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Matrix metalloproteinase inhibitors reduce collagen gel contraction and α-smooth muscle actin expression by periodontal ligament cells

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Background and Objective: Orthodontic tooth movement requires remodeling of the periodontal tissues. The matrix metalloproteinases (MMPs) degrade the extracellular matrix components of the periodontal ligament, while the tissue inhibitors of metalloproteinases (TIMPs) control their activity. Synthetic MMP inhibitors have been developed to inhibit MMP activity. In this study, periodontal ligament cells in contracting collagen gels served as a model for enhanced periodontal remodeling. The effect of MMP inhibitors on gel contraction and on MMP and TIMP expression was analyzed.

Material and Methods: Human periodontal ligament cells were cultured in three-dimensional collagen gels and incubated with the MMP inhibitors BB94, CMT-3, doxycycline and Ilomastat. Gel contraction was determined using consecutive photographs. The relative amounts of MMPs and TIMPs were analyzed using substrate zymography and mRNA expression using quantitative polyermase chain reaction.

Results: All MMP inhibitors reduced MMP activity to about 20% of the control activity. They all reduced contraction, but CMT-3 and doxycycline had the strongest effect. These inhibitors also reduced MMP-2, MMP-3 and α-smooth muscle actin mRNA expression. The expression of MMP-1 mRNA seemed to be increased by CMT-3. No effects were found on the amounts of MMPs and TIMPs.

Conclusion: Synthetic MMP inhibitors strongly reduced gel contraction by periodontal ligament cells. This was primarily caused by an inhibitory effect on MMP activity, which reduces matrix remodeling. In addition, α-smooth muscle actin expression was reduced by CMT-3 and doxycycline, which limits the contractile activity of the fibroblasts.

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The periodontal ligament is the connective tissue that attaches the tooth to the alveolar bone. It plays a crucial role in the adaptation of the teeth to physiological forces as well as to orthodontic forces (1). Its ability to remodel rapidly allows the periodontal ligament to adapt quickly to these forces (2). The mechanical strength of the periodontal

ligament is mainly derived from collagen type I, which is one of the major proteins in the extracellular matrix (3).

Fibroblasts are the main cells in the periodontal ligament and they are responsible for the production and degradation of most extracellular matrix components. They produce matrix metalloproteinases (MMPs), which represent a major family of enzymes that are able to degrade the extracellular matrix (4). The naturally occurring tissue inhibitors of metalloproteinases (TIMPs), which are also produced by the periodontal ligament fibroblasts, counteract the activity of MMPs (5,6). A balance between MMPs activity and the synthesis of new extracellular matrix components is thought to be essential for maintaining the periodontal structures during physiological turnover and orthodontic tooth movement.

Next to the naturally occurring TIMPs, synthetic MMP inhibitors have been developed. They are thought to have therapeutic applications in diseases that involve extensive matrix degradation, such as periodontitis (7). About seven synthetic inhibitors are currently under study for clinical use and one has already been approved for therapeutic use in periodontitis (7,8). Matrix metalloproteinase inhibitors have been shown to inhibit orthodontic tooth movement in rats (9,10) and might be used to prevent relapse after orthodontic treatment. The production of MMPs and TIMPs by human fibroblasts has mainly been studied in two-dimensional culture systems (11–13). These studies showed that fibroblasts produce MMP-1, -2, -3, -9, -13 and -14, and TIMP-1 and -2. Periodontal ligament cells were shown to produce similar types of MMPs and TIMPs in two-dimensional cultures (14–16). Synthetic inhibitors have been reported to decrease the production of MMP-2 and -9 by porcine periodontal ligament cells (17). However, other studies have shown the stimulation of expression of MMP-2 by human periodontal ligament cells (18) and of MMP-9 by keratinocytes (19).

The morphology, proliferation and cytoskeletal organization of fibroblasts in three-dimensional matrices is more physiological than in a two-dimensional culture (20–23). The production of MMP-1, -2, -3 and -9 is up-regulated when (myo)fibroblasts are cultured in a three-dimensional collagen matrix compared with a two-dimensional matrix (24,25). Periodontal ligament cells cultured in a collagen gel express MMP-1, -2 and -9, and TIMP-1 and -2 (26,27).

Generally, fibroblasts cultured in a collagen gel remodel and contract this artificial tissue (28). Previous studies showed that α-smooth muscle actin and MMP-1, -2 and -3 are required for contraction (29–31). By contrast, TIMP-1 seems to reduce gel contraction (32).

