

Matrix metalloproteinases and chemokines in the gingival crevicular fluid during orthodontic tooth movement

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SUMMARY Matrix metalloproteinases (MMPs) and monocyte chemoattractants are key modulators of the biological mechanisms triggered in the periodontium by mechanical forces. The gingival crevicular fluid (GCF) provides a non-invasive method to assess longitudinally the release of inflammatory mediators during orthodontic tooth movement. The goal of this study was to examine the GCF levels of MMP-3, MMP-9, and MMP-13 and of the chemokines macrophage inflammatory protein (MIP)-1 β , monocyte chemoattractant protein (MCP)-1, and regulated on activation normal T cells expressed and secreted (RANTES) at different time points during orthodontic tooth movement. Fourteen subjects (three males and 11 females, 18.8 ± 4.8 years of age; range from 12 to 28 years) had their maxillary canines retracted. Thirty-second GCF samples were collected from the tension and pressure sides 7 days prior to the activation of the orthodontic appliance, on the day of activation, and after 1 and 24 hours, and 14, 21, and 80 days of constant force application. The volume of GCF was measured and samples analysed using a multiplexed bead immunoassay for the content of the six target molecules. Differences in the mean GCF volumes and mean level for each analyte over time were assessed using the Friedman test, and differences between the tension and pressure sides at each time point with the Mann–Whitney test.

The mean levels of the three MMPs changed significantly over time but only at the compression side ($P < 0.05$, Friedman test). The GCF levels of the three chemokines were not affected by the application of mechanical stress. The levels of MMPs in GCF at the pressure side are modulated by the application of orthodontic force.

Introduction

In orthodontic tooth movement, there is a delicate and well-balanced remodelling process of the extracellular matrix in the adjacent periodontium in order to allow tooth movement, while the functional integrity of the periodontium is maintained. Among the myriad of inflammatory mediators potentially involved in this process, matrix metalloproteinases (MMPs) have been implicated in orthodontic tooth movement. Using a dog model, Redlich *et al.*, (1996) demonstrated an increase in messenger RNA (mRNA) levels and activity of MMP-1 in the compression side of the gingivae, during orthodontic tooth movement. An increased expression of MMP-8 and MMP-13 mRNA in the periodontal ligament (PDL) of rats during active tooth movement has also been demonstrated (Takahashi *et al.*, 2003). Orthodontic tooth movement can be delayed or prevented in mice by the use of MMP inhibitors (Holliday *et al.*, 2003). In addition, human studies have quantified the presence of MMPs in the gingival crevicular fluid (GCF) during orthodontic tooth movement and have reported alteration in their levels as a consequence of the application of orthodontic forces (Apajalahti *et al.*, 2003; Cantarella *et al.*, 2006).

During early tooth movement, the blood supply at the compression side in the PDL is reduced, resulting in a zone of aseptic necrosis or hyalinization. Indirect bone resorption in the medullary spaces release the pressure and capillaries can grow into the hyaline zone and the necrotic tissue can be removed by macrophages. Newly formed osteoclasts will later resorb the bone surface directly and tooth movement will resume, while the periodontal membrane is reformed (Masella and Meister, 2006). Chemokines involved in the migration of monocytes to the PDL, where they will be differentiated into osteoclasts and macrophages, are crucial for orthodontic tooth movement (Nahm *et al.*, 2004). In a study using Wistar rats and *in situ* hybridization, the number of cells expressing monocyte chemoattractant protein (MCP)-1, regulated on activation normal T cells expressed and secreted (RANTES), and macrophage inflammatory process (MCP)-2 mRNA peaked 3 days after the beginning of tooth movement. The results strongly suggest a role for these chemokines in the remodelling process of the PDL and alveolar bone during orthodontic tooth movement (Alhashimi *et al.*, 1999). Despite the relevance of monocyte chemoattractants in the biology of orthodontic tooth movement, these mediators have not been studied in humans.

