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Characterization of progressive periodontal lesions in chronic periodontitis patients: levels of chemokines, cytokines, matrix metalloproteinase-13, periodontal pathogens and inflammatory cells

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Abstract

Background and aims: Periodontitis is an infection with an episodic nature of tissue support destruction. The aim of this work was to determine the levels of chemokines, cytokines, matrix metalloproteinase-13, periodontal pathogens and inflammatory cells in periodontal sites characterized by active periodontal connective tissue destruction.

Material and Method: Fifty-six patients with moderate or advanced severity of chronic periodontitis were selected. Periodontitis was characterized by at least six sites with probing depth $\geqslant 5$ mm, clinical attachment level $\geqslant 3$ mm and radiographic bone loss. Periodontitis progression was determined by the tolerance method. Receptor activator for nuclear factor κ B-ligand (RANK-L), monocyte chemoattractant protein-1 (MCP-1), tumour necrosis factor-α (TNF-α), IL-1β, MMP-13, *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, *Tannerella forsithia* and inflammatory cells levels were determined. Statistical analysis was performed using the Stata® 7.0 software. Data were expressed as mean $\pm$ SD and paired samples *t*-test and χ^2 tests were used.

Results: Higher RANK-L, IL-1β and MMP-13 activity levels were observed in active sites ($p < 0.05$). The proportion of *P. gingivalis*, *A. actinomycetemcomitans*, *T. forsythia* and the number of $CD4^+$ T were higher in active than in inactive sites ($p > 0.05$).

Conclusion: The detection of periodontopathic bacteria, host matrix metalloproteinases and cytokines in periodontitis patients with lesions undergoing episodic attachment loss could partially explain the mechanisms associated with the destruction of the supporting tissues of the tooth.

Key words: cytokines; inflammatory cells; matrix metalloproteinases; periodontal pathogens; progressive periodontitis

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Conflict of interest and source of funding statement

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Chronic periodontitis is an inflammatory disease of the supporting tissues of the teeth caused by groups of specific microorganisms, resulting in progressive destruction of the periodontal ligament and alveolar bone with pocket formation, recession, or both (Armitage 1999).

Chronic peridontitis is usually related to polybacterial infection, including, among others, *Porphyromonas gingivalis, Actinobacillus actinomycetemcomitans* and *Tannerella forsithia* (Eley & Cox 2003). Destruction of the osseous support of the dentition is a hallmark of chronic periodontitis (Listgarten 1986). The infiltrate present in periodontal diseases contains mononuclear cells, mainly transmigrated mononuclear phagocytes and lymphocytes. Whereas T lymphocytes predominate in the established chronic lesion, the proportion of B cells and plasma cells increases with disease progression (Saglie et al. 1988, Zappa et al. 1991).

The tissue destruction appears to result from a complex interaction between these bacteria and the host's immune and inflammatory responses. Proteolytic activities of these bacteria, including collagenase, may participate in collagen degradation (Sela et al. 2003). Activation and over-expression of host matrix metalloproteinases (MMPs) caused by periodontal pathogens such as *A. actinomycetemcomitans* and *P. gingivalis*, and by inflammatory cytokines have been reported (DeCarlo et al. 1997, Okamoto et al. 1997, Ryan & Golub 2000, Chang et al. 2002, Claesson et al. 2002). Interstitial collagenase activity in gingival tissue extracts and gingival crevicular fluid (GCF) from periodontitis sites are pathologically elevated when compared with the periodontally healthy gingival tissue and GCF (Ingman et al. 1994, Golub et al. 1997). Considerable effort has been made to study the cytokine released by different host cells when exposed to components of periodontopathogenic bacteria (Offenbacher 1996). These studies have demonstrated that a number of pro-inflammatory cytokines are synthesized in response to periodontopathogenic bacteria and their products, hence inducing and maintaining an inflammatory response in the periodontium (Page 1991).

Goodson et al. (1982) have presented evidence indicating that periodontal disease has dynamic states of exacerbation and remission and can be described in terms of patterns of progression and regression of the disease. In the present study, we investigated the prevalence and proportions of *P. gingivalis, A. actinomycetemcomitans and T. forsithia* in active sites, where clear bone tissue destruction occurred, and compared these samples with inactive sites from patients undergoing progression of chronic periodontitis. Bacterial culture was used for the detection and quantitative evaluation of periodontophatic bacteria. The total amount an concentration of receptor activator for nuclear factor κ B-ligand (RANK-L), monocyte chemoattractant protein-1 (MCP-1), tumour necrosis factor-α (TNF-α), IL-1β and MMP-13 activity were measured in samples of GCF. We also identified the cell populations present in lesions undergoing episodic attachment loss from gingival tissue biopsies.