The periodontal ligament also undergoes extensive remodeling during orthodontic tooth movement. We used contracting collagen gels with periodontal ligament cells as a model for increased periodontal ligament remodeling. The specific aim was to analyse the effects of MMP inhibitors on gel contraction and on MMPs and TIMPs. To study this, we used the tetracycline-based MMP inhibitors CMT-3 and doxycycline, and the hydroxymate-based MMP inhibitors Batimastat (BB94) and Ilomastat (GM6001).

Material and methods

MMP inhibitors

The following MMP inhibitors were used: BB94 (Batimastat; British Biotech., Inc., Oxford, UK), 4-dedimethylamino sancycline (CMT-3; CollaGenex Pharmaceuticals, Inc., Newtown, CT, USA), GM6001 (Ilomastat; Chemicon International, Inc., Billerica, MA, USA) and doxycycline (Sigma-Aldrich Chemie, GmbH, Steinheim, Germany). All inhibitors were dissolved in dimethylsulfoxide (ICN Biomedicals, Inc., Aurora, OH, USA).

Cell culture

Human periodontal ligament tissue was obtained from fully erupted healthy molars that were extracted from three subjects as part of an overall dental treatment plan. All subjects gave their consent according to the guidelines of the local ethical committee. After extraction, the molars were washed in saline and stored at 4°C in phosphate-buffered saline (Gibco, Paisley, UK) containing 100 U/mL of penicillin and 100 μg/mL of streptomycin (Gibco). Explants were obtained by cutting the periodontal ligament tissue from the lower half of the root, until 2 mm from the apex. The explants were washed extensively in fresh culture medium consisting of Dulbecco's modified Eagle's medium (Gibco), 10% fetal calf serum (Gibco), 100 U/mL of penicillin and 100 μg/mL of streptomycin. The explants were incubated in one well of a 24-well culture plate at 37°C in a humidified atmosphere of 5% CO_2 in air. The culture medium was changed three times per week. After the outgrowth of fibroblasts, the cultures were trypsinized with 0.25% trypsin (Gibco) and consecutively passaged to culture flasks of 25 and 75 cm^2. After a culture period of about 3 wk, the cells (passage 4) were frozen in 7.5% dimethylsulfoxide (ICN Biomedicals, Inc.) in culture medium and stored in liquid N_2. The cells were considered as periodontal ligament cells based on the specific location of harvesting and their alkaline phosphatase activity. The alkaline phosphatase activity of the periodontal ligament cells was determined using *p*-nitrophenyl phosphate as a substrate (Sigma-Aldrich) and was found to be consistently higher than that of control oral fibroblasts. Prior to the experiments, the cells were rapidly thawed at 37°C, diluted in culture medium and centrifuged at 400 *g* for 5 min. The cell pellet was resuspended in culture medium and seeded in a 75-cm^2 culture flask. At 80–90% confluence, the cells were passaged to two 175-cm^2 flasks. At 80–90% confluence the cells were trypsinized and thus ready for use in the experiments.

Experimental design

Based on the results of a pilot experiment, 5 μM BB94, 25 μM CMT-3, 25 μM Ilomastat and 50 μM doxycycline were

used in the final experiments. For these experiments, 4×10^5 cells were incorporated in gels prepared from 3 mL of collagen solution and incubated with the inhibitors. The controls did not contain MMP inhibitors. All gels were used to determine the gel contraction. Four gels from each inhibitor group were used to determine the DNA content, and the conditioned media of these gels were used for zymography. To obtain enough mRNA to carry out the quantitative polymerase chain reaction (PCR), eight gels were pooled for each inhibitor.

Collagen gels

The culture plates were precoated with 1% bovine serum albumin in phosphate-buffered saline to obtain free-floating gels. The mixture for the collagen gels was prepared on ice and contained 1.2 mg/mL of collagen type I from rat tail (Serva Electrophoresis, Heidelberg, Germany), 10% (v/v) minimal essential medium (10×; (Gibco), 0.1 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; Gibco), 30 mM $NaHCO_3$ (Gibco) and 2 mM NaOH. A cell suspension was added total mixture to obtain a concentration of 1.33×10^5 cells/mL. In each well, 3 mL of gel solution (4×10^5 cells) was carefully dispensed. The gels were placed in an incubator at 37°C in a humidified atmosphere of 5% CO_2 in air to gelate for 1.5 h. Thereafter, 2 mL of culture medium, with or without MMP inhibitors, was added to give final concentrations of 25 μM CMT-3, 25 μM Ilomastat, 5 μM BB94 and 50 μM doxycycline. The final concentrations of the additives in the cultures were 1% fetal calf serum (Gibco), 100 U/mL of penicillin and 100 μg/mL of streptomycin. The gels were incubated at 37°C for 24 h. Samples of the culture medium were taken at 24 h for zymography. Separate gels were used for RNA extraction and to determine the total DNA content.

