European Journal of Orthodontics 31 (2009) 529–535
doi:10.1093/ejo/cjn127
Advance Access Publication 18 March 2009

Matrix metalloproteinases and tissue inhibitors of metalloproteinases in gingival crevicular fluid during orthodontic tooth movement

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SUMMARY Orthodontic tooth movement requires extensive re-modelling of the periodontium. Matrix metalloproteinases (MMPs) degrade the extracellular matrix during re-modelling, while their activity is regulated by the tissue inhibitors of metalloproteinases (TIMPs). The aim of this study was to investigate differences in MMP and TIMP levels in the gingival crevicular fluid (GCF) at the resorption and apposition sides of orthodontically moved teeth, and to compare these with control teeth.

GCF samples were collected from eight orthodontic patients wearing fixed appliances with superelastic nickel-titanium coil springs. The samples were analysed by gelatin zymography, which allows detection of both active and latent MMPs, and reverse zymography for analysis of TIMPs. Western blotting was performed to confirm the identity of MMPs. The data were analysed using either the one-way analysis of variance or the Kruskal–Wallis test.

In general, higher levels of MMPs and TIMPs were found at both the resorption and apposition sides compared with the control teeth. Remarkably, partially active MMP-1 was found in GCF from both the resorption and the apposition side but was barely present at the control teeth. TIMP-1 was strongly increased at the apposition side. Gelatinases were mainly present at the resorption side, while gelatinolytic fragments were exclusively detected at the apposition side. MMP-9, which is known to be involved in bone degradation, and a 48 kDa gelatinase were increased at the resorption side. The small increase in TIMP-1 at the resorption side might stimulate bone resorption, whereas the large increase at the apposition side reduces bone resorption. The analysis of MMPs and TIMPs may contribute to the improvement of orthodontic treatment regimens.

Introduction

Orthodontic tooth movement requires extensive re-modelling of the periodontium. Re-modelling is thought to be initiated in the periodontal ligament (PDL; Kawarizadeh *et al.*, 2005). A family of proteases that play a key role in the re-modelling of the extracellular matrix (ECM) are the matrix metalloproteinases (MMPs). To date, 23 human MMPs have been described (Snoek-Van Beurden and Von den Hoff, 2005). Based on their molecular structure and substrate preference, the MMPs can be divided into several groups: collagenases (MMP-1, -8, and -13), gelatinases (MMP-2 and -9), stromelysins (MMP-3, -10, and -11), membrane-type MMPs (MMP-14, -15, -16, and -17), and a group of miscellaneous MMPs (Snoek-Van Beurden and Von den Hoff, 2005). MMPs are secreted as inactive pro-enzymes, which can be activated by proteolytic processing in the ECM (Snoek-Van Beurden and Von den Hoff, 2005). The activity of MMPs is controlled by the tissue inhibitors of metalloproteinases (TIMPs; Verstappen and Von den Hoff, 2006). MMPs and TIMPs play an important role in the physiological re-modelling of the periodontium (Ejeil *et al.*, 2003) as well as in the response to mechanical forces during orthodontics (Ingman *et al.*, 2005). The inhibition of MMPs by synthetic MMP inhibitors has been shown to reduce orthodontic tooth movement (Holliday *et al.*, 2003; Bildt *et al.*, 2007).

Experimental orthodontic tooth movement in animals, mostly rats, shows an increased expression of MMP-1, -2, -8, -9, and -13 and TIMP-1 and -3 in the PDL and alveolar bone (Domon *et al.*, 1999; Takahashi *et al.*, 2003, 2006; Leonardi *et al.*, 2007). MMP-1 was also increased in the gingiva during tooth movement in dogs (Redlich *et al.*, 2001). In the gingival crevicular fluid (GCF) of orthodontic patients, MMP-2 and -8 are increased, and sometimes also MMP-1 (Apajalahti *et al.*, 2003; Ingman *et al.*, 2005; Cantarella *et al.*, 2006). In the PDL of orthodontic patients, MMP-1 messenger RNA (mRNA) expression is upregulated at both the resorption and the apposition sides, whereas TIMP-1 mRNA is increased only at the apposition side (Garlet *et al.*, 2007).

