THE PRESENCE OF PERIODONTOPATHOGENS ASSOCIATED WITH THE TUMOUR NECROSIS FACTOR-α EXPRESSION IN PATIENTS WITH DIFFERENT PERIODONTAL STATUS

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ABSTRACT

The purpose of this study was to investigate the relationship between P. gingivalis, T. forsythia, T. denticola, P. intermedia, and A. actinomycetemcomitans in the sulci or pockets of patients with gingivitis (G), mild chronic periodontitis (MiCP), moderate chronic periodontitis (MoCP) and severe periodontitis (SP), and the expression of TNF-α in gingival tissue associated with clinical parameters. Six patients with G, 7 with MiCP, 23 with MoCP and 7 with SP were recruited. Pathogens obtained from the sulci or pockets were identified by PCR, and expression of TNF-α from gingival tissue was analysed. Probing depth (PD), clinical attachment level (CAL) and loss of bone were recorded. P. gingivalis was detected at the following rates: 16.6% in subjects with G, 57.1% in MiCP, 57.8 % in MoCP and 58.1% in SP ($p<0.05$). P. intermedia was not identified in subjects with G. A. actinomycetemcomitans was only identified in subjects with MoCP (31.5%) and SP (42.8 %). T. denticola and T. forsythia were identified in all subject groups. Bacterial combinations were identified as follows: P. denticola + P. intermedia and P. intermedia + T. forsythia were associated ($p = 0.04$, $p = 0.02$) with the presence of TNF-α mRNA in 20% and 25% of subjects, respectively. P. gingivalis + A. Actinomycetemcomitans and A. actinomycetemcomitans + T. forsythia were associated with severe PD and CAL, respectively. The association between the presence of P. intermedia and expression levels of TNF-α was significant ($p = 0.05$). These results indicate that the proportion of patients with P. gingivalis increases with the progression of disease. We observed that the presence of P. intermedia may trigger the expression of TNF- α and cause a worsening of the patient's clinical status.

Key words: periodontal diseases,microbiology- Tumor Necrosis Factor alpha-periodontal index.

PRESENCIA DE PERIODONTOPATOGENOS ASOCIADOS CON EXPRESIÓN DE FACTOR NECROSIS TUMORAL-α EN PACIENTES CON DIFERENTE ESTADO PERIODONTAL

RESUMEN

El propósito de este trabajo fue investigar la relación entre P. gingivalis, T. forsythia, T. denticola, P.intermedia, y A. actinomycetemcomitans presentes en surcos y/o bolsas de pacientes con gingivitis (G), periodontitis crónica leve (MiCP), periodontitis crónica moderada (MoCP) y periodontitis severa (PS) y la expresión de TNF-α en tejido gingival según el estado clínico periodontal. Para ello se seleccionaron seis pacientes con G, 7 con MiCP, 23 con MoCP y 7 con PS. Los patógenos extraídos de los surcos y/o bolsas se identificaron mediante PCR con cebadores específicos para cada especie, Se detectó la expresión de TNF-α en tejido gingival. Se registraron los siguientes parámetros clínicos: profundidad al sondaje (PD), perdida de inserción clínica (CAL) y perdida de hueso. Se detectó P. gingivalis con la siguiente frecuencia: 16,6% en sujetos con G, 57,1% en MiCP, 57.8% en MoCP y 58.1% en PS ($p < 0,05$). P. intermedia no fue detectada en pacientes con G y A. actinomycetemcomitans fue solamente identificado en MoCP (31,5%) y PS (42.8%) T denticola y T. forsythia se identificaron en todos los grupos. Las combinaciones bacterianas P. denticola + P. intermedia y P. intermedia + T. forsythia se identificaron asociadas significativamente ($p = 0,04$, $p = 0,02$) con la presencia de mRNA TNF-α en 20% y 25% de los sujetos, respectivamente. P. gingivalis + A. actinomycetemcomitans y A. actinomycetemcomitans + T. forsythia se asociaron con valores de PD y CAL de gravedad. La asociación entre la presencia de P. intermedia y los niveles de expresión de TNF-α fue significativa ($p = 0,05$). Estos resultados indican que la proporción de pacientes con P. gingivalis aumenta con la progresión de la enfermedad. Observamos que la presencia de P.intermedia desencadenaría la expresión de TNF-α y provocaría un empeoramiento del estado clínico del paciente.