Measurement of gel contraction

The surface area of the gels was measured at 0, 1, 2, 4, 6, 12 and 24 h. Standardized pictures were taken using the digital camera of a Gel Doc 2000 system (Bio-Rad, Hercules, CA, USA). The gel surface areas were subsequently measured using QUANTITY ONE software (Bio-Rad). The gel surface area at each time-point was expressed as the percentage of the gel surface at $t = 0$, and plotted against time.

Quantitative PCR

RNA isolation and quantitative reverse transcription (RT)-PCR was performed to measure the mRNA level of different MMPs, TIMPs and α-smooth muscle actin. First, the periodontal ligament cells were lysed by adding RLT buffer (Qiagen, Hilden, Germany) and 10 μL/mL of β-mercaptoethanol (ICN Biomedicals, Inc.) to the gels. The gels were then homogenized through a 21 gauge needle, and stored at −80°C until use. RNA was extracted using the RNeasy Kit (Qiagen), according to the guidelines of the manufacturer, and this included a DNAse I digestion step (Qiagen) to degrade genomic DNA. Total RNA was quantified by measuring the absorption at 260 and 280 nm. For the RT reaction, about 1.5 μg of RNA was used. One microlitre of deoxyribonucleotide triphosphates (dNTPs) containing 10 mM of each deoxyribonucleotide (dATP, dCTP, dGTP and dTTP) and 1 μL of 0.5 μg/μL of random primers (Promega, Madison, WI, USA) were added and incubated at 65°C for 5 min. Then, the samples were put on ice and 4 μL of SuperScript™ II Reverse Transcriptase buffer [SuperScript™ II RT, 5× first-strand buffer (250 mM Tris–HCl, pH 8.3, 375 mM KCl, 15 mM $MgCl_2$)] and 2 μL of 0.1 M dithiothreitol (Gibco) were added. The samples were heated for 2 min at 25°C, 200 U of SuperScript™ II reverse transcriptase was added and the samples were incubated for a further 10 min at 25°C. The RT reaction was performed at 42°C for 50 min and was stopped by heating the samples to 70°C for 15 min. Thereafter, the samples were stored at −20°C.

The PCR reaction was performed in a final volume of 25 μL containing 12.5 μL of SYBR®Green Supermix (Bio-Rad), about 5 μg of cDNA, 4.5 μL of RNAse-free water, 1.5 μL of 2.5 M forward primer and 1.5 μL of 2.5 M reverse primer. The primers for MMP-2, TIMP-1 and TIMP-2 were obtained from TNO (Leiden, the Netherlands). The primer sequences are shown in Table 1. In order to quantify the gene-expression levels, the cDNA content of the samples was normalized and the linear range of amplification was determined for each primer set. Glyceraldehyde-3-phosphate dehydrogenase was used as a reference gene. The amount of mRNA was expressed as ΔC_t ($= C_t$, gene of interest$-C_t$, glyceraldehyde-3-phosphate dehydrogenase), where C_t is the threshold cycle. All cycles up to 40 were included in the calculations, although cycles between 35 and 40 were interpreted with caution. Cycles of 40 and more were not included. The fold change in gene expression by the MMP inhibitors was expressed, relative to the control, as $2^{-\Delta Ct}$. The gene expression in the control was set to 100%.

DNA assay

The amount of DNA in each gel was determined using the PicoGreen® dsDNA Quantitation kit (Molecular Probes, Inc., Eugene, OR, USA). PicoGreen is a fluorescent stain for double-stranded DNA. The fluorescent signal was measured in an FL600 Microplate Fluorescence Reader (Bio-Tek Instruments, Inc., Winooski, VT, USA) at excitation 485 nm and emission 520 nm. Bacteriophage lambda DNA was used as a standard, ranging from 0 to 1000 ng/mL. The total amount of DNA in each gel was calculated.

