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Matrix metalloproteinases, their physiological inhibitors and osteoclast factors are differentially regulated by the cytokine profile in human periodontal disease

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Abstract

Objective: Inflammatory reactions raised in response to periodontopathogens are thought to trigger pathways of periodontal tissue destruction. We therefore investigated the expression of matrix metalloproteinases (MMPs) and the osteoclastogenic factor receptor activator of nuclear factor-κB ligand (RANKL), their respective tissue inhibitors of metalloproteinases (TIMPs) and osteoprotegerin (OPG) in different forms of human periodontal diseases (PDs), and the possible correlation with the expression of inflammatory and regulatory cytokines.

Material and Methods: Quantitative polymerase chain reaction (real-time PCR) was performed with gingival biopsies mRNA from aggressive (AP) and chronic periodontitis (CP) patients.

Results: Periodontitis patients exhibit higher expression of all analyzed factors when compared with healthy tissues. The expression of MMPs and RANKL were similar in AP and CP, as well as the expression of TNF-α. On the other hand, the expression of TIMPs and OPG was higher in CP, and was associated with lower IFN-γ and higher IL-10 expression, compared with AP.

Conclusion: It is possible that the pattern of cytokines expressed determines the stable or progressive nature of the lesions and regulates the severity of PD, driving the balance between MMPs and TIMPs, RANKL and OPG expression in the gingival tissues controlling the breakdown of soft and bone tissues and, consequently, the disease severity.

Key words: cytokines; matrix metalloproteinases; osteoprotegerin; periodontal disease; RANKL; tissue inhibitors of metalloproteinases

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Periodontal diseases (PDs) are considered to be infectious diseases, where putative periodontopathogens trigger chronic inflammatory and immune responses that are thought to determine the clinical outcome of the disease. Periodontitis is characterized by an intense inflammatory infiltrate associated with irreversible loss of alveolar bone and/or connective tissue attachment in the periodontium, which ultimately results in the loss of teeth. This tissue breakdown is thought to be the result of activation of host cells by inflammatory mediators, such as arachidonic acid metabolites, cytokines and enzymes. These factors in turn trigger the resorption of alveolar bone and the

generation of proteases that degrade extracellular matrix (ECM), resulting in tissue destruction (Genco 1992, Baker 2000, Kinane & Lappin 2001, Seymour & Gemmel 2001).

Among host proteases that target the ECM, matrix metalloproteinases (MMPs – a family of zinc- and calcium-dependent proteases) play a role in both degradation and remodeling of matrix proteins during different physiologic and pathological processes (Birkedal-Hansen et al. 1993). MMPs comprise four major subclasses based on their substrate specificity and sequence homology: collagenases such as MMP-1 or interstitial collagenase are active against fibrillar collagen; gelatinases, also called type IV collagenases (A or MMP-2 and B or MMP-9), which present high activity against denaturated collagens; stromelysins, which degrade non-collagen components of ECM; and membrane-type MMPs (Werb 1997).

The activation of MMPs is regulated by a group of endogenous proteins named tissue inhibitors of metalloproteinases (TIMPs) that are each capable of inhibiting almost every member of the MMP family in a non-specific way (Baker et al. 2002). Usually, the TIMPs are in balance with the MMPs and the matrix is remodeled in a highly regulated fashion. However, in many disease processes the levels of MMPs are elevated without a concomitant increase in TIMPs, resulting in tissue destruction (Dean et al. 1989, Murphy et al. 1991, Reynolds 1996, Nawrocki et al. 1997). It is thought that MMPs and TIMPs are involved in the physiological turnover of periodontal tissues, and MMPs appear to be involved in tissue destruction in PDs (Reynolds et al. 1994, Van der Zee et al. 1997, Golub et al. 2001). However, there are contradictory results regarding levels of MMPs in pathological versus healthy gingival samples (Aiba et al. 1996, Ingman et al. 1996, Kubota et al. 1996, Nomura et al. 1998, Dahan et al. 2001), and their contribution towards the pathogenesis of periodontitis is still not well understood.

In addition to the destruction of connective tissue, alveolar bone loss is a key event in PD. The integrity of bone tissues depends on maintaining a delicate equilibrium between bone resorption by osteoclasts and bone formation by osteoblasts. The major regulatory mechanism of osteoclasts activity is driven by some members of the TNF family of receptors RANK (receptor activator of nuclear factor-κB) and osteoprotegerin (OPG), and the ligand RANKL (Teitelbaum 2000, Katagiri & Takahashi 2002). RANK is expressed on osteoclastic precursors and on mature osteoclasts while its ligand, RANKL, a transmembrane protein, is expressed particularly on osteoblast and activated T cells, in addition to being produced in soluble form. The interaction between RANK and RANKL is required for differentiation and activation of osteoclasts, an event regulated by OPG, a decoy receptor of RANKL, that strongly inhibits bone resorption by preventing RANK–RANKL engagement (Teitelbaum 2000, Katagiri & Takahashi 2002). The imbalances of this system are pivotal components of the etiology of some bone disorders (Rodan & Martin 2000, Romas et al. 2002, Sezer et al. 2002).

