endothelial cell and macrophage. But Portik-Dobos et al.[39] demonstrated that

MMP induction and activation system exists in human arterial vasculature and that is downregulated in diabetes. They commented that decreased MMP activity(MMP-1, -2 and -9) may contribute to increased collagen deposition and pathological remodeling in diabetes. Rittie et al.[40] studied the influence of collagen glycation on matrix metalloproteinase production by dermal fibroblasts using the model of lattice culture. Contraction of glycated collagen lattices was strongly reduced and fibroblast synthesized lower amount of interstitial collagenase (MMP-1). These results demonstrate that nonenzymatic glycation of type I collagen could greatly impair healing by decreasing the collagen remodeling capacity of fibroblast.

In diabetic patient, further evaluation is needed to investigate the relationship between MMP-1 and other MMP system and the influence of inflammatory mediator (cytokine) on MMP-1 expression. Understanding expression patterns and levels of MMPs in periodontal tissues is essential for monitoring the course of periodontitis as well as the effects of various treatment modalities.

V. Summary

The purpose of this study was to quantify and compare the level of MMP-1 in the healthy or inflamed gingival tissue of patients with or without type 2 diabetic mellitus. We investigated whether mean amount of MMP-1 was changed by chronic periodontitis and type 2 DM.

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 divided into the three group. Group 1(n=8) was clinically healthy gingiva without bleeding and no evidence of bone resorption or periodontal pockets, obtained from systemically healthy 8 patients. Group 2(n=8) was inflamed gingiva from patients with chronic periodontitis. Group 3(n=8) was inflamed gingiva from patients with chronic periodontitis and type 2 diabetes. Tissue samples were prepared and analyzed by Western blotting. The quantitative analysis of MMP-1 was performed using a densitometer and statistically analyzed by ANOVA.

MMP-1 was expressed in all samples and an increased MMP-1 level was observed in group 2 compared to group 1 and decreased MMP-1 level was found group 3 compared to group 2, but the differences among 3 groups were not statistically significant.

In conclusion, this study demonstrated that MMP-1 levels of inflamed gingiva of systemically healthy patient(group 2) were higher than normal gingiva of systemically health patients and although the severity of gingival inflammation in group 2 and 3 were similar, MMP-1 expression was decreased in diabetic patients than systemically healthy periodontal patients.

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-Abstract-

단순만성치주염환자와 2형 당뇨환자의 만성치주염에서 Matrix metalloproteinase-1의 발현양상

이 재 목

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본 연구의 목적은 전신적으로 건강한 치주질환자를 대조군으로 하여 제 2형 당뇨병을 동반한 치주질환자의 치은조직에서 MMP-1의 발현양상을 관찰, 비교하는 것으로 당뇨병을 동반한 경우 MMP-1의 발현양상이 변화되는지의 여부를 연구하였다.

경북대학교 병원 치주과에 내원한 환자 중 검사 및 수술에 동의한 환자로 전신 질환이 없고 부착 소실이 없거나 안정되어 있으며 치은 염증 소견이 없는 환자를 정상조직군, 임상적 치주낭 깊이가 5 mm 이상이고 방사선 사진상 치조골 소실이 분명한 환자를 만성 치주염 환자군, 심각한 전신적 합병증, 감염등의 위험요인이 없고 2형 당뇨병으로 진단받은 환자로서 만성 치주염으로 진단된 환자군 각 8명을 대상으로 하였다. 만성 치주염 환자와 당뇨병을 가진 만성 치주염 환자에서 치은 염증 소견을 보이는 치은조직을 채득하고 액화질소에 넣어 급속 동결고정시킨 후 MMP-1의 발현 양상을 western blot analysis를 통해 관찰하였고, densitometer를 이용하여 상대적 발현을 정량, 각 조직의 β-actin을 이용하여 표준화하여 각 군의 평균치를 비교하였다. 각 군간의 차이를 one way ANOVA test로 분석하였다.

모든 군에서 분자량 53 kDa의 MMP-1에 상응하는 띠가 나타났으며 정량결과 전신적으로 건강한 치주염 환자군에서 MMP-1의 발현이 당뇨병을 동반한 치주염 환자군과 정상조직군의 치은조직에서보다 높게 나타났으나 통계적으로 유의한 차이는 나타나지 않았다.

치은염증의 존재시 MMP-1의 발현이 다소 증가됨을 관찰하였으나 통계적으로 유의한 수준은 아니였으며, 당뇨병을 동반한 치주염 환자군에서 전신적으로 건강한 치주염 환자군에서 보다 MMP-1의 발현이 감소되는 경향을 보였다.

주요어 : 치주염, 당뇨병, Matrix metalloproteinase, MMP-1, 치은조직