Gelatin zymography

Gelatinases (MMP-2 and -9) in the conditioned media were analyzed by gelatin zymography. All samples were analyzed in quadruplicate. The polyacrylamide gel (7.5%) contained 3.5 mM sodium dodecyl sulphate and 1 mg/mL of gelatin, as described by others (33). A 1:1 mixture of sample and sample buffer was then electrophoresed for 1.5 h at 80 mA. A Broad Range

Table 1. Forward and reverse primers for the matrix metalloproteinases (MMPs), tissue inhibitors of metalloproteinases (TIMPs) and α-smooth muscle actin (ASMA)

Gene	Forward primer	Reverse primer
MMP-1	GGGAGATCATCGGGACAACTC	GGGCCTGGTTGAAAAGCAT
MMP-0033	TGGCATTCAGTCCCTCTATGG	AGGACAAAGCAGGATCACAGTT
MMP-8	CCAAGTGGGAACGCACTAACTTGA	TGGAGAATTGTCACCGTGATCTCTT
MMP-9	TGGGGGGCAACTCGGC	GGAATGATCTAAGCCCAG
TIMP-3	CCAGGACGCCTTCTGCAAC	CCTCCTTTACCAGCTTCTTCCC
ASMA	GCTCACGGAGGCACCCCTGAA	TCCAGAGTCCAGCAGATG
GAPDH	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG

GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Marker (Bio-Rad), 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, CA, USA) was used as a reference sample. After electrophoresis, the gels were washed in 2.5% Triton X-100 (Sigma-Aldrich) to remove the sodium dodecyl sulphate, 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% Triton X-100, at 37°C for 18 h. They were stained for 45 min with 2.5 g/L of Coomassie Brilliant Blue R250 (Imperial Chemical Industries PLC, London, UK), 10% acetic acid, and 40% methanol in water, and thereafter destained with 10% acetic acid and 40% methanol in water. The MMPs appear as bright bands within the stained gel. The gels were scanned using an HP ScanJet 4C/T, and the bands were analyzed using QUANTITY ONE software (Bio-Rad). The amount of enzyme in the bands was calculated as average density × mm^2 per ng of DNA. 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 were calculated to obtain the relative amount of MMPs.

Reverse zymography

TIMPs in the conditioned media were analyzed using reverse zymography. This technique is similar to gelatin zymography, except that conditioned medium from baby hamster kidney cells (BHK-21; ATCC, Rockville, MD, USA) was included in the gels. This medium contains mainly MMP-2. The MMP-2 degrades all gelatin in the gel, except where TIMPs are present. The polyacrylamide gel (15%) contained 3.5 mM sodium dodecyl sulphate, 1 mg/mL of gelatin and 10% conditioned medium from BHK-21 cells. A 1:1 mixture of sample and sample buffer was then electrophoresed for 3 h at 80 mA. A Broad Range Marker (Bio-Rad) was included to determine the molecular weight of the TIMPs. Recombinant human TIMP-1 and -2 (Oncogene) were used as references. After electrophoresis the gels were washed in 2.5% Triton X-100 (Sigma-Aldrich) to remove the sodium dodecyl sulphate. The gels were then incubated in 50 mM Tris–HCl (pH 7.5), 5 mM $CaCl_2$, 0.1% Triton X-100 and 0.2 M NaCl at 37°C for 18 h. They were stained and destained, following the same method as described for gelatin zymography. The TIMPs were visible as dark blue bands within the destained gel. Thereafter, the gels were scanned and analyzed as described for gelatin zymography.

MMP activity assay

MMP activity in the conditioned media was analyzed in quadruplicate using an MMP assay. In short, the degradation of the fluorogenic MMP substrate TNO211-F (TNO) was measured using a fluorimeter (36). TNO211-F consists of a fluorophore and a light-absorbing group (quencher) attached to an amino acid sequence that serves as a substrate for MMPs. Through enzymatic cleavage by MMPs, the quencher is eliminated and fluorescence can be measured. It is mainly cleaved by MMP-1, -2, -3, -8, -9 and -13. The fluorescent signal was measured in a Cytofluor II fluorimeter (PerSeptive Biosystems, Framingham, MA, USA), and expressed as relative fluorescence units per second. The MMP activity in the samples was presented as relative fluorescence units/s per ng of DNA.