It is also important to establish the factors that regulate the breakdown of homeostasis of connective and osseous tissue that takes place in PD. Inflammatory mediators, such as prostaglandins (PGE2), IL-1 and TNF-α, and the Th1-type cytokine IFN-γ, have been described as positive regulators of osteoclastogenesis and of expression of MMPs (Harris et al. 2002, Katagiri & Takahashi 2002). The reverse effect is exerted by the Th2-type cytokines such as IL-4, IL-10 and IL-13 (Harris et al. 2002, Katagiri & Takahashi 2002). It has been suggested that, in periodontal lesions, the balance between the expression of Th1- and Th2-type cytokines and chemokines in a mixed inflammatory immune response is a relevant factor to the outcome of disease (Seymour & Gemmel 2001, Taubman & Kawai 2001, Ukai et al. 2001, Teng 2002, Garlet et al. 2003). However the impact of different trends in the production of Th1 and Th2 cytokines and chemokines upon the expression of MMP, TIMPS, RANKL and OPG and the attendant repercussions in osteoclastogenesis is not known.

In the present study, we investigated the pattern of expression of mRNAs encoding for MMPs, TIMPs, RANKL and OPG by quantitative polymerase chain reaction (real-time PCR), and further correlated them with the profile of cytokine expression in gingival biopsies from aggressive periodontitis (AP) and chronic periodontitis (CP) patients. Our data showed similar expression of MMPs and RANKL in AP and CP groups. On the other hand, the expression of TIMPs and OPG were increased in CP and associated with a lower expression of IFN-γ and higher expression of IL-10 when compared with AP patients.

Material and Methods

Study population and clinical examination

Patients and controls were submitted to anamnesis and to clinical, periodontal and radiographic examination. Prior to the beginning of the study, all subjects received supragingival prophylaxis to remove gross calculus and allow probing access. All teeth, with the exception of third molars were scored for probing depth and clinical attachment level, at six sites per tooth. Measurements were made at mesiobuccal, buccal, distobuccal, distolingual, lingual and mesiolingual positions. Dichotomous measurement of bleeding on probing (BOP) was performed at six sites per tooth. A second measurement was made 3–4 weeks after the initial basic periodontal therapy.

The patients were categorized according to the classification of the American Academy of Periodontology into control, AP or CP groups. Inclusion criteria comprised partially or fully dentate patients (at least 14 natural teeth, including 10 posterior teeth, excluding third molars), systemically healthy with no evidence of known systemic modifiers of PD (type 1 and 2 diabetes mellitus, osteoporosis, and medications known to influence periodontal tissues). Exclusion criteria comprised patients who did not give informed consent; patients with a significant medical history indicating evidence of known systemic modifiers of PD as described above; pregnant or lactating females; and patients who had taken systemic antibiotic, anti-inflammatory, hormonal or other assisted drug therapy in the last 6 months prior to the study, or those who had been submitted to periodontal therapy in the last 2 years. Smokers were not specifically excluded.

CP patients had moderate-to-advanced PD (at least 1 teeth per sextant with probing depth >6 mm and attachment loss >3 mm and radiographic evidence of extensive bone loss). AP patients are characterized by the aggressive and typical alveolar bone loss localized at the first molars and incisors (localized AP), or aggressive and

extensive generalized bone loss affecting at least three teeth other than molars and incisors (generalized AP), evidence from dental history that the onset of disease occurred when they were less than 35 years old, with a high probing depth (at least one teeth per sextant with probing depth >6 mm and attachment loss >3 mm), and extensive radiographic evidence of bone loss.

All patients were scheduled for periodontal treatment at the Department of Periodontics, University of Ribeirão Preto Dental School (UNAERP) and after the diagnostic phase, they received basic periodontal therapy, which consisted in oral hygiene instruction, scaling and root planing. Biopsies of gingival tissue (comprising junctional epithelium, gingival crevicular epithelium and connective gingival tissue) were obtained during surgical therapy of the sites that exhibited persistent BOP and that showed no improvement in clinical condition (i.e. higher probing depth) 3–4 weeks after the basic periodontal therapy. One sample of gingival tissue was obtained from each one of the 16 AP and 20 CP patients.

The control group was comprised of 10 subjects presenting clinical healthy gingival tissues (low scores of BOP – under 10% of the sites; no sites with probing depth >3 mm or presenting attachment loss) from whom biopsies of gingival tissue were taken during surgical procedures for wisdom teeth removal (all the sampled sites showed no BOP and probing depths <3 mm). The clinical features of the groups are summarized in Table 1.

Table 1. Clinical features of the control, AP and CP groups (mean ± SD)

	Control	AP	CP
distribution	(10) 6f/4m	(16) 7f/9m	(20) 11f/9m
age	28.5 ± 12.11	26 ± 4.32	46.25 ± 8.09
probing depth (mean)	1.92 ± 0.65	3.55 ± 1.37	3.38 ± 1.81
probing depth (sampled site)	1.74 ± 0.55	7.15 ± 0.89	6.05 ± 1.27
attachment loss (sampled site)	0	3.80 ± 1.62	3.6 ± 1.35
% BOP sites (mean)	4.92 ± 3.38	52.41 ± 12.6	57.17 ± 10.9
% BOP sites (sampled site)	0	100	100

AP, aggressive periodontitis; f, female; m, male; CP, chronic periodontitis; BOP, bleeding on probing.