During its passage from the capillaries through the periodontal tissues into the gingival sulcus, the GCF carries a multitude of inflammatory mediators present in the periodontal tissues. This easily collected sample has been used to study the levels of several molecules released during orthodontic tooth movement in humans. Most of these studies have confirmed the involvement of several inflammatory cytokines (Uematsu *et al.*, 1996; Iwasaki *et al.*, 2001), host-derived enzymes (Perinetti *et al.*, 2005; Batra *et al.*, 2006), and chemokines (Tuncer *et al.*, 2005; Basaran *et al.*, 2006) in the biological mechanism triggered by orthodontic forces. A major limitation of GCF studies is the small sample volume for analysis, typically, less than 0.5 μ l is obtained from healthy sites. The small volume of GCF limits the number of analytes that can be conveniently studied by traditional enzyme linked immunosorbent assays. The introduction of the Multianalyte Microsphere Assay affords the simultaneous quantification of several targets in the same assay. Therefore, this study aimed to measure the levels of three distinct MMPs, MMP-3, MMP-9, and MMP-13 and three chemokines, MIP-1 β , MCP-1, and RANTES in the GCF of maxillary canines from subjects undergoing orthodontic treatment, using multiplexed bead assays.

Subject and methods

Fourteen subjects (three males and 11 females, 18.8 ± 4.8 years of age; range from 12 to 28 years) requiring maxillary first premolar extractions as part of their orthodontic treatment were enrolled in this study. The subjects were informed of the characteristics and objectives of the research and signed or, if the subject was a minor, had a responsible adult sign an informed consent form (1636-CEP/HUPE). The protocol was approved by the Dental School of Rio de Janeiro State University, Rio de Janeiro, Brazil. Exclusion criteria were autoimmune diseases, pregnancy, lactation, and use of any medication that could interfere with orthodontic tooth movement, or had adverse effects directly on the periodontium or interfered with the inflammatory process, 6 months prior to the study (e.g. antibiotics, antihistamines, cortisone, and hormones). Seven days prior to the application of orthodontic force, the subjects received oral hygiene instruction and began to rinse with chlorhexidine gluconate 0.12 per cent twice a day for four consecutive weeks.

The premolars were extracted at least 20 days prior to the retraction of the canines, applying the method described by Iwasaki *et al.* (2005) using a force of 150 g. Samples of GCF were collected from the mesial (tension) and distal (pressure) sides of the maxillary canines of each subject at seven time points. The first collection took place 7 days before the orthodontic force was first applied (–7 days), the second was undertaken on the day of force application, denominated time 0, and third, 1 hour, the fourth, 24 hours, the fifth, two

weeks, the sixth, three weeks (21 days), and the seventh, 80 days after the orthodontic force was first applied.

GCF sampling

Following removal of supragingival plaque, the sites were isolated with cotton rolls to prevent contamination with saliva and gently air-dried. Thirty-second GCF samples were collected from the mesial and distal sites of the maxillary canines, using filter strips (Periopaper®, IDE Interstate, Amityville, New York, USA) gently inserted 1–2 mm into the gingival sulcus (Figure 1A and 1B). GCF volumes were determined using a pre-calibrated Periotron 8000™ (Periotron 8000, IDE Interstate). The paper strips were individually placed in microcentrifuge tubes and stored at –80°C until assay. Samples visibly contaminated with blood or saliva were discarded.