Palabras clave: enfermedades periodontales, microbiologia-Factor de necrosis tumoral alfa-índice periodontal.

INTRODUCTION

Periodontitis is a bacterial-driven disease that leads to a destructive inflammatory disease of the periodontium and causes tooth loss. Chronic periodontitis has a slow progression rate and is often associated with local biofilm retentive factors, such as difficult to brush molar areas, overhanging restorations and overcrowded teeth[1]. Some authors speculate that chronic periodontitis is an inflammatory-immune response to a selected group of oral bacteria[2], such as *Aggregatibacter actinomycetemcomitans (A. actinomycetemcomitans)*, *Porphyromonas gingivalis (P. gingivalis)* and *Tannerella forsythia (T. forsythia)*. Other bacteria are also considered putative periodontopathogens, including *Treponema denticola* (*T. denticola*) and *Prevotella intermedia (P. intermedia*) [3].

Thus, the clinical manifestations of the different stages of periodontitis will result from interactions involving the ability of the subject's defence mechanisms, external factors (oral hygiene, smoking, systemic diseases, among others) and the virulence of the microorganisms[4].

Supporting the data from human studies, experimental periodontal disease models in rats and primates clearly demonstrate that TNF-α plays a central role in inflammatory reaction, alveolar bone resorption and the loss of connective tissue attachment [5-8]. Knowledge of the patterns of certain pathogenic flora that inhabits periodontal pockets or sulci should help to understand the ecologic shifts that occur in the transition from health to periodontal disease.

The purpose of this study was to describe the composition of microbiota in sulci or pockets in gingivitis and at different stages of periodontitis using specific oligonucleotide PCR detection. In addition, this study aimed to detect the expression of TNF-α mRNA in biopsies of active sites of infection to correlate the periodontopathogenic description with TNF-α mRNA detection, and to associate how clinical parameters are affected in patients with different degrees of periodontal disease. The goal was to contribute to the general understanding of the complex mechanisms that maintain the chronic inflammatory response in periodontitis.

MATERIALS AND METHODS

Patient selection and clinical examination

Forty-three individuals were recruited from Periodontal Department of the National University of Cordoba. Subjects were systemically healthy with normal oral hygiene (brushing at least once a day) and with clinical and diagnostic radiographs of gingivitis and/or chronic periodontitis. Potential subjects who were pregnant, smokers, or who had used antibiotics and/or anti-inflammatory drugs and local antimicrobial agents within the preceding 6 months were excluded. The selected subjects had no history of periodontal treatment within the last year. This protocol was previously approved by the Institutional Committee of Ethics in Dental Research, School of Dentistry. Periodontal examinations were performed by a single dentist, MMU.

The following parameters were assessed in periodontal pockets of upper and lower teeth from proximal, distal and mesial positions, using a manual periodontal probe (Hu-Friedy, Chicago, Illinois, USA): 1) presence or absence of bleeding on probing (BOP), either spontaneously or after gentle probing; 2) presence or absence of suppuration (SUP); 3) presence or absence bacterial plaque (BP) along the supragingival margin; 4) probing depth (PD, mm), which is the distance between the gingival margin and the bottom of the sulcus pocket; 5) clinical attachment level (CAL) distance between cement-enamel junction and the bottom of the sulcus pocket; and 6) loss of bone (LB). Full-mouth periapical radiographs (Insight dental films, Eastman Kodak Company, Argentine) were obtained for each subject using the paralleling technique. The LB was measured using the cement-enamel junction as a reference point.

