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Analysis of Matrix Metalloproteinase (MMP-8 and MMP-2) Activity in Gingival Crevicular Fluid from Children with Down’s Syndrome

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Running title: MMP production in Down’s syndrome patients

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Abstract

Background and Objectives: High levels of colonization by periodontopathic bacteria and a high prevalence of chronic inflammatory periodontal disease have been reported in children with Down's syndrome. Matrix metalloproteinases (MMPs) are mediators of extracellular matrix degradation and remodeling, and are deeply involved in the course of periodontal disease. To clarify the relationship between Down's syndrome and periodontitis, we investigated levels of MMP-2 and MMP-8 in gingival crevicular fluid (GCF) and detection of periodontopathic bacteria from subgingival plaque.

Materials and Methods: GCF and plaque samples were isolated from central incisors. Levels of MMPs were evaluated by ELISA and periodontopathic bacteria were detected by polymerase chain reaction.

Results: Levels of MMP-2 and MMP-8 in Down's syndrome patients were higher than those in healthy controls. In the Down’s syndrome group, increases in these MMPs were observed in GCF from patients with an Oral Hygiene Index score of less than 2 and in GCF from bleeding on probing-negative sites. Detection rate of periodontopathic bacteria in Down's syndrome patients was higher than that in the controls. MMP-2 levels in sites harboring Porphyromonas gingivalis or Aggregatibacter (Actinobacillus) actinomycetemcomitans were lower than those without these microorganisms.

Conclusion: These results suggest an increase in MMP-2 and MMP-8 in Down's syndrome patients, regardless of whether inflammation of periodontal tissue is present or not.
INTRODUCTION

Down’s syndrome (DS) is an autosomal chromosomal anomaly resulting from trisomy of chromosome 21 (1). Several reports have described a high prevalence of chronic inflammatory periodontal disease in children with DS (2-5). In DS, the frequency of periodontitis lesions with alveolar bone loss is higher around the mandibular incisors than around the first maxillary molars (4). Several systemic deficiencies, including defective neutrophil chemotaxis and a reduction in IgM production have been reported in DS (2, 6).

Matrix metalloproteinases (MMPs) are a group of endogenous proteinases that contribute to the degradation of the extracellular matrix and basement membrane components (7). MMPs are believed to act as mediators of extracellular matrix degradation and remodeling and be deeply involved in the course of periodontal disease (8). Human periodontal tissue cells such as fibroblasts, keratinocytes, macrophages, polymorphonuclear leukocytes (PMNs) and endothelial cells are all capable of expressing and releasing MMPs, and a number of studies have suggested that MMP expression and levels are good indicators for a clinical diagnosis of periodontal disease (9, 10). MMP-2 is a gelatinase which mainly cleaves type IV collagen and degrades native fibrillar interstitial collagens (11-13). Gelatinases are believed to play an important role in tissue destruction in periodontitis (14-18). MMP-8 is a PMN-type collagenase involved in periodontal tissue degradation in periodontal disease (10). PMN-type collagenase is stored in specific granules within PMNs, and is released rapidly when PMNs are triggered. Sorsa et al. (19) demonstrated that the major collagenase involved in periodontitis was MMP-8.

Recent studies have reported significantly higher levels of MMP-8 in saliva in DS children than in controls (20). MMP-2 levels in cultured gingival fibroblasts from Down’s
syndrome patients were significantly higher than in controls (21). In addition, *Porphyromonas gingivalis*, *Tannerella forsythia*, *Treponema denticola*, *Prevotella nigrescens*, *Campylobacter rectus*, *Aggregatibacter (Actinobacillus) actinomycetemcomitans* and *Capnocytophaga* were detected with significantly greater frequency in Down’s syndrome patients (22, 23). Periodontal microorganisms have been reported to induce release of MMP-8 and MMP-9 in the gingival crevice via activation of host immune response (24, 25). However, the reason for this increase in level of MMPs in DS patients remains to be clarified.