Statistical analyses

For the pilot experiment, repeated-measures analysis of variance was used to compare gel contraction in the experimental groups. One-way analysis of variance was used to compare the gel surface areas and the percentage of live cells in the cytotoxicity test. For the final experiment, differences in gel contraction were analyzed using a repeated-measures analysis of variance. One-way analysis of variance was used to analyze gel contraction at the 12-h time-point, the amounts of MMPs or TIMPs in the conditioned media, and the amounts of α-smooth muscle actin, MMP and TIMP mRNA. Dunnett's test was used as a *post hoc* test. Differences were considered to be significant at a p-value of < 0.05.

Results

Gel contraction

A pilot experiment showed that optimal inhibitor concentrations were 5 μM for BB94, 25 μM for CMT-3 and Ilomastat, and 50 μM for doxycycline (data not shown). As the pilot experi-

ment showed no further contraction of the collagen gels after 24 h, this was chosen as the end point for the final experiment. Figure 1 shows the results of the final gel-contraction experiments. The gel surface area was measured at t = 1, 2, 4, 6, 12 and 24 h. The graphs show that contraction of the gels started mostly after a lag phase of 1 h. All MMP inhibitors reduced contraction up to t = 12 (p = 0.027, 0.001, < 0.001 and 0.005 for BB94, CMT-3, Ilomastat and doxycycline respectively). At t = 24, the reduction was still significant for CMT-3 and doxycycline (p = 0.006 and < 0.001, respectively). The right panel of Fig. 1 shows the effect of all inhibitors on the gel surface area at t = 12 h.

mRNA expression

The results of the quantitative RT-PCR are shown in Fig. 2. Most data were obtained from threshold cycles between 15 and 25, but for MMP-9 mRNA this was mostly between 35 and 40. Therefore, these results have to be interpreted with caution. CMT-3 significantly reduced MMP-2, α-smooth muscle actin and TIMP-2 mRNA expression (p = 0.025, 0.010, and 0.021 respectively). MMP-3 mRNA expression was reduced by doxycycline (p = 0.027), which also tended to inhibit α-smooth muscle actin mRNA expression. There was a trend of increased MMP-1 mRNA expression by CMT-3 and doxycycline.