Subgingival biofilm was collected from each patient by inserting sterile endodontic paperpoints (ISO 40) into a representative deepest pocket or sulcus site within each quadrant. Six paper points were inserted into selected periodontal pockets for 15 s and then placed in a sterile Eppendorf tubes for molecular determination of bacteria.

Next, gingival tissues were obtained from periodontal biopsies taken from 33 periodontitis patients during the surgical removal of wisdom teeth. The base incisions were made 1-2mm subgingivally; therefore the specimens consisted of the gingival margin, sulcular epithelium, and gingival connective[9]. The biopsies were washed with phosphate buffered saline (PBS) prepared with diethylpyrocarbonate (DEPC) to eliminate debris and traces of blood and immediately stored at -70 °C until use[10].

The patients were classified as follows: Gingivitis (G), patients with PD ≥ 4 mm, positive BOP,

without CAL and LB; and Periodontitis (P) with PD ≥ 5 mm. The latter group includes three categories: Mild Chronic Periodontitis (MiCP), patients with CAL ≤ 2 mm and horizontal LB reaching the first third of the root crown, Moderate Chronic Periodontitis (MoCP) patients with CAL ≤ 4 mm, horizontal LB comprising two thirds of the root (including coronary and middle third, and Severe Periodontitis (SP) with CAL ≥ 5mm and vertical LB at any third[11] (International Workshop for Classification of Periodontal Diseases and Conditions).

Identification of bacteria by PCR amplification

DNA extraction: sterile water (200 µl) was added to Eppendorf tubes containing the paperpoints impregnated with the periodontal material. The tubes were then incubated at 37 °C for 10 min and centrifuged at 14,000 g for 5 min[10]. The sequence-specific for each bacterial species in the supernatant was determined as previously described[12]. The amplification was performed in a Perkin Elmer Cetus thermocycler. Each reaction was performed in duplicate, with a negative control without DNA, and a positive control. PCR products were analyzed by electrophoresis on 1.6% agarose gels in Tris-Borate-EDTA buffer. The gel was stained with 0.5 µg/ml ethidium bromide and visualised with UV light[10].

Sampling of gingival tissue for TNF-α mRNA detection

Total RNA from biopsies was extracted using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Total RNA was transcribed using Improm II™ Reverse Transcription System (Promega) according to the manufacturer's instructions. TNF-α was amplified from 0.2 µg of cDNA using 1x buffer (Promega), dNTPs 0.2 mM (Pharmacia), 2 mM $MgCl_2$ (Promega), 0.8 µM specific oligonucleotides and 1.25 U Taq DNA polymerase (Promega) in a final volume of reaction of 13 µl [12]. Amplification reactions were conducted in a Perkin Elmer Cetus thermal cycler. The PCR products were separated and visualised using electrophoresis on 1.6% agarose gels in Tris-Borate-EDTA buffer.

Histological processing of gingival tissue samples

To confirm the presence of inflammatory infiltrate, 3 biopsies were taken at random from patients with G, MiCP and MoCP. The histological assessment was carried out following routine hematoxylin and eosin staining.

Statistical analysis

The patients were considered as evaluation units and $p< 0.05$ was considered to be statistically significant. The data were analyzed to determine whether statistically significant relationships existed between the disease-state category and the presence of each periodontopathogens or whether there were associations among the pathogens and the presence of TNF-α mRNA. To test the hypothesis that the presence of specific bacteria is related to a certain disease-state category or is associated with varying clinical assessments, one-way analyses of variance (ANOVAs) were performed. A non-parametric Kruskall-Wallis test was used to detect differences between clinical parameters of disease-state categories. Differences in the prevalence of microorganisms and associations between the numbers of species within and between the three study groups were detected with a chi-square test. An analysis of variance factor was used to determine the differences in the prevalence of TNF-α mRNA. The chi-square test was carried out to establish an association between bacteria and clinical parameters according to the disease groups; the aim of this analysis was to identify any association between the presence/absence of periodontopathogens and clinical parameters (CAL, PD) and the presence/absence of TNF-α. mRNA. The software used was Infostat.

