Background: Periodontal tissues remodel rapidly, which enables quick adaptation to mechanical changes. Matrix metalloproteinases (MMPs) are involved in these remodeling processes under control of tissue inhibitor of metalloproteinases (TIMPs). In periodontitis, overactivity of MMPs results in pathologic tissue degradation. The aim of this study was to analyze MMPs and TIMPs in healthy and diseased gingiva, periodontal ligament (PDL), and gingival crevicular fluid (GCF).

Methods: Samples of gingiva, PDL, and GCF were obtained from healthy controls (gingiva: n = 18; PDL: n = 15; GCF: n = 8) and subjects with periodontitis (gingiva: n = 11; PDL: n = 18; GCF: n = 12). MMPs and TIMPs were analyzed by gelatin-, collagen-, and reverse zymography and by Western blotting. Total MMP activity was analyzed using a fluorogenic substrate.

Results: TIMP-1 and -2, active and pro-MMP-2 and -9, and active MMP-1 and -8 were present in all samples. Large amounts of active MMP-2 complexes and collagenolytic fragments were also found. Their levels were higher in PDL and GCF from subjects with periodontitis. In general, TIMP levels were lower in diseased periodontal tissues. Especially diseased GCF contained more MMPs. Surprisingly, some MMPs were more abundant in healthy gingiva and PDL than in diseased tissue.

Conclusions: Unexpected variations in MMP and TIMP levels in gingiva, PDL, and GCF may result from differences in subject characteristics and disease activity. The levels of active MMP-2 complexes and collagenolytic fragments are higher in the periodontium of subjects with periodontitis and might contribute significantly to periodontal destruction. J Periodontol 2008; 79:1704-1711.

KEY WORDS
Matrix metalloproteinase; periodontal ligament; periodontitis; tissue inhibitor of metalloproteinase.

Periodontal tissues remodel rapidly compared to other connective tissues, which allows adaptation of the teeth during function. The matrix metalloproteinases (MMPs) are believed to be crucial in these remodeling processes. Twenty-three human MMPs are known. Their activity is controlled by tissue inhibitor of metalloproteinases (TIMPs). MMPs and TIMPs are important in the physiologic remodeling of the periodontium and its adaptation to mechanical forces, as in orthodontics. In periodontitis, a relative overactivity of MMPs results in the breakdown of periodontal structures. This can be enhanced by orthodontic forces.

The total amounts of MMP-1, -2, -3, and -9 seem to be increased in inflamed gingiva. Ejeil et al. found higher levels of the latent and active forms of MMP-2 in inflamed gingiva, but the total amount of MMP-2 was similar to healthy gingiva. Smith et al. found more pro-MMP-9 in diseased gingiva and equal amounts of latent and active MMP-2, although these findings were not quantified. MMP-1 mRNA was reported to be increased in inflamed gingival tissue, but another study showed similar levels of MMP-1, -2, and -14 mRNA in healthy and diseased gingiva. According to the investigators, this might have been due to the large individual variation. TIMP levels in inflamed gingiva have been found to be reduced, similar, or even increased compared to healthy gingiva. In the first study, TIMPs

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were extracted from gingival tissue, whereas in the latter two they were analyzed in culture medium. Aiba et al.15 found that the levels of TIMP-1 mRNA were similar in healthy and diseased tissue, but protein levels were not analyzed. In summary, there is no consensus on MMP and TIMP levels in healthy and diseased gingiva.

More active and latent MMP-1, -3, -8, and -9 were found in gingival crevicular fluid (GCF) from deep pockets than from healthy sulci.2,13,18,19 The total MMP-2 content was also reported to be higher,13 whereas other investigators9 found no MMP-2. This might be due to the use of different analytical methods. The levels of TIMP-1 and -2 are reduced in the GCF of periodontitis patients,13,19 but periodontal treatment does not seem to normalize them.9

In contrast to gingiva and GCF, little data are available on MMP and TIMP levels in healthy and diseased periodontal ligament (PDL). However, it is known from histologic studies20,21 in rats that MMPs are present.