Material and Methods

Patients

This is a longitudinal clinical study in which moderate to severe chronic periodontitis patients were followed until they developed periodontitis progression. Fifty-six patients were selected from the Center of Diagnostic and Treatment of Northern Metropolitan Health Services, Santiago, Chile, and consecutively enrolled between March 2005 and January 2006. The criteria for entry were a minimum of 14 natural teeth, excluding third molars, and including at least 10 posterior teeth. Patients with chronic periodontitis had moderate to advanced periodontitis (at least six teeth had sites with probing depth (PD) $\geqslant$5 mm with attachment loss $\geqslant$3 mm and extensive bone loss in radiography ($>$30% of sites involved), according to a classification of the severity periodontal disease based on the location of the alveolar crest) and had received no periodontal treatment at the time of examination. Subjects did not suffer from systemic illness and had not received antibiotics or non-steroid anti-inflammatory therapy during the 6-month period before the study. Before the study, all subjects received supragingival prophylaxis to remove gross calculus for allowing PD. The protocol was clearly explained to all patients, and Institutional Reviews Board-approved informed consents were signed. The protocol stated that, within 2 weeks of the detection of disease activity, all patients would be provided with periodontal treatment. Periodontal therapy consisted of scaling, root planing and oral hygiene instructions.

Clinical measurement and determination of periodontitis progression

Clinical parameters were evaluated in all teeth, excluding third molars, and included PD, clinical attachment loss (CAL), dichotomous measurements of supragingival plaque accumulation (PI) and bleeding on probing to the base of the crevice (BOP). Six sites were examined for each tooth: mesiobuccal, buccal, distobuccal, distolingual, lingual and mesiolingual. One calibrated examiner monitored the patients and collected the clinical reports.

All clinical measurements were taken at baseline, 7 days and 2 months: PD, clinical attachment loss, supragingival PI and BOP to the base of the crevice. Disease activity was defined by the tolerance method (Haffajee et al. 1983). At the site level, active sites were considered if they exhibited attachment loss $\geqslant$2.0 mm during the following 2-month period. Inactive sites were defined as those sites with CAL, PD and BOP equivalent to active sites, but without attachment loss during the same period. At the patient level, at least two active sites were needed to consider the patient as undergoing disease progression. Measurements of clinical parameters were monitored at baseline and at 2 months samples were collected from one active and one inactive sites simultaneously, on each patient undergoing progression, before the periodontal therapy was carried out.

Samples of GCF, subgingival plaque and a gingival biopsy were collected from one active site and one inactive site from each patient undergoing attachment loss. Eighteen patients demonstrating disease activity were included in the present study. One inactive site sample, characterized by no attachment loss, was taken as

control from each patient undergoing attachment loss.

Collection of GCF

After isolating the tooth with a cotton roll, supragingival plaque was removed with curettes (Hu Friedy, Chicago, IL, USA) without touching the marginal gingiva. The crevicular site was then dried gently with an air syringe. GCF was collected on paper strips (ProFlow, Amityville, NY, USA). The strips were placed in the sulcus/pocket until mild resistance was sensed and then left in place for 30 s. Strips contaminated with saliva or blood were excluded from the sampled group. After GCF collection, the volume of the sample on the paper strips was measured using a calibrated Periotron 8000 (ProFlow). The readings from the Periotron 8000 were converted to an actual volume (μl) by reference to the standard curve. GCF strip samples were obtained from one active site and one inactive site in each patient undergoing periodontitis progression. After GCF collection, strips obtained from the same site were placed in Eppendorf vials and kept at -80°C. GCF was extracted by centrifugation at 18.000 g for 5 min. at 4°C in 100 μl of elution buffer containing 50 mM Tris HCl (pH 7.5), 0.2 M NaCl, 5 mM $CaCl_2$ and 0.01% Tritón X-100. The elution procedure was repeated twice, and 18 samples from each site were stored at -80°C until further analysis.

