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Chemically modified tetracyclines stimulate matrix metalloproteinase-2 production by periodontal ligament cells

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Background and Objective: The purpose of this study was to investigate the effects of chemically modified tetracyclines (CMTs) on the production of gelatinases [matrix metalloproteinase (MMP)-2 and -9] by human periodontal ligament (PDL) cells, and on the activity of recombinant gelatinases.

Material and Methods: Human PDL cells were cultured with CMT-1, -3, -5, -7 or -8 in concentrations of 0, 1, 5, 10, 20, 50, 100, 200 and 500 μM. Gelatin zymography was used to determine MMP-2 and -9 production of the cells. The amount of DNA present in the cultures was analyzed using a fluorescent assay. The cytotoxicity of the CMTs was also determined. Recombinant human MMP-2 and -9 were incubated with the CMTs (0–500 μM) and their activity was analyzed using an internally quenched fluorogenic substrate.

Results: MMP-2 production was stimulated up to sevenfold by CMT-1, -3, -7 and -8 at low concentrations (10–200 μM). No significant amounts of MMP-9 were produced. In contrast, MMP-2 and -9 activity was reduced by ≈10–40-fold at higher concentrations (200–500 μM). CMT-5 had no effect on the production or on the activity of MMP-2 and -9. Only CMT-3 and -8 had cytotoxic effects on the PDL cells at the highest concentrations.

Conclusion: Surprisingly, CMTs are able to stimulate MMP-2 production at relatively low concentrations. However, at higher concentrations they exert a much stronger inhibitory effect on gelatinase activity. A possible stimulatory effect of CMTs on MMP production should be considered in their clinical use.

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The primary function of the periodontal ligament (PDL) is to attach the teeth to the alveolar bone. In addition, the PDL provides resistance against tensional and compressive forces, and it regulates mastication through mechanoreceptors (1). The PDL is a highly cellular tissue that contains several cell types, such as fibroblasts, cementoblasts, osteoblasts, osteoclasts, epithelial cells and progenitor cells (2). The fibroblasts are the predominant cell type and they are responsible for the synthesis and degradation of the extracellular matrix molecules. The PDL has a relatively high turnover rate compared with other connective tissues (3). It can therefore adapt quickly to mechanical forces, such as during orthodontic tooth movement (4,5). Matrix metalloproteinases (MMPs) play an important role in the degradation of the extracellular matrix of the PDL during remodeling (6,7). At

present, 23 human MMPs are known (7–9). Besides their role in physiological remodeling of the periodontium and its adaptation to orthodontic tooth movement (10), they also play a role in pathological tissue degradation, such as in periodontitis (11). Several MMPs have been detected in crevicular fluid, and MMP-2, -3, -8, -13 and -14 were found to be elevated in fluid samples from teeth that were affected by periodontitis (11–13). Elevated MMP levels have also been detected in the crevicular fluid of orthodontic patients (14). Proteolytic enzymes, such as urokinase- and tissue-type plasminogen activators, can activate MMPs (15,16). Furthermore, MMP-14, a membrane-bound MMP, can activate MMP-2 (17). At the transcriptional level, MMPs are regulated by factors, such as cytokines and fibronectin (18).

During the late 1980s, it was discovered that tetracyclines have an inhibitory effect on MMP activity (19). This led to the development of chemically modified tetracyclines (CMTs), which have lost their antimicrobial activity but retain their anti-MMP activity (20). The mechanism of action of CMTs is still not completely clear. Several mechanisms for the inhibition of activity of MMPs have been postulated. CMTs may down-regulate MMP expression, inhibit pro-MMP activation, or directly inhibit active MMPs, by binding to the active Zn^{2+} site and the Zn^{2+} at a secondary site of the MMP molecule (21). Moreover, it has become clear that CMTs, by inhibiting MMPs, also disturb the balance between MMPs and their naturally occurring inhibitors, the tissue inhibitors of matrix metalloproteinases (TIMPs) (22). Several authors have investigated the effects of CMTs on MMP activity *in vitro* (23–26). CMT-3 and -8 appear to be the most potent inhibitors of MMP activity. CMT-1 also affects MMP activity, but it is less powerful. CMT-5 has no inhibitory effects on MMP activity and is therefore often used as a control CMT. Only a few studies have focused on the effects of CMTs on the production of MMPs *in vitro*. A marked decrease of MMP-2 mRNA levels is found in keratinocytes by doxycycline, CMT-1 and CMT-8 (27). Others have shown that CMT-3 is able to inhibit the production of MMP-2 by human colon carcinoma cells (28). PDL cells play an important role in both the periodontal tissue breakdown during periodontitis and the remodeling of the periodontium during orthodontic tooth movement. Therefore, PDL cells were used in this study. Despite their therapeutic potential in dentistry, little is known about the effect of CMTs on MMP production by human PDL cells. Therefore, the aim of this study was to investigate the effect of CMT-1, -3, -5, -7 and -8 on the gelatinases (MMP-2 and -9) produced by human PDL cells.

