Introduction

The initial phase of orthodontic tooth movement usually involves many inflammatory-like reactions characterized by vascular changes and migration of leucocytes out of the periodontal ligament (PDL) capillaries (Rygh et al., 1986). These changes lead to cellular activation and expression of biologically active substances, such as enzymes and cytokines, within the periodontium (Sandy et al., 1992; Iwasaki et al., 2001; Redlich et al., 2001). Recent studies have shown that the molecular equilibrium between collagen synthesis and degradation in the periodontium is disturbed after force application (Kyrkanides et al., 2000). However, the mechanism which converts mechanical stress to cellular response and finally to tooth movement is poorly understood.

In order to monitor orthodontic tooth movement non-invasively in humans, changes in the profile and levels of various cytokines, growth factors, proteoglycans, and enzymes in gingival crevicular fluid (GCF) have been examined. The levels of prostaglandins, interleukins, tumour necrosis factor-α, transforming growth factor-β, and epidermal growth factor in GCF have been demonstrated to be responsive to orthodontic force in humans (Grieve et al., 1994; Uematsu et al., 1996). Cytokine levels in GCF have also been shown to be less responsive to orthodontic force in adults than in juveniles (Ren et al., 2002). Individual differences in interleukin-1β and interleukin-1 receptor antagonist ratios in GCF have been found to correlate with interindividual differences in the amount of tooth movement (Iwasaki et al., 2001). Elevated levels of proteoglycans, such as chondroitin sulphate, in human GCF during orthodontic tooth movement have been suggested to indicate active alveolar bone and PDL remodelling (Baldwin et al., 1999; Waddington and Embery, 2001). Total collagenase activity, i.e. type I collagenolysis, in the GCF of orthodontic patients undergoing fixed appliance treatment has been shown to be 10-fold that of control GCF (Sorsa et al., 1992). Recently, increased levels of cathepsin B in human GCF during orthodontic tooth movement have been demonstrated (Sugiyama et al., 2003).

Matrix metalloproteinase-1 and -8 in gingival crevicular fluid during orthodontic tooth movement: a pilot study during 1 month of follow-up after fixed appliance activation

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SUMMARY The role of matrix metalloproteinases (MMPs) in response to mechanical forces in orthodontic tooth movement has only been partially clarified. In the present in vivo pilot study, the presence, levels, and degree of activation of MMP-1 and -8 were measured daily for 1 month in gingival crevicular fluid (GCF) of patients treated with orthodontic fixed appliances. GCF samples were collected from five orthodontic patients and three controls from one upper or lower central incisor or from one upper canine before fixed appliance activation and every 24 hours for 1 month thereafter. The molecular forms and activation degrees of MMP-1 and -8 in GCF were analysed by Western blotting, and MMP-8 levels determined by immunofluorometric assay (IFMA).

IFMA revealed, during the study period, on average 12-fold higher levels (56 ± 50 versus 4.6 ± 4 µg/l) of MMP-8 in orthodontic GCF than in control GCF. The MMP-8 levels in orthodontic GCF were lower than those detected in gingivitis and periodontitis GCF, but significantly higher than in control GCF. IFMA analysis was confirmed by Western blot analysis showing elevated MMP-8 levels from orthodontic GCF relative to control GCF. Forty-one per cent of total MMP-8 immunoreactivities were high-molecular weight complexes (>100 kDa), 32 per cent in the 75 kDa pro-polymorphonuclear (PMN)-MMP-8 form, 14 per cent in the 60 kDa active-PMN-MMP-8 form, and 13 per cent in the 55 kDa fibroblast-type pro-MMP-8 form. In the GCF of orthodontic patients no MMP-1 immunoreactivities were detected. MMP-8 and -1 levels in the control GCF were low and not detectable.