Quantification of inflammatory mediators

Levels of MMP-3, -9, and -13 and MIP-1 β , MCP-1, and RANTES were determined using a multiplexed bead immunoassay (BioSource International, Camarillo, California, USA). The assay was performed in a 96-well filter plate as previously described (Vignali, 2000). Briefly, the filter plate was pre-wetted with washing buffer and the solution was

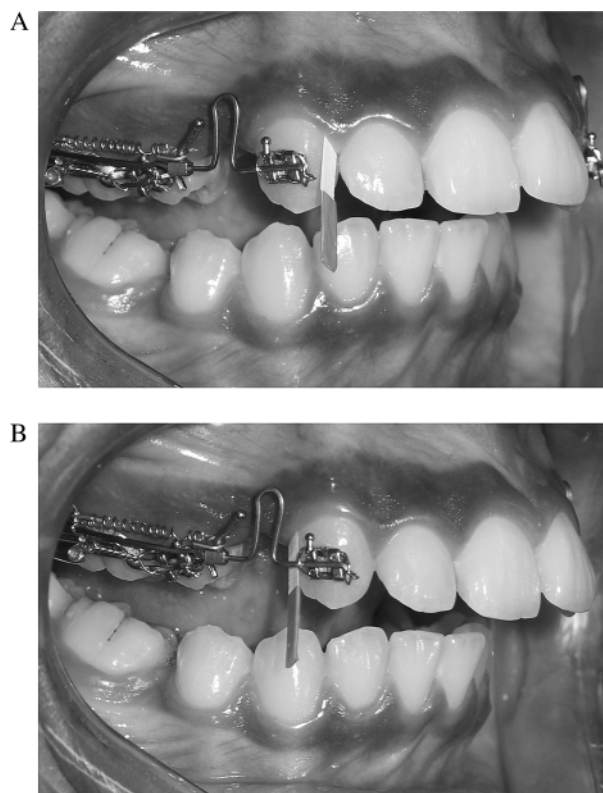


Figure 1 Retraction of the canines using nickel–titanium springs in an archwire with 150 g of force. Samples of gingival crevicular fluid were collected from the mesial/tension (A) and distal/pressure (B) sides of the maxillary canines of each subject.

aspirated from the wells using a vacuum manifold (Millipore Corporation, Billerica, Massachusetts, USA). Microsphere beads coated with monoclonal antibodies against different target analytes were added to the wells. Two sets of five assays were performed, one using the Human 3-Plex MMP Antibody Bead Kit (BioSource International) for the quantification of MMP-3, MMP-13, and MMP-9 and a second set to quantify the chemokines, prepared by multiplexing single bead kits for MIP-1 β , MCP-1, and RANTES (BioSource International). Samples and standards were pipetted into the wells and incubated for 2 hours with the beads. The wells were washed using the vacuum manifold (Millipore Corporation) and biotinylated secondary antibodies were added. After incubation for 1 hour, the beads were washed followed by incubation of 30 minutes with streptavidin conjugated to the fluorescent protein, R-phycoerythrin (streptavidin-RPE). After washing, to remove the unbound streptavidin-RPE, the beads (minimum of 100 per analyte) were analysed in the Luminex 100™ instrument (Luminex Corporate, Austin, Texas, USA). The concentrations of the unknown samples (antigens in GCF samples) were estimated from the standard curve using linear regression analysis.

Data analysis

Data available for each subject were the volume of GCF in microlitre and the levels of MMP-3, MMP-9, MMP-13, MIP-1 β , MCP-1, and RANTES for up to four sites per subject at day -7, baseline, 1 and 24 hours, and 14, 21, and 80 days after the beginning of tooth movement. The data for each analyte were expressed as picogram per site, averaged within each subject for the tension and pressure sides independently and then averaged across subjects at each time point separately. Significance of the differences in the mean volume of GCF and mean levels for each analyte over time was assessed using the Friedman test, while differences between the tension and pressure sides at each time point were determined using the Mann-Whitney test.

Results

Changes over time in the volume of GCF

All 14 subjects completed the study. Figure 2 illustrates the mean volume of GCF at the tension and pressure sides for each subject. At both sides, the volume of GCF changed significantly over time ($P < 0.01$). There were no significant differences between the tension and pressure sides at most of the time points, with the exception of 21 days, when the pressure side had a statistically significantly higher volume of GCF than the tension side ($P < 0.05$).