The aim of this study was to investigate MMP-2 and MMP-8 expression in gingival crevicular fluid (GCF) in DS patients without periodontitis to clarify which factors were involved in increased levels of MMPs in DS patients.

**MATERIALS AND METHODS**

**Patients.** Fourteen children with DS (9 boys and 5 girls; mean age 12.7 years, range 6 to 18 years) and 14 healthy children (8 boys and 6 girls; mean age 12.2 years, range 7 to 17 years) were enrolled in this study. The DS children were selected at random from the Department of Pediatrics, Dental Faculty, Tokyo Dental College and a school for physically handicapped children at Chiba University. None of the DS patients or healthy children were on any medication over the 3-month period prior to sampling, and were otherwise healthy. Informed consent was obtained from the parents of the children, and approval for the study from the Tokyo Dental College Ethical Committee.

**Clinical Examination of Periodontal Status.** All clinical examinations were performed after collection of GCF. Clinical examination consisted of evaluation according to the Oral
Hygiene Index (OHI, (26)), probing pocket depth (PD), and bleeding on probing (BOP). After GCF collection, PD and BOP in all teeth were recorded and the presence or absence of gingivitis determined. PD was defined as the distance from the free gingival margin to the base of the periodontal pocket for each tooth. BOP was scored as presence or absence of bleeding within 30 sec of probing. Any patients manifesting disease at the time of examination were excluded from the study.

**Analysis of metalloproteinase production** Maxillary central incisors were chosen for sampling of GCF as this reduced the risk of contamination of saliva. GCF was collected by means of durapore filter membranes (pore size: 0.22 µm; Millipore Corp., Bedford, MA, USA) without touching the marginal gingival. GCF samples were collected first to avoid any tendency the site might have for bleeding on/after plaque sampling and probing. The site was isolated with cotton rolls, and the surface gently dried to avoid contamination by saliva. A durapore membrane was inserted 1 mm into the sulcus and left in place for 5 minutes. The membrane was then placed in a microcentrifuge tube containing 500 µl phosphate buffered saline (pH 7.4) and stored at -80°C until use in determining MMP-2 and MMP-8 levels. Where there was visible contamination with blood, the strips were discarded. In the case of visible contamination, GCF samples were collected on another day.

The samples were thawed at room temperature before determination of MMP levels. Levels of MMP-2 and MMP-8 were determined using the Human Biotrack ELISA system (GE Health Care, Tokyo, Japan) according to the manufacturer’s instructions, and the results were expressed as ng/membrane. The detection limits for MMP-2 and MMP-8 with the kits used were given by the manufacturer as 1.5-24 ng and 0.25-4 ng, respectively.
Detection of bacteria from subgingival plaque. Subgingival plaque was collected from the maxillary central incisors. After careful removal of supragingival plaque deposits, sampling sites were isolated with cotton rolls, gently air-dried and subgingival plaque was collected with 2 sterile paper points. Samples were isolated from the maxillary central incisors, and 3-4 samples were collected from each person. Paper points from all 3 experimental sites were pooled in 200 µl boiling buffer containing 20mM Tris-HCl buffer (pH8.5), 2mM EDTA and 1% triton X-100. The suspension was treated at 100°C for 10 min and supernatant was obtained by centrifugation. Genomic DNA from the supernatant was isolated by phenol extraction. Polymerase chain reaction (PCR) was used to detect microorganisms. *P. gingivalis* and *A. actinomycetemcomitans* were detected according to the method of Ashimoto et al (27). The primers used for *Treponema denticola* were 5'-GCGAACAGATATTTGACATAACTAGGAAG-3' and 5'-CTATTCTTTCGCTTGACCATATTATTGTCC-3'; amplicon length was 155 bp. The primers used for *Campylobacter rectus* were 5'-AACTTCTCTATCCGATTACCGCTTAAG-3' and 5'-TACTAGCCAAGGCATCCACCATTAC-3'; amplicon length was 203 bp. Primers were designed based on the 16S and 23S rRNA sequences of *T. denticola* and *C. rectus*, respectively, in GenBank at the National Center of Biotechnology Information (Bethesda, MD, USA). The specificity of these primers was confirmed against 39 oral bacterial species. PCR for these microorganisms was performed as previously reported for *Fusobacterium nucleatum/periodonticum* (28). Obtained products were analyzed using 2% agarose gel electrophoresis.

