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Expression of metalloproteinases and their tissue inhibitors in inflamed gingival biopsies

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Objectives: Matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) are known to be involved in the periodontal disease process. Results of in vivo MMPs and TIMPs gene expressions in the gingiva, though, are still controversial. In the present study, we compared the gene expression of MMP-1, -2, -9, -13 and TIMP-1, -2 in healthy and inflamed gingiva.

Methods: 38 gingival samples were collected from gingivitis (n = 10), advanced chronic periodontitis (n = 10), generalized aggressive periodontitis (n = 8) and periodontally healthy individuals (n = 10). Total RNA isolated from those samples was subjected to reverse transcription followed by amplification by polymerase chain reaction (RT-PCR). Products were visualized in agarose gels and quantified by optical densitometry. Samples were also processed for gelatin zymography and Western blotting for MMP-2 and MMP-9 in order to assess for post-transcriptional MMP regulation at the protein level.

Results: The frequencies and levels of transcripts encoding MMPs and TIMPs were found to be not significantly different among groups ($p > 0.05$, Fisher's Exact and Kruskall-Wallis tests). There is a trend towards higher MMP-2 and -9 gelatinase activities in the inflamed samples, although not statistically significant. In contrast, zymography and Western blotting studies show that MMP-2 is virtually absent in the chronic periodontitis group.

Conclusion: These results could reflect a complex regulation of MMPs and TIMPs' gene expression in the course of gingival inflammation. They also reveal a great biological diversity even among individuals with similar periodontal status.

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Matrix metalloproteinases (MMPs) are a family of structurally related zinc-dependent endopeptidases that are collectively capable of degrading the different macromolecular components of the extracellular matrix (ECM). They play an important role in a variety of biological processes, including tissue remodeling, wound healing, cancer invasion and inflammation. Their expression is influenced by cytokines, growth factors, cell–cell and cell–matrix interactions in the local tissue environment. Matrix metalloproteinase activity is additionally regulated by specific inhibitors (tissue inhibitors of metalloproteinases; TIMPs), which contribute to maintaining the balance between synthesis and degradation of the ECM (1).

Periodontal diseases are driven by interactions between pathogenic bacterial biofilms present on the root surface/periodontal pocket and host-derived inflammatory cells and molecules (2). In fact, many studies have investigated the relationship of MMPs

and TIMPs to the loss of connective tissue seen in the chronically inflamed gingiva. For instance, MMP-1, -2, -3, -4, -7, -8, -9, -13, -25 and -26 as well as TIMP-1 and -2 have been detected in gingival biopsies and crevicular fluid of patients with diverse periodontal conditions (3–17). The increased activity/levels of proteinases during experimental gingivitis (18) and their decrease after periodontal and/or low-dose doxycycline therapy (19–22) further suggest the involvement of certain MMPs in periodontal tissue destruction. Nevertheless, it is not clear which of those enzymes are prominent in the course of gingivitis and periodontitis. Results of gene expression studies are still controversial, depending on the experimental method employed and the sample studied (3,5,8,23,24). In the present study, we aimed to analyze the expression of MMP-1, -2, -9, and -13 and TIMP-1 and -2 in gingival biopsies of individuals suffering from gingivitis, chronic or aggressive periodontitis.

Material and methods

Patient selection and sample collection

Patients seeking or under treatment in the School of Dentistry of the Federal University of Espírito Santo (UFES) or in the School of Dentistry of the Federal University of Rio de Janeiro (UFRJ), Brazil, were recruited from March 2004 to December 2005. To participate in this study, individuals signed an informed consent to a research protocol that had been reviewed and approved by the UFES' Ethics Committee. All subjects were systemically healthy and were not smokers. Moreover, none had received periodontal treatment in the last 2 years or taken antibiotics in the previous year. Clinical parameters were recorded with a North Carolina periodontal probe (UNC-15, Hu-Friedy, Chicago, IL, USA) and included probing pocket depth (PPD), clinical attachment level (CAL) and bleeding on probing (BOP). Generalized aggressive periodontitis was diagnosed in patients younger than 35 years of age, who presented four sites BOP on different teeth with PPD ≥ 6 mm and CAL ≥ 5 mm, and extensive generalized bone loss affecting at least three teeth other than molars and incisors. Chronic periodontitis was diagnosed in patients older than 35 years of age, presenting four sites BOP on different teeth with PPD ≥ 6 mm and CAL ≥ 5 mm with extensive presence of calculus. Those patients were treated in the graduate periodontics clinic and received a full-mouth debridement, including a single course of scaling and root planing according to van Winkelhoff *et al.* (25), as well as surgical access to the deepest palatal sites, preferentially. Patients without periodontitis but presenting sites with bleeding on probing were assigned a diagnosis of gingivitis. Samples from individuals suffering from periodontitis or gingivitis were collected during the periodontal treatment or crown-lengthening procedures, respectively. Clinically non-inflamed biopsies from periodontally healthy individuals obtained during pre-prosthetic surgery were used as control samples. All samples included part of the oral and pocket epithelium and the adjacent connective gingival tissue.