Changes over time in the levels of MMP

For all MMPs, changes over time were only statistically significant at the pressure side ($P < 0.05$ for MMP-3 and

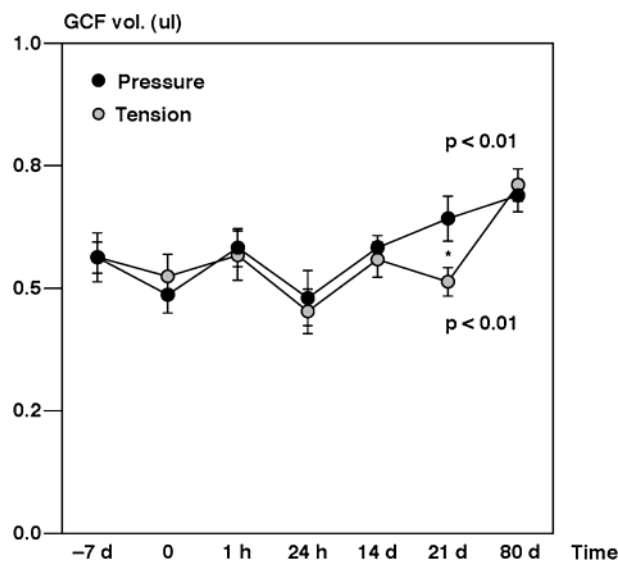


Figure 2 Plots of the mean volume of gingival crevicular fluid in microlitre (\pm the standard error of the mean, SEM) at day -7, baseline, 1 and 24 hours, and 14, 21, and 80 days after the beginning of tooth movement. The circles are the mean values and the whiskers the SEM. The black circles represent the data for the pressure side, and the grey circles the data for the tension side. The levels of each marker were measured at two sites per subject, averaged within a subject and then averaged across all subjects at the tension and pressure sides for each time point. Significance of differences over time was tested using the Friedman test and differences between sides at each time point using the Mann-Whitney test.

MMP-13 and $P < 0.01$ for MMP-9). The changes in the levels of all three mediators at the pressure side followed the same trend. There was an increase in their levels 1 hour after activation of the orthodontic appliance, followed by a sharp decrease after 1 day of tooth movement. Thereafter, there was a steady increase in GCF content of MMPs until the end of the observation period. The values for MMP-9 are reported in fluorescence intensity as they could not be converted to picograms per site (Figure 3). This was the result of GCF samples with higher values of fluorescence than the highest value in the standard curve (upper limit of quantification).

Changes over time in the levels of chemokines

Table 1 shows the mean levels of the three chemokines for the tension and pressure sides for all 14 subjects. There were no statistically significant changes over time in the levels of any of the chemokines on either side. There were also no statistically significant differences between the tension and pressure sides for any of the chemokines at any time point.

Discussion

In the present study, changes over time in GCF volume and levels of MMPs and monocyte chemoattractants at sites