Material and methods

Chemically modified tetracyclines

The CMTs 4-dedimethylamino tetracycline (CMT-1), 4-dedimethylamino sancycline (CMT-3), tetracycline pyrazole (CMT-5), 12a-dehydroxy-4-dedimethylamino tetracycline (CMT-7) and 4-dedimethylamino doxycycline (CMT-8), were kindly provided by CollaGenex Pharmaceuticals, Inc. (Newton, PA, USA).

Cell culture

Human PDL tissue was obtained from an extracted, fully erupted, healthy third molar. After extraction, the third molar was washed in saline and stored at 4°C in phosphate-buffered saline (PBS) (Gibco, Paisley, UK) containing 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco). Explants were obtained by cutting the PDL 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 (DMEM; Gibco), 10% fetal calf serum (FCS; Gibco), and 100 U/ml penicillin and 100 μg/ml 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, 75 and 175 cm^2. After a culture period of ≈3 wk, the cells (passage 4) were frozen in 7.5% dimethylsulfoxide (DMSO; ICN Biomedicals, Inc., Aurora, OH, USA) in culture medium and stored in liquid N_2. The cells tested positive for alkaline phosphatase activity, indicating that these cells were indeed PDL cells and not from gingival origin.

Incubation with CMTs

Before 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, 75-cm^2 flasks. At 80–90% confluence, they were trypsinized and transferred to 24-well culture plates (100,000 cells/well) for the MMP assays and to 96-well culture plates (25,000 cells/well) for the cytotoxicity assay. All cells were then cultured in medium with DMEM, but without phenol red, for 1 d. Then, the cells were washed with DMEM without phenol red. The following 24 h, quadruple cultures were incubated with increasing concentrations of CMT-1, -3, -5, -7 and -8. Each CMT was dissolved in DMSO and diluted to give final concentrations of 0, 1, 5, 10, 20, 50, 100, 200 and 500 μM in DMEM containing 2% DMSO, but without phenol red. DMSO at 2% did not affect cell viability. DMSO concentrations up to 2% also do not affect MMP production by cultured cells, as shown previously (29).

Gelatin zymography

Gelatinases in the culture media were analyzed by gelatin zymography. Zymograms of samples of recombinant MMPs incubated with CMTs (0–500 μM) showed no inhibitory activity of CMTs during zymography. The polyacrylamide gel (7.5%) contained 3.5 mM sodium dodecyl sulphate (SDS) and 1 mg/ml gelatin, as described previously (30). All samples from the quadruple cultures of each

CMT were loaded onto four gels and each gel contained the full series of all concentrations of that CMT. A mixture of 10 µl of culture medium and 10 µl of sample buffer was electrophoresed. A broad-range marker (Biorad, Hercules, CA, USA), ranging from 6.4 to 203 kDa, was included to determine the molecular weight of the MMPs. Recombinant human pro-MMP-9 (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, St Louis, MO, USA) to remove all SDS. They were then incubated in 50 mM Tris-HCl (pH 7.8), containing 5 mM $CaCl_2$ and 0.1% Triton X-100, at 37°C for 18 h. The gels were stained with 2.5 g/l Coomassie Blue and destained with 10% acetic acid and 40% methanol in water. The MMPs appear as bright bands within the stained gel. Thereafter, the gels were scanned with an HP ScanJet 4C/T (Hewlett-Packard Company, Palo Alto, CA, USA) and the bands were analyzed using QUANTITY ONE software (Biorad). The amount of gelatinolytic activity of each band was calculated as average density × mm^2/ng of DNA. The pro-MMP-9 served as a reference sample, enabling comparison of the four gels of each CMT. The amount of gelatinolytic activity of the pro-MMP-9 reference sample was arbitrarily set to 100% in each gel. All experimental samples were expressed relative to this reference. Then, the incubations with CMTs were expressed relative to the control (0 µM) and the results of the quadruple gels were averaged.