Following collection, gingival specimens were immediately rinsed in sterile saline, immersed in Dulbecco's modified Eagle's medium (DMEM, Sigma, St Louis, MO, USA) and kept on ice until used (same day processing). For RNA isolation, gingival tissues were rinsed with RPMI 1640 medium (Sigma), dissected into small pieces using a sterile surgical blade and immersed in a 4 M guanidinium isothiocyanate solution (Life Technologies Inc., Gaithersburg, NY, USA), containing 2-mercaptoethanol and phenol. For Western blotting and zymography assay, parts of those biopsies were snap frozen in liquid nitrogen followed by lyophilization under vacuum.

Isolation and purification of RNA

Total RNA was isolated by the guanidinium isothiocyanate–phenol–chloroform procedure according to the method of Chomczynski & Sacchi (26). The final RNA precipitate was washed in 70:30 ethanol:water, previously treated with diethylpyrocarbonate (DEPC) to eliminate RNAse contamination, and dissolved in DEPC-treated water. The concentration of RNA was estimated by spectrophotometry at 260 nm (Genesys 5, Spectronic Institute, Rochester, NY, USA). Purity was considered acceptable when the ratio of absorbance values at 260 nm/280 nm equaled 1.6–1.8. The RNA solutions were stored at −70°C.

Synthesis of cDNA

A volume of 10 μL of RNA solution (3.0 μg of RNA) was heated to 65°C and then placed in an ice bath. A negative control (containing 10 μL of H_2O–DEPC) was included. Synthesis of cDNA was achieved by adding 9.8 μL cDNA mix [0.8 μL deoxynucleotide triphosphates (dNTPs) of a 25 mM solution containing equimolar amounts of dATP, dCTP, dGTP and dTTP (Invitrogen, Paisley, UK); 2 μL of 0.1 M dithiothreitol (DTT, Invitrogen); 1 μL of a Random Hexamer primers 100 pM/solution (Invitrogen); 4 μL 5X-Buffer (Invitrogen); 1 μL of 200 μg/μL Moloney Murine Leukemia Virus reverse transcriptase (Invitrogen) and 1 μL of 33 U/μL RNAse inhibitor (Amersham Bioscience, Freiburg, Germany)]. Final volumes (20 μL per tube) were kept at room temperature for 10 min followed by incubation at 42°C for 40 min. Reactions were terminated by heating at 65°C for 5 min. The cDNAs were stored at −20°C.

Polymerase chain reactions (PCRs)

Two microlitres of cDNA were mixed with 30 μL of each MMP, TIMP and GAPDH mixes [1 mL of each mix = 125 μL of 10X Taq Pol buffer (Ludwig Biotecnologia, São Paulo, Brazil); 10 μL of 25 mM dNTPs with equimolar amounts of dTTP, dCTP, dGTP and dATP (Invitrogen); 62.5 μL of 25 mM $MgCl_2$; 5 μL of primer sense and 5 μL of primer antisense (100 pmol/μL of each primer, Gene Link, USA) and 792.5 μL of water]. Further, 0.3 μL of a Taq polymerase (Ludwig Biotecnologia) solution (5 U/μL) was added to the reaction mix

Table 1. Primers and experimental conditions for PCR amplification

Gene	Primer	Sequence 5′ → 3′	PCR protocol	Fragment size (bp)	References
MMP-1	Sense	TGC TCA TGC TTT TCA ACC	1 cycle of 98°C (1 min), 96°C (30 s); 30 cycles of 96°C (30 s), 49.6°C (45 s), 72°C (1 min); 1 cycle of 72°C (5 min); 4°C ∞	511	Constructed
	Antisense	GAA CAG CCC AGT ACT TAT TCC			
MMP-2	Sense	GTG CTG AAG GAC ACA CTA AAG AAG A	1 cycle of 96°C (1 min), 96°C (1 min); 30 cycles of 96°C (1 min), 55°C (1 min), 72°C (2 min); 1 cycle of 73°C (5 min); 4°C ∞	606	Liu *et al.* (31)
	Antisense	TTG CCA TCC TTC TCA AAG TTG TAG G			
MMP-9	Sense	GGA GAC CTG AGA ACC AAT CTC	1 cycle of 96°C (1 min), 96°C (1 min); 40 cycles of 96°C (1 min), 52°C (1 min), 72°C (2 min); 1 cycle of 73°C (5 min); 4°C ∞	296	Yang *et al.* (32)
	Antisense	TCC AAT AGG TGA TGT TGT GGT			
MMP-13	Sense	TAT CCC TTG ATG CCA TTA CC	1 cycle of 98°C (1 min), 96°C (30 s); 30 cycles of 96°C (30 s), 53°C (45 s), 72°C (1 min); 1 cycle of 72°C (5 min); 4°C ∞	526	Constructed
	Antisense	CAT GAC GCG AAC AAT ACG			
TIMP-1	Sense	AGT CAA CCA GAC CAC CTT ATA CCA	1 cycle of 98°C (1 min), 96°C (30 s); 30 cycles of 96°C (30 s), 58°C (45 s), 72°C (1 min); 1 cycle of 72°C (5 min); 4°C ∞	386	Kubota *et al.* (24)
	Antisense	TTT CAG AGC CTT GGA GGA GCT GGT C			
TIMP-2	Sense	GGA AGT GGA CTC TGG AAA CGA CAT T	1 cycle of 98°C (1 min), 96°C (30 s); 30 cycles of 96°C (30 s), 54°C (45 s), 72°C (1 min); 1 cycle of 72°C (5 min); 4°C ∞	496	Kubota *et al.* (24)
	Antisense	CTC GAT GTC GAG AAA CTC CTG CTT G			
GAPDH	Sense	ATC ACC ATC TTC GAG GAG CG	1 cycle of 96°C (1 min), 96°C (30 s); 30 cycles of 96°C (30 s), 57.5°C (45 s), 72°C (1 min); 1 cycle of 72°C (5 min); 4°C ∞	571	Coutinho *et al.* (33)
	Antisense	CCT GCT TCA CCA CCT TCT TG			