Quantification of cytokine RANK-L, MCP-1, IL-1β, TNF-α

Aliquots of each GCF samples were assayed by enzyme-linked immunosorbent assay to determine the levels of RANK-L, MCP-1, IL-1β and TNF-α according to the manufacturer's recommendations (American Laboratory Products Company, ALPCO Diagnostic, Windham, NH, USA). Briefly, 50 μl of standards and GCF samples were added in the respective wells in duplicate. Fifty microliters of detection antibody was added to all wells, except blank, mixed gently, covered with strips with plastic film and incubated overnight (16–24 h) at 4°C. Plates were washed 5 × and incubated with 200 μl of conjugate for 50 min. at room temperature (18–26°C). The plates were washed 5 × and 200 μl of substrate was added and incubated for 30 min. at room temperature (18–26°C) in the dark. The reaction was stopped by the addition of 50 μl of stop solution, and colour was measured in an automated microplate spectrophotometer (Bio-Tek Instruments Inc., Elx 800, Highland Park, VT, USA).

The amount of RANK-L, MCP-1, IL-1β and TNF-α was determined in picograms (pg). Cytokine levels in the GCF were calculated with a standard curve (3.9–500 pg) obtained with recombinant cytokines. Sample cytokine concentration values lower than the detection limit (below 3.9 pg in our assay) were considered undetectable. Cytokine concentration (pg/μl) was calculated from the volume of GCF estimated from the calibration unit reading, according to the following formula: cytokine concentration (pg/μl) = total cytokine (pg)/volume GCF (μl).

Subgingival plaque samples

Subgingival plaque samples were collected from the active and inactive sites in each patient and 18 samples from each site were collected. After isolating the area with cotton rolls and gently air drying, supragingival deposits were carefully removed with curettes (Hu Friedy). Subgingival microbial samples were obtained by inserting two standardized N 30 sterile paper points (Johnson & Jonson, Tokio, Japan) into the deepest part of the periodontal pocket for 20 s. The samples from each patient were placed in a vial containing 1 ml of cold sterilized prereduced transport fluid (RTF) without ethylenediamine-tetraacetic acid. Vials with samples were transported at 4°C to the Microbiological Laboratory of the Faculty of Dentistry, University of Chile, and processed immediately.

Microbiological procedures

Subgingival plaque samples were dispersed by mixing (Thermolyne maxi mix II type 37,600, IA, USA) for 45 s followed by a 10-fold serial dilution of the bacterial suspension in RTF. Aliquots of 100 μl of the appropriate dilution (10^{-3}, 10^{-4} and 10^{-5}) were plated on non-selective Columbia blood agar (5% defibrinated sheep blood, 1 mg/l hemin, and 0.5 mg/l menadione) for total anaerobic cell counts (CFU/ml) and for the detection and quantification of *P. gingivalis* and *T. forsithia*. The plates were incubated anaerobically at 35°C for 7–14 days in a jar containing gas generator envelopes for the production of an anaerobic atmosphere (Oxoid Limited, Hampshire, UK).

Bacteria were primarily identified by colony morphology under a stereoscopic microscope (Stemi 2000-C, Zeiss, Jena, Germany), pigment production, Gram staining and rapid enzymatic tests for oxidase, trypsin-like (BANA) and α-glucosidase detection. In addition, black pigmented colonies of *P. gingivalis* were tested for red-fluorescence under UV light (360 nm): negative for *P. gingivalis* and positive for *P. intermedia/nigrescens* (18). The anaerobic isolates were further identified by the BBL-Crystal anaerobe ID System. *A. actinomycetemcomitans* was also primarily identified by colony morphology (star-like inner structure or ridges) but also on selective trypticase soy-serum bacitracin-vancomycin (TSBV) medium (trypticase, 10% horse serum, bacitracin and vancomycin). One hundred microlitres of undiluted and 10^{-1} diluted samples were inoculated on the agar plates and incubated at 35°C for 2–3 days in CO_2 candle jars. The percentage of *A. actinomycetemcomitans* was obtained using the number of CFU on TSBV as a percentage of the total anaerobic counts. Gram staining and catalase production were also used for *A. actinomycetemcomitans* identification. Plates were incubated anaerobically as mentioned above, for 2–3 days.

Gingival tissue biopsies and preparation of gingival cells

Gingival tissues biopsies were obtained from periodontitis patients with disease activity. The incisions were made 1–2 mm subgingivally; therefore, specimens consisted of the gingival margin, sulcular epithelium and gingival connective tissue. Soft issue biopsy specimens were washed extensively in phosphate buffer saline (PBS) and immediately placed in a vial containing 5 ml of cold sterilized transport media: RPMI 1640 supplemented with 50 UI/ml penicillin, 50 μg/ml streptomycin and L-glutamine 200 mM (Sigma Chemical Co., St Louis, MI, USA). Vials with the samples were transported at 4°C to the Periodontal Biology Laboratory of the Faculty of Dentistry, University of Chile and processed immediately. Samples of gingival tissues were weighed, minced into approximately 1 mm^3 pieces and incubated in tissue digestion medium in a water bath at 37°C for 90 min. The

tissue digestion medium consisted of (RPMI 1640) supplemented with 50 UI/ml penicillin, 50 μg/ml streptomycin and L-glutamine 200 mM, plus 200 U/ml of type IV collagenase (Gibco Invitrogen Corporation, Grand Island, NY, USA), in a relation of ml of tissue digestion medium by 50 mg of gingival tissue.