Statistical analysis. Quantitative differences between the DS children group and the
healthy control group were determined using the Student \( t \)-test. Differences in MMPs were determined by a one-way ANOVA followed by the Student-Newman-Keuls test to make multiple comparisons among groups in DS patients separated by oral condition and in controls.

**RESULTS**

**Clinical findings** A summary of the OHI, PD and BOP in both the DS and control groups is given in Table 1. In the DS group, one patient had 5-mm deep gingival pockets. No significant difference was found in PD between the DS patients and the controls. OHI in DS patients was significantly higher (\( p < 0.05 \)) than that in the controls. The level of BOP-positive sites was significantly higher (\( p < 0.05 \)) in the DS patients than in the controls. The mean and SD of probing depth of sampling sites in DS patients and controls were 2.9 ± 0.58 and 2.9 ± 0.56, respectively. These values were close to average of full mouse as shown in Table 1.

**Production of MMP-2 and MMP-8** In the GCF samples, levels of MMP-2 and MMP-8 were significantly higher in the DS patients (\( p < 0.01 \)) than in the controls (Fig 1). It is possible that this difference in MMP levels between the two groups was influenced by OHI and BOP values. Therefore, MMP levels were compared after dividing the DS patients based on levels of OHI and BOP. Fig. 2 shows levels of MMP-2 revealed after dividing the DS patients into high and low OHI groups. When the DS group was separated into two groups, one with an OHI score of 2 or less and the other with a score of more than 2, levels of MMP-2 and MMP-8 in both groups were significantly higher than those in control patients.
No statistically significant difference was observed in MMP level between high and low OHI in DS patients (p < 0.01). Levels of MMP2 and MMP-8 at BOP-positive and -negative sites in DS patients were higher than those in the control patients. No statistically significant difference, however, was found in MMP2 and MMP-8 levels between BOP-positive and -negative sites in DS patients (Fig. 3, p<0.001).

**Bacterial Prevalence in DS and Controls** We compared prevalence of subgingival bacterial species between the DS group and the controls (Table 3). The detection rate for all periodontopathic bacteria tested was higher in DS patients than in the control group, although this was only statistically significant for *C. rectus*. MMP levels in DS patients with or without each periodontopathic bacterium are shown in Fig. 4. Levels of MMP-2 were significantly lower in DS patients harboring *P. gingivalis* or *A. actinomycetemcomitans* than in those who were not (P<0.001).

**DISCUSSION**

A high susceptibility to and prevalence of periodontitis in DS patients has been reported (29). However, the reason for this high susceptibility and prevalence remains to be clarified. In the present study, we showed an increase in levels of MMP-2 and MMP-8 in DS patients compared with in healthy controls. This result agrees with that of a previous report in DS patients (20, 21). MMP-2 and MMP-8 were reported to show an increase in chronic periodontitis and aggressive periodontitis patients (18, 19, 30). It is possible that an increase in MMPs in gingival tissue induces periodontal destruction. In the present study, we evaluated levels of MMP-2 and -8 using a membrane filter and expressed as ng/membrane. An increase in the vascular permeability of blood vessels following gingival stimulation was reported (31). This suggests that MMP levels in GCF are influenced by
stimulation during sampling. Therefore, MMP levels in unstimulated GCF may better reflect MMPs levels in gingival tissue. Although collection of GCF using filter paper is frequently used (30, 32, 33), further study employing measurement of real concentrations of MMPs in unstimulated GCF is required.