∞ refers to the 'forever' module in the PCR machine last reaction step.

(final volume = 32.3 μL). Efficiency of cDNA amplification was monitored with primers for the amplification of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Two positive cDNA controls were used for each reaction: (1) cDNA made from patient samples known to be positive for the target mRNA; and/or (2) cDNAs made from cell lines that express the target gene as described in the references cited in Table 1. Additionally, negative controls for PCR amplifications were employed that lacked RNA in the reverse transcription step, or lacked cDNAs in the amplification step. All amplifications were carried out with a thermocycler (T gradient, Biometra, Göttingen, Germany) under conditions listed in Table 1. Reaction products were eletrophoresed in 2% agarose gels. For estimation of the PCR results, digital photographs of the gels were analyzed by the NIH Scion Image Program (version 1.62). Comparisons between different reverse transcriptase (RT)-PCR assays were made after normalization of the MMPs or TIMPs densitometric scans by dividing each optical density value by the amplified GAPDH product optical density value (internal control). It is important to consider that all RT-PCRs of each sample were made from the same cDNA. The relative expression levels among groups were then compared.

Gelatin zymography

Lyophilized samples were reconstituted in 70 μL of DMEM, and protein content was measured using the Advanced protein assay reagent (Cytoskeleton Inc., Denver, CO, USA). Fifteen micrograms of protein sample were mixed with 2X non-reducing sample buffer (0.5 M Tris-HCl, pH 6.8, 20% glycerol, 0.2% SDS, 0.0005% Bromophenol Blue) and electrophoresed on 0.75-mm-thick 8% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) containing 0.1% gelatin as a protease substrate. Following electrophoresis, gels were washed in 2.5% Triton X-100, 50 mM Tris-HCl, pH 7.5, for 1 h to remove SDS and then washed with 50 mM

Tris-HCl, pH 7.5. Subsequently, gels were incubated for 20 h at 37°C in incubation buffer (50 mM Tris-HCl, pH 7.5, 1 mM $CaCl_2$ and 1 mM $ZnCl_2$) on a rotary shaker. Afterwards, gels were stained in 30% methanol, 10% acetic acid and 0.2% (w/v) Coomassie Brilliant Blue for 1 h, followed by destaining in 30% methanol and 10% acetic acid. Gelatinolytic activity was manifested as horizontal white bands on a dark blue background. Gels were scanned (VersaDoc, Bio-Rad, Hercules, CA, USA), and bands were quantified by optical densitometry.

Western blot analysis

Lyophilized samples were reconstituted in 70 μL of DMEM, and protein content was measured using the Advanced protein assay reagent (Cytoskeleton Inc.). Protein samples (15 μg each) in sample buffer (62.5 mM Tris, 10% glycerol, 2% SDS and 5% β-mercaptoethanol) were subjected to 10% SDS-PAGE and Western blotting with antibodies specific for the proteins of interest. Proteins were transferred to polyvinylidene difluoride membranes overnight in blotting buffer (0.025 M Tris, 0.192 M glycine and 20% methanol). Membranes were blocked with blocking solution containing 5% non-fat milk in Tris Buffered Saline-Tween 20 (TBS-T) (20 mM Tris-HCl, pH 7.5, 150 mM NaCl and 0.1% Tween) for 1 h followed by incubation with blocking solution in the presence of primary anti-MMP-2 or anti-MMP-9 antibodies (1:1000; Cell Signaling Tecnology, Danvers, MA, USA) overnight at 4°C with mild shaking. Membranes were then washed with TBS-T, three times for 10 min each. Membranes were next subjected to incubation in the presence of secondary anti-rabbit horseradish peroxidase-conjugated antibodies (1:2000; Cell Signaling Tecnology) in 5% non-fat milk in TBS-T for 2 h at room temperature with mild shaking. Membranes were washed again with TBS-T three times for 10 min each. Chemiluminescent detection of bound horseradish peroxidase-conjugated secondary antibodies was determined using the Amersham ECL™ Western Blotting Detection Reagents (GE Healthcare Bio-Sciences Co., Piscataway, NJ, USA) and exposed to film.