Cells obtained were washed twice with PBS and resuspended in cellular culture medium RPMI 1640 supplemented with 50 UI/ml penicillin, 50 μg/ml streptomycin, L-glutamine 200 mM and 10% foetal bovine serum (Gibco Invitrogen Corporation). Cell counting was performed in a Neubahuer chamber using phase contrast microscopy (Axiovert 100, Zeiss Co., Germany). Cell viability was equal to or greater than 90% as calculated by Trypan blue dye exclusion.

Flow cytometry analysis

In order to quantify the immune cells that infiltrate the gingival tissues, 200,000 total cells in 50 μl of PBS were incubated separately with 10 μl of phycoerythrin (PE) – and fluorescein isothiocyanate (FITC)-conjugated anti-CD4 ($CD4^+$ T cells), anti-CD8 ($CD8^+$ T cells) and anti-$CD19^+$ (B cells) monoclonal antibodies (mAb) (Biosciences, San Diego, CA, USA) for 30 min. at 4°C in the dark. Cells were washed once in PBS and resuspended in 300 μl of PBS to be analyzed by flow cytometry (FACS Calibur Machine, Becton Dickinson, San Jose, CA, USA). Cells were gated according to their forward and side-scatter characteristics and their specific CD marker. FITC- and PE-conjugated isotype-matched control mAb were used to determine the positive and negative population.

MMP-13 activity measurements

Aliquots of each GCF samples were assayed by the ''Fluorokine E'' activity fluorescent assay (R&D Systems Inc., Minneapolis, MN, USA) to determine the basal activity of MMP-13, according to the manufacturer's recommendations. Activity was expressed as nanograms of fluorescent product (ng FP) per site and nanograms of FP per μl of GCF.

Data analysis

Clinical and experimental data were statistically analysed using software stata 7.0 and expressed as subject means ± standard deviation. χ^2 test and Student's *t*-test were used to compare parameters between the clinical periodontitis groups and microbiological data. In the flow cytometry analysis differences were evaluated using the ANOVA test. The fraction of positive cells was expressed as a percentage. Cytokines, chemokines and MMP-13 activity values at each site were recorded and the unpaired Student's *t*-test was used to analyze differences in levels between active and inactive sites. Statistical significance was considered when $p < 0.05$. The significance of differences was assessed using the Tukey test.

Results

The clinical characteristics of the 18 patients studied with progressive chronic periodontitis in this study are grouped in Table 1 (2 months data). No statiscal difference existed between both groups. Five males and 13 females were studied, with age range 35–56 years (mean age 45.66 ± 6.67). The mean plaque index was 100%, mean BOP was 100%, mean PD was 4.42 ± 0.46 mm mean attachment level was 6.20 ± 0.76 in active sites and mean probing depth was 4.64 ± 0.62 mm, and mean attachment level was 6.42 ± 0.68 in inactive sites ($p > 0.05$) (Table 1).

Table 2 describes the microbiological findings in subgingival plaque samples of active and inactive sites from patients undergoing progression of chronic periodontitis. Active sites showed higher mean percentages for *P. gingivalis* of the total anaerobic than inactive sites, this difference being statiscally significant (17.9 *versus* 1.9, $p < 0.05$).

One of the main concerns in chronic periodontitis is the definition of active sites, where tissue destruction, and probably secretion of certain cytokines and MMPs is produced. Active sites are identified by an increase in attachment

Table 1. Clinical characteristics from baseline measurements of active and inactive sites from periodontitis patients (Mean ± SD)

Clinical parameters (2 months data)	Active sites ($n = 18$)	Inactive sites ($n = 18$)	*p* value
Mean probing depth (mm)	4.42 ± 0.46	4.64 ± 0.62	> 0.05
Mean attachment level (mm)	6.20 ± 0.76	6.42 ± 0.68	> 0.05
% sites with plaque	100	100	
% sites with bleeding on probing	100	100	
Age (years)	45.66 ± 6.67 (35–56)		
Females (%)	72.22 (13/18)		