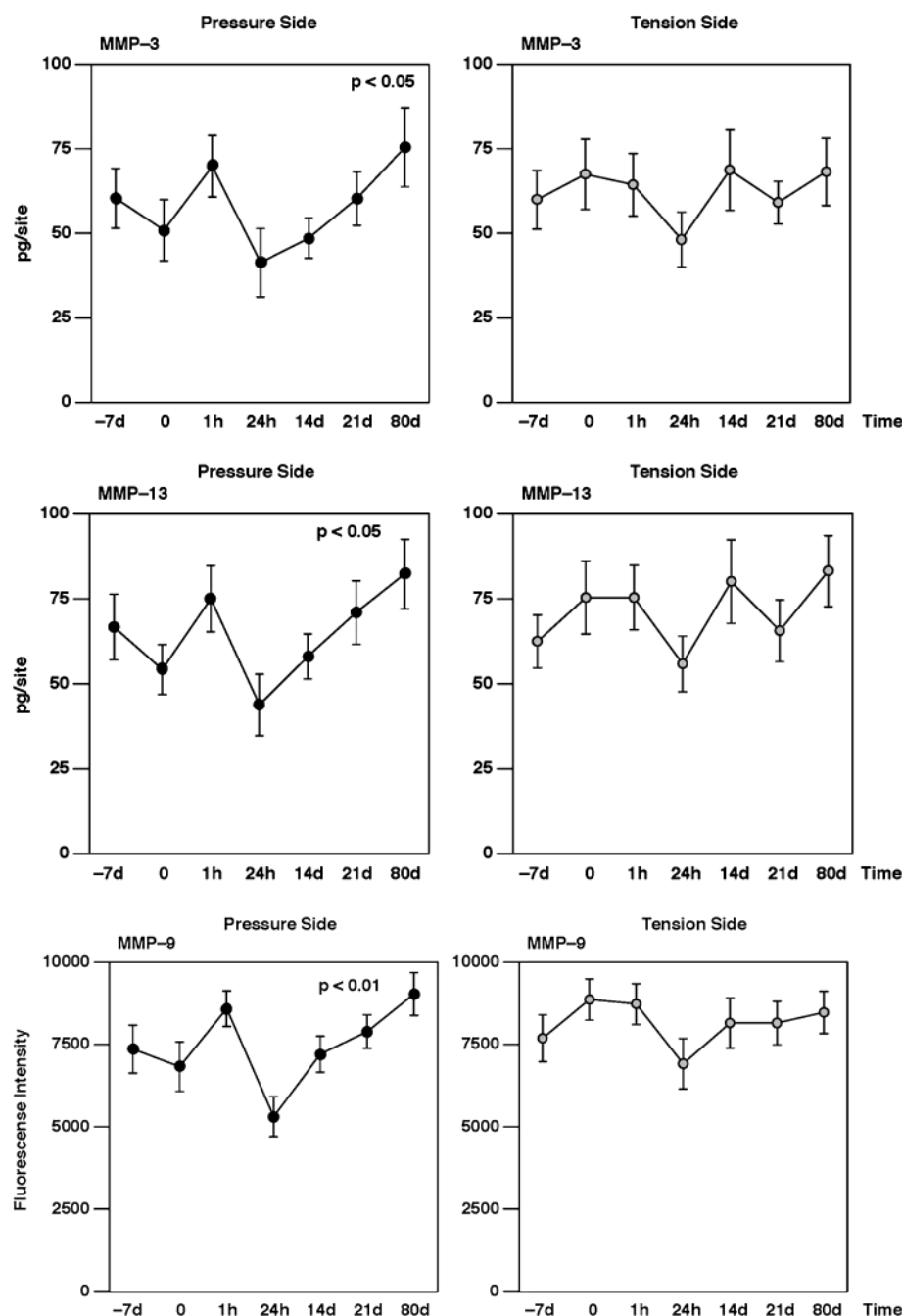


Figure 3 Plots of the mean levels [\pm the standard error of the mean (SEM)] of matrix metalloproteinases (MMPs)-3, MMP-13 (pg/site), and MMP-9 (fluorescence) at day -7, baseline, 1 and 24 hours, and 14, 21, and 80 days after the beginning of tooth movement. The circles represent the mean values and the whiskers the SEM. The levels of each marker were measured at two sites per subject, averaged within a subject and then averaged across all subjects at the tension and pressure sides for each time point. The graph on the left presents the data for the pressure side (black circles), and that the right the data for the tension side (grey circles). Significance of differences over time was tested using the Friedman test and differences between sides at each time point using the Mann-Whitney test.

subjected to orthodontic force were examined. Since GCF volume and composition are directly influenced by plaque-induced inflammation of the gingiva, the subjects underwent professional cleaning followed by 4 weeks of chemical plaque control in order to minimize the impact of

gingivitis. In addition, since the nature of the mechanical stress (tension versus compression) can differently influence the expression of inflammatory mediators (Perinetti *et al.*, 2005; Tuncer *et al.*, 2005; Cantarella *et al.*, 2006), GCF volume and levels of the target analytes were compared

Table 1 Mean levels (\pm the standard error of the mean) and prevalence of positive sites, for macrophage inflammatory protein 1 β (MIP-1 β), monocyte chemoattractant protein-1 (MCP-1) and regulated on activation normal T cells expressed and secreted (RANTES).