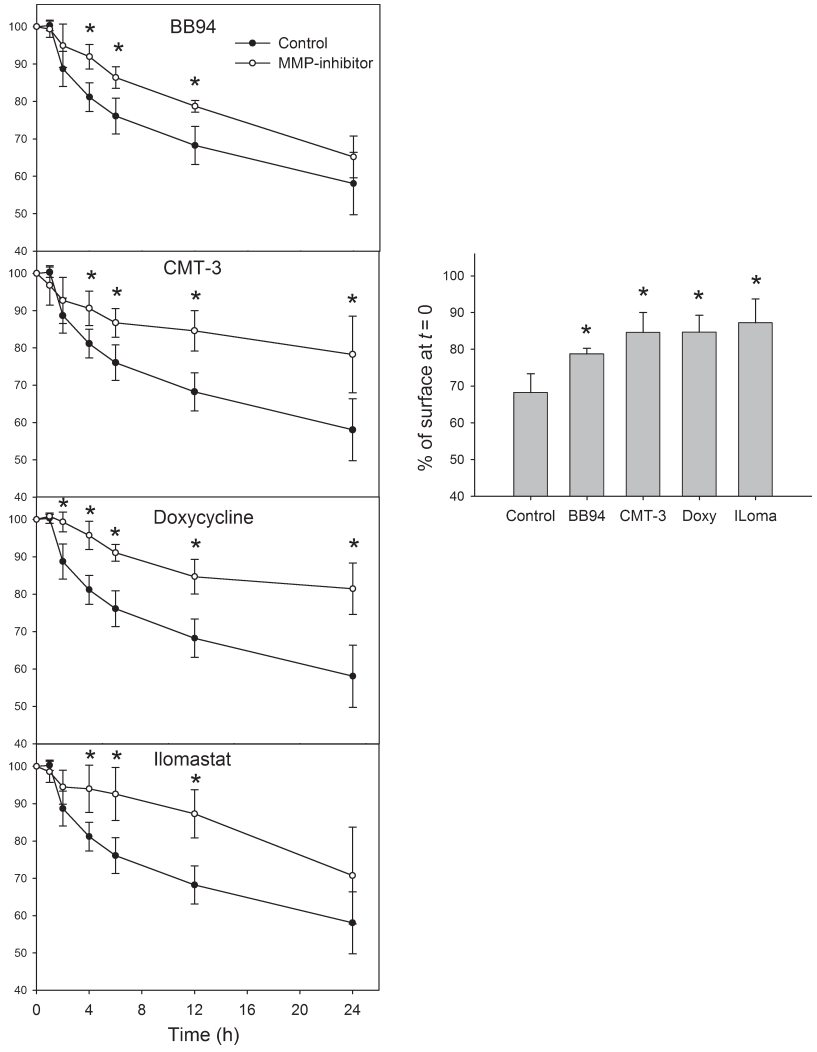

Fig. 1. Collagen gel contraction. The gel surface areas were measured at t = 1, 2, 4, 6, 12 and 24 h. Data are expressed as means (± standard deviation). From t = 0 to 12, all matrix metalloproteinase (MMP) inhibitors showed a significant reduction of gel contraction compared with the control. At t = 24, the reduction was still significant for CMT-3 and doxycycline. The right panel shows the gel surface area at t = 12. The surface areas are expressed as a percentage of the gel surface areas at t = 0 (means ± standard deviation). Asterisks indicate a significant difference compared with the control (p < 0.05). Doxy, doxycycline; Iloma, Ilomastat.

Relative amounts of MMPs and TIMPs

Representative zymograms are shown in Fig. 3. The gelatin zymograms show that the periodontal ligament cells produced gelatinase activity in bands at 92, 82, 74, 69 and 62 kDa. Also, minor amounts of MMP complexes were found at around 130 kDa. A pro-MMP-2 reference, used to compare the zymograms, also showed the complexed form at 130 kDa, the latent form at 69 kDa and the active form at 62 kDa. The bands at 92 and 82 kDa were probably the latent and active forms of MMP-9, respectively. These forms have been described previously (34). The nature of the 74-kDa band remained unknown and was not further analyzed. The reverse zymograms show that the periodontal ligament cells produced mainly TIMP-1. The bands at 21 kDa, representative for TIMP-2, were mostly faint. None of the MMP inhibitors had a significant effect on the relative amounts of MMPs or TIMPs.

MMP activity

General MMP activity in the conditioned medium was analyzed using an MMP activity assay. As expected, all four MMP inhibitors significantly reduced MMP activity (Fig. 4). BB94 reduced the MMP activity to 23 ± 3.8% (p = 0.021), CMT-3 reduced the MMP activity to 24 ± 2.8% (p = 0.023), doxycycline reduced the MMP activity to 18 ± 5.5% (p = 0.014) and Ilomastat reduced the MMP activity to 25 ± 4.6% (p = 0.025).