Statistical analysis

Differences in proportions of the investigated target gene expressed in control and diseased samples (gingivitis, chronic and aggressive periodontal disease) were analyzed using the Fisher's exact test. The transcript level for each MMP and TIMP and the gelatinase activity of MMP-2 and -9 were compared between groups using the non-parametric Kruskal–Wallis test. All tests were performed using the software SPSS, version 11.5 (SPSS Inc., Chicago, IL, USA). Differences were considered significant at an α level of 5%.

Results

Demographic and clinical data are listed in Table 2. Accordingly, probing pocket depth and clinical attachment level means were higher in the periodontitis groups. The mean age of the aggressive periodontitis patients was lower than that of the chronic periodontitis subjects, as expected.

Prevalence of mRNA expression of MMPs and TIMPs

Frequencies of mRNA expression for MMPs and TIMPs are presented in Table 3. The collagenase-1 (MMP-1) mRNA was detected in 50% of diseased samples and 30% of control samples. In contrast, expression of MMP-2 transcripts was virtually absent in the periodontitis groups (detected in just one sample of the aggressive group), while it was detected in 40% of gingivitis samples and 30% of control samples. The MMP-9 transcripts were more frequently detected in periodontitis-affected tissues (50% of aggressive and 40% of chronic peridontitis), compared with the 10% of gingivitis and 20% of control samples. Overall, the transcripts of gelatinases MMP-2 and MMP-9 showed less positivity (21 and 28.9%, respectively). Transcripts of MMP-13 and TIMP-2 were detected in 76.3% of the samples analyzed. The presence of

Table 2. Age and clinical parameters of experimental groups

	Experimental groups			
Parameters[a]	Control (n = 10)	Gingivitis (n = 10)	Chronic periodontitis (n = 10)	Aggressive periodontitis (n = 8)
Age (years)	27.3 ± 11.2	32.9 ± 9.0	41.8 ± 5.5	25.0 ± 9.9
Number of sites	13.1 ± 3.5	10.9 ± 2.2	9.5 ± 1.9	14.9 ± 2.6
PPD (mm)	2.3 ± 0.1	2.3 ± 0.2	6.1 ± 0.2	7.3 ± 0.5
CAL (mm)	2.3 ± 0.1	2.3 ± 0.2	7.4 ± 0.5	8.5 ± 0.7
BOP (% of sites)	0.0 ± 0.0	56.7 ± 12.7	74.0 ± 13.7	97.9 ± 2.1

Values are expressed as means ± SD. [a] Clinical parameters refers to the biopsied sites.

Table 3. Prevalence of transcripts in the experimental groups

	Experimental groups					
Transcripts	Control (n = 10; %)	Gingivitis (n = 10; %)	Chronic periodontitis (n = 10; %)	Aggressive periodontitis (n = 8; %)	Total (n = 38; %)	p[a]
MMP-1	30	50	50	50	44.7	0.755
MMP-2	30	40	0	12.5	21	0.122
MMP-9	20	10	40	50	28.9	0.251
MMP-13	60	90	70	87.5	76.3	0.403
TIMP-1	40	60	40	62.5	50	0.709
TIMP-2	60	90	70	87.5	76.3	0.403

[a]Fisher's exact test.