Time	-7 days	0	1 h	24 h	14 days	21 days	80 days
Tension side							
MCP (pg)	0.12 \pm 0.14	0.10 \pm 0.20	0.06 \pm 0.09	0.12 \pm 0.28	0.09 \pm 0.17	0.04 \pm 0.06	0.11 \pm 0.13
MCP % + sites	43	21	21	29	21	21	43
MIP (pg)	1.74 \pm 2.74	1.07 \pm 2.66	1.28 \pm 3.36	1.04 \pm 1.34	1.52 \pm 3.02	0.53 \pm 0.57	0.65 \pm 0.77
MIP % + sites	71	50	57	64	64	64	57
RANTES (pg)	0.14 \pm 0.19	0.11 \pm 0.22	0.09 \pm 0.23	0.11 \pm 0.25	0.15 \pm 0.23	0.03 \pm 0.07	0.11 \pm 0.24
RANTES % + sites	43	21	21	21	43	14	29
Pressure side							
MCP (pg)	0.11 \pm 0.13	0.07 \pm 0.08	0.08 \pm 0.11	0.05 \pm 0.08	0.05 \pm 0.07	0.06 \pm 0.13	0.09 \pm 0.17
MCP % + sites	36	43	36	29	21	14	29
MIP (pg)	1.47 \pm 2.69	1.12 \pm 2.38	0.58 \pm 0.84	0.69 \pm 0.66	0.63 \pm 0.86	0.80 \pm 1.61	0.56 \pm 1.14
MIP % + sites	64	43	57	71	64	57	43
RANTES (pg)	0.36 \pm 0.46	0.04 \pm 0.12	0.15 \pm 0.19	0.18 \pm 0.39	0.07 \pm 0.12	0.18 \pm 0.34	0.11 \pm 0.30
RANTES % + sites	57	7	43	29	21	29	21

The levels of each marker were measured at two sites per subject, averaged within a subject and then averaged across all subjects at the tension and pressure sides for each time point.

between the tension and pressure sides of the moved teeth. The results demonstrated a statistically significant change in GCF volume over time at both the pressure and tension sides. With the exception of day 21, the volume of GCF was similar for both sides. These data confirm previous reports that have demonstrated the impact of orthodontic forces on the volume of GCF (Basaran *et al.*, 2006; Karacay *et al.*, 2007). However, the effects of orthodontic forces on GCF volume are not a consistent finding since some studies did not demonstrate a significant influence of mechanical stress on GCF flow (Apajalahti *et al.*, 2003; Ingman *et al.*, 2005; Bildt *et al.*, 2009).

In this study, the aim was to quantify three different MMPs, representing three different subgroups: MMP-3 of the stromelysin group, MMP-9 of the gelatinase group, and MMP-13 of the collagenase group. These MMPs were chosen due to their involvement in different stages of collagen remodelling and canalization of the initial degradation of most extracellular matrix proteins. Levels of MMP-9 were much higher than those of MMP-3, which were greater than the levels of MMP-13. The levels of MMP-9 were above the highest value of the standard curve in the Human 3-Plex MMP antibody bead kit used in the Luminex 100™ assay. Therefore, the results for this MMP were expressed in fluorescence intensity and were not converted to picograms. Since the statistical tests employed in the analyses were non-parametric, based on ranks rather than actual values, the reported findings for this mediator should be accurate. This high amount of MMP-9 is probably associated with the presence of neutrophils in the gingival sulcus.