These results demonstrate that in vivo in human GCF, elevation and partial activation of multiple species of PMN- and fibroblast-type MMP-8 reflect periodontal remodelling during orthodontic tooth movement.
Matrix metalloproteinases (MMPs) are a gene family of at least 25 proteolytic enzymes contributing to the degradation and remodelling of the extracellular matrix (Kyrkanides et al., 2000). MMP-8 (human neutrophil collagenase, collagenase-2) is produced not only by polymorphonuclear (PMN) leucocytes, but also by certain non-PMN lineage cells, such as gingival fibroblasts, bone and plasma cells (Hanemaaijer et al., 1997; Wahlgren et al., 2001; Sasano et al., 2002). MMP-8 is the most effective in hydrolysing type I collagen (Hasty et al., 1987) and is the major interstitial collagenase in inflamed human gingiva (Kiili et al., 2002). MMP-1 (interstitial collagenase, collagenase-1), synthesized and secreted ubiquitously by connective tissue cells (fibroblasts) and macrophages, is more often associated with normal tissue remodelling, and hydrolyses mainly type III collagen (Welgus et al., 1981).

Whereas morphological and histochemical changes in PDL cells have been thoroughly investigated (Bolcato-Bellemin et al., 2000), few studies have focused on the in vivo and in vitro expression of MMPs in the periodontium in response to mechanical force. Total collagenase activity in the GCF of orthodontic patients treated with fixed appliances has been shown to be 10-fold that of control GCF (Sorsa et al., 1992). Human gingival and periodontal fibroblasts have shown increased MMP-1 and -2 production under continuous and cyclic stretch in vitro, while the expression of MMP-9 and MT1-MMP in these cells was not induced by mechanical stress (Carano and Siciliani, 1996; Bolcato-Bellemin et al., 2000). The effect of orthodontic force on MMP-1 mRNA and MMP-1 production has been analysed in dogs during experimental tooth movement: orthodontic force significantly elevated both MMP-1 mRNA and protein activity in the gingival tissues (Redlich et al., 2001). Recently, increased expression of both MMP-8 and -13 mRNA in the PDL of rats during active tooth movement has been demonstrated (Takahashi et al., 2003). In addition, orthodontic tooth movement has been shown to be inhibited with the use of MMP inhibitors in mice (Holliday et al., 2003).

This in vivo pilot study measured the presence, molecular forms, levels, and activation degree of MMP-1 (collagenase-1) and MMP-8 (collagenase-2) daily during 1 month in the GCF of patients undergoing orthodontic treatment with fixed appliances.

**Materials and methods**

**Patients and samples**

GCF samples were collected from five orthodontic patients (three females and two males, aged 11, 12, 13, 13, and 36 years). All were undergoing fixed appliance treatment (Mini-mat brackets, 0.018 inch slot; Ormco, Orange, California, USA) at a private dental office in Helsinki, Finland. An initial aligning archwire (Respond, Ormco) was placed at the same visit, when the GCF sample collection was started. The samples came from the upper or lower central incisor or from the upper canine just before and every 24 hours after fixed appliance activation for 1 month. All patients were healthy, except for one with type 1 diabetes which was under control. Patients with controlled diabetes have been found to exert collagenase activities or MMP-8 levels in their GCF comparable with those of healthy controls (Ryan et al., 1999). The GCF control samples were collected from one upper central incisor from each of three healthy females (mean age 36 years) who had not undergone orthodontic treatment. Both orthodontic and control patients had clinically healthy periodontal tissues, as assessed by careful clinical, microbial, and radiological examination of the periodontium. For comparison, GCF samples were also collected from patients with gingivitis and periodontitis, as described by Mäntylä et al. (2003). The protocol for collecting GCF samples was approved by the ethics committee of the Institute of Dentistry, University of Helsinki, Finland. All subjects gave their informed consent.

**GCF collection**

After the initial examination, GCF samples were collected from the periodontal sulcus. The surface of each tooth was dried gently with air and kept dry with cotton wool rolls. Two filter paper strips were placed into the sulcus for 3 minutes. The GCF flow volume was measured by weighing the strips in polypropylene tubes (Mettler AJ 100/GWB scale, Mettler, Greifensee, Switzerland). The absorbed fluid was eluted from each strip into 25 µl of 0.2 M NaCl, 10 mM CaCl₂, 50 mM Tris-HCl, pH 7.5. The samples were then frozen and kept at –20°C until assayed.