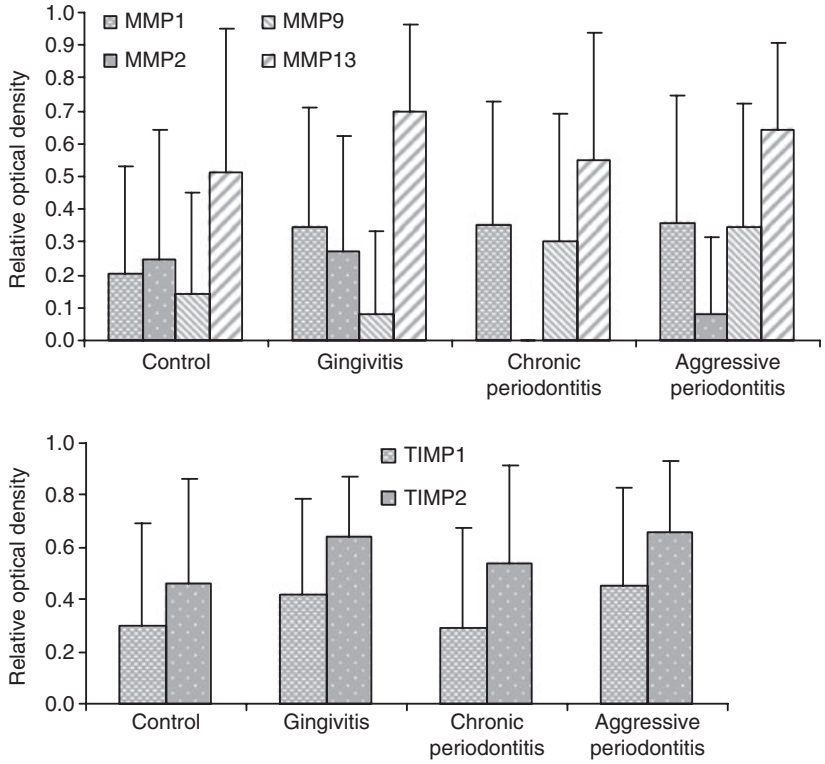

Fig. 1. Quantitative analysis of gene expression of MMPs and TIMPs in control gingiva and in gingiva affected by gingivitis, chronic and aggressive periodontitis. Total RNA isolated from the experimental samples was subjected to reverse transcription followed by amplification by polymerase chain reaction. Products from those reactions were visualized in agarose gels and quantified by optical densitometry. All results were normalized for the GAPDH mRNA concentrations and represent the means + SD. The results show that there are no differences in levels of expression for the transcripts of MMP-1, -2, -9 and -13 and TIMP-1 and -2 among gingival tissues of healthy individuals and those suffering from gingivitis, chronic or aggressive periodontitis ($p > 0.05$, Kruskal–Wallis test).

TIMP-1 mRNA was observed in 50% of all samples (40% in control and chronic periodontitis groups, and 60% in aggressive periodontitis and gingivitis samples). No statistically significant difference was detected among the four experimental groups, for any transcript.

Semi-quantitative analysis of mRNA expression of MMPs and TIMPs

The relative amounts of MMPs and TIMPs transcripts in control and diseased tissues are shown in (Fig. 1). The expressions of MMP-1, MMP-13 and TIMP-2 were slightly higher in the diseased samples. Expression of MMP-9 was lower in gingivitis-affected tissues, and MMP-2 levels were nearly undetectable in the periodontitis groups. In contrast, the expression of TIMP-1 was slightly higher in aggressive periodontitis samples. No statistically significant difference was seen for any MMPs or TIMPs among groups. Moreover, our data showed a great heterogeneity in the expression of mRNAs encoding for each MMP and TIMP within groups (large standard deviation).

Gelatin zymography assay

A representative zymogram and the comparison of data among experimental groups are depicted in Fig. 2A and B, respectively. One can notice that MMP-2 and MMP-9 gelatinase activities vary among groups. There is also a discrepancy between both, particularly in the chronic periodontitis group. In fact, there is a twofold difference between control and diseased groups for MMP-9, although statistical significance was not reached. Regarding MMP-2, a similar higher enzymatic activity in the inflamed groups can be observed, with the exception of the chronic periodontitis group where the MMP-2 bands can barely be visualized. The densitometry analysis, combining the proMMP-2 (72 kDa) and the active form (64 kDa), revealed a statistically significant ($p < 0.01$) lower value for the chronic periodontitis compared with the gingivitis group (4,100 ± 5,354 vs. 27,071 ± 6,246).

Western blotting for MMP-2 and MMP-9

A qualitative analysis of the Western blots indicated that MMP-2 and MMP-9 protein expression is regulated in parallel to their enzymatic activity. For instance, gingivitis and aggressive periodontitis groups presented the greatest protein expression for MMP-9, whereas the control group had the lowest. Likewise, MMP-2 protein level was higher in gingivitis, followed by the aggressive periodontitis group. Moreover, MMP-2 was found at very low levels in the chronic periodontitis and healthy groups (Fig. 2C).