To the best of our knowledge, MMP and TIMP levels in gingiva, PDL, and GCF of subjects with periodontitis and healthy subjects have not been compared directly in one study. This might clarify some of the controversies found in the literature. In our study, substrate zymography and Western blotting were used to analyze MMPs and TIMPs in gingiva, PDL, and GCF of subjects with periodontitis and healthy controls. The analysis of MMPs and TIMPs in healthy and diseased periodontium might contribute to the development of therapies to reduce tissue degradation in periodontitis.

MATERIALS AND METHODS

Gingiva
Healthy gingiva (score 0 according to the Dutch Periodontal Screening Index [DPSI]22) was obtained from gingiva correction after the extraction of healthy third molars. This tissue was obtained from 18 subjects aged 17 to 41 years (mean: 23.2 ± 5.8 years; nine males and nine females). Inflamed gingiva was obtained from discarded material during periodontal flap surgery (DPSI >3–). The inflamed tissue was obtained from 11 subjects aged 25 to 71 years (mean: 51.6 ± 10.4 years; five males and six females) who had chronic periodontitis. All subjects from each group gave their oral consent according to the guidelines of the local ethical committee. All samples were collected at the Departments of Periodontology and Oral and Maxillofacial Surgery of Radboud University Nijmegen Medical Center from January to October 2005. The samples were washed in saline and temporarily stored at −80°C in phosphate buffered saline (PBS)† containing 100 U/ml penicillin and 100 µg/ml streptomycin.¶

PDL
PDL was obtained from teeth that were extracted during routine dental treatment. All subjects gave their consent according to the guidelines of the local ethical committee. PDL samples from healthy periodontium (DPSI 0) were derived from fully erupted third molars of 15 subjects aged 20 to 41 years (mean: 25.9 ± 6.2 years; four males and 11 females). Diseased PDL samples (DPSI >3–) were obtained from teeth affected by chronic periodontitis that were extracted because of severe bone loss (18 patients aged 38 to 69 years [mean: 52.0 ± 14.9 years; 10 males and eight females]). After extraction, the teeth were washed in saline and stored at 4°C in PBS containing 100 U/ml penicillin and 100 µg/ml streptomycin. PDL was cut from the lower half of the root, excluding the last 2 mm of the apex, and stored at −80°C.

GCF
GCF samples were taken from volunteers with a healthy periodontium (DPSI 0) and subjects with chronic periodontitis (progressive disease, DPSI >3–). All subjects gave their consent according to the guidelines of the local ethical committee. Only subjects not on any medication were included. The healthy group consisted of eight volunteers aged 27 to 63 years (mean: 41.4 ± 14.3 years; three males and five females), and the periodontitis group consisted of 12 subjects aged 38 to 66 years (mean: 49.0 ± 10.2 years; seven males and five females). The teeth at the sampling sites were isolated with cotton rolls and gently dried with air. Thereafter, a 1-µl microsyringe§ was carefully inserted into the sulci or pockets to collect GCF. The GCF samples were immediately placed on ice and stored at −80°C until use. For analysis, healthy and diseased GCF samples were pooled separately.

Tissue Extraction
The tissue extraction method was a modification of the procedure of Robinson et al.23 The individual gingiva or PDL samples were put in homogenization buffer containing 10 mM CaCl2 and 0.25% detergent† on ice. The tissues were homogenized with a homogenizer with a 5 × 75-mm generator.¶ The homogenates were centrifuged for 45 minutes at 13,000 × g and 4°C. The supernatants were removed, pooled, and stored at −80°C. This procedure was repeated. The pellet was homogenized again in extraction buffer containing 50 mM Tris-HCl pH 7.5, 150 mM NaCl2, and 100 mM CaCl2. Heat extraction was performed at 60°C for 5 minutes while shaking firmly, and the samples were placed on ice. The samples were centrifuged for 45 minutes at 13,000 × g and 4°C, and the supernatant was stored at −80°C. The supernatants from the first and third extraction step were pooled because they contained the highest concentrations of MMPs.