Western blotting

The identity of the bands in the gelatin zymograms was confirmed by western blotting. Concentrated samples of conditioned medium were processed for western blotting according to standard procedures (31). In short, a 10-µl sample was dissolved in 10 µl of sample buffer and electrophoresed through a 7.5% polyacrylamide gel until the bromophenol blue front reached the bottom of the gel. Then, the proteins were blotted onto a nitrocellulose membrane (Biorad) and the membrane was incubated with blockbuffer, which consisted of 3% bovine serum albumin (BSA; Sigma-Aldrich) and 0.1% Tween 20 (Sigma) in PBS. Thereafter, the blots were incubated with the first antibodies (dilution 1 : 200) at 4°C for 18 h. A monoclonal antibody recognizing the latent and active forms of MMP-2, and a monoclonal antibody recognizing the latent, intermediate and active forms of MMP-9 (both Oncogene) were used. After washing with PBS containing 0.1% Tween 20, the blots were incubated, for 1 h, with a peroxidase-conjungated goat anti-mouse immunoglobulin G (IgG) (GAMPO; BD Biosciences, San Jose, CA, USA), diluted 1 : 4000 in PBS containing 1% BSA and 0.1% Tween. After washing, the membrane was incubated with ECL Plus reagent (Amersham, Chicago, IL, USA) for 4 min for detection by chemiluminescence. Excess ECL reagent was removed and the membrane was exposed to a Kodak Biomax film (Eastman Kodak Company, Rochester, NY, USA) for 1 h and developed thereafter.

MMP-activity assay

The MMP-activity assay was performed to analyze the effect of CMTs on the activity of recombinant gelatinases. CMT-1, -3, -5, -7 and -8 were dissolved in DMSO and diluted in DMEM without phenol red to give final concentrations of 0, 1, 10, 100 and 500 µM in 2% DMSO. A standard amount of 1 nM of recombinant MMP-2 and -9 were incubated with these CMTs at 37°C for 1 h. The MMP-activity assay was performed according to the method of Beekman *et al.* (32). In short, the fluorogenic substrate, TNO211-F (TNO, Leiden, the Netherlands) was used to determine the activity of recombinant MMP-2 and -9 (Oncogene) in the presence and absence of CMTs. 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 the MMPs, the quencher is eliminated and the fluorescence can be measured. The fluorescent signal was measured in a Cytofluor II fluorimeter (PerSeptive Biosystems, Framingham, MA, USA) at emission 485 nm and excitation 530 nm, and expressed as relative fluorescence units (RFU) per second.

DNA assay

The PicoGreen®dsDNA Quantitation reagent (Molecular Probes, Inc., Eugene, OR, USA) was used to determine the amount of DNA in the 24-well plates. PicoGreen is a fluorescent nucleic acid stain for double-stranded DNA. The assay was performed according to the protocol of the manufacturer. The fluorescent signal was measured in an FL600 Microplate Fluorescence Reader (Bio-Tek Instruments Inc., Winooski, VT, USA) at excitation 485 nm, emission 520 nm. Standards ranging from 0 to 1000 ng/ml of bacteriophage lambda DNA were used. The total amount of DNA in each well was calculated.

Cytotoxicity assay

To determine the cytotoxicity of the CMTs, the Live/Dead®Viability/Cytotoxicity assay (Molecular Probes) was used on the cells in the 96-well plates. In this assay, calcein acetoxymethyl and ethidium homodimer-1 are simultaneously used to determine the percentages of viable and dead cells. The assay was performed according to the protocol of the manufacturer. The fluorescence was measured in a FL600 Microplate Fluorescence Reader (Bio-Tek Instruments Inc.) at excitation 485 nm and emission 530 nm for calcein and excitation 530 nm and emission 645 nm for ethidium homodimer-1. The data were expressed as the percentage of live and dead cells.

Statistical analysis

Data are presented as mean ± standard deviation (SD). The effect of CMTs on the production of MMP-2 was evaluated by a Kruskall–Wallis one-way analysis variance (ANOVA) followed by Dunnett's post hoc test. The effect of CMTs on gelatinase

activity was evaluated by a one-way ANOVA, followed by the Bonferroni test. The effect of DMSO was tested with the Student's *t*-test. Differences were considered statistically significant at $p < 0.05$.