**MMP-8 immunofluorometric assay (IFMA)**

Concentrations of MMP-8 in the collected GCF samples were determined using a time-resolved IFMA. Monoclonal MMP-8-specific antibodies 8708 and 8706 served as the catching and tracing antibody, respectively. The tracer antibody was labelled with europium chelate. The assay buffer contained 20 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 5 mM CaCl₂, 50 µM ZnCl₂, 0.5 per cent bovine serum albumin, 0.05 per cent sodium azide, and 20 mg/l diethylaminetrimetaphenacetic acid (DTPA). The samples were diluted in assay buffer and incubated for 1 hour, followed by incubation for 1 hour with the tracer antibody. Enhancement solution was added, and after 5 minutes fluorescence was measured with a 1234 Delfia Research fluorometer (Wallac, Turku, Finland). The results were expressed as µg/l. The specificity of the
monoclonal antibodies against MMP-8 corresponded to that of polyclonal MMP-8 (Hanemaaijer et al., 1997; Sorsa et al., 1999; Mäntylä et al., 2003).

Western blot analysis

The molecular forms of MMP-1 and -8 in the GCF of orthodontic patients and the controls were analysed by Western blotting. The samples were treated with Laemmli buffer at pH 6.8, and heated for 5 minutes at 100°C. Low-range pre-stained sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) standards (Bio-Rad, Richmond, California, USA) served as a molecular weight marker. The samples were separated on 8–10 per cent SDS–PAGE and electrophoretically transferred to nitrocellulose membranes. To block non-specific binding sites on the nitrocellulose membranes, they were diluted in 3 per cent gelatin in 10 mM Tris-HCl, pH 7.5, 0.05 per cent Triton X-100, 22 mM NaCl (TTBS) for 1 hour at 37°C, after which the membranes were washed four times with TTBS, for 15 minutes each. The membranes were incubated with monoclonal mouse anti-human MMP-1 (1:250 dilution in TTBS; Oncogene, Boston, Massachusetts, USA) and polyclonal rabbit anti-human MMP-8 (Hanemaaijer et al., 1997) (1:500 dilution in TTBS), at 20°C for 24 hours. After four 15 minute washes with TTBS, the membranes were incubated with goat anti-mouse IgG for MMP-1 and goat anti-rabbit IgG for MMP-8 alkaline phosphatase conjugates (1:1000 and 1:500 dilutions in TTBS, respectively; Sigma, St. Louis, Missouri, USA) for 1 hour. After four washings with TTBS for 15 minutes, one 15 minute wash with 10 mM Tris-HCl, pH 7.5, 22 mM NaCl, and one 15 minute wash with alkaline phosphatase buffer (100 mM NaCl, 5 mM MgCl2, 100 mM Tris, pH 9.5), the immunoblots were visualized by the addition of nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate diluted in N-N-dimethyl formamide (Sigma) in 100 mM Tris-HCl, 5 mM MgCl2, 100 mM NaCl, pH 9.5 (Ingman et al., 1994). If required, the molecular analyst/PC program of the imaging densitometer (model GS-700, Bio-Rad) was used for quantitative analysis of the Western blots (Kiili et al., 2002). Human PMN and rheumatoid synovial culture media were used as positive controls for MMP-8 and -1, respectively (Hanemaaijer et al., 1997; Kiili et al., 2002).

Results

Clinical parameters and GCF flow

For all the studied sites, plaque accumulation was minimal throughout the investigation and gingival health was excellent. Probing depths were less than 2 mm at all times during the experiment. GCF flow was not significantly changed by orthodontic treatment (data not shown).