Table 2. Primers sequences and reaction properties

Target	Sense and anti-sense sequences	tA (°C)	tM (°C)	bp
MMP-1	TGGACCTGGAGGAAATCTTGC AGAGTCCAAGAGAATGGCCGA	58	79	155
MMP-2	CTGATGGCACCCATTTACACCT GATCTGAGCGATGCCATCAAA	60	82	186
MMP-9	AGAGATGCGTGGAGAGTCGAA AAGGTTTGGAATCTGCCCAGG	65	85	162
TIMP-1	ACTGCAGGATGGACTCTTGCA TTTCAGAGCCTTGGAGGAGCT	30	82	206
TIMP-2	CAAGTTCTTCGCCTGCATCAA TCGAAACCCTTGGAGGCTT	61	84	155
TIMP-3	TTCTCAGCGAGGATGGCACTT AAACACGGTTCAGGATGCTGG	60	81	200
RANKL	CAGAAGATGGCACTCACTGCA CACCATCGCTTTCTCTGCTCT	65	73	203
OPG	GGAACCCCAGAGCGAAATACA CCTGAAGAATGCCTCCTCACA	57	77	225
TNF-α	AAGCCTGTAGCCCATGTTGT CAGATAGATGGGCTCATACC	56	79	330
IFN-γ	AT GAAATATACA AGTTATATCATG TGTTTCGAGGTCGAAGAGCATCCC	58	77	501
IL-4	GCGATA TCACCTTACA GGAG TGTCCTGTG AAGGAAGCCAAC	58	82	308
IL-10	AGATC TCCGAGATGC CTTCA CCGTGGAGCAGGTGAAGAAT	58	85	307
β-actin	ATGTTTGAGACCTTCAACA CACGTCAGACTTCATGATGG	56	75	495

tA, annealing temperature; tM: melting temperature; bp, base pairs of amplicon size; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; RANKL, receptor activator of nuclear factor-κB ligand; OPG, osteoprotegerin.

RNA extraction

Total RNA from gingival tissue biopsies was extracted using the Trizol reagent (Life Technologies, Grand Island, NY, USA) according to the directions supplied by the manufacturer. Briefly, Trizol (1 ml/mg tissue) was added to the biopsy, shaken for 30 s and incubated at room temperature for 5 min. To each milliliter of the suspension, 0.2 ml of chloroform (Sigma, St Louis, MO, USA) was added, vortexed and then centrifuged at 12,000 $\times$ *g* by 15 min for 4°C. The aqueous phase was transferred for a new tube, to which the same volume of isopropanol (Sigma) was added. The sample was vortexed, incubated for 20 min at -20°C and centrifuged. The pellet was washed in 100% ethanol and dried at room temperature. RNA samples were resuspended in 50 μl of diethylpyrocarbonate (DEPC)-treated water and stored at -70°C. The concentration of RNA was determined from the optical density at a wavelength of 260 nm, using the GeneQuant (Pharmacia Amersham Biosciences, Piscataway, NJ, USA).

Real-time-PCR reactions

Complementary DNA (cDNA) was synthesized using 3 μg of RNA through a reverse transcription reaction (Superscript II, Gibco Life Technologies, Grand Island, NY, USA). Real-time-PCR quantitative mRNA analyses were performed in an ABI Prism 5700 Sequence Detection System using the SYBR-green fluorescence quantification system (Applied Biosystems, Warrington, UK) for quantitation of amplicons. The standard PCR conditions were 95°C (10 min), and then 40 cycles of 94°C (1 min), 56°C (1 min) and 72°C (2 min), followed by the standard denaturation curve. The sequences of human primers were designed using the PrimerExpress software (Applied Biosystems) using nucleotide sequences present in the GenBank database. The primers sequences, predicted amplicon sizes, the annealing and melting temperatures are depicted in Table 2.

PCR conditions for each target were conscientiously optimized with regard

to primer concentration, absence of primer dimer formation, and efficiency of amplification of target genes and housekeeping gene control. SYBR Green PCR Master Mix (Applied Biosystems), 400 nM specific primers, and 2.5 ng of cDNA were used in each reaction. Threshold for positivity of real-time PCR was determined based in negative controls. The prevalence of subjects from each study group for positive expression of the message for the genes of interest is shown in Table 3. Calculations for determining the relative level of gene expression were made according to the instructions from User's Bulletin (P/N 4303859) from Applied Biosystems, by reference to the β-actin in the sample, using the cycle threshold (Ct) method. Briefly, Ct is the point at which the exponential increase in signal (fluorescence) crosses a somewhat arbitrary signal level (usually 10 times background). The mean Ct values from duplicate measurements were used to calculate expression of the target gene, with normalization to an internal control (β-actin), and then compared with the target–internal control in control subjects to calculate fold increase expression, using the $2-\Delta Ct$ formula, also according to User's Bulletin. Negative controls without RNA and without reverse transcriptase were also performed. Results show one experiment representative of three.