Results

Gelatinase production

The gelatin zymograms show that PDL cells mainly produce gelatinase activity in bands at 69 and 62 kDa (Fig. 1A). Bands at ≈92 kDa were rarely seen and, if so, were faint. Sometimes, minor amounts of MMP complexes were found at > 120 kDa. A pro-MMP-9 reference, used to compare the zymograms, shows the latent form (92 kDa), the intermediate form (85 kDa, faint) and the active form (68 kDa, faint). Western blotting with an antibody against MMP-2 shows that the 69- and 62-kDa bands are the latent and the active forms of MMP-2, respectively (Fig. 1B). MMP-9 was not detected in the medium (Fig. 1B).

The quantified data of the experimental samples are shown in Fig. 2. CMT-1 induced a significant increase, of 222 ± 64%, in the amount of active MMP-2 at 200 μM compared with the control. CMT-3 increased the amount of latent MMP-2 at 50 and 100 μM with 383 ± 171% and 722 ± 422%, respectively. No significant effect on the amount of active MMP-2 was found, although the trend was similar to its latent form. CMT-5 had no significant effect on the amount of both forms of MMP-2, although there seemed to be a small increase at 50–100 μM. The amount of active MMP-2 was increased to 151 ± 10% by CMT-7 at 50 μM and its latent form was increased at 10, 50 and 100 μM to 294 ± 56, 275 ± 24 and 210 ± 60%, respectively. The amount of active MMP-2 was increased at 200 μM, by CMT-8, to 174 ± 13%, and the latent form was increased at 5 and 200 μM to 140 ± 23% and 141 ± 15%, respectively. No significant overall effect of DMSO on gelatinase production was found ($p < 0.05$).

Gelatinase activity

CMT-1, -3, -5, -7 and -8 were tested for their ability to inhibit recombinant human MMP-2 and MMP-9. A control without DMSO was used to evaluate the effect of DMSO on MMP-2 activity. MMP-2 activity was not significantly influenced by DMSO (Fig. 3). MMP-2 activity was significantly reduced to 42 ± 11% and 5.5 ± 2.0%, at 200 and 500 μM CMT-1, respectively, compared to the control without CMT. MMP-9 activity was reduced to 13 ± 2.9% at 500 μM CMT-1. CMT-3 induced a reduction of MMP-2 activity to 67 ± 6.1, 36 ± 3.6 and 1.7 ± 0.7% at 100, 200 and 500 μM, respectively. This CMT showed the strongest inhibitory effect of all CMTs tested. MMP-9 activity was found to be reduced to 40 ± 21 and 2.9 ± 0.6% at 200 and 500 μM CMT-3, respectively. CMT-5 had no effect on MMP-2 or MMP-9 activity. CMT-7 showed an inhibitory effect on both gelatinases; MMP-2 activity was reduced to 37 ± 4.0% at 200 μM and to 2.1 ± 0.2% at 500 μM, and MMP-9 activity was reduced to 49 ± 11% at 200 μM and to 8.2 ± 5.8% at 500 μM. With CMT-8, the activity of both gelatinases was significantly reduced only at 500 μM. MMP-2 activity was reduced to 9.9 ± 8.8%, and MMP-9 activity to 21 ± 20%.

Cytotoxicity

Cytotoxic effects of the CMTs were assessed using a cytotoxicity assay (Fig. 4). CMT-1, -5 and -7 showed no significant cytotoxic effects on the PDL cells, although CMT-5 showed a decreasing trend in the percentage of viable cells. CMT-7 showed a peak in the percentage of viable cells at 500 μM, but this was caused by the yellow color of the CMT itself. CMT-3 and -8, however, showed a significant reduction of viable cells at the highest concentrations.