† Gibco, Paisley, U.K.
‡ Gibco.
§ Hamilton, Reno, NV.
¶ Triton X-100, Sigma-Aldrich, St. Louis, MO.
§ Pro 200, Pro Scientific, Monroe, CT.
This yielded four pooled samples for healthy and diseased gingiva and PDL tissue. As a control for differences in extraction efficiency between healthy and diseased tissues, we determined the extraction efficiency of MMP-2 in healthy and inflamed gingiva. The extraction efficiency was defined as the percentage of MMP-2 in the first and third supernatant compared to the total in all three fractions. The extraction efficiency of total extractable MMP-2 was 77% ± 5% for healthy gingiva and 83% ± 6% for diseased gingiva, which was not significantly different. The extraction efficiency of total protein was determined as the percentage of protein present in the extract compared to the total protein in the tissue (extracts and pellet) using the bicinchoninic acid (BCA) protein assay. This differed significantly for diseased PDL (92% ± 2%) and healthy PDL (48% ± 6%), but not for diseased gingiva (69% ± 9%) and healthy gingiva (71% ± 10%). Because the results were expressed per nanogram of protein, the data were corrected for differences in protein-extraction efficiency.

**Gelatin Zymography**

Gelatinases (MMP-2 and -9) in the pooled samples were analyzed in quadruple by gelatin zymography. The polyacrylamide gel (7.5%) contained 3.5 mM sodium dodecyl sulfate (SDS) and 1 mg/ml gelatin. A (1:1) mixture of sample and sample buffer was electrophoresed for 1.5 hours at 80 mA. A broad-range marker was included to determine the molecular weight of the MMPs. Recombinant human pro-MMP-2 was used as a reference sample. After electrophoresis, the gels were washed in 2.5% detergent. The gels were incubated at 37°C for 18 hours in activation buffer containing 50 mM Tris-HCl (pH 7.8), 5 mM CaCl₂, and 0.1% detergent; stained for 45 minutes in stain (2.5 g/l), 10% acetic acid, and 40% methanol in water; and destained. The MMPs appear as bright bands within the stained gel. The gels were scanned and the bands were analyzed with software. The amount of enzyme in the bands was represented as average density × square millimeter per nanogram of total tissue protein. The amount of protein in the samples was determined by BCA protein assay. The amount of enzyme in the GCF was represented as average density × square millimeter per microliter 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. The corresponding bands on the different gels were averaged, and standard deviations were calculated.

**Collagen Zymography**

Collagenases (MMP-1, -8, and -13) in the pooled samples were analyzed in quadruple by collagen zymography. The polyacrylamide gel (10%) contained 3.5 mM SDS and 5 mg/ml collagen type I from calf skin. A (1:1) mixture of sample and sample buffer was electrophoresed. A broad-range marker was included on the gels. Recombinant human pro-MMP-2 was used as a reference. After electrophoresis, the gels were washed in 2.5% detergent. The gels were incubated in activation buffer at 37°C for 43 hours. They were stained and destained as described for the gelatin zymography. Thereafter, the gels were scanned and analyzed as described for the gelatin zymography.

**Reverse Zymography**

TIMPs in the pooled samples were analyzed in quadruple by reverse zymography. This technique is similar to gelatin zymography, except that conditioned medium from baby hamster kidney cells was included in the gels. This 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 SDS, 1 mg/ml gelatin, and 10% conditioned medium. A (1:1) mixture of sample and sample buffer was electrophoresed for 3 hours at 80 mA. A broad-range marker was included. Recombinant human TIMP-1 and -2 were used as standards. After electrophoresis the gels were washed in 2.5% detergent. The gels were incubated in 50 mM Tris-HCl (pH 7.5), 5 mM CaCl₂, 0.1% detergent, 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. TIMPs appear as dark blue bands within the destained gel. Thereafter, the gels were scanned and analyzed as described for gelatin zymography, except that TIMP-2 was used as a reference to enable comparison of the corresponding bands on different gels.