Statistical analysis

The data regarding the positivity of expression of the investigated target gene between control groups and patients, and between AP and CP groups, was analyzed by the Fischer exact test. To access possible differences in the intensity of mRNA expression between control subjects and patients from both clinical groups, ANOVA followed by Bonferroni multiple comparison test was done. The differences in the intensity of fold increase mRNA in relation to the control group, and normalized by the housekeeping gene expression, between the patients from the two clinical groups were analyzed by the Mann–Whitney test. Spearman's analysis was used to test possible correlations between the levels of expression of MMPs, TIMPs, RANKL and OPG compared with levels of cytokines IFN-γ, TNF-α, IL-4 and IL-10, and clinical parameters of disease severity (probing depth and loss of insertion) in diseased groups (AP and CP) were derived. For all the tests used, values of $p<0.05$ were considered statistically significant. All statistical tests were performed with the GraphPad InStat 3.05 and GraphPad Prism 3.0 software (GraphPad Software Inc.).

Table 3. Prevalence of positive subjects for the expression of MMPs, TIMPs, RANKL, OPG and cytokines in control, AP and CP groups

	Control (10)	AP (16)	CP (20)
MMP-1	8 (80)	16 (100)	19 (95)
MMP-2	8 (80)	15 (93)	18 (90)
MMP-9	9 (90)	16 (100)	20 (100)
TIMP-1	9 (90)	16 (100)	20 (100)
TIMP-2	9 (90)	14 (87)	18 (90)
TIMP-3	8 (80)	15 (93)	18 (90)
RANKL	2 (20)	15 (93) ($^*p=0.0002$)	19 (95) ($^*p<0.0001$)
OPG	7(70)	16 (100)	20 (100)
TNF-α	2 (20)	15 (93) ($^*p=0.0002$)	19 (95) ($^*p<0.0001$)
IFN-γ	1 (10)	14 (87) ($^*p=0.0002$)	15 (75) ($^*p=0.0014$)
IL-4	1 (10)	5 (31)	11 (55) ($^*p=0.0235$)
IL-10	3 (30)	12 (75) ($^*p=0.0426$)	16 (80) ($^*p=0.0147$)

AP, aggressive periodontitis; CP, chronic periodontitis; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; RANKL, receptor activator of nuclear factor-κB ligand; OPG, osteoprotegerin. Data are shown as number of positive subjects (and percentage).
*Statistical significance compared with the control group (Fisher's exact test).

Results

Quantitative analysis of MMPs and TIMPs mRNA expression

In order to evaluate the role of MMPs and TIMPs balance in the different clinical forms of PD, we first evaluated the expression of their mRNA in periodontal tissues from patients with AP and CP. There were no significant differences between controls and patients, and between the AP and CP groups in the prevalence of expressed message for MMPs and TIMPs (Table 3). However, quantitative analysis showed weak messages for all of the MMPs (MMP-1, MMP-2, MMP-9) and TIMPs (TIMP-1, TIMP-2, TIMP-3) in gingival tissues from control subjects, statistically different from the strong expression that was found in patients from both AP and CP groups (Fig. 1). The intensity of mRNA expression for MMP-1, MMP-2, MMP-9 and TIMP-2 was similar in both AP and CP groups (Fig. 1). Conversely, the expression of TIMP-1 ($p<0.05$) and TIMP-3 ($p<0.05$) was more intense in CP than in AP patients. In spite of the lack of statistical significance, the expression of MMP-2 was slightly higher in biopsies from AP patients than in those from CP patients (Fig. 1).

Quantitative analysis of expression of mRNA for osteoclast factors RANKL and OPG

We next evaluated the expression of the osteoclastogenic factor RANKL and its antagonist, OPG. Healthy controls presented with a low frequency of positive reactions for RANKL, which, when detected, was weak message (Table 3). Regardless of their clinical group, PD patients presented with a high prevalence (Table 3) of strong messages for RANKL ($p<0.05$) (Fig. 1). Positive reactions for OPG were equally prevalent in controls and patients (Table 3), but a more intense expression was found in diseased groups ($p<0.05$). When comparisons were performed between the two clinical groups, we found that the expression of RANKL was similar between them, while the expression of OPG is higher in CP than in AP patients ($p<0.001$) (Fig. 1).

Quantitative analysis of cytokines mRNA expression

We next asked if the differences in the expression of MMPs, TIMPs, RANKL and OPG could be due the differential expression of cytokines that are known to be regulators of their expression. We thus examined the expression of the cytokines TNF-α, IFN, IL-4 and IL-10 in gingival tissues. The expression of TNF-α, IFN-γ and IL-10 was more prevalent (Table 3) and higher ($p<0.05$) in the AP and CP groups when compared with the controls. Messages for IL-4 were predominantly found in CP biopsies. Quantitative