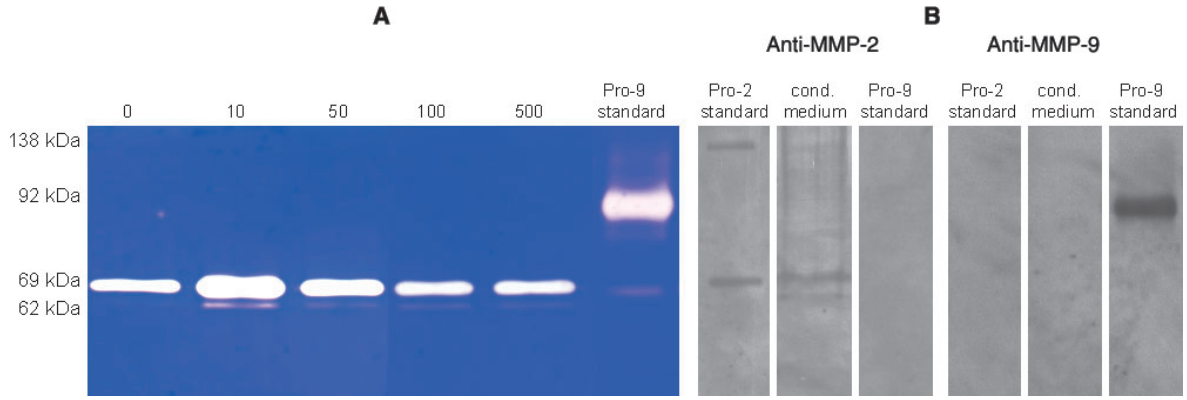

Fig. 1. Representative zymogram and western blots. (A) Gelatin zymography. Human periodontal fibroblasts were incubated with chemically modified tetracycline-3 (CMT-3) at concentrations ranging from 0 to 500 μM. Gelatin zymography of the conditioned medium shows bands at 62 and 69 kDa. The last lane of the zymogram represents a control with recombinant pro-matrix metalloproteinase-9 (pro-MMP-9). (B) Western blotting on concentrated culture media, using an antibody against human MMP-2, confirmed the bands at 62 and 69 kDa to be the active and latent forms of MMP-2. The band of ≈138 kDa in the sample with recombinant pro-MMP-2 probably represents a dimer of pro-MMP-2. The antibody against MMP-9 detected the MMP-9 standard, but MMP-9 was not found in the conditioned media.

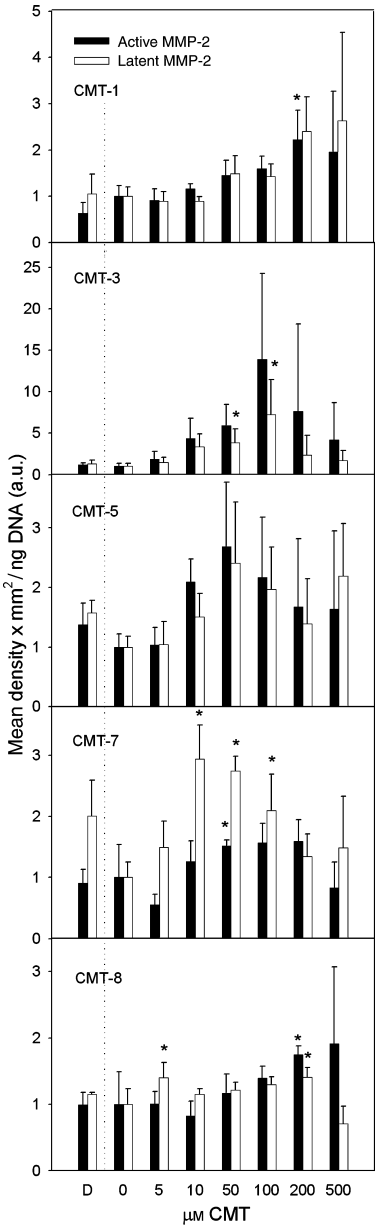

Fig. 2. Quantification of matrix metalloproteinase-2 (MMP-2) production in the culture media. MMP-2 production was analyzed with gelatin zymography and expressed as mean density × mm^2/ng of DNA relative to the MMP-9 reference sample. The MMP-2 production in the control was arbitrarily set to 1. The other experimental samples were expressed relative to the control. *Significant difference compared with the control. D represents the control without dimethylsulfoxide (DMSO). a.u., arbitrary units; CMT, chemically modified tetracycline.