None of the methods used differentiates between the active and latent forms of the MMPs. Previous studies point towards an increase in MMPs, specifically collagenases and TIMPs in orthodontic tooth movement at the apposition as well as the resorption side. At both sides, extensive re-modelling takes place to allow orthodontic tooth movement. The aim of this study was to investigate differences in latent and active MMPs and TIMPs in the

GCF at the resorption and apposition side of orthodontically moved teeth and to compare these with control teeth. The samples were analysed by zymography, which allows the detection of both active and latent MMPs.

Subjects and methods

Collection of GCF

GCF samples were collected from eight orthodontic patients [two males, six females, aged 10–18 years (mean 13.6 ± 2.6 years)]. All patients were treated at an academic orthodontic clinic. They all had fixed appliances with superelastic nickel-titanium coil springs (GAC international Inc., Bohemia, New York, USA) that gave light continuous forces of approximately 150 cN. The following inclusion criteria were applied: no use of medication, good periodontal health (score 0 according to the Dutch Periodontal Screening Index; Van Rossum and Zeegers, 1999), and the presence of one or more teeth that were not orthodontically moved. The study was approved by the local medical ethics committee (CMO, Arnhem-Nijmegen, The Netherlands). All patients gave their written consent to participate in the study. GCF was collected at the apposition and resorption sides of teeth that were orthodontically moved and around control teeth. The type and number of teeth differed between the patients depending on the treatment plan. In most cases, both upper first premolars were distalized. Sampling was performed just before a control visit with all orthodontic appliances *in situ*. All experimental teeth had been moved for about 4 weeks before sampling. The teeth at the sampling sites were isolated with cotton rolls and gently dried with air. Thereafter, a 1 μl microsyringe (Hamilton, Reno, Nevada, USA) was carefully inserted into the sulci to collect GCF. This was carried out three times per sampling site with an interval of 2 minutes. The GCF samples were immediately placed on ice and stored at −80°C until use. The GCF samples were pooled to obtain a pool from the resorption sides, a pool from the apposition sides, and a control pool. These were run in triplicate (gelatin and reverse zymography) or duplicate (collagen zymography).

Gelatin zymography

Gelatinases (MMP-2 and -9) in the samples were analysed in triplicate by gelatin zymography. The polyacrylamide gel (7.5 per cent) contained 3.5 mM sodium dodecyl sulphate (SDS) and 1 mg/ml gelatin, as described by Waas *et al.* (2002). A (1:1) mixture of the sample and sample buffer was then electrophoresed for 1.5 hours at 80 mA. A Broad Range marker (Biorad, Hercules, California, USA) ranging from 6.4 to 203 kDa was included to determine the molecular weight of the MMPs. Recombinant human pro-MMP-2 (oncogene, CN Biosciences, San Diego, California, USA) was used as a reference sample. After electrophoresis, the gels were washed in 2.5 per cent Triton X-100 (Sigma-Aldrich, St. Louis, Missouri, USA) to remove the SDS, and the marker bands were indicated. The gels were then incubated in activation buffer, containing 50 mM Tris–HCl (pH 7.8), 5 mM $CaCl_2$, and 0.1 per cent Triton X-100, at 37°C for 18 hours. They were stained for 45 minutes with 2.5 g/l Coomassie Brillantblue R250 (Imperial Chemical Industries Plc, London, UK), 10 per cent acetic acid, and 40 per cent methanol in water and thereafter destained with 10 per cent acetic acid and 40 per cent methanol in water. The MMPs appear as bright bands within the stained gel. The gels were scanned with a Hewlett Packard ScanJet 4C/T, and the bands were analysed with Quantity One software (Biorad). The amount of enzyme in the bands was represented as average density × mm^2/μl GCF. The pro-MMP-2 reference sample enabled comparison of the corresponding bands on different gels. First, within each gel, the amount of enzyme in the reference sample was arbitrarily set to 1, and all other bands were calculated relative to it. Then, the corresponding bands on the different gels were averaged and standard deviations (SDs) were calculated. The amount of pro-MMP-9 in the samples was too high for a reliable analysis. Therefore, it was repeated after dilution of the samples.