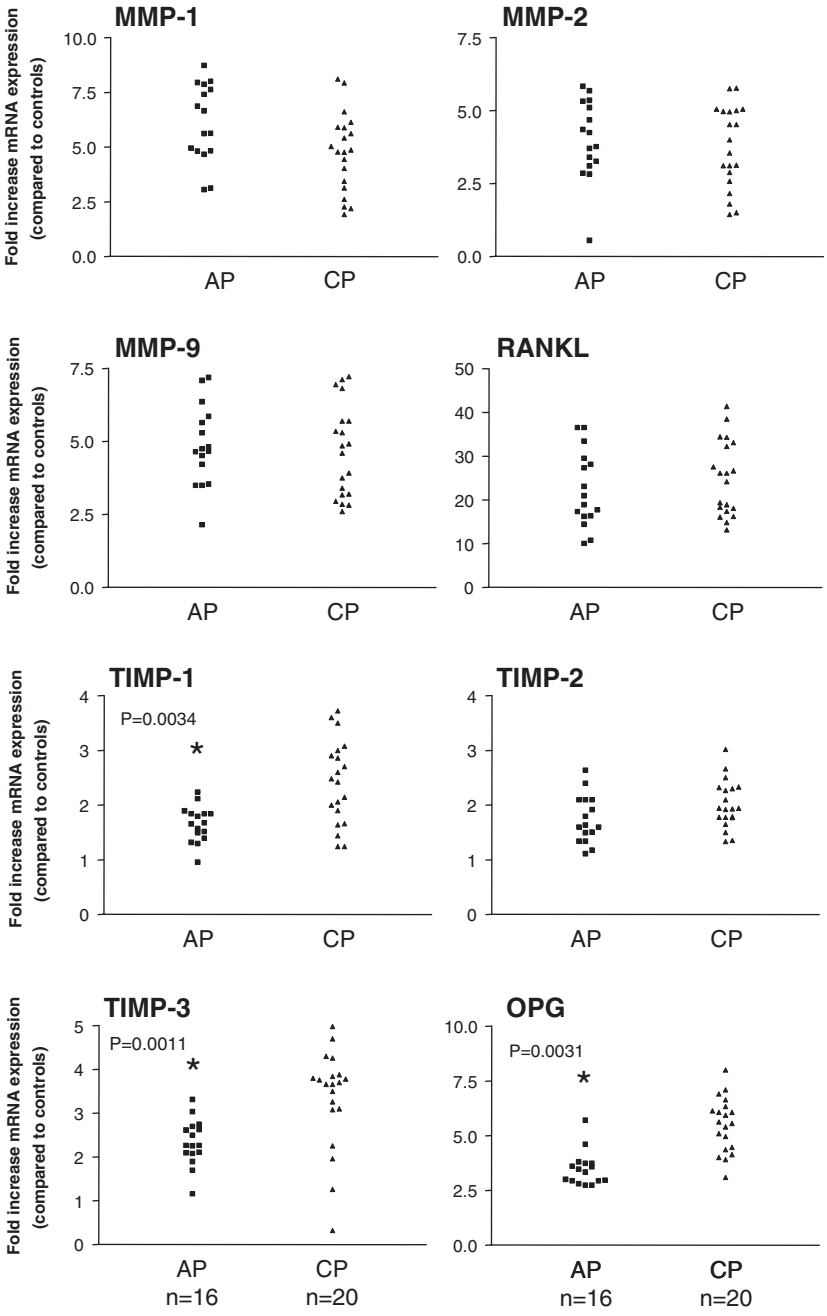

Fig. 1. Quantitative expression of matrix metalloproteinases (MMPs), tissue inhibitors of metalloproteinases (TIMPs), receptor activator of nuclear factor-κB ligand (RANKL) and osteoprotegerin (OPG) in aggressive periodontitis (AP) and chronic periodontitis (CP) patients. Total RNA was extracted, and levels of MMPs, TIMPs, RANKL and OPG mRNA were measured quantitatively by real-time-PCR SYBR-Green System. The results are presented as the fold increase of expression of the individual mRNAs, with normalization to β-actin, when compared with the target–internal control of control subjects using the cycle threshold (Ct) method. The results shown are from one experiment representative of three. All controls were significantly different from the AP and CP patients. *Statistical significance comparing AP versus CP patients.

analysis did not show any differences in the expression of TNF between patients presenting with AP and CP, while the expression of IFN-γ mRNA was higher in AP patients ($p<0.05$) (Fig. 2). Moreover, while the expression of IL-4 mRNA in the AP group was not significantly different from that seen in the CP group of patients, IL-10 mRNA expression was significantly lower in AP than with CP patients ($p<0.01$) (Fig. 2).

Correlation analysis of the expression of MMP, TIMPs, RANKL and OPG with expression of cytokines in diseased periodontal tissues

We also investigated the possible correlations between the levels of expression of MMPs, TIMPs, RANKL and OPG and the levels of cytokines expressed in diseased periodontal tissues of both clinical groups. Positive correlations were found between expression of IFN-γ and that of MMP-1 ($p<0.001$, $R=0.5522$), IL-10 and TIMP-1 ($p<0.01$, $R=0.1747$), IL-10 and TIMP-2 ($p<0.05$, $R=0.3467$) and IL-10 and OPG ($p<0.001$, $R=0.5832$). Negative correlations were found between IFN-γ and TIMP-1 ($p<0.05$, $R=-0.3406$) and IL-10 and MMP-2 ($p<0.001$, $R=-0.2060$) (Fig. 3). There was no correlation between expression of either MMPs, TIMPs, RANKL or OPG and the clinical parameters of disease severity (probing depth and loss of insertion) in any of the periodontitis groups studied.