MMP-8 (collagenase-2) levels and molecular forms in the GCF of orthodontic patients and controls during a follow-up period of 1 month

IFMA revealed, on average, 12-fold higher (56 ± 50 versus 4.6 ± 4 µg/l) levels of MMP-8 in orthodontic GCF than in the control GCF during the 1 month of follow-up testing (Figure 1). MMP-8 levels in orthodontic GCF were higher than those in healthy control GCF, but lower than MMP-8 levels in gingivitis and periodontitis GCF (Mäntylä et al., 2003). The IFMA analysis was confirmed by Western blotting, which also showed more MMP-8 immunoreactivity in the GCF of orthodontic patients than in control GCF during the whole month (Figure 2A). The experimental samples revealed that 41 per cent of the total MMP-8 immunoreactivity was of a high-molecular weight complex (>100 kDa). The 75 kDa pro-PMN-MMP-8, i.e. the non-activated PMN leucocyte MMP-8, represented 32 per cent of the total MMP-8 immunoreactivity. Of the total MMP-8, 14 per cent was in the 60 kDa active-PMN-MMP-8 form and 13 per cent in the 55 kDa fibroblast-type pro-MMP-8 species (Table 1). The immunoreactivities of 60 kDa...
active-PMN-MMP-8 were smaller than those of high-molecular weight complexes (>100 kDa) during the study period. Only on days 12 and 22 from the activation of the fixed appliances were 60 kDa active-PMN-MMP-8 levels higher than those of high-molecular weight complexes (>100 kDa) (Figure 2A). MMP-8 levels in control samples were hardly detectable with Western blotting (Figure 2C).
Moreover, increased expression of both MMP-8 and -13 has also been reported in human orthodontic patients. The levels of these mediators in GCF have been well demonstrated to be responsive to orthodontic forces. The levels of MMP-1 in the GCF of orthodontic patients during 1 month of follow-up showed lower in orthodontic GCF than in adult periodontitis GCF. The percentage of 60 kDa active-PMN-MMP-8 was also lower in orthodontic GCF than in adult periodontitis GCF (14 versus 43.9 per cent) (Kiili et al., 2002). The MMP-8 IFMA assay indicated total levels of MMP-8 immunoreactivities in orthodontic GCF were in >100 kDa high-molecular weight complexes: 41 versus 5.6 per cent detected in adult periodontitis GCF. The percentage of 60 kDa active-PMN-MMP-8 was also lower in orthodontic GCF than in adult periodontitis GCF (14 versus 43.9 per cent) (Kiili et al., 2002). 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<table>
<thead>
<tr>
<th>Bands</th>
<th>Molecular weight (kDa)</th>
<th>Mean</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complex</td>
<td>&gt;100</td>
<td>41</td>
<td>0–65.7</td>
</tr>
<tr>
<td>Pro-PMN-MMP-8</td>
<td>75</td>
<td>32</td>
<td>0–45.5</td>
</tr>
<tr>
<td>Active-PMN-MMP-8</td>
<td>60</td>
<td>14</td>
<td>0–58.2</td>
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<tr>
<td>Fibroblast-type pro-MMP-8</td>
<td>55</td>
<td>13</td>
<td>0–28.5</td>
</tr>
<tr>
<td>Fragments</td>
<td>&lt;30</td>
<td>0</td>
<td>0–30</td>
</tr>
</tbody>
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PMN, polymorphonuclear.

### MMP-1 (collagenase-1) levels in the GCF of orthodontic patients during 1 month of follow-up

MMP-1 levels in orthodontic (Figure 2B) and control (Figure 2D) GCF samples were not detectable by Western blotting.

### Discussion

Previous research has suggested that local mediators, such as prostaglandins, interleukins and growth factors, play an important role in bone remodelling induced by orthodontic forces. The levels of these mediators in GCF have been well demonstrated to be responsive to orthodontic force in humans. However, only a few studies have focused on the MMP-dependent remodelling of human periodontal tissues during orthodontic tooth movement (Sorsa et al., 1992; Redlich et al., 2001). The present pilot in vivo study demonstrated significantly higher MMP-8 levels in the GCF of human orthodontic patients when compared with control GCF during a 1 month time period.