Collagen zymography

Collagenases in the samples were analysed in duplicate by collagen zymography. The polyacrylamide gel (10 per cent) contained 3.5 mM SDS and 5 mg/ml collagen type I from calf skin (Sigma-Aldrich). A (1:1) mixture of the sample and sample buffer was then electrophoresed. A broad range marker (Biorad) ranging from 6.4 to 203 kDa was included to determine the molecular weight of the MMPs. Recombinant human pro-MMP-1 and -8 (oncogene) were used as standards. After electrophoresis, the gels were washed in 2.5 per cent Triton X-100 (Sigma-Aldrich) to remove the SDS. The gels were then incubated in activation buffer at 37°C for 43 hours. They were stained and destained as described for the gelatin zymography. The MMPs appear as bright bands within the stained gel. Thereafter, the gels were scanned and analysed as described for the gelatin zymography. The collagenolytic bands at 44 and 41 kDa were identical to those seen in gelatin zymography. For quantification, only the results of the gelatin zymograms were used.

Reverse zymography

TIMPs in the samples were analysed in triplicate by reverse zymography. This technique is similar to gelatin zymography, except that conditioned medium from baby hamster kidney cells (BHK-21, ATCC, Rockville, Maryland, USA) was included in the gels. This medium mainly contains MMP-2. The MMP-2 degrades all gelatins in the gel, except where TIMPs are present. The polyacrylamide gel (15 per cent) contained 3.5 mM SDS, 1 mg/ml gelatin, and 10 per cent conditioned medium from BHK-21 cells. A (1:1) mixture of the sample and sample buffer was then electrophoresed for

3 hours at 80 mA. A Broad Range Marker (Biorad) was included to determine the molecular weight of the TIMPs. Recombinant human TIMP-1 and -2 (oncogene) were used as standards. After electrophoresis, the gels were washed in 2.5 per cent Triton X-100 (Sigma-Aldrich) to remove the SDS. The gels were then incubated in 50 mM Tris–HCl (pH 7.5), 5 mM $CaCl_2$, 0.1 per cent Triton X-100, and 0.2 M NaCl at 37°C for 18 hours. They were stained and destained, following the same method as described for gelatin zymography. The TIMPs appear as dark blue bands within the destained gel. Thereafter, the gels were scanned and analysed as described for gelatin zymography, except that TIMP-2 was used as a reference to enable comparison of the corresponding bands on different gels.

Western blotting

GCF samples were analysed by Western blotting according to standard procedures (Burnette, 1981). In short, a 10 µl sample was dissolved in 10 µl sample buffer and electrophoresed on a 7.5 per cent polyacrylamide gel (room temperature, 80 mA). The proteins were blotted onto a nitrocellulose membrane (pore size 0.45 µm, Biorad) at 400 mA and 4°C for 1 hour. The membrane was then incubated in block buffer [5 per cent milk powder (Elk, Campina, The Netherlands) and 0.1 per cent Tween 20 (Sigma-Aldrich) in phosphate-buffered saline (PBS)]. Thereafter, the blots were incubated with monoclonal antibodies against MMP-1, -2, -8, -9, or -13 (all oncogenes), dilution 1:200 (except for MMP-2; dilution 1:500) at 4°C for 18 hours. After washing with 0.1 per cent Tween 20 in PBS, the blots were incubated with a peroxidase-conjungated goat anti-mouse IgG (BD Biosciences, San Jose, California, USA), diluted 1:5000 in 1 per cent bovine serum albumin, 0.1 per cent Tween in PBS, for 1 hour. After washing, the membranes were incubated with ECL Plus™ reagent (GE-Healthcare, Chicago, Illinois, USA) for chemiluminesence detection. Excess ECL reagent was removed, and the membrane was exposed to a Kodak Biomax film (Eastman Kodak Company, Rochester, New York, USA) for 1 hour and developed.

Statistical analysis

The difference in levels of MMPs and TIMPs between the resorption, apposition, and control samples was evaluated by one-way analysis of variance (ANOVA). Tukey's Honesty Significant Difference was used as a *post hoc* test. The Kruskal–Wallis test was used in case of a non-normal distribution or if there was a considerable amount of difference in variance. This was the case in the data for the pro-MMP-2, MMP-2 complex, pro-MMP-9, and the 48 kDa gelatinase. For clarity, all results are displayed as the mean and SD. The Mann–Whitney *U*-test was used after the Kruskal–Wallis test. When multiple ANOVAs were performed, the Bonferroni correction was applied. Differences were considered significant at $P<0.05$.