**MMP Activity Assay**

MMP activity in the pooled samples was analyzed in quadruple by an MMP assay. In short, the experimental fluorogenic substrate was used to determine the total activity of the MMPs present in the gingiva, PDL, and GCF samples. The substrate 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

# Pierce Biotechnology, Rockford, IL.
** Bio-Rad, Hercules, CA.
†† Triton X-100, Oncogene, San Diego, CA.
†† Sigma-Aldrich.
‡‡ Sigma-Aldrich.
§§ HP ScanJet 4C/T, Hewlett-Packard, Amstelveen, The Netherlands.
¶¶ Quantity One, Bio-Rad.
#* Bio-Rad, Hercules, CA.
** Bio-Rad, Hercules, CA.
††† American Type Culture Collection, Manassas, VA.
†††† Oncogene.
measured in a fluorimeter§§§ and expressed as relative fluorescence units (RFUs) per second. The MMP activity in the gingiva and PDL samples was represented as RFUs/second per nanogram of total tissue protein. The amount of protein was determined in the sample by the BCA protein assay. The MMP activity in the GCF was represented as RFUs/second per microliter of GCF.

**Western Blotting**

Pooled GCF and concentrated pooled tissue extracts were analyzed by Western blotting according to standard procedures.26 In short, 10 μl sample was dissolved in 10 μl sample buffer and electrophoresed on a 7,5% polyacrylamide gel (room temperature, 80 mA). The proteins were blotted onto a nitrocellulose membrane (pore size 0.45 μm) at 400 mA and 4°C for 1 hour. The membrane was incubated in block buffer (5% milk powder¶¶¶ and 0.1% Tween 20### in PBS). Thereafter, the blots were incubated with monoclonal antibodies against MMP-1, -2, -8, -9, or -13 and TIMP-1 or -2,**** dilution 1:200 at 4°C for 18 hours. After washing with 0.1% Tween20 in PBS, the blots were incubated with a peroxidase-conjugated goat anti-mouse immunoglobulin G,†††† diluted 1:4,000 in 1% bovine serum albumin, 0.1% Tween in PBS for 1 hour. After washing, the membranes were incubated with reagent‡‡‡‡ for chemiluminescence detection. Excess reagent was removed, and the membrane was exposed to film§§§§ for 1 hour and developed.

**Statistical Analysis**

The differences in amounts of MMPs and TIMPs and MMP activity between the healthy and diseased tissues were evaluated with the Student t test. The Mann-Whitney U test was used in case of a non-normal distribution or a considerable amount of difference in variances. This was necessary for data for MMP-1 and the collagenolytic fragments in PDL. For clarity, all results are displayed as mean ± SD. Differences were considered significant if P<0.05. When multiple t tests were performed, the Bonferroni correction was applied to adjust the P value.

**RESULTS**

**Zymography**

The gelatin zymograms showed that the periodontium contained gelatinolytic activity in bands around 130, 92, 69, and 62 kDa (Fig. 1A). Also, a faint band around 85 kDa was found in all samples, except the inflamed gingiva. The bands at 69 and 62 kDa were the latent and active form of MMP-2 as confirmed by Western blotting. The band at 130 kDa probably represented the complexed form of MMP-2, because it was also found in the MMP-2 reference sample (Fig. 1A, lane 7). However, this could not be clearly confirmed by Western blotting (Fig. 1A, lane 8). The bands at 92 and 85 kDa represented the latent and active forms of MMP-9. The latent form was also shown by Western blotting (Fig. 1A, Western blotting lane 4). The active form of MMP-9 was lacking in diseased gingiva. This active form has also been described as the “intermediate” form, because MMP-9 has another active form of 67 kDa.27 We refer to the 85-kDa form as the active form because we did not find the 67-kDa form.
The collagen zymograms showed bands around 55 and 43 kDa and wider bands at 30 to 35 kDa (Fig. 1B). The 55-kDa band was identified as MMP-8 by Western blotting only in gingiva. The bands at 30 to 35 kDa were probably fragments of MMP-8, as shown by Western blotting for gingiva, and other fragments. This band was only faintly present in healthy PDL and GCF. The band at 43 kDa could not be identified by Western blotting, but it most likely was MMP-1 based on its molecular weight. No bands were found at the molecular weights of latent MMP-1 (58 kDa) and -8 (64 kDa) or latent and active MMP-13 (60 and 48 kDa, respectively) by zymography or Western blotting.