In the present study, the ratio of BOP-positive sites in DS patients was somewhat higher than that in healthy controls, indicating gingival inflammation in DS patients; although average PD in DS patients was the same as that in the controls. MMP-8 is a neutrophil-derived collagenase. It is possible that the higher levels of MMP-2 and MMP-8 seen in DS patients in this study were a result of inflammation caused by poorer oral hygiene than that in the healthy controls. To eliminate the effects of level of oral hygiene or inflammation on increase in MMP production in DS patients, the DS patients were divided by OHI or BOP score at the sampling site. DS patients with an OHI score of less than 2 also showed significantly higher levels of MMP-2 and MMP-8 than healthy subjects. Levels of these MMPs showed no statistically significant difference between patients with an OHI score of less than 2 and those with a score of 2 or more in DS patients. MMP-2 and MMP-8 levels in DS patients at BOP-negative sites were significantly higher than those in the controls. No difference was found in these levels between BOP-positive and -negative sites in DS patients. These results suggest a difference in production of MMP-2 and MMP-8 in DS patients, regardless of inflammatory response. MMP-2 was reported to be constitutively expressed at low levels in the periodontium (34). The increase in MMP-2 observed in this study agrees with that seen in a previous report by Komatsu et al. (21). They reported an increase in MMP-2 by increase in MMP-2 mRNA expression in fibroblasts from DS patients in vitro. It is possible that there is a difference in MMP-2 production between DS patients and healthy controls. MMP-8 levels usually reflect inflammation level, as MMP-8 is a
neutrophil-derived proteinase (8). The present results showed no significant difference in MMP-8 levels between DS patients with an OHI score of less than 2 and an OHI score of 2 or more, or between BOP-positive and BOP-negative sites in DS patients. This suggests that a factor other than infiltration by neutrophils during inflammation is involved in inducing an increase in levels of MMP-8. These observations suggest that level of oral hygiene and inflammation are not the only cause of increase in MMPs in DS patients.

Detection rates of periodontopathic bacteria in DS patients were higher than those in the control patients. The profile was similar to that in a report by Amano et al. (22). Interestingly, if the DS patients were separated by detection of microorganisms, levels of MMP-2 and MMP-8 were lower in DS patients harboring A. actinomycetemcomitans and P. gingivalis. Differences in MMP-2 were statistically significant (P < 0.001). Lipopolysaccharide of A. actinomycetemcomitans was reported to increase production of MMP-2 in fibroblasts (35). P. gingivalis significantly up-regulated MMP-2 and MMP-9 mRNA expression by oral epithelial cells (36). The present results disagree with those of these earlier reports. However, Garlet et al. (37) reported a correlation between an increase in MMP-1, -2, and -9 and interleukin 1β, tumor necrosis factor α and interferon γ in A. actinomycetemcomitans-infected mice. The expression of MMP-2 in their study showed a progressive increase from 24 hr until 15 days after infection, with a decrease at 30 and 60 days. P. gingivalis and A. actinomycetemcomitans have factors which induce an immunoresponse, including lipopolysaccharide and immunosuppressive factors (38-40). Immune dysfunction was also reported in Down’s syndrome (41). It is possible that immunomodulation by these microorganism or immune dysfunction affected production of MMP-2. However, further analysis with a larger number of samples is required to confirm this reduction. Levels of MMP-8 in subjects infected by periodontopathic bacteria were
slightly lower or the same as those in subjects free of infection. Most MMP-8 in periodontal lesions is released from neutrophils, and MMP-8 has been reported to be associated with severity of chronic periodontitis (10). Defect in and dysfunction of bactericidal capacities have been described (6, 42). These types of dysfunction may affect levels of MMP-8 in DS patients. In addition, colonization by periodontopathic bacteria showed no effect on MMP levels. This also suggests that inflammation caused by periodontopathic bacterial colonization does not involve an increase in MMP-2 and MMP-8 in DS patients. Colonization by P. gingivalis and A. actinomycetemcomitans have been reported to induce immune response in many studies (43). This discrepancy also suggests immune dysfunction. To clarify the dysfunction of the immune response in DS patients, further analysis is required comparing condition of periodontal tissue and immune response in DS patients.