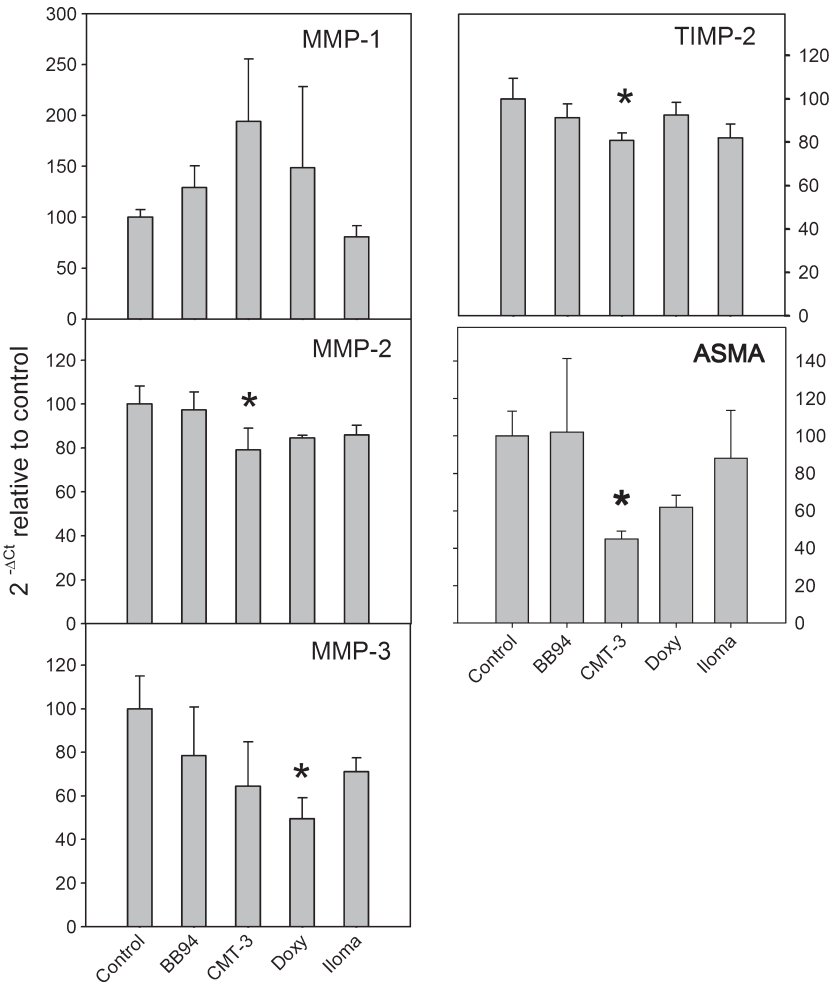

Fig. 2. Effect of matrix metalloproteinase (MMP) inhibitors on the mRNA expression of MMPs, α-smooth muscle actin and tissue inhibitors of metalloproteinases (TIMPs). The panels show the effect of BB94, CMT-3, Ilomastat and doxycycline on the mRNA expression of MMP-1, -2, -3, α-smooth muscle actin, and TIMP-2. The fold change in gene expression induced by the MMP inhibitors was expressed as $2^{-\Delta Ct}$ relative to the control, which was set to 100%. Doxy, doxycycline; Iloma, Ilomastat.

Discussion

In this study, free-floating three-dimensional collagen gels with periodontal ligament cells were used as a model for increased periodontal ligament remodeling. MMP inhibitors might be used to reduce matrix degradation. All MMP inhibitors used in this study were able to inhibit gel contraction by periodontal ligament cells, as also shown for other cell types (30,35,36). Contraction involves the attachment of fibroblasts to the collagen by integrins and collagen degradation by MMPs. The major integrins are α1β1 and α2β1, of which binding of the latter stimulates MMP1 expression (37,38). In addition, MMP-3 and -13 seem to be involved (39,40). Contraction itself may result from the repeated formation of cellular lamellipodia, and cytoskeletal re-arrangement by α-smooth muscle actin and myosin II-B (41,42).

Messenger RNA analyses revealed that periodontal ligament cells express a wide variety of MMPs (MMP-1, -2, -3, -8 and -9), TIMPs (TIMP-1, -2 and -3) and α-smooth muscle actin. Only few significant effects of the MMP inhibitors on mRNA expression were found. Doxycycline reduced MMP-3 mRNA expression to about 50% of that of the control, and the same trend was found for CMT-3. Earlier studies have shown that MMP-3 production is decreased in mechanically stimulated gels (43,44). *In vitro*, specifically MMP-3 has been shown to release transforming growth factor-β from the extracellular matrix by proteolytic cleavage of the latent transforming growth factor-β binding protein (45). This might be more relevant for oral fibroblasts than for skin fibroblasts as they produce much more MMP-3 (39). Transforming growth factor-β subsequently stimulates α-smooth muscle actin expression, which enhances gel contraction and cell migration (39,46). In our study, α-smooth muscle actin mRNA was decreased by CMT-3, and the same trend was found for doxycycline. The down-regulation of MMP-3 may have contributed to the inhibition of gel contraction via the reduction of transforming growth factor-β-induced α-smooth muscle actin expression. Also, MMP-3 has been described as a strong activator of latent MMPs (47,48). Its down-regulation may therefore reduce contraction through multiple mechanisms.

By contrast, both CMT-3 and doxycycline seem to up-regulate MMP-1 mRNA expression. In a previous study, we found an up-regulation of MMP-2 protein expression by several CMTs, including CMT-3, in two-dimensional cultures (18). An up-regulation of MMP-2 was also shown for periosteal cells incubated with other synthetic inhibitors (49). These data indicate that MMP inhibitors might have unexpected stimulatory effects on MMP production via positive feedback mechanisms. In the present experiments, CMT-3 showed a reduction of MMP-2 and TIMP-2 mRNA expression, but no effect on the amounts of protein. This might be a result of the difference between the two-dimensional and the three-dimensional culture systems. In our study, we found that periodontal ligament cells cultured in collagen gels produced latent and active MMP-2 as well as a complexed form of MMP-2. Also, TIMP-1 protein was present, and in some samples small amounts of TIMP-2 were found. Remarkably, the periodontal ligament cells produce MMP-9 and a 74-kDa related proteinase,

