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Periodontopathic bacteria and herpesviruses in chronic periodontitis

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SUMMARY

Periodontal disease involves complex interactions of microorganisms and host defenses. This work investigated the associations between putative bacterial pathogens, herpesviruses and chronic periodontitis. Subgingival samples were collected from 40 periodontally healthy individuals and from 40 patients with chronic periodontitis with probing depths of ≤3 mm or ≥6 mm. Multiplex and nested polymerase chain reactions were used to identify bacterial pathogens and herpesviruses. *Porphyromonas gingivalis*, *Tannerella forsythia*, Epstein–Barr virus (EBV) type 1, cytomegalovirus (CMV), *Aggregatibacter actinomycetemcomitans* and EBV type 2 were detected in, respectively, 95, 75, 72.5, 50, 12.5 and 10% of sites with probing depths ≥6 mm. *P. gingivalis*, *T. forsythia*, EBV-1 and CMV were statistically associated with probing depths ≥6 mm. *A. actinomycetemcomitans* and EBV-2 showed no association with periodontitis sites, and no significant associations were found for any of the test infectious agents and probing depths ≤3 mm. Our results confirm an association between *P. gingivalis*, *T. forsythia*, EBV-1 and CMV, and chronic periodontitis. These infectious agents may play an important synergistic role in the pathogenesis of chronic periodontitis.

INTRODUCTION

One of the more obscure issues in periodontology concerns the molecular events that trigger progression of gingivitis to periodontitis. Periodontitis is an infectious disease, but the specific mechanisms by which tooth-supportive tissues are lost are only beginning to be understood. Deciphering the pathogenesis of periodontitis is hampered by the complex interactions of a range of infectious agents and numerous cellular and humoral immune responses. Nonetheless, it is generally believed that progressive periodontitis is related to specific bacterial and viral pathogens (Slots, 2005).

Porphyromonas gingivalis, *Tannerella forsythia* and *Aggregatibacter actinomycetemcomitans* show a close relationship to severe periodontitis (Haffajee & Socransky, 1994), and although these organisms have been associated with the etiopathogenesis of the disease (Ezzo & Cutler, 2003), there is a suggestion that bacterial activities alone may not explain several clinical features of destructive periodontal

disease (Saygun *et al.*, 2004). Herpesviruses, especially Epstein–Barr virus (EBV) and cytomegalovirus (CMV), infect 80–90% of most adult populations worldwide (Slots, 2009), and a major oral reservoir for CMV, and probably also for some other members of the herpesvirus family, seems to be periodontal disease sites (Sahin *et al.*, 2009). Herpesviruses can infect or alter structural cells or host defense cells of the periodontium, and thereby reduce the ability of periodontal tissues to resist bacterial insults. Conceivably, the presence of herpesviruses may promote the initiation and progression of some type of periodontitis and herpesviruses may depend upon a co-infection with periodontal bacteria to evoke periodontitis (Botero *et al.*, 2007). The aim of the present study was to evaluate the prevalence and association between putative bacterial pathogens and herpesviruses in patients with chronic periodontitis.

METHODS

Sampling

Forty periodontally healthy individuals (mean age 42.0 ± 12.0 years) and 40 patients with chronic periodontitis (mean age 40.9 ± 12.2 years) were included in the study. The study subjects received dental care at the School of Dentistry, Isfahan University of Medical Sciences, Iran. The subjects gave written informed consent before participation in the study. Periodontitis was defined as the presence of at least two sites with periodontal probing depth >6 mm and attachment loss of more than 5 mm.

All subjects were systemically healthy and had no history of periodontal treatment or any type of antibiotic therapy for at least 6 months prior to the present study. A total of 160 subgingival plaque samples were collected from two probing depths (≥6 mm and ≤3 mm), and two periodontal sites were sampled in each subject. After removing supragingival plaque with sterile cotton pellets, a sterile periodontal curette was gently inserted to the bottom of the test periodontal pocket, and subgingival material was removed by a single stroke (Saygun *et al.*, 2005).

Polymerase chain reaction analyses

Subgingival specimens were suspended in 0.5 ml sterile TE buffer (10 mM Tris–HCl, 1 mM ethylenediaminetetraacetic acid, pH 8), homogenized by vigorous vortexing, and stored at −70°C. Genomic DNA was extracted from thawed samples by a phenol–chloroform method (Moghim *et al.*, 2007). The extracted DNA in TE buffer was stored at −70°C. The quality of the DNA was evaluated by polymerase chain reaction (PCR) using beta-globin specific primers (Borisch *et al.*, 1993). A multiplex PCR method was used for the identification of major periodontopathic bacteria, and the primers, based on the sequences of 16S ribosomal RNA, have been described elsewhere (Tran & Rudney, 1999). Each multiplex PCR was carried out in a total volume of 50 μl containing 1 U *Taq* polymerase (Sinagene, Tehran, Iran), 1 ×PCR buffer with 1.5 mM $MgCl_2$, 1 mM mixture of dNTPs, 12.5 pM of each forward primer, 25 pM of reverse primer and 5 μl template DNA. A nested-PCR method was used to identify EBV and CMV (Chalabi *et al.*, 2008). The primers for EBV-1, EBV-2 and CMV have previously been described (Borisch *et al.*, 1993; Tukimatsu *et al.*, 1995). Each amplification reaction was carried out in a total volume of 50 μl containing 1.5 U *Taq* polymerase (Sinagene, Tehran, Iran), with 1 mM $MgCl_2$, 0.1 mM dNTPs, and 10 pM each primer. The PCR conditions for multiplex PCR were as follows: 95°C for 10 min; 30 cycles at 94°C for 1 min, 59°C for 45 s and 72°C for 1 min, with a final extension at 72°C for 3 min. The nested-PCR conditions were identical except that the annealing step was at 63.5°C for 1 min and PCR was for 34 cycles. Positive and negative controls were included in all runs. Purified genomic DNA samples from *P. gingivalis*, *T. forsythia* and *A. actinomycetemcomitans* were used as positive controls and DNA-free water served as the negative control. Isolated DNA from the cell lines B95-8, Razi (Pasteur Institute, Tehran, Iran) and AD169 (Tarbiat Modares, University of Iran) were used as controls for EBV-1, EBV-2 and CMV, respectively. PCR products were analysed using agarose gel electrophoresis and ethidium bromide staining.

Statistics

Pearson chi-square test, confirmed by McNemar test and independent *t*-test, were used to determine the association of tested pathogenic agents with each other. *P*-values equal to or <0.05 were considered statistically significant. Logistic regression was employed to determine whether individual pathogens

or a combination of pathogens were associated with chronic periodontitis. Statistical analyses were carried out using SPSS 11.5 (SPSS for Windows 11.5, SPSS Inc. 1982–2002, LEADTOOLS© 1991–2002, LEAD Technologies, Washington, DC).

RESULTS

The age and sex distributions of the study subjects are shown in Table 1. Bleeding on probing was detected in 36