Discussion

Local inflammatory immune reactions of the host in response to periodontal pathogens trigger tissue destruction by the activation of bone resorpting mechanisms and by the generation of proteases that degrade the ECM (Kinane & Lappin 2001, Seymour & Gemmel 2001, Taubman & Kawai 2001). However, the relevance of regulatory cytokines in driving the balance between MMPs and TIMPs, and between RANKL and OPG, and the potential consequences to the disease outcome, as well as possible variations between different clinical forms of PD are unclear.

The results presented here show that, although the prevalence of expression of MMPs and TIMPs was similar in the gingival tissues of control subjects and patients (Table 3), the intensity of their expression was more intense in patients with periodontitis than in healthy subjects. The expression of MMPs and TIMPs is regularly found in healthy periodontal tissues and are supposed to be involved in the physiological turnover of the ECM of periodontium, or are the result of a low subclinical inflammatory state in the tissues (Sodek & Overall 1992, Ryan et al. 1996). Indeed, MMPs are expressed at low levels in several types of tissues even in the

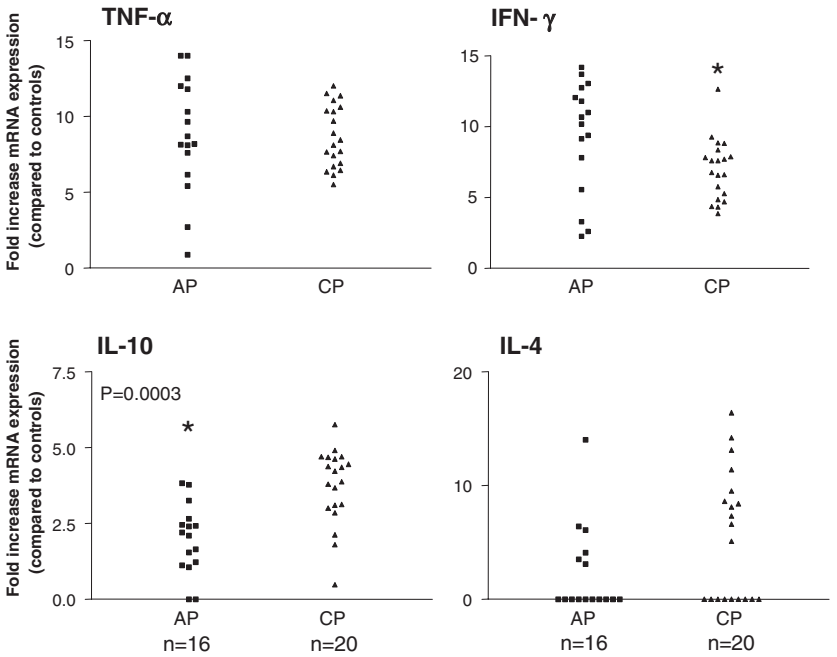

Fig. 2. Quantitative expression of TNF-α, IFN-γ, IL-10 and IL-4 in aggressive periodontitis (AP) and chronic periodontitis (CP) patients. Total RNA was extracted, and levels of TNF-α, IFNγ, IL-10 and IL-4 mRNA were measured quantitatively by the real-time-PCR SYBR-Green System. The results are presented as the fold increase of expression of the individual mRNAs, with normalization to β-actin, when compared with the target–internal control of normal subjects, using the cycle threshold (Ct) method. The results shown are from one experiment representative of three. All controls were significantly different from the AP and CP patients. *Statistical significance comparing AP versus CP patients.

absence of clinical inflammation or other pathological processes (Vu & Werb 2000).

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 even bone that results in some of the clinical symptoms of PD (Birkedal-Hansen 1993, Aiba et al. 1996, Ingman et al. 1996). Indeed, in animal models the inhibition of MMPs results in less severe PD (Ramamurthy et al. 2002) and it is proposed as an adjuvant therapy in human disease (Ryan et al. 1996, Novak et al. 2002). On the other hand, data regarding the expression of TIMPs in healthy versus diseased tissues are contradictory. Some studies show a decrease in the levels of TIMPs in diseased periodontal tissues, a finding which supports the idea that an imbalance in the levels of TIMPs/MMPs takes place in PD and results in tissue destruction (Alexander & Damoulis 1994, Soell et al. 2002, Tuter et al. 2002). However, other studies detected an increase in the expression of TIMPs in diseased periodontal tissues (Haerian et al. 1995, Nomura et al. 1998, Alpagot et al. 2001), which could reflect an attempt to maintain the tissue homeostasis, in the view of the increased expression of MMP. However, such upregulation of TIMPs may not be enough to compensate for the upregulation of MMPs, and such imbalance results in periodontal destruction.