With time, changes in the levels of MMP-9, MMP-13, and MMP-3 were observed. The data demonstrated that their levels oscillated during the period of tooth movement at the pressure side, while only minor non-statistically significant fluctuations were noted at the tension side

(Figure 3). There was a decrease in the three MMPs from day -7 to baseline. This decrease could be the result of a reduction in marginal gingivitis as a consequence of the chemical plaque control. From baseline (time 0) to 1 hour, there was a trend for an increase in the levels of MMPs, which could be the result of the application of orthodontic force. From time 1 to 24 hours, there was a trend for a sharp decrease in the levels of all MMPs. Thus, an immediate consumption of enzymes related to the degradation of collagen induced by the orthodontic force could be confirmed. From 24 hours to 14 days and 21 to 80 days, there was a progressive increase in MMP levels. After day 21, canine retraction continued, but the subjects interrupted the use of chlorhexidine, potentially with gingival inflammation overriding the mechanical stress as the stimulus to the secretion of the proteolytic enzymes.

Figure 3 shows similar profiles of changes over time for MMP-3, MMP-9, and MMP-13, suggesting a temporal coordination in the expression of these MMPs. Since MMPs are involved in extracellular cleavage and activation of other MMPs and are responsible for different stages of degradation of the extracellular matrix, the temporal coordination of their expression was expected. For instance, MMP-3 has the ability to activate other MMPs, including MMP-8 and -9 (Beklen *et al.*, 2006). Collagenases (e.g. MMP-13) dissolve native fibrillar collagens, while gelatinases (e.g. MMP-9) cleave denatured collagen (gelatin), complementing the degradation of collagen. In addition, the concomitant increased expression of MMP-1, MMP-2, and MMP-9 in the gingival tissues of dogs, following the application of orthodontic forces has been reported (Redlich *et al.*, 2001).

This appears to be the first study on these three MMPs in GCF during orthodontic tooth movement. Previous research has concentrated on the levels of MMP-1, -2, and -8.

Apajalahti *et al.* (2003) demonstrated an increase in the mean concentration of MMP-8 in the GCF of orthodontically treated teeth 4–8 hours after mechanical stress compared with baseline values and control teeth but did not detect MMP-1 in immunoblots of GCF from the orthodontically moved teeth. The same researchers later reported on the GCF levels of MMP-1 and MMP-8, measured over 28 days of orthodontic movement. Levels of MMP-8 were higher around orthodontically moved teeth compared with control teeth for the entire observation period, while MMP-1 was not detected (Ingman *et al.*, 2005). In another study, the levels of MMP-1 and -2 were measured at the compression and tension sides of retracted canines 1, 2, 3, 4, and 8 hours after activation of an orthodontic appliance. MMP-1 levels increased at the pressure side after 1 hour, remained elevated for up to 3 hours after force application, and then subsided. At the tension side, MMP-1 was only elevated after 1 hour of tooth movement. The expression of MMP-2 was induced in the compression side and increased significantly over time, peaking at 8 hours. At the tension side, MMP-2 peaked at 1 hour after activation and gradually returned to baseline levels (Cantarella *et al.*, 2006). To ascertain whether MMP-3 is up-regulated *in vivo* by orthodontic force, Chang *et al.* (2008) examined human bone samples at the compression site by realigning the angulated molars. Immunohistochemical staining revealed MMP-3 distributed along the compressive site of the bony region within 3 days of orthodontic force compression. Leonardi *et al.* (2007) using Sprague–Dawley rats observed immunolabelling of MMP-13 initially on the compression side and then on both the compression and tension sides. Since this increase in MMP-13 immunolabelling occurred very early following force application in both the PDL and alveolar bone, this would indicate that MMP-13 might play an important role during tooth movement.