Results

Gelatin zymography

The gelatin zymograms showed that GCF contained gelatinolytic activity in bands around 132, 92, 82, 68, 62, 44, and 41 kDa (Figure 1A). In the apposition and resorption samples, a band at 48 kDa was also detected. Only the apposition sample showed bands at 33 and 34 kDa. The reference samples indicated that the bands at 132, 68, and 62 kDa were complexed MMP-2, pro-MMP-2, and active MMP-2, respectively. The bands at 92 and 82 kDa (faint) were most likely pro-MMP-9 and active MMP-9, respectively. The band at 92 kDa was identified as active MMP-9 by Western blotting, but the 82 kDa band was not detected. The band at 48 kDa was only found on the gelatin zymograms and not on the collagen zymograms, suggesting that it is a gelatinase.

Reverse zymography

The reverse zymograms (Figure 1B) showed two distinct bands around 28 and 30 kDa, representing two forms of TIMP-1, as described earlier (Kirk *et al.*, 1995). The standard TIMP-2 showed a band at 21 kDa; however, this band was not present in the GCF samples.

Collagen zymography

The collagen zymograms showed bands comparable with those in the gelatin zymograms at 44 and 41 kDa (Figure 1C). The control lacked a distinct band at 44 kDa but showed a faint wide band of collagenolytic activity. The MMP-1 standard indicated that the bands around 44 and 41 kDa were active forms of MMP-1 as confirmed by Western blotting. These are referred to as 'partially active' (44 kDa) and 'fully active' (41 kDa) MMP-1 as described by Rao *et al.* (1999). The standard MMP-1 showed two distinct bands around 37 kDa, whereas in the samples, only one faint wide band was present. MMP-8 and -13 were not detected by Western blotting.

Quantitative analysis of MMPs and TIMPs

The quantified data of the gelatin and reverse zymography are shown in Figure 2. The collagen zymograms were not quantified because there was too little sample for multiple analysis. Significantly more active- and pro-MMP-2 were present at both experimental sides compared with the control teeth ($P=0.03$ and 0.032, respectively, Figure 2A). Also, more active MMP-9 was present at the resorption side compared with the control ($P=0.003$, Figure 2B). More 48 kDa gelatinase was present at both sides compared with the control ($P<0.0001$ and $P=0.005$, respectively, Figure 2C). The resorption side contained more gelatinase than the apposition side ($P=0.029$). At the apposition and resorption side, more partially activated MMP-1 was present (both $P<0.0001$ compared with the control, Figure 2D).