Notwithstanding these discrepancies in the literature, interesting results were obtained when we compared AP and CP patients. Although we did not find significant differences between AP and CP in the levels of expression of MMPs, the expression of TIMP-1 and TIMP-3 was lower in AP than in CP. These results suggest that a more severe imbalance in the expression of MMPs and TIMPs takes place in AP, which could account for the early onset and more rapid progression of this clinical form of the disease. In fact, imbalances in the MMPs/TIMPs system (i.e. lower levels of TIMPs and/or higher levels of MMPs) are involved in the pathogenesis of several diseases (Giannelli et al. 2002, Lanchou et al. 2003, Schulze et al. 2003), including rheumatoid arthritis (Kong et al. 1999, Golbach et al. 2000, Yoshihara et al. 2000, Romas et al. 2002, Katrib et al. 2003), which share several features with PD, including the chronic nature of the inflammatory reaction and tissue destruction activity (Mercado et al. 2001).

In addition to the destruction of ECM by MMPs, the alveolar bone resorption driven by osteoclasts is another key feature of PD. In view of this, we also examined the expression of the osteoclastogenic factors RANKL and its inhibitor, OPG. The expression of RANKL was more prevalent in patients than in control subjects, while there were no differences between these groups in the prevalence of the expression of OPG (Table 3). Additionally, the intensity of expression of both RANKL and OPG was augmented in diseased tissues. When comparing the results obtained from the diseased groups (AP and CP), we found that, while the expression of RANKL was similar in both patients groups, the expression of OPG was higher in biopsies from patients presenting with CP than with AP. As RANKL is closely associated with bone resorption (Teitelbaum 2000, Ritchlin et al. 2003), and the blockade of RANKL by OPG leads to a reduction in the loss of alveolar bone (Teng et al. 2000, Liu et al. 2003), we suggest that the higher expression of OPG in CP than in AP could control, at least in part, the alveolar bone loss driven by RANKL, attenuating the progression rate and the severity of this form of PD. Conversely, its lower expression in AP may result in a more severe disease.

Since our data show a significant reduction in the expression of TIMPs and OPG in AP compared with CP, we investigated the expression of cytokines, which could regulate the expression of such factors (Birkedal-Hansen et al. 1993, Teitelbaum 2000, Ritchlin et al. 2003). We have previously shown that in these same groups of patients, the expression of Th1-type chemokines and chemokine receptors predominate in AP, leading to a higher expression of IFN-γ, while the predominant expression of Th2 chemokines in CP is related to a higher expression of IL-10 (Garlet et al. 2003). In the present study, we used real-time PCR to evaluate the expression of the cytokines TNF-α, IFN-γ, IL-4 and IL-10 in diseased tissues, and performed correlation analyses in order to address their role in the regulation of MMPs, TIMPs, RANKL and OPG.