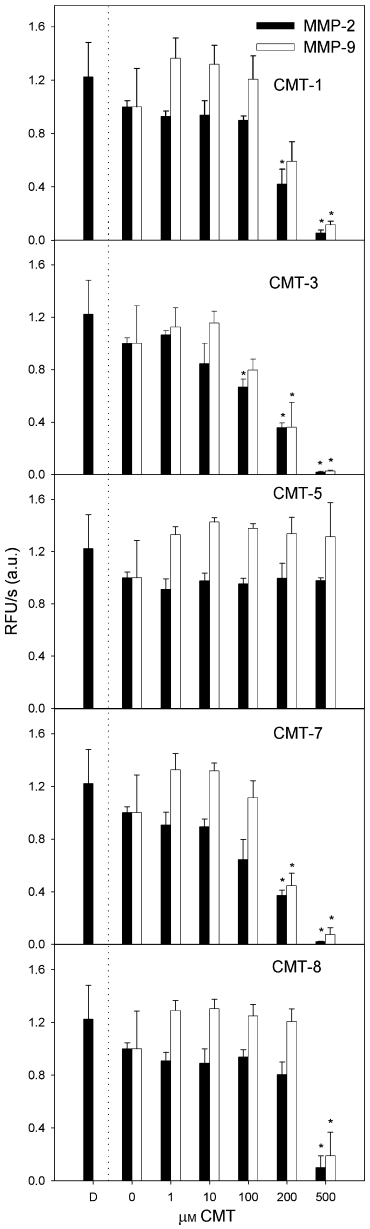

Fig. 3. The effect of chemically modified tetracyclines (CMTs) on gelatinase activity. Recombinant matrix metalloproteinase (MMP)-2 and -9 were incubated with CMTs and the degradation of a fluorescent substrate was expressed as relative fluorescence units (RFU)/s. A control without dimethylsulfoxide (DMSO) (D) showed that DMSO alone did not have a significant effect on MMP-2 activity. *Significant difference compared with the control. a.u., arbitrary units.

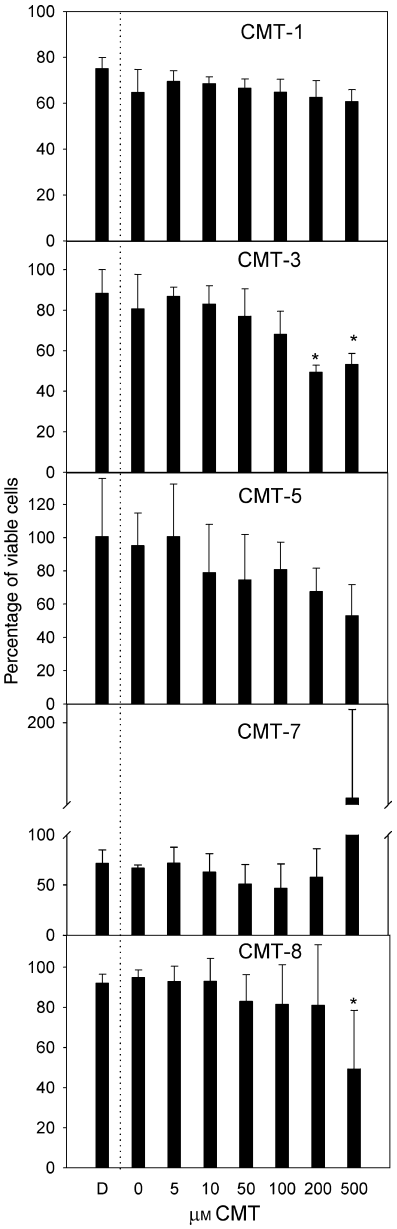

Fig. 4. Cytotoxicity of chemically modified tetracyclines (CMTs). The viability of cells was analyzed with a cytotoxicity assay and expressed as percentage viable cells. *Significant difference compared with the control. D, control without dimethylsulfoxide (DMSO).

Discussion

In this study, the effects of CMTs on PDL gelatinases were investigated. Gelatin zymography of the culture media showed bands at 62 and 69 kDa that were shown, by western blotting, to be the active and latent forms of MMP-2. MMP-9 was not detected in the media. The gelatin zymograms also showed minor amounts of MMP complexes at higher molecular weights. An earlier study also showed only the latent and active forms of MMP-2, and no MMP-9 in cultures of PDL cells (33). However, Wu *et al.* (34) found

gelatinase activity at 92, 70, 68, 57 and 52 kDa in cultured PDL cells from primary and permanent teeth, using gelatin zymography. The band at 92 kDa was probably the latent form of MMP-9. It is known that the active forms of MMPs occur mainly in situations with high tissue turnover, such as in inflammatory processes or as a response to mechanical forces (14,18). The active form of MMP-2 was found mainly in gingival fibroblasts from patients with periodontitis (35). In our study, the latent form of MMP-2 was most abundant, indicating that the periodontium of the molar used for this study was healthy.