Table 2. Microbiological finding in subgingival plaque from active and inactive sites

Bacteria	Active sites				Inactive sites			
	isolation frequency		% of total count		isolation frequency		% of total count	
	N	%	mean ± SD	range	N	%	mean ± SD	range
Actinobacillus actinomycetemcomitans	3	16.6	0.6	0–5	1	5.5	0.1	0–1
Porphyromonas gingivalis	6	33.3	17.9*	0–82	8	44.4	1.9*	0–11.3
Tannerella forsythensis	3	16.6	0.6	0–5	1	5.5	0.1	0–1

*% of total count *P. gingivalis*, active *versus* inactive sites $p < 0.05$.

loss during the length of study. Active sites showed a significantly higher total amount of cytokine RANK-L and IL-1β (125.9 ± 22.2 *versus* 91.8 ± 10.7; 186.84 ± 20.2 *versus* 123.78 ± 19.6) ($p = 0.0001$ and 0.037, respectively) than the inactive sites (Table 3). The mean variations of basal activity of MMP-13 expressed as nanograms of fluorescent product per site and per microlitre of GCF (FP/site and ng FP/μl GCF, respectively) from active and inactive sites are shown in Table 3. Active sites, identified by an increase in attachment loss during the length of the study and associated with periodontal tissue destruction, contained higher basal activity levels of MMP-13 than those found in inactive sites (1.2 ng *versus* 0.7 ng FP/site and 0.3 ng FP/μl *versus* 0.2 ng FP/μl, respectively; $p < 0.05$).

Levels of $CD4^+$, $CD8^+$ and $CD19^+$ lymphocyte cells are more abundant in active sites than in inactive sites, although differences were not so clear ($p > 0.05$) (Fig. 1). Figure 1 shows the flow cytometry of a representative sample of periodontitis patients with destructive periodontitis. Higher levels of $CD4^+$, $CD8^+$ and $CD19^+$ lymphocyte cells are associated with active sites (12.81 *versus* 11.41; 10.26 *versus* 9.06; 5.24 *versus* 4.41, respectively).

Discussion

This study examined the prevalence and proportions of *P. gingivalis, A. actinomycetemcomitans* and *T. forsithia*, the total amount and concentration of RANK-L, MCP-1, TNF-α, IL-1β and measured MMP-13 activity in samples of GCF, and also identified the cell

Table 3. Cytokine levels of RANK-L, MCP-1, IL-1β, TNF-α and MMP-13 activity in gingival crevicular fluid (GCF) in active and inactive sites (Mean $\pm$ SD)

	Active total amount (pg)	Inactive total amount (pg)	*p* value	Active concentration (pg/μl)	Inactive concentration (pg/μl)	*p* value
RANK-L	125.95 ± 22.2	91.80 ± 10.7	0.0001	131.17 ± 35.1	108.80 ± 18.9	0.001
MCP-1	101.22 ± 18.2	92.13 ± 22.2	>0.05	122.15 ± 18.4	106.12 ± 19.6	>0.05
IL-1β	186.84 ± 20.2	123.78 ± 19.6	0.037	205.95 ± 20.4	147.78 ± 24.6	>0.05
TNF-α	66.18 ± 14.6	47.86 ± 16.8	>0.05	73.87 ± 16.6	57.12 ± 18.4	>0.05
	basal activity (ngFP/site)	basal activity (ngFP/site)	*p* value	basal activity (ngFP/μl)	basal activity (ngFP/μl)	*p* value
MMP-13	1.207 ± 0.429	0.7945 ± 0.205	0.020	0.312 ± 0.091	0.247 ± 0.043	0.01
GCF (μl)	0.99 ± 0.15	0.86 ± 0.15	>0.05			

MMP-13, matrix mettaloproteinase-13; RANK-L, receptor activator for nuclear factor κ B-ligand; MCP-1, monocyte chemoattractant protein-1; TNF-α, tumor necrosis factor-α.