The reverse zymograms showed bands around 28 and 21 kDa, representing TIMP-1 and -2, which is indicated by the reference samples (Fig. 1C, lanes 7 and 8). The band of TIMP-1 in GCF seemed to be divided into two fractions.

Quantitative Analysis of MMPs and TIMPs

The quantified data of all experimental samples are shown in Figure 2. Significantly more pro- and complexed MMP-2 were present in the healthy gingiva compared to diseased gingiva ($P = 0.045$ and $P < 0.001$, respectively). In healthy PDL, more active and pro-MMP-2 was present than in diseased PDL ($P < 0.003$ and $P = 0.001$, respectively). In contrast, the amount of complexed MMP-2 was higher in diseased PDL ($P = 0.036$). The amounts of all three forms of MMP-2 were higher in diseased GCF than in healthy GCF ($P < 0.0001$, $P < 0.0001$, and $P = 0.042$, respectively).

Significantly more active and pro-MMP-9 was present in the healthy gingiva compared to diseased gingiva ($P < 0.001$ and $P = 0.004$, respectively). In diseased PDL, more active and pro-MMP-9 was present than in the healthy tissue ($P = 0.032$ and $P = 0.001$, respectively). The amount of active MMP-9 was also higher in diseased GCF ($P < 0.0001$).

Diseased gingiva contained more MMP-1 than healthy tissue ($P = 0.011$). There was no significant difference in the amount of MMP-8 or collagenolytic fragments. The fragments might be partly derived from MMP-8 as shown for gingiva in Figure 1B. More active MMP-1 and -8 were present in healthy PDL than in diseased tissue ($P = 0.029$ and $< 0.001$, respectively). However, the amount of collagenolytic fragments was higher in the diseased tissue ($P = 0.029$). In diseased GCF, more active MMP-8 and collagenolytic fragments were present than in healthy GCF ($P < 0.001$ and 0.04, respectively). There was no significant difference in MMP-1.

Healthy gingiva contained significantly more TIMP-1 than diseased gingiva ($P = 0.011$). The same trend was observed for TIMP-2. Higher amounts of TIMP-1 and -2 were found in healthy PDL compared to diseased PDL ($P = 0.004$ and 0.015, respectively). Healthy GCF contained more TIMP-1 than diseased GCF ($P = 0.029$), but there was no significant difference in the amount of TIMP-2.

MMP Activity

The results of the MMP activity assay are shown in Figure 3. MMP activity was three times higher in healthy gingiva than in diseased gingiva ($P < 0.0001$). Also, the MMP activity of healthy PDL was higher than diseased PDL ($P = 0.029$), although this difference was
small (1.2-fold increase). In contrast, there was a large difference between healthy and diseased GCF ($P = 0.029$). MMP activity was increased 250-fold in diseased GCF compared to healthy GCF.

DISCUSSION

In this study, MMPs and TIMPs in healthy and diseased gingiva, PDL, and GCF were analyzed to investigate tissue degradation in periodontitis. MMPs and TIMPs in gingiva and PDL were analyzed after extraction of tissue samples, and those in GCF were studied directly. We found a difference in extraction efficiency of total protein between healthy and diseased PDL. More protein was extracted from diseased PDL than from healthy PDL (92% versus 48%). Surprisingly, the extraction efficiencies of healthy and diseased gingiva were similar (71% versus 69%). This might be due to a difference in the course of the inflammatory process or the different composition and architecture of PDL and gingiva. Because the MMP content was expressed per nanogram total protein, we corrected for this difference.