These findings on MMP immunoreactivity are in agreement with previous results that have shown significantly elevated total collagenase activity in the GCF of orthodontic patients at 24 hours after retractor activation (Sorsa et al., 1992). In the present study, orthodontic treatment did not affect GCF flow. Similarly, an in vivo study in dogs during experimental tooth movement showed significantly increased mRNA MMP-1 levels in gingival tissue (Redlich et al., 2001). Moreover, an in vitro study on human PDL and gingival fibroblasts demonstrated a significant increase in the levels of MMP-1 and -2 (Bolcato-Bellemin et al., 2000). Recently, increased expression of both MMP-8 and -13 mRNA in the PDL of rats during active tooth movement was found in vivo (Takahashi et al., 2003). These results on MMP-1 and -8 activities are consistent with the present findings that showed low immunoreactivities for MMP-1 in the GCF of some patients, although the role of MMP-8 in humans seems to be more essential than that of MMP-1 during orthodontic tooth movement. Furthermore, orthodontic tooth movement was delayed or inhibited with the use of MMP inhibitors (Holliday et al., 2003).

During orthodontic treatment with fixed appliances, a clinically healthy periodontium with no plaque accumulation is important. In the present study, the patients exposed to orthodontic force and the controls all had a clinically healthy periodontium. Various isoenzyme forms of MMP-8 are derived from multiple host cellular sources; PMN degranulate highly glycosylated 75–80 kDa MMP, and various mesenchymal, epithelial, plasma, and bone cells all de novo express 40–55 kDa less glycosylated MMP-8 species (Weiss, 1989; Hanemaaijer et al., 1997; Sasano et al., 2002). The pattern of MMP-8 species in orthodontic GCF also differs from MMP-8 immunoreactivities detected in GCF and dental plaque extracts from chronic adult periodontitis patients (Kiili et al., 2002). The orthodontic GCF showed low levels of immunoreactivity for 80 kDa pro-PMN, 55 kDa fibroblast-type pro-MMP-8 or <30 kDa fragments, as in adult periodontitis GCF (Kiili et al., 2002). Most of the MMP-8 immunoreactivities in orthodontic GCF were in >100 kDa high-molecular weight complexes: 41 versus 5.6 per cent detected in adult periodontitis GCF. The percentage of 60 kDa active-PMN-MMP-8 was also lower in orthodontic GCF than in adult periodontitis GCF (14 versus 43.9 per cent) (Kiili et al., 2002). The MMP-8 IFMA assay indicated total levels of MMP-8 immunoreactivity lower than those detected in chronic adult periodontitis and gingivitis GCF (Mäntylä et al., 2003), but higher than in healthy control GCF. Overall, these data may reflect a more aggressive breakdown of the periodontal extracellular matrix in periodontitis in relation to tissue resorption/apposition events characteristic of orthodontic tooth movement. It remains to be seen whether the relatively high immunoreactivities for >100 kDa high-molecular weight complexes in orthodontic GCF compared with those in adult periodontitis GCF may be due to a greater imbalance between the activated MMPs and their natural endogenous inhibitors (tissue inhibitor of matrix metalloproteinases, TIMP-1 and -2 or α2-macroglobulin) in relation to adult periodontitis GCF (Ingman et al., 1996; Kiili et al., 2002). Increased down-regulation of MMPs by TIMPs during orthodontic tooth movement than in periodontitis may, at least in part, explain this difference (Ryan and Golub, 2000; Kiili et al., 2002).

### Conclusions

The results of the present pilot study demonstrate, that in a 1 month follow-up, multiple species of both MMP-8 isoforms evidently take part in tissue destruction/remodelling during human orthodontic tooth movement in vivo. Second, changes in MMP-8 levels are clearly reflected in the GCF of orthodontic patients.
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