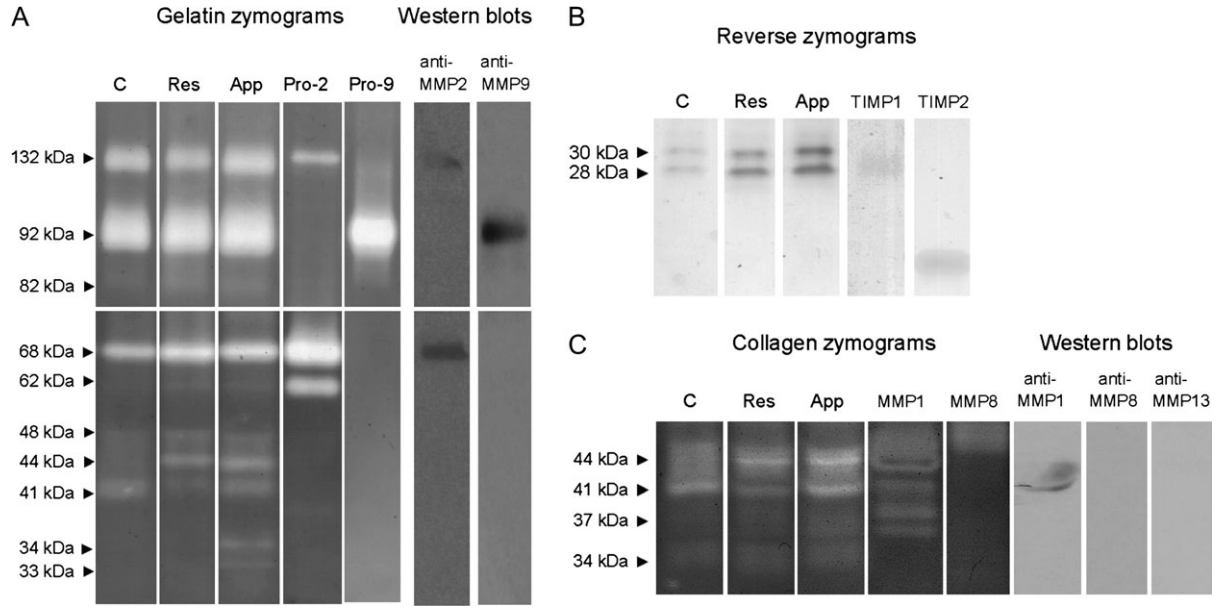

Figure 1 Representative zymograms and Western blots of the resorption and apposition sides of orthodontically moved teeth as well as for the control teeth. The molecular weights of the bands are indicated by the arrows. (A) Representative gelatin zymograms of samples from the control teeth (C, lane 1), the resorption side (Res, lane 2), the apposition side (App, lane 3), a pro-matrix metalloproteinase 2 (MMP-2) reference (lane 4), and a pro-MMP-9 standard (lane 5). Because the proteolytic activity of pro-MMP-9 in the samples was too high for reliable analysis, it was repeated after dilution (upper part). Western blots for MMP-2 (lane 6) and -9 (lane 7) are shown for gingival crevicular fluid (GCF) of orthodontically moved teeth. (B) Representative reverse zymograms of samples of the control teeth (lane 1), the resorption side (lane 2), the apposition side (lane 3), a standard sample of tissue inhibitors of metalloproteinase (TIMP)-1 (lane 4), and a standard sample of TIMP-2 (lane 5). (C) Representative collagen zymograms for the same samples as in panels A and B. Lanes 4 and 5 contained MMP-1 and -8 as standard samples. The Western blots show that GCF of orthodontically moved teeth contain MMP-1 (lane 6).

The amount of active MMP-1 was less at the resorption side compared with the apposition side ($P=0.022$, Figure 2D). The 28 and 30 kDa forms of TIMP-1 were higher at the apposition side compared with the resorption side ($P<0.0001$ and $P=0.11$, respectively) and the control teeth ($P<0.0001$ and $P=0.001$, respectively, Figure 2E).

Discussion

The expression of MMPs and TIMPs was studied at the apposition and resorption sides of orthodontically moved teeth in comparison with control teeth of the same patients. A common problem in the analysis of GCF is the small volume available. In order to have sufficient for the analyses, samples from the same site were pooled (Sari *et al.*, 2004). The differences between the sites represent true differences in MMP and TIMP content since the volume of GCF does not change during orthodontic tooth movement (Serra *et al.*, 2003; Sari *et al.*, 2004; Ingman *et al.*, 2005).

Only a few studies have reported on MMPs and TIMPs during orthodontic tooth movement in humans, and they mainly focused on the initial phase (Apajalahti *et al.*, 2003; Cantarella *et al.*, 2006). In the present investigation, MMPs and TIMPs were analysed after long-term tooth movement. In general, an increased expression of MMPs and TIMP-1 was found at the resorption side as well as the apposition side. TIMP-2 was not detected. Garlet *et al.* (2007) also found higher MMP-1 mRNA at the resorption and apposition side compared with the control and most TIMP-1 mRNA expression at the tension side. This corresponds with the present data. In another study in humans, MMP-1 levels were elevated at the apposition side, but only temporarily elevated at the resorption side, whereas MMP-2 levels remained elevated at the resorption side (Cantarella *et al.*, 2006). However, that study described MMP levels during the initial phase of orthodontic tooth movement, whereas the GCF in the present study was collected during the linear phase. In an immunofluorometic assay, MMP-8 was also detected in the GCF of orthodontic patients (Ingman *et al.*, 2005). This MMP was not found in the present study, which might be due to differences in analysis methods. In two studies of orthodontic tooth movement, no MMP-1 was detected by Western blotting (Apajalahti *et al.*, 2003; Ingman *et al.*, 2005). One of these only investigated MMPs during initial tooth movement (Apajalahti *et al.*, 2003), while MMP-1 might only be increased during linear tooth movement.