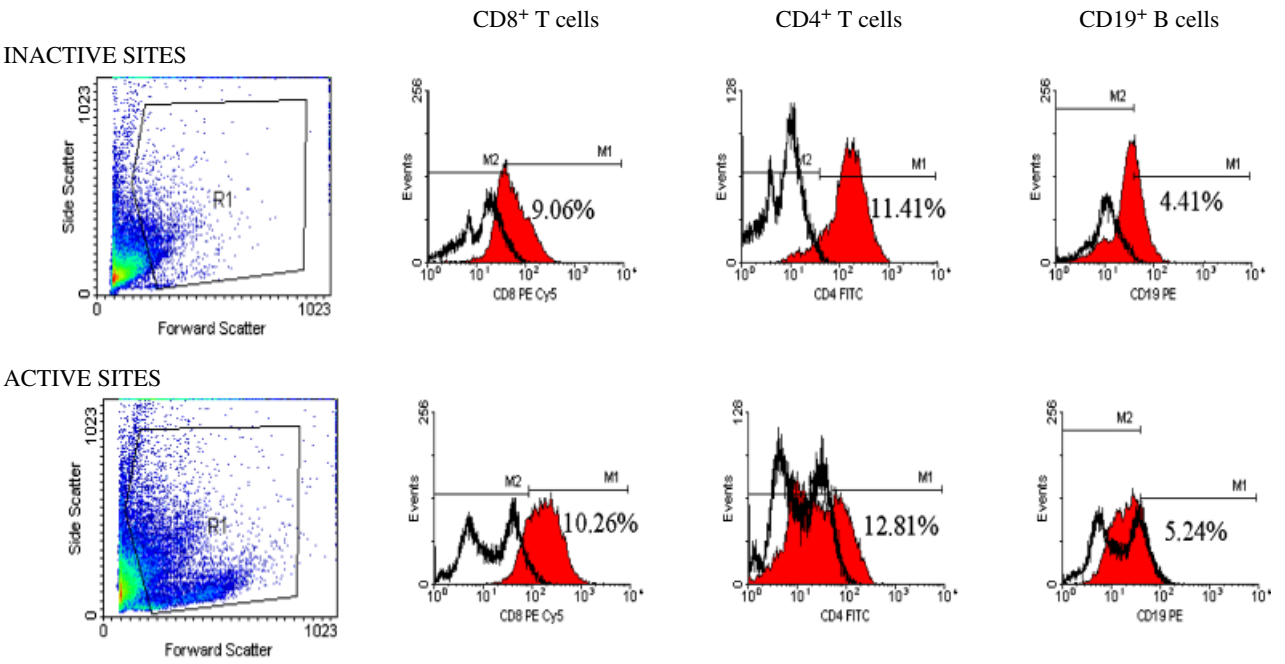

Fig. 1. Immunotypification by the flow cytometry of gingival cells obtained by enzymatic tissue digestion of gingival samples. The fraction of positive cells was expressed as a percentage. M1 = marker of positive cells. M2 = marker of negative cells. The analysis was made with the red-filled curve; which represents labelled cells with specific antibody. The unfilled curve represents the unlabelled cells used as control. Active sites $CD8^+$ T cells 10.26%, $CD4^+$ T cells 12.81% and $CD19^+$ B cells 5.24%. Inactive sites $CD8^+$ T cells 9.06%, $CD4^+$ T cells 11.41% and $CD19^+$ B cells 4.41%.

populations present in lesions undergoing episodic attachment loss and compared active sites with inactive sites from patients undergoing progression of chronic periodontitis. Our results demonstrate that the total amount of cytokine RANK-L, IL-1β and MMP-13 activity present in GCF of sites undergoing progression of periodontitis in levels is significantly higher than in inactive sites. Active sites showed a higher proportion of *P. gingivalis*, *A. actinomycetemcomitans*, *T. forsithia* and $CD4^+$ T cells. Our data demonstrate that the detection of periodontophatic bacteria, host matrix metalloproteinases, cytokines and population of infiltrate might correlate with episodic attachment loss and could partially explain the mechanisms associated with the destruction of the supporting tissues of the tooth.