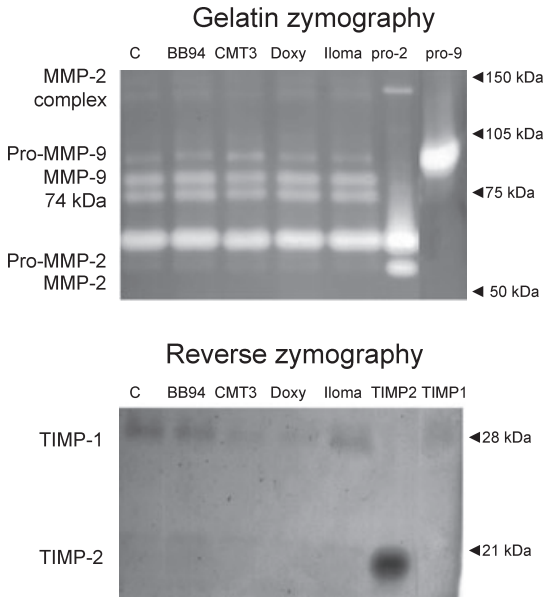

Fig. 3. Effect of matrix metalloproteinase (MMP) inhibitors on the amounts of MMP and tissue inhibitor of metalloproteinase (TIMP). Representative zymograms. The molecular weights of the markers are indicated by the arrows. The upper panel represents a gelatin zymogram, showing proteolytic activity of complexed MMP-2, pro-MMP-9, active MMP-9, a 74 kDa gelatinase, pro-MMP-2 and active MMP-2. The lower panel represents a reverse zymogram, showing bands of TIMP-1 and -2. Doxy, doxycycline; Iloma, Ilomastat.

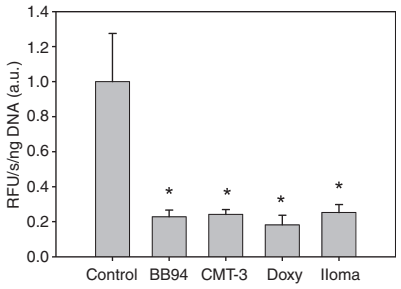

Fig. 4. The effect of BB94, CMT-3, Ilomastat and doxycycline on general matrix metalloproteinase (MMP) activity in the conditioned media. The degradation of a fluorescent substrate was expressed as relative fluorescence units (RFU)/s/ng of DNA. The asterisks indicate a significant difference compared with the control ($p < 0.05$). Doxy, doxycycline; Iloma, Ilomastat.

whereas in a two-dimensional culture they do not (18). The 74-kDa proteinase has not previously been described in periodontal ligament cells, but it was identified as an activated form of MMP-9 in fibrosarcoma cells (50) and in mast cells (51). Although it is not known whether the 74-kDa proteinase also occurs *in vivo*, it might play a role in periodontal ligament remodeling.

Periodontal ligament cells produce MMP-2, but no significant amounts of MMP-9, in a two-dimensional culture (16,18). Others showed that MMP-9 production increased when fibroblasts or trophoblasts were transferred from a two-dimensional to a three-dimensional environment (52,53). This might be a result of the interaction of the cells with the collagen matrix through integrins (38). It is known that this interaction triggers signal transduction, which leads to metabolic changes in the cell (54). MMP-9 is probably required for the migration of cells in a three-dimensional matrix (55).

In summary, periodontal ligament cells seem to produce different MMPs in three-dimensional cultures compared with two-dimensional cultures. This is probably caused by the interaction with collagen. Periodontal ligament cells also responded differently to incubation with MMP inhibitors. As a three-dimensional culture system seems to mimic the *in vivo* situation much better, it is more suitable for studying periodontal ligament remodeling. The activity of MMPs produced by periodontal ligament cells in a three-dimensional culture can be greatly reduced by synthetic MMP inhibitors, thereby inhibiting collagen gel contraction. This might be enhanced by the specific inhibition of MMP-3 expression, which reduces transforming growth factor-β-mediated α-smooth muscle actin expression. All inhibitors reduced MMP activity to an equal extent, but CMT-3 and doxycycline showed the largest effect on gel contraction.

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