In contrast to others, the different forms of MMPs were also investigated in the present study. Partially activated MMP-1 was clearly present at both sides of the experimental teeth, but minimally around the control teeth, whereas active MMP-1 was present at all sites. The partially activated MMP-1 might serve as a reservoir of rapidly available MMP-1 during re-modelling. In total, more MMP-1 was

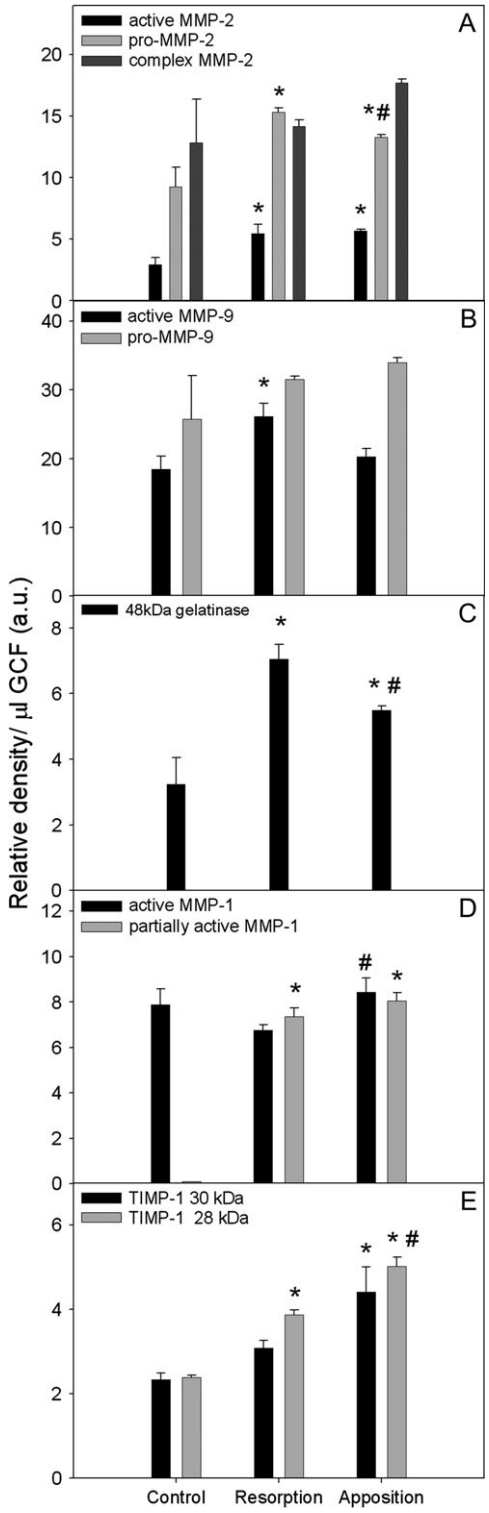

Figure 2 Quantification of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinase (TIMP)-1. MMPs and TIMP-1 were analysed by zymography, quantified, and expressed as mean density × mm^2/µl gingival crevicular fluid (GCF) relative to the reference sample concerned. *Significantly different to the control. #Significantly different to the resorption side (differences were considered significant at $P<0.05$). (A) The different forms of MMP-2. (B) The different forms of MMP-9. (C) The 48 kDa gelatinase. (D) The different forms of MMP-1. (E) The different forms of TIMP-1.

present at both experimental sides than at the control side. This indicates that MMP-1 is involved in increased re-modelling in the PDL and alveolar bone during orthodontic tooth movement. A higher level of active MMP-1 was also found at the apposition side compared with the resorption side. At the apposition side, the fibres of the PDL are stretched and partly torn. They are rapidly re-modelled and lengthened, which involves an upregulation of collagen types I and III (Takahashi *et al.*, 2003). The ratio of collagen type III to type I is increased (Duncan *et al.*, 1984), as in healing PDL (Ivanovski *et al.*, 2000). Type III collagen is thought to play a role in the regulation of the ECM assembly. Finally, some of these fibres are replaced by type I fibres (Christau *et al.*, 2007). This requires MMPs and TIMPs. MMP-1, -8, and -13 and, to a lesser extent, MMP-2 which are known to cleave collagen types I, II, and III into a quarter and a three-quarter fragment (Lazarus *et al.*, 1972; Nagase *et al.*, 2006). The denatured collagen, also known as gelatin, is then degraded by MMP-2 and -9. Remarkably, gelatinolytic fragments of 33 and 34 kDa were exclusively found at the apposition side. Although their exact origin is unclear, they may be involved in the re-modelling processes *in vivo*, since they clearly demonstrate proteolytic activity. The relatively higher expression of MMP-1 at the apposition side might be induced by TIMP-1, which was also increased. TIMP-1 is known to stimulate the secretion of MMP-1 from skin fibroblasts (Clark *et al.*, 1994).