Overall, the present results are in agreement with the above reports and suggest that orthodontic forces modulate the expression of MMPs within the periodontal tissues and that the changes in the levels of these mediators can be detected in GCF. Further, they indicate that the effects of the orthodontic forces differ depending of the nature of the mechanical stress, i.e. pressure or tension. No statistically significant differences in the levels of the three MMPs between the tension and pressure sides at any time point could be demonstrated in the present study. However, the clearly different profiles of MMP expression at the tension and pressure sides suggest that the MMPs quantified also responded differently to different mechanical stimuli.

MCP-1, MIP-1 β , and RANTES chemokines were detected in the gingival sulcus at all time intervals, at both the tension and pressure sides (Table 1), confirming the sensitivity to the multiplexed bead immunoassay for the identification of these molecules. However, the level of these chemokines in the GCF did not seem to have been altered by the orthodontic forces. Only a few studies have examined the association of chemokines with tooth movement. An increase in the levels

of interleukin IL-8 at the tension side during the initial stages of force application was reported, whereas there was no increase at the pressure side (Tuncer *et al.*, 2005). In an animal model using Wistar rats and *in situ* hybridization, MCP-1, RANTES, and MIP-2 chemokines showed maximum induction at the compression side 3 days after the application of orthodontic force. Macrophages were detected in close proximity to MCP-1, RANTES, and MIP-1 expressing cells, suggesting that the mRNA was translated into biologically active chemokines (Alhashimi *et al.*, 1999).

It has been proposed that as a result of the application of mechanical stress, the biological events that occur in the PDL would result in sufficient amounts of inflammatory mediators to diffuse into the GCF. According to this view, changes in the levels of cytokines and other substances in the GCF, during the application of orthodontic force, would reflect changes in the deeper periodontal tissues (Uematsu *et al.*, 1996). Alternatively, the alterations detected in GCF composition during orthodontic tooth movement might reflect events that take place within the gingival tissues. Hence, since monocyte chemoattractants coordinate events that occur in the PDL, a localized increase in their levels might not ‘spill over’ into the GCF; these mediators would find their receptors and be consumed within the PDL.

In contrast to collagen in bone and in the PDL, supracrestal gingival collagen fibres are not completely resorbed or degraded during orthodontic tooth movement, they are rather compressed or retracted during tooth dislocation (Redlich *et al.*, 1996). This mechanism is probably important in the maintenance of a healthy periodontium during orthodontic movement. The constant presence of supracrestal collagen during tooth movement might restrict the diffusion of mediators from the PDL to the gingival margin. It has been proposed that MMPs play a role in the remodelling of supracrestal fibres during tooth movement. According to this hypothesis, alterations in the GCF levels of MMPs reflect biological mechanisms taking place within the marginal gingival tissue, rather than within the PDL and alveolar bone. In support of this mechanism, it has been demonstrated that the expression of mRNA for MMP-1 in the gingival tissues of dogs was up-regulated by the application of orthodontic force. Conversely, removal of the force caused a significant decrease in MMP-1 gene expression, suggesting a strong correlation between mechanical stress and the induction of MMP-1 (Redlich *et al.*, 2001).

Increased levels of the chemokines MCP-1 and RANTES in the GCF of subjects with periodontitis compared with periodontally healthy controls have also been demonstrated (Gamonal *et al.*, 2000; Kurtis *et al.*, 2005). In contrast to orthodontically moved teeth, in periodontally involved teeth, the inflammatory infiltrate is contiguous with the periodontal pocket and the supracrestal fibres are disintegrated. This condition affords access of tissue generated inflammatory mediators to the sulcus/pocket

environment and the GCF, possibly explaining the association between these biomarkers and periodontal inflammation. In orthodontic tooth movement, these chemokines are released and exert their biological activity within the PDL, not reaching the GCF.

Conclusions

1. Statistically significant fluctuations during orthodontic tooth movement could be detected for MMP-3, MMP-9, and MMP-13 but only on the compression side. The levels of these MMPs increased after 1 hour of force application and decreased sharply over the following 24 hours.
2. The levels of MCP-1, MIP-1 β , and RANTES in GCF did not seem to be altered by orthodontic force application.

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