RESULTS

Subject characteristics

The participants in this study formed a homogeneous group. The clinical conditions of the patients in the study are shown in Table 1.

Bacteria were identified in 43 patients. Cytokine expression analysis was conducted on 33 biopsies from 43 patients because not all patients agreed to have biopsies taken, and some samples were not preserved in good enough condition for mRNA determination.

Identification of pathogens by PCR

Table 2 shows the distribution of the frequencies of bacteria detected according to each group of patients.

Table 1: Clinical characteristics of the study population.

	G	MiCP	MoCP	SP
Mean Age	34.33	41.35	37.71	36.21
(years) (range)	(28-41)	(32-51)	(28-48)	(27-46)
Gender (female/male)	6/0	3/4	13/10	3/4
BP (%)	100	100	100	100
BOP (%)	100	100	100	100
SUP (%)	0	0	15.8	20.3
PD (mm)	4.67±0.31	4.43±0.29	6±0.16	6.3±1.2
CAL (mm)	0±0.47	2±0.44	4±0.24	5.2±0.7
LB %	0	33	>50	89

G: Gingivitis; MiCP: Mild Chronic Periodontitis; MoCP: Moderate Chronic Periodontitis; SP: Severe Periodontitis; BP: Bacterial plaque; BOP: Bleeding on probing; SUP: suppuration; PD: Probing depth; CAL: Clinical attachment level; LB: Loss of bone. Data are presented as means ± standard deviations.

Table 2: Frequencies of subgingival microorganisms detected by PCR in Gingivitis, Mild Chronic Periodontitis, Moderate Chronic Periodontitis and Severe Periodontitis.

Bacteria	G	MiCP	MoCP	SP
P. gingivalis	16.6% (1)a*	58.1% (4)b	57.8% (11)b	58.1% (4)b
T. forsythia	83.3% (5)a	71.4% (5)a	84.2% (16)a	85.7% (6) a
T. denticola	50% (3)a	42.8% (3)a	47.3% (9)a	57.1% (4) a
A. actinomycetemcomitans	0 a	0 a	31.5% (6)b**	42.8% (3)b**
P. intermedia	0 a*	28.6% (2)b	26.3% (5)b	28.6% (2)b

G: Gingivitis; MiCP: Mild Chronic Periodontitis; MoCP: Moderate Chronic Periodontitis; SP: Severe Periodontitis. The data represent the percentage of individuals with each bacterium; the numbers of positive subjects for each bacterium are shown in parentheses. The different letters indicate significant differences between groups (* $p < 0.05$, ** $p < 0.01$).

In the bacterial association analysis, we observed that *P. gingivalis* + *T. forsythia* was the main association in MiCP, MoCP and SP patients (p=0.05), and *P. gingivalis* + *T. denticola* was observed in all groups of patients (p=0.05). The prevalence of this association (*P. gingivalis* + *T. denticola*) was greater in advanced disease states. *P. gingivalis* + *A. actinomycetemcomitans* and *T. forsythia* + *A. actinomycetemcomitans* were present in MoCP and SP (p=0.01). *P. gingivalis* + *T. denticola* + *T. forsythia* (members of the red complex) was detected at 16% in G, 28.6 % in MiCP, 42.1% in MoCP and 52.3 % in SP patients; this difference is significant (p=0.05).

Determination of TNF-α mRNA expression in gingival biopsies

We analyzed 6 biopsies from G, 7 from MiCP, 13 from MoCP and 7 from SP and compared the expression of TNF-α mRNA among the groups; using β-actin as the normalizing gene. TNF-α was detectable in 3 samples from G patients, 4 (57%) patients with MiCP, 11 (85 %) patients with MoCP and 5 (71%) patients with SP. There was no statistical difference in TNF-α mRNAs presence/absence among the groups (figure not shown).