At the apposition side, extensive bone deposition takes place. The increased strain of the PDL fibres is transferred to the bone. It has been proposed that small forces on the bone result in fluid flow through the canaliculi in the bone, which is detected by osteocytes (Melsen, 2001; Rubin *et al.*, 2006). They release nitric oxide, which stimulates bone formation (Burger and Klein-Nulend, 1999). Osteoblasts deposit new osteoid, which is subsequently mineralized (Rodan, 1992). In the present investigation, the highest TIMP-1 levels were found at the apposition side. Previous studies show that TIMP-1 is highly expressed in osteoblasts during bone formation (Bord *et al.*, 1999). It might protect the newly formed bone from degradation by metalloproteinases.

At the resorption side, the compression of the PDL causes a slackening of the fibres in the PDL. The degradation of these fibres is highly increased, and they are replaced by thin collagen type III fibres. Moreover, a change in orientation of the PDL fibres occurs. These non-functional collagen fibres are disorganized and mainly run parallel to the bone surface (Von Böhl *et al.*, 2004). The re-modelling of the PDL requires an upregulation of both MMPs and TIMPs (Takahashi *et al.*, 2006). Bone resorption by osteoclasts is enhanced at this side of the tooth (Andrade *et al.*, 2007; Bildt *et al.*, 2007). This might be induced by the absence of strain in the bone, as the PDL fibres are non-functional (Melsen, 2001). An increase in MMP-9 expression at the resorption

side was found in the present study. MMP-9 is known to stimulate osteoclast activity by enhancing their migration (Samanna *et al.*, 2007). Also, a 48 kDa gelatinase fragment, which might be a proteolytic fragment of MMP-2 (Lee *et al.*, 2002), was upregulated at the resorption side. Since it displays gelatinolytic activity, it might also play a role in the breakdown of gelatin *in vivo*. In the present study, the level of TIMP-1 was lower at the resorption side than at the apposition side, but both were higher than at the control teeth. This might be explained by the dual effects of TIMPs on bone resorption. At low concentrations, they stimulate bone resorption by directly stimulating osteoclasts (Shibutani *et al.*, 1999; Sobue *et al.*, 2001). At higher concentrations, they reduce bone resorption by inhibiting MMP activity (Geoffroy *et al.*, 2004). This is consistent with the present data.

Conclusions

Orthodontic tooth movement induces extensive changes in MMP and TIMP levels in GCF. The main difference was an increase in MMP and TIMP levels between the experimental and the control teeth. This probably reflects the general increase in re-modelling activity during tooth movement. The differences between the resorption and apposition side are caused by the different nature of re-modelling. At the resorption side, bone resorption takes place and extensive re-modelling of the PDL. MMP-1, 2, and 9, a 48 kDa gelatinase, and TIMP-1 are upregulated. At the apposition side, only bone apposition takes place and more limited PDL re-modelling. MMP-1, 2, and the 48 kDa gelatinase are upregulated. However, pro-MMP-2 and the 48 kDa gelatinase levels are significantly lower at the apposition side compared with the resorption side. TIMP-1 levels on the other hand are higher at the apposition side. TIMP-1 might play a dual role by stimulating bone resorption at low concentrations at the resorption side and reducing it at higher concentrations at the apposition side. Analysis of MMPs and TIMPs in orthodontics may contribute to more predictable treatment regimens in the future. It may serve as a diagnostic aid to predict the rate of tooth movement, the severity of root resorption, and the relapse. Chair-side MMP tests have already been developed to monitor periodontitis progression (Mäntylä *et al.*, 2003).

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