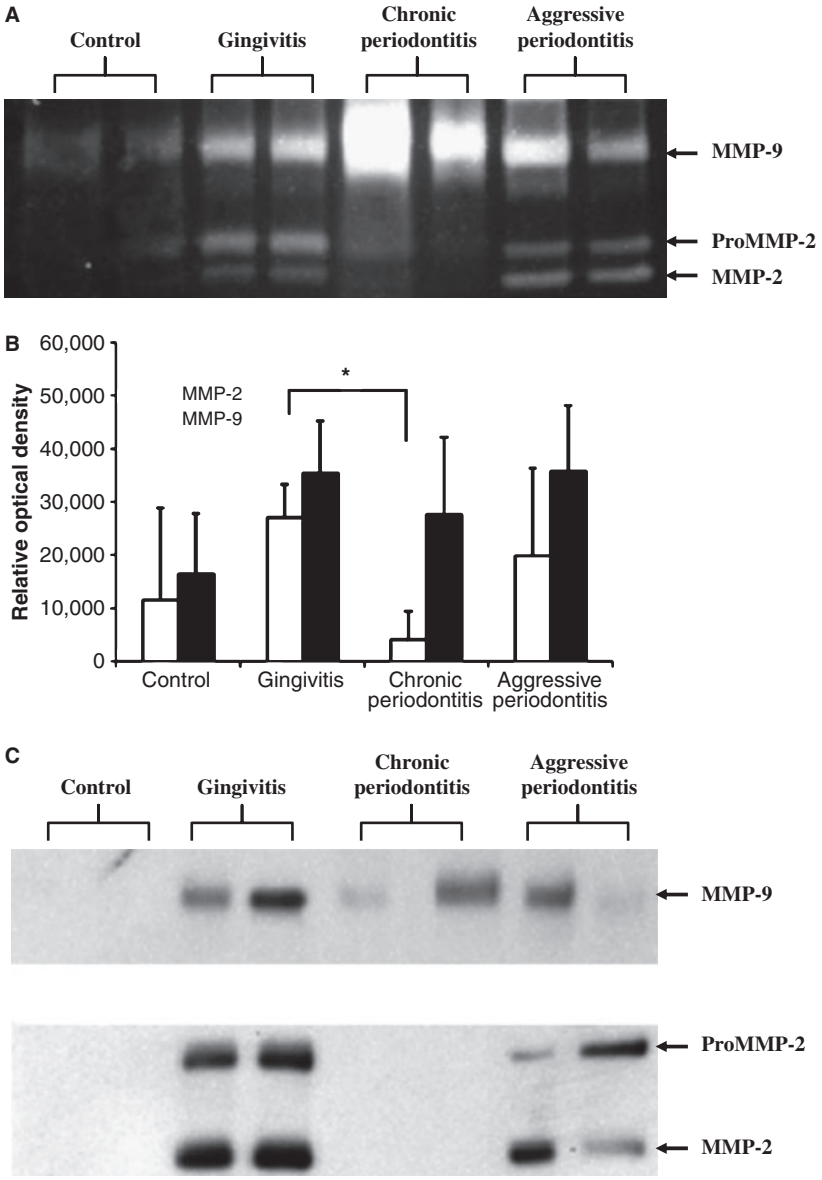

Fig. 2. Equal amounts of protein were loaded onto SDS-PAGE and separated by electrophoresis as described in the Material and methods. (A) Representative gelatinase zymogram. Bands correspond to 92 kDa (MMP-9); 72 kDa (proMMP-2) and 64 kDa (active MMP-2) in control, gingivitis, chronic and aggressive periodontitis groups. (B) Zymogram gels were scanned, and bands were quantified by optical densitometry. Results represent means + SD. * $p < 0.01$, Kruskal–Wallis test. (C) Representative Western immunoblots. Bands correspond to 92 kDa (MMP-9); 72 kDa (proMMP-2) and 64 kDa (active MMP-2) in control, gingivitis, chronic and aggressive periodontitis groups.

Discussion

Extracellular matrix remodeling is a complex and crucial process for the development, homeostasis and repair of connective tissues. Temporal, concerted regulation of genes coding MMPs and TIMPs is required for those processes to occur (27). Increased expression of MMPs in diseased periodontal tissues seems to be the consensus in the literature and is thought to account for the destruction of soft and mineralized tissues that results in some of the clinical symptoms of periodontal disease (3,5,7,8,24). In the present study, we sought to compare the expression of some transcripts of MMPs and TIMPs, along with the protein expression and gelatinolytic activity of MMP-2 and -9 in gingivitis-, advanced chronic- or aggressive periodontitis-affected samples obtained from subjects undergoing periodontal therapy or pre-prosthetic surgical procedures.

The results presented here indicate that neither the frequencies nor the levels of transcripts were statistically different among gingivitis, advanced chronic or aggressive periodontitis and control groups. It could be argued that the lack of difference among groups could be related to the cyclical nature of loss of periodontal attachment. This might be associated with sudden activation of latent enzymes stored in the ECM rather than an increase in their synthesis and/or release to the inflam-

mation sites, according to the model proposed by van der Zee *et al*. (28). Besides regulation of the mRNA, several mechanisms, such as blocking of intracellular mobilization, neutralization by metalloproteinases or even translational modulation, might affect the protein level in tissues. Likewise, it is important to consider that the levels of MMP-8 and -9 molecules are related to neutrophil degranulation induced by cytokines and bacterial virulence factors rather than to *de novo* gene transcription (29). Therefore, we also carried out Western blotting and gelatin zymographic assays to test whether the gene expression for some of those MMPs could have been regulated at the translational or post-translational level. Based on those assays, there seemed to be a trend of higher, although not statistically significant, gelatinase activity in most of the inflamed samples compared with the healthy control samples. Moreover, a divergence between MMP-2 and MMP-9 was seen in the chronic periodontitis group, which presented a statistically significant lower level of total MMP-2 enzymatic activity. This is in contrast to other studies (5, 8) but is in line with the RT-PCR and Western Blotting results, and with a recent report of diminished MMP-2 gene expression and gelatinolytic activity following inflammatory stimulus in an animal model of experimental periodontitis (30).