The present results showed an increase in MMP-2 and MMP-8 in DS patients. Recently, Bildt reported that pro-MMP-2, active MMP-2 and the MMP-2 complex were higher in patients with chronic periodontitis than in healthy subjects, suggesting the involvement of these metalloproteinases in periodontitis (44). Involvement of MMP-8 production in periodontitis has also been reported (8). However, the present results indicate an increase in MMPs, regardless of whether inflammation of gingival tissue is present or not. Taken together with the results of earlier studies, the results of the present study suggest that an increase in MMP-2 and MMP-8 levels is involved in higher susceptibility to and prevalence of periodontitis in DS patients, although further analysis with a larger population will be required for clarification.

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There is no conflict of interest with this paper.
Reference


35. Bodet C, Andrian E, Tanabe S, Grenier D. *Actinobacillus actinomycetemcomitans*


Table 1. Clinical data (mean ± SD) from DS patients and controls

<table>
<thead>
<tr>
<th></th>
<th>Down’s (n = 14)</th>
<th>Control (n = 14)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oral Health Index (OHI)</td>
<td>1.95 ± 1.1</td>
<td>1.68 ± 0.62</td>
<td>$P &lt; 0.05$</td>
</tr>
<tr>
<td>Average Probing Depth (PD) (mm)</td>
<td>2.89 ± 0.43</td>
<td>2.57 ± 0.44</td>
<td>N. S.</td>
</tr>
<tr>
<td>Number of subjects with PD more than 5 mm</td>
<td>1</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Number of teeth with BOP (+)/total teeth (%)</td>
<td>14.9 ± 16.7</td>
<td>3.51 ± 2.82</td>
<td>$P &lt; 0.05$</td>
</tr>
</tbody>
</table>
Table 2  Detection rate (%) of specific periodontal pathogens in subgingival plaque samples

<table>
<thead>
<tr>
<th>Species</th>
<th>Down's Syndrome (n=14)</th>
<th>Controls (n=14)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. gingivalis</em></td>
<td>42.9</td>
<td>21.4</td>
</tr>
<tr>
<td><em>A.. actinomycetemcomitans</em></td>
<td>64.3</td>
<td>57.1</td>
</tr>
<tr>
<td><em>C. rectus</em></td>
<td>35.7*</td>
<td>0</td>
</tr>
<tr>
<td><em>T. denticola</em></td>
<td>35.7</td>
<td>7.1</td>
</tr>
</tbody>
</table>

*p<0.05 by Fisher's exact test
Figure legend

Fig. 1. MMP-2 and MMP-8 levels (ng/membrane ± SE) in GCF.

A. MMP-2 levels in GCF from DS patients and controls
B. MMP-8 levels in GCF from DS patients and controls.
C. MMP-2 levels in GCF by BOP. ($p < 0.001$ compared to control)
D. MMP-8 levels in GCF by BOP. ($p < 0.001$ compared to control)
E. MMP-2 levels (ng/membrane ± SD) in GCF by OHI. Patients were separated into two group by score of OHI 2 and less or more than 2. ($p < 0.001$ compared to control)
F. MMP-8 levels (ng/membrane ± SD) in GCF by OHI. Patients were separated into two group by score of OHI 2 and less or more than 2. ($p < 0.001$ compared to control)

A and B were analyzed by t-test (\(^*p < 0.001\))
C-E was analyzed by one-way repeated measures ANOVA for inter-group comparisons.
Student-Newman-Keuls test was used for multiple comparisons ($\$p < 0.001$).

Fig. 2. Comparison of MMP-2 and MMP-8 production (ng/membrane ± SE) based on detection of periodontopathic bacteria ($*p < 0.001$ by t-test compared with control).
Fig. 2

MMP-2

- P. gingivalis
- A. actinomycetemcomitans
- T. denticola
- C. rectus

MMP-8

- P. gingivalis
- A. actinomycetemcomitans
- T. denticola
- C. rectus