Association analysis between the more commonly observed bacterial combinations and TNF-α mRNA in relation to PD and CAL

We observed that the bacterial combinations *T. denticola* + *P. intermedia* and *P. intermedia* + *T. forsythia* were statistically associated with the presence of TNF-α mRNA. These bacterial associations are not associated with PD and CAL values of disease severity. *P. gingivalis* + *A. actinomycetemcomitans* and *A. actinomycetemcomitans* + *T. forsythia* were associated with more condition values of CAL (p=0.02) and PD (p=0.01) (Table 3).

Association between *P. intermedia* and *A. actinomycetemcomitans* and TNF-α mRNA in relation to clinical parameters

We observed an association among the presence of *P. intermedia*, the presence of TNF-α mRNA and PD and CAL values. No significant association was found between the presence of *A. actinomycetemcomitans*, the presence of TNF-α mRNA and periodontal clinical parameters (Table 4).

Table 3: Associations among each bacteria pair, TNF-α mRNA presence/absence and CAL and PD values.

Bacteria Combinations	TNF-α mRNA % (p value)	CAL (p value)	PD (p value)
T. denticola + P. intermedia	20% (0.0464)*	0.1292	0.5227
P. intermedia + T. forsythia	25% (0.0230)*	0.1050	0.3391
P. gingivalis + A. actinomycetemcomitans	5% (0.6462)	0.0213*	0.0150*
A. actinomycetemcomitans + T. forsythia	5% (0.6462)	0.0213*	0.0150*

Percentage of positive subjects for each pair of bacteria and associated clinical parameters. P values are shown within parentheses. * Statistically significant. TNF-α mRNA: RNA Messenger of Tumor Necrosis Factor-α; CAL: clinical attachment level; PD: Probing depth.

Table 4: Associations among the presence/absence of P.intermedia and A. actinomycetemcomitans, the presence/absence of TNF-α mRNA and PD and CAL scores.

		TNF-α mRNA						
Bacteria		**P**			**A**			
		n (%)	PD	CAL	n (%)	PD	CAL	*p*
P. intermedia	P	15 (40)	5.6	5.6	0	---	---	*0.05*
	A	7 (25)	5.4	3.9	11(35)	4.5	2.5	
A. actinomycetemcomitans	P	4(5)	6	8	4(5)	6	6	*ns*
	A	15(60)	5.2	3.4	10(30)	4.3	2	

Values in parentheses represent the percentage of the subjects analyzed. PD: probing depth as the mean ± SD; CAL: Clinical attachment level, mean ± SD. P: presence, number of patients positive for this parameter; A: absence, number of patients negative for this parameter. p<0.05
shows a positive association between the variables. ns: no significant association. TNF-α mRNA: RNA Messenger of Tumor Necrosis Factor-α.

In a comparison between clinical parameters and the presence/absence of TNF-α mRNA, a highly significant difference (p <0.01) was observed between the presence/absence of TNF-α mRNA and severity of PD, with the highest value of PD associated with the presence of TNF-α mRNA. The same trend was observed with the CAL values (p <0.05). These clinical parameters behave differently in the presence/absence of *A. actinomycetemcomitans*.

Inflammatory infiltrate from gingival biopsies

No significant inflammatory cell infiltrate was found in the connective tissue of patients with periodontal disease (data not shown).

DISCUSSION

The present investigation examined subgingival samples from subjects with G, MiCP, MoCP and SP to provide a more comprehensive picture of the microbial profiles in different degrees of the disease and to correlate the presence of bacteria with presence/absence of TNF-α in the host in relation to clinical symptoms determined by CAL and PD measurement.

First, we detected a significant prevalence (p <0.05) of *P. gingivalis* in patients with MiCP (58.1%), MoCP (57.89%) and SP (58.1%) compared to G (16.6%); additionally, in G patients, we did not detect *P. intermedia.* This would indicate that *P.gingivalis* and *P. intermedia* are pathogens that belong to the subgingival microflora in individuals with PD ≥ 5mm. We could speculate that the presence of these pathogens may be involved in the transition from gingivitis to periodontitis.