MMPs and TIMPs were analyzed by zymography, which allows the detection of latent and active MMPs. We found latent and active forms of MMP-2 and -9, active MMP-1 and -8, and TIMP-1 and -2 in gingiva, PDL, and GCF. These MMPs and TIMPs were also found in previous studies on gingiva and GCF. Moreover, we found considerable amounts of MMP-2 complexes in all samples. These have been described as MMP-2 dimers but were never quantified. The MMP-2 complexes were also found in GCF samples, which indicates that they are present in vivo, although this was not clearly confirmed by Western blotting. The collagen zymograms showed collagenolytic fragments around 30 kDa in gingiva and PDL but relatively few in GCF. Some of these were identified as MMP-8 fragments in gingiva. These fragments were described previously and so were fragments of MMP-131 and -1. The MMP-8 fragments in GCF described by other investigators seemed to disappear after treatment. The fragments may originate from autolysis or the activity of other MMPs. The binding of TIMPs to these fragments seems to be reduced, so they may be active in the presence of TIMPs. Because the collagenolytic fragments and MMP-2 complexes clearly showed proteolytic activity, they might also play a role in the tissue degradation in periodontitis.

The zymographic analyses showed that diseased GCF contained greater amounts of MMP-2, active MMP-9 and -8, and collagenolytic fragments than the healthy fluid; this was also reported previously. A chairside test for MMP-8 was developed to support the diagnosis of periodontitis and to monitor treatment outcome. The total MMP activity in diseased GCF was ~250 times greater than in healthy fluid. This seemed to be mainly caused by MMP-2 because it had greater activity toward the fluorescent substrate in this activity assay than other MMPs. Other studies also found a higher level of total MMP activity in GCF of periodontitis-affected teeth. In addition, the authors and other investigators found decreased TIMP-1 content in diseased GCF.

To our knowledge, this is the first report to describe MMPs and TIMPs from human PDL tissue. In diseased PDL, more MMP-2 complexes, MMP-9, and collagenolytic fragments were found than in healthy tissue. The amount of collagenolytic fragments was six times higher in diseased PDL than in healthy tissue. In addition, TIMPs were reduced by approximately half in PDL. Unexpectedly, we found higher levels of active and pro-MMP-2 and active MMP-1 and -8 in healthy PDL. The total MMP activity was also slightly higher in the healthy tissue.

In our study, some profound differences existed between the healthy and diseased subjects. The subjects with periodontitis were about twice as old as the healthy group (mean age: 52.0 versus 25.9 years). This is a common problem in periodontitis studies, because the disease usually occurs at a later age. General age-related differences have been described for PDL. These include differences in composition, architecture, and width of PDL and impaired regeneration. To the best of our knowledge, this is the first report of age-related changes in MMP levels in PDL. Studies on other tissues, like cartilage and myocardial tissue, showed a decrease in MMPs with aging. In contrast, other investigators showed an increase in MMPs in aortic tissue. The healthy group consisted mainly of women, whereas the diseased group consisted of equal numbers of men and women. Although not much is known about gender differences and MMP levels in humans, rat studies showed that estradiol promotes MMP expression.
In gingiva, 36% more MMP-1 was found in diseased tissue than in healthy tissue. The presence of MMP-1 in gingival epithelium was shown previously by in situ hybridization. In addition to MMP-8, MMP-1 was recently associated with periodontal inflammation. Higher TIMP-1 levels were present in healthy gingiva. Unexpectedly, pro- and complexed MMP-2 and both forms of MMP-9 were also higher in healthy gingiva, as was total MMP activity. Diseased gingiva was derived from subjects who displayed ongoing attachment loss despite good oral hygiene. It is conceivable that the gingiva no longer showed profound signs of clinical inflammation, whereas the inflammatory process in the depth of the pockets persisted. In these cases there may be a subclinical inflammation that maintains periodontal breakdown. In addition, there was a considerable age difference between the healthy (mean age: 23.2 years) and diseased subjects (mean age: 51.6 years).

CONCLUSIONS

MMPs and TIMPs in the healthy and diseased periodontium have been the focus of many studies. In this study, in addition to other MMPs and TIMPs, collagenolytic fragments and MMP-2 complexes were quantified in the gingiva, PDL, and GCF. Their function in vivo is not clear, but they exert profound proteolytic activity and might also contribute to tissue destruction in periodontitis. In addition, this study showed that subject characteristics, such as age, gender, and degree of inflammation, might explain the apparent controversial results found in this and previous studies.

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