Three specific pathogens have been repeatedly identified as etiologic agents in the periodontal destruction associated with chronic periodontitis: *A. actinomycetemcomitans*, *P. gingivalis* and *T. forsithia* (Zambon 1996). However, evaluation of these three pathogens as risk factors for identification of attachment loss over time has resulted in conflicting evidence. Three studies indicated that none of these pathogens was useful in predicting periodontal disease progression (Wennstrom et al. 1987, MacFarlane et al. 1988, Listgarten et al. 1991). In another study, *T. forsithia* was predictive of tooth loss among subjects with little or no periodontitis at baseline (Machtei et al. 1999). In a recent prospective longitudinal study, *T. forsithia* was identified as a risk marker for attachment loss in a population with low prevalence and severity of chronic periodontitis (Tran et al. 2001). *T. forsithia* and *P. gingivalis* were found to be associated with disease progression in established periodontal patients (Machtei et al. 1997) and were also found to be associated with alveolar bone loss (Grossi et al. 1995). Perhaps most relevant to the ability of *A. actinomycetemcomitans* to evade the innate defences and survive mechanical periodontal therapy is its ability to invade gingival tissues (Christersson et al. 1998) and, in particular, to invade epithelial cells (Meyer et al. 1991). *T. forsithia* possesses several virulence traits, including the production of a trypsin-like protease and lipopolysaccharide, but more recently its ability to penetrate host cells or induce apoptosis has received attention (Arakawa et al. 2000). *P. gingivalis* is one of the best characterized of the opportunistic oral pathogens that inhabit the oral biofilm. *P. gingivalis* expresses three major virulence factors:fimbriae, gingipains and lipopolysaccharides (Genco et al. 1991, Wingrove et al. 1992, Cutler et al. 1995). The three main commonalities of *A. actinomycetemcomitans*, *T. forsithia* and *P. gingivalis* include the following: all are Gram-negative, and therefore produce lipopolysaccharide, which can modulate the local inflammatory response in host cells that express pattern recognition receptors; all appear capable of invasion of the mucosal barrier to infection and possibly of being sequestered inside epithelial cells; all produce factors that enable them to evade the antibacterial functions of the innate immune response either passively or actively (Ezzo & Cutler 2003).

Our results showed a difference in the level of the microbial component in active and inactive sites. However, the difference in clinical appearance may be caused by factors other than the microbiological composition of subgingival plaque. Bacteria are essential, but insufficient to cause disease; a susceptible host is also essential and host factors are determinative (Page et al. 1997). Periodontitis is a family of related diseases that differ in etiology, natural history, disease progression and response to therapy, but with common shared pathways of tissue destruction (Page & Beck 1997). The shared events in the pathobiology are influenced by disease modifiers (also known as risk factors and indicators), both genetic and environmental or acquired, which may differ from stage and form of disease to another (Page & Beck 1997). The clinical picture observed is a result of the complex interplay among microbial challenge, shared events and disease modifiers. The modifying factors are major determinants of the differences observed in different periodontal conditions (Page et al. 1997).

Longitudinal studies on the progression of periodontitis indicate that the rate of periodontal tissue destruction is low and that advanced forms of the disease occur in comparatively few individuals and few tooth sites (Lindhe et al. 1989, Hugosan & Laurell 2000). Attention has been focused in the past on the possibility that periodontal disease may not be a continuous process, but may be characterized by episodes of activity, followed by periods of relative quiescence (Goodson et al. 1982). Episodic periodontal probing attachment loss may be associated with variations in the supracrestal inflammatory cell populations where significantly higher numbers of mast cells, monocytes/macrophages and plasma cells are present in active sites as compared with inactive sites (Zappa et al. 1991). However, the contribution of acquired immune cells in the progression of periodontal disease has long been controversial, with its exact role in the protection *versus* destruction of the host's periodontium being unclear (Zappa et al. 1990, Ebersole & Taubman 1994). Our results showed higher levels of $CD4^+$ helper T cells in the context of periodontal disease progression. Recent studies show that interactions between the cell-mediated responses, in particular, $CD4^+$ T cells, are critical for the development of alveolar bone loss (Theill et al. 2002). Other studies demonstrated that $CD4^+$ mononuclear cells were relatively prominent (constituting approximately 27% of recoverable mononuclear cells) and that the $CD8^+$ cells were relatively enriched compared with the composition of peripheral blood or normal gingival tissues (Taubman et al. 1984, Stoufi et al. 1987). Our results confirm that there is a higher degree of inflammation and an increased number of B cells in the inflammatory infiltrate from biopsies of periodontitis patients. $CD19^+$ cells are also abundant in active and inactive sites, whereas the difference was not so evident for T cells. Other authors suggest that the episodic loss of connective tissue in periodontitis patients is related to the massive presence of B cells and macrophages in the inflammatory infiltrate (Zappa et al. 1990, Seymour 1991).