The expression of TNF-α, IFN-γ and IL-10 was more intense in periodontitis

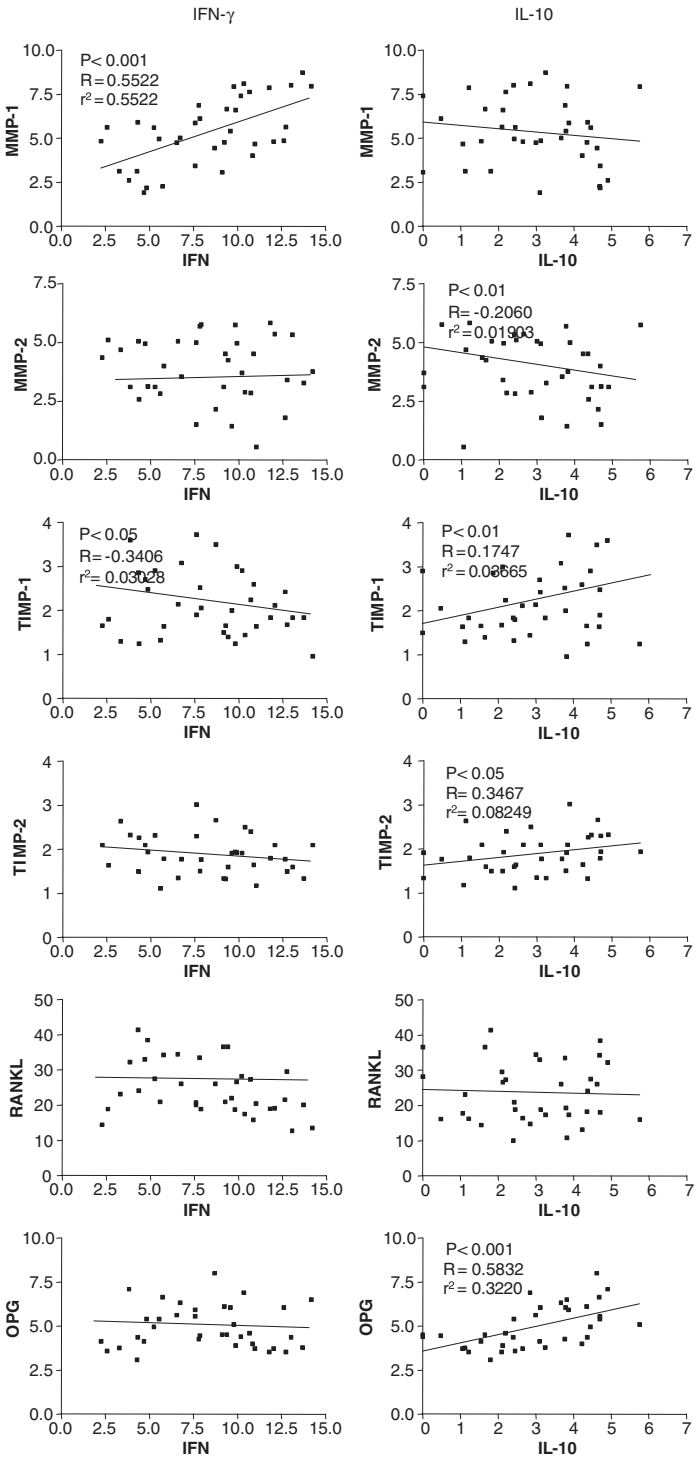

Fig. 3. Correlation between the expression of mRNA for matrix metalloproteinases (MMPs), tissue inhibitors of metalloproteinases (TIMPs), receptor activator of nuclear factor-κB ligand (RANKL) and osteoprotegerin (OPG) and mRNA for cytokines in diseased periodontal tissues. Spearman' analysis was used to test the correlations between the levels of expression of MMPs, TIMPs, RANKL and OPG compared with levels of the cytokines IFN-γ and IL-10 in periodontal tissues from AP and CP groups. Total RNA was extracted, and the individual levels of mRNA were measured quantitatively by a real-time-PCR SYBR-Green System, with normalization to β-actin, and then compared with the target–internal control in control subjects using the cycle threshold (Ct) method. The Spearman correlation test was performed with the GraphPad Prism 3.0 software (GraphPad Software Inc.) and values considered statistically significant are identified in the graphs.

patients than in healthy subjects, in accordance with previous studies (Kinane & Lappin 2001, Seymour & Gemmel 2001). The frequency of positive reactions for TNF-α, IFN-γ and IL-10 was similar between AP and CP, while expression of IL-4 was more frequent in CP patients. Moreover, the quantitative analysis of the expression of cytokines showed that the levels of TNF-α were similar between AP and CP, while the expression of IFN-γ was increased in AP. Conversely, biopsies taken from CP patients presented higher expression of IL-10 and IL-4 when compared with those with AP. These data support our previous findings (Garlet et al. 2003) and suggest that the differential expression of MMPs/TIMPs and of RANKL/OPG detected in PD could be due to the different patterns of cytokines produced in the tissues. Accordingly, we found positive correlations between the expression of IFN-γ and MMP-1, IL-10 and TIMP-1, and TIMP-3 and OPG in diseased periodontal tissues. Negative correlations were found between the expression of IFN-γ and TIMP-1, and IL-10 and MMP-2. Therefore, the expression of MMPs and RANKL may result in tissue destruction and disease progression, while increased expression of TIMPs and OPG, possibly induced by IL-10, could be responsible, at least in part, for the control of the tissue destruction.

It is possible that the differences found between patients groups were due to the time of onset and duration of the pathological process, reflecting different stages of the disease chronicity, or even the time elapsed between the conservative treatment and surgical treatment may reflect a site in healing process. This questions can only be answered by the study of the kinetics of disease development, that may show different patterns of cytokines during the evolution of the disease, and the possible correlations with the expression of MMPs/TIMPs and RANKL/OPG.

Despite controversies regarding the role of cytokines in PDs, Th1 and Th2 cytokines have been shown to be associated with, respectively, activation and suppression of bone resorption (Eastcott et al. 1994, Kawashima & Stashenko 1999, Kawai et al. 2000, Sasaki et al. 2000). IFN-γ appears to be the predominant cytokine produced by T cells in PD, and an increase in IFN-γ-producing cells in the lesions is correlated with the progression of disease (Roberts et al.

1997, Ukai et al. 2001). In contrast, Th2 cytokines (IL-4 and IL-10) are widely expressed in diseased periodontal tissues (Tokoro et al. 1997, Lappin et al. 2001) and are associated with suppression of bone resorption (Kawashima & Stashenko 1999, Sasaki et al. 2000).

However, mixed patterns of immune response is shown to take place in PD (Ukai et al. 2001, Teng 2002, Garlet et al. 2003), suggesting that the balance of Th responses could account for the control of infection with minimal damage to host tissues (Taubman & Kawai 2001, Garlet et al. 2003). In agreement with this possibility, we showed concomitant expression of IFN-γ, TNF-α, IL-10 and IL-4 in diseased tissues. Moreover, we provide new data, filling the lack of information regarding the diversity in the expression of MMP/TIMP and of the osteoclast factors RANKL and OPG, between different clinical forms of disease, as well as the correlations between their expression and that of up- and downregulatory cytokines.

The first steps in solving the puzzle of the pathological mechanisms of PD have been achieved, but further studies are required to understand the role of cytokines in the regulation of osteoclastogenesis and in the MMPs/TIMPs system, and, consequently, their role in the immunopathogenesis of PD. Such knowledge might allow us to develop preventive and therapeutical strategies to control the inflammatory process, to improve the removal of microorganisms and to promote the repair of tissues, while limiting damage.

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