Different from others studies (3,5,8,24), biopsies were collected during a single course of full-mouth scaling and root planing in an attempt to avoid healing events that might be influenced by activity of MMPs/TIMPs (4). Moreover, considering that subjects belonging to the same group exhibited similar periodontal conditions, it is intriguing that a large variation in the expression pattern of the analyzed transcripts is seen; however, this is in accordance with data presented by Dahan *et al*. (5). One should be aware of the possibility that some of the patients could have been mistakenly assigned to a particular group based on their periodontal parameters. This would be particularly relevant for those rendered a diagnosis of aggressive periodontitis if subjected to a previous periodontal treatment. In that case, the present episode of recurrent periodontitis could have been of a chronic nature and influenced the results presented here. However, this is unlikely to be true because no patient reported any history of periodontal therapy (data not shown).

In summary, in the present paper we provide data on RT-PCR analysis of several TIMPs and MMPs in gingivitis- and periodontitis-affected tissue samples. Particularly relevant, data on the expression of MMP-13, a peculiar collagenase that has a wide range of substrate specificity, were observed for the first time in different periodontal conditions. Moreover, we observed a general trend for a higher MMP-2 and -9 gelatinase activity in the inflamed tissues. However, transcripts for MMP-2, as well as its protein expression and gelatinase activity, were not detected or barely detected in chronic periodontits-affected tissues. In conclusion, these results could reflect a complex regulation of gene expression of MMPs and TIMPs in the course of gingival inflammation. They could also reveal a great biological heterogeneity, even among individuals with similar periodontal status.

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References

1. Sorsa T, Tjaderhane L, Konttinen YT *et al*. Matrix metalloproteinases: contribution to pathogenesis, diagnosis and treatment of periodontal inflammation. *Ann Med* 2006;**38:**306–321.
2. Kornman KS, Page RC, Tonetti MS. The host response to the microbial challenge in periodontitis: assembling the players. *Periodontol 2000* 1997;**14:**33–53.
3. Aiba T, Akeno N, Kawane T *et al*. Matrix metalloproteinases-1 and -8 and TIMP-1 mRNA levels in normal and diseased human gingivae. *Eur J Oral Sci* 1996;**104:** 562–569.
4. Tervahartiala T, Pirila E, Ceponis A *et al*. The in vivo expression of the collagenolytic matrix metalloproteinases (MMP-2, -8, -13, and -14) and matrilysin (MMP-7) in adult and localized juvenile periodontitis. *J Dent Res* 2000;**79:**1969–1977.
5. Dahan M, Nawrocki B, Elkaim R *et al*. Expression of matrix metalloproteinases in healthy and diseased human gingiva. *J Clin Periodontol* 2001;**28:**128–136.
6. Ejeil AL, Gaultier F, Igondjo-Tchen S *et al*. Are cytokines linked to collagen breakdown during periodontal disease progression? *J Periodontol* 2003;**74:**196–201.
7. Smith PC, Munoz VC, Collados L *et al*. *In situ* detection of matrix metalloproteinase-9 (MMP-9) in gingival epithelium in human periodontal disease. *J Periodont Res* 2004;**39:**87–92.
8. Garlet GP, Martins W Jr, Fonseca BA *et al*. Matrix metalloproteinases, their physiological inhibitors and osteoclast factors are differentially regulated by the cytokine profile in human periodontal disease. *J Clin Periodontol* 2004;**31:**671–679.
9. Pozo P, Valenzuela MA, Melej C *et al*. Longitudinal analysis of metalloproteinases, tissue inhibitors of metalloproteinases and clinical parameters in gingival crevicular fluid from periodontitis-affected patients. *J Periodont Res* 2005;**40:** 199–207.
10. Emingil G, Tervahartiala T, Mantyla P *et al*. Gingival crevicular fluid matrix metalloproteinase (MMP)-7, extracellular MMP inducer, and tissue inhibitor of MMP-1 levels in periodontal disease. *J Periodontol* 2006;**77:**2040–2050.
11. Emingil G, Kuula H, Sorsa T *et al*. Gingival crevicular fluid matrix metalloproteinase-25 and -26 levels in periodontal disease. *J Periodontol* 2006;**77:**664–671.
12. Kumar MS, Vamsi G, Sripriya R *et al*. Expression of matrix metalloproteinases (MMP-8 and -9) in chronic periodontitis patients with and without diabetes mellitus. *J Periodontol* 2006;**77:**1803–1808.
13. Hernandez M, Valenzuela MA, Lopez-Otin C *et al*. Matrix metalloproteinase-13 is highly expressed in destructive periodontal disease activity. *J Periodontol* 2006;**77:**1863–1870.
14. Ilgenli T, Vardar-Sengul S, Gurkan A *et al*. Gingival crevicular fluid matrix metalloproteinase-13 levels and molecular