We identified *A. actinomycetemcomitans* only in patients with MoCP and SP (p <0.01), which might indicate that this species is part of the putative periodontopathogens that may characterize the progression of periodontal damage. *T. denticola* and *T. forsythia* are considered pathogenic species involved in periodontal disease, but our results suggest that they are not related to disease progression, because they were detected in the same proportions in the three groups of patients.

Periodontitis is a mixed infection involving several species of bacteria that act cooperatively or synergistically[4]. In our study, we observed that the potential for partnerships between bacteria increases with disease progression. We mentioned earlier that *T. forsythia* was found in high proportion in all groups of patients. A study by Takemoto et al.[13] in 1997 showed that *T. forsythia* is usually found in mixed infections. This might explain the fact that we found *T. forsythia* in patients with gingivitis without it causing serious injury because the combination with *P. gingivalis* is very low in these patients; only one patient had both bacteria. However, the association of *T. forsythia* + *P. gingivalis* is significantly higher (P <0.05) in patients who have more severe clinical conditions. *T. forsythia* was also found associated with *A. actinomycetemcomitans* only in MoCP (31%) and SP (46%). Moreover, the association found between *P. gingivalis* and *A. actinomycetemcomitans* in patients with MoCP and SP may contribute to the worsening of the clinical picture, based on our finding of a significant association between the bacterial combination and higher scores CAL and PD scores for disease severity. We note that the presence of *P. intermedia* and *A. actinomycetemcomitans* and the presence of TNF-α in the host tissue are positively associated with CAL and PD, regardless of the disease group.

Bascones and Caballero[14] associate *P. gingivalis* with periodontal inflammation and increase in probing depth, poor oral hygiene and attachment loss.

For *A. actinomycetemcomitans*, the observations are contradictory; in our results, the presence of *A. actinomycetemcomitans* was associated with increased CAL and PD scores indicating increased severity of disease. Wolf et al.[15] saw no significant difference in gingival index, probing depth or attachment level between cultures that were positive or negative for *A. actinomycetemcomitans*. However, Ebersole et al.[16], showed an association between this pathogen and increased pocket depth and attachment loss.

Several authors have demonstrated high concentrations of TNF-α in gingival crevicular fluid at sites with active periodontal destruction[17]. We detected a positive association among the presence of *P. intermedia*, the presence of TNF-α mRNA and increased CAL and PD parameters. This association could indicate that the presence of this bacterium might induce the expression of TNF-α mRNA and that this cytokine contributes to tissue deterioration, causing an increase in CAL and PD scores. Górska R, et al.[18], demonstrated positive associations between severe CAL and high levels of TNF-α, IFN-γ and IL-2 in gingival tissue from periodontal patients who had pocket depths exceeding 5 mm. We note that the presence of *P. intermedia* and *A. actinomycetemcomitans* is positively associated with CAL and PD, regardless of the patient group involved. However, only *P. intermedia* is associated with TNF- α presence in the host tissue.

Moreover, the detection of *A. actinomycetemcomitans* in the depth of periodontal pockets, either alone or in combination with other bacteria, was not statistically associated with the presence of host TNF-α mRNA. Therefore, we can speculate that *A. actinomycetemcomitans* caused periodontal tissue degradation and increased PD and CAL clinical parameters by a mechanism that does not involve the participation of pro-inflammatory cytokine TNF-α, whereas *P. intermedia* induces the synthesis of TNF-α, thus worsening the patient's periodontal status. A recent study from Andrukhov et al.[19], reported that due to differences in the bacterial load, different types of periodontal disease could be associated with specific cytokine profiles and therefore specific risk factors. The study of Lima PM et al.[20], are in line with our observations because they recently showed distinct cellular sources of TNF-α, IL-4 and IL-10 in patients with aggressive periodontitis compared to those with chronic periodontitis. Further studies with inhibitors of TNF-α or its receptor are necessary, especially in those patients who have not responded positively to periodontal treatment.

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