In this study, all CMTs, except CMT-5, had a strong inhibitory effect on the activity of MMP-2 and -9. This is consistent with the results of others, although the extent of inhibition was found to vary (23,25,36–38). CMT-3 was the most potent inhibitor of gelatinase activity in our study, which was also shown in these earlier studies. Surprisingly, CMT-7 was the second best inhibitor of MMP activity in our study. Not much is known about the effects of CMT-7 *in vitro*, but when tested in a rat model for periodontitis, CMT-7 was the least effective of six CMTs (39). *In vitro*, CMTs act directly on cells, whereas *in vivo* systemic properties, like the uptake of the agent from the digestive tract, also play an important role.

In our study, all CMTs, except CMT-5, had a stimulatory effect on gelatinase production of up to sevenfold at lower concentrations. Their effect on gelatinase activity, however, was much larger. MMP-2 activity was reduced maximally to 1.7% and MMP-9 activity to 2.9%, depending on the CMT tested. MMP-2 production was stimulated only about two- to threefold. A significant reduction of gelatinase activity only occurred at concentrations of 200 and 500 μM, except for CMT-3, which already showed a reduction at 100 μM. Significant effects on MMP production, on the other hand, were found at concentrations of < 200 μM. Therefore, the net result of CMTs in our experiments was an inhibitory effect on gelatinase activity at higher concentrations, whereas at lower concentrations the stimulation of gelatinase production was more prominent.

To date, no data are available about the clinical concentrations of CMTs in the periodontium during treatment. However, doxycycline, a synthetic tetracycline closely related to CMTs, was found at concentrations of up to 10 μg/ml in crevicular fluid at therapeutic oral doses (40). During periodontal pocket irrigation, tetracycline concentrations in crevicular fluid ranged from $\approx$1500 μg/ml after 2 h to $\approx$20 μg/ml 2 wk later (41). The concentrations of CMTs used in our experiments were $\approx$2–200 μg/ml, which is within the expected clinical range. As low concentrations may stimulate MMP production, topical application of CMTs are expected to be more suitable. To date, very little data have been reported on the effects of CMTs on MMP production. Mäkela *et al*. (26) also reported a stimulation of MMP-9 protein expression by cultured human gingival keratinocytes with CMT-3 at lower concentrations. An earlier study (27), on the other hand, described an inhibitory effect on MMP-2 and -9 protein expression by porcine PDL epithelial cells at similar concentrations. Moreover, other synthetic MMP inhibitors have been shown to increase MMP-2 mRNA and protein expression at low concentrations in rabbit periosteal cell cultures (42). The increase in MMP-2 expression by PDL cells, as shown in our study, has not been previously reported. The mechanism by which CMTs might stimulate MMP production is not known. The cells might sense a decrease of extracellular MMP activity and in response increase their MMP production. However, CMT-5 also showed a trend towards stimulation of production of MMP-2 at the intermediate concentrations, whereas it did not show any significant effect on gelatinase activity, as also shown earlier (43). This suggests that an MMP-independent mechanism could be responsible for the observed increase in MMP production.

This study further shows that CMT-3 and -8 have cytotoxic effects on PDL cells at $\approx$500 μM. In contrast, CMT-1, -5 and -7 did not have any significant cytotoxic effect in our experiments. Others also found CMT-1 not to be toxic at similar concentrations when added to PDL epithelial cells (27). Bettany *et al*. (44) found that CMT-3 had cytotoxic effects on monocytes already at lower concentrations, whereas CMT-2, -5 and doxycycline had not.

In conclusion, our study shows that CMTs up-regulate gelatinase production of PDL cells at lower concentrations, but strongly reduce gelatinase activity at higher concentrations. The clinical application of CMTs aims to inhibit MMP-mediated tissue degradation, as occurs during periodontitis. They also have a potential therapeutic role in the prevention of relapse after orthodontic tooth movement. However, the stimulatory effect of CMTs on the production of certain MMPs might have unexpected consequences in the clinical situation.

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