forms in various types of periodontal diseases. *Oral Dis* 2006;**12:**573–579.
15. Soder B, Airila Mansson S, Soder PO *et al*. Levels of matrix metalloproteinases-8 and -9 with simultaneous presence of periodontal pathogens in gingival crevicular fluid as well as matrix metalloproteinase-9 and cholesterol in blood. *J Periodont Res* 2006;**41:**411–417.
16. Liu KZ, Hynes A, Man A *et al*. Increased local matrix metalloproteinase-8 expression in the periodontal connective tissues of smokers with periodontal disease. *Biochim Biophys Acta* 2006;**1762:**775–780.
17. Maeso G, Bravo M, Bascones A. Levels of metalloproteinase-2 and -9 and tissue inhibitor of matrix metalloproteinase-1 in gingival crevicular fluid of patients with periodontitis, gingivitis, and healthy gingiva. *Quintessence Int* 2007;**38:**247–252.
18. Kowashi Y, Jaccard F, Cimasoni G. Increase of free collagenase and neutral protease activities in the gingival crevice during experimental gingivitis in man. *Arch Oral Biol* 1979;**24:**645–650.
19. Choi DH, Moon IS, Choi BK *et al*. Effects of sub-antimicrobial dose doxycycline therapy on crevicular fluid MMP-8, and gingival tissue MMP-9, TIMP-1 and IL-6 levels in chronic periodontitis. *J Periodont Res* 2004;**39:**20–26.
20. Emingil G, Atilla G, Sorsa T *et al*. Effectiveness of adjunctive low-dose doxycycline therapy on clinical parameters and gingival crevicular fluid laminin-5 γ2 chain levels in chronic periodontitis. *J Periodontol* 2004;**75:**1387–1396.
21. Figueredo CM, Areas A, Miranda LA *et al*. The short-term effectiveness of non-surgical treatment in reducing protease activity in gingival crevicular fluid from chronic periodontitis patients. *J Clin Periodontol* 2004;**31:**615–619.
22. Tuter G, Kurtis B, Serdar M *et al*. Effects of phase I periodontal treatment on gingival crevicular fluid levels of matrix metalloproteinase-3 and tissue inhibitor of metalloproteinase-1. *J Clin Periodontol* 2005;**32:**1011–1015.
23. Ingman T, Tervahartiala T, Ding Y *et al*. Matrix metalloproteinases and their inhibitors in gingival crevicular fluid and saliva of periodontitis patients. *J Clin Periodontol* 1996;**23:**1127–1132.
24. Kubota T, Nomura T, Takahashi T *et al*. Expression of mRNA for matrix metalloproteinases and tissue inhibitors of metalloproteinases in periodontitis-affected human gingival tissue. *Arch Oral Biol* 1996;**41:**253–262.
25. Van Winkelhoff AJ, Van der Velden U, de Graaff J. Microbial succession in recolonizing deep periodontal pockets after a single course of supra- and subgingival debridement. *J Clin Periodontol* 1988; **15:**116–122.
26. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987;**162:**156–159.
27. Palmon A, Roos H, Edel J *et al*. Inverse dose- and time-dependent effect of basic fibroblast growth factor on the gene expression of collagen type I and matrix metalloproteinase-1 by periodontal ligament cells in culture. *J Periodontol* 2000;**71:**974–980.
28. van der Zee E, Everts V, Beertsen W. Cytokines modulate routes of collagen breakdown. Review with special emphasis on mechanisms of collagen degradation in the periodontium and the burst hypothesis of periodontal disease progression. *J Clin Periodontol* 1997;**24:**297–305.
29. Ding Y, Uitto VJ, Haapasalo M *et al*. Membrane components of Treponema denticola trigger proteinase release from human polymorphonuclear leukocytes. *J Dent Res* 1996;**75:**1986–1993.
30. Silva JA, Lorencini M, Peroni LA, De La Hoz CL, Carvalho HF, Stach-Machado DR. The influence of type I diabetes mellitus on the expression and activity of gelatinases (matrix metalloproteinases-2 and -9) in induced periodontal disease. *J Periodont Res* 2008;**43:**48–54.
31. Liu W, Kunishio K, Matsumoto Y *et al*. Matrix metalloproteinase-2 expression correlates with cavernous sinus invasion in pituitary adenomas. *J Clin Neurosci* 2005;**12:**791–794.
32. Yang J, Wei D, Liu J. Repressions of MMP-9 expression and NF-κB localization are involved in inhibition of lung carcinoma 95-D cell invasion by (–)-epigallocatechin-3-gallate. *Biomed Pharmacother* 2005;**59:**98–103.
33. Coutinho CC, Fonseca RN, Mansure JJ *et al*. Early steps in the evolution of multicellularity: deep structural and functional homologies among homeobox genes in sponges and higher metazoans. *Mech Dev* 2003;**120:**429–440.