Microorganism-triggered induction of RANK-L expression on $CD4^+$ T cells and RANK-L-mediated osteoclast activation and bone loss represent the molecular explanation for the alveolar bone destruction observed in periodontal infections. Moreover, it is known that the expression of pro-inflammatory cytokines (i.e., IL-1) can also regulate the balance of RANK-L/OPG in the bone microenvironment and/or mesenchymal tissue adjacent to bone (Hofbauer et al. 1999), thus contributing to bone destruction. These cytokines and inflammatory mediators are capable of acting alone or together to stimulate

alveolar bone resorption and collagen destruction via tissue-derived MMPs, a major pathway for the breakdown of bony and soft connective tissue associated with periodontal disease activity (Offenbacher 1996). However, the pathological mechanisms underlying the progression of gingivitis to early periodontitis lesions remain unclear and will require further investigation. For example, periodontal pathogens like *A. actinomycetemcomitans*, *P. gingivalis* or *T. forsithia* are capable of stimulating the pro-inflammatory cytokines production (i.e., RANK-L, IL-1β) for local tissue destruction. They can then further activate naïve T- and/or B cells for subsequent adaptive immune responses (Zambon 1996).

Our findings demonstrate that the total amount of RANK-L is significantly higher in active sites *versus* inactive sites, considered as a marker of bone resorption. Disease activity is generally accepted to be loss of soft or hard tissue attachment to the tooth; a change in probing attachment level may represent a true change in the attachment level or a change in tissue tone or a combination of the two (Jeffcoat & Reddy 1991). The presence of RANK-L in the GCF of sites with episodic loss of connective tissues implies their potential role in the mechanisms of tissue destruction associated with destructive periodontitis. There are two molecules considered essential and sufficient to support osteoclastogenesis: macrophage colony-stimulating factor (M-CSF) and RANK-L. The process of bone resorption is initiated with a resorptive stimulus (Rodan & Martin 2000). These stimulators typically affect bone resorption through the activation of M-CSF or RANK-L (Rodan & Martin 2000). In the present study, we demonstrated that the total amounts of RANK-L present in the GCF of periodontitis subjects were higher than those in healthy subjects. There are few studies in the literature relating RANK-L and its potential role in periodontal tissue. Recently, RANK and RANK-L have been found in dental tissues and cells in human deciduous teeth (Lossdörfer et al. 2002) and RANK-L has also been associated with alveolar bone tissue destruction during periodontal infection using an animal model (Rodan & Martin 2000). Furthermore, microbial stimulation with *A. actinomycetemcomitans* induced RANK-L expression on the surface of CD4+ cells and in vivo inhibition of RANK-L function, and using the decoy receptor OPG diminished alveolar bone destruction and reduced the periodontal osteoclasts after microbial challenge (Lossdörfer et al. 2002). These results indicate that the alveolar bone destruction observed in periodontitis is due, at least in part, to the action of osteoclasts and is mediated by RANK-L.

Our data demonstrate that MMP-13 is present in the GCF of adult patients with progressive chronic periodontitis, and significantly higher activity levels in active sites might correlate the presence of the active form of this enzyme with alveolar bone loss and teeth loss occurring during this disease (Mancini et al. 1999). MMP-13 (collagenase-3), a highly expressed collagenolytic MMP in developing bone and cartilage, has been assigned a role in the joint tissue destruction that is a major feature of various forms of human arthritis (Pelton et al. 1990, Murphy et al. 2002). MMP-13 has critical roles in embryonic development and remodelling of the skeleton in humans. These roles are reflected in the collagenase-mediated destruction of bone and cartilage in several forms of human inflammatory diseases (Mitchell et al. 1996). The molecular weights of pro-MMP-13 and active MMP-13 are reported to be about 60 and 48 kDa, respectively (Neuhold et al. 2001). Our findings demonstrate that MMP-13 amounts in periodontitis patients are similar, but the level of activation is increased in active sites compared with inactive ones and basal activity levels are significantly higher in active sites than in inactive sites. During periodontitis progression, the main changes between active and inactive sites could occur at the level of pro-MMP-13 activation, determining significant increases of enzyme activity in active sites, hence resulting in bone and periodontal ligament destruction. MMP-13 is considered as a marker of activity progression, and this could be a preliminary approach, which will help to determine the function and relevance of this metalloproteinase during the progression of periodontitis.

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Clinical Relevance

Scientific rationale for the study: Periodontitis is an infection with an episodic nature of tissue support destruction, in which short periods of destruction are followed by long periods of remission and healing.

Principal findings: In summary, we hypothesized that disease progression is due to a combination of several factors, including the presence of periodontopathic bacteria, high levels of pro-inflammatory cytokines, matrix metalloproteinases and higher levels of $CD4^+$ T cells.

Practical implications: The presence of IL-1β, RANK-L, MMP-13 and higher levels of $CD4^+$ T cells appears to be a useful tool for knowledge on the mechanisms and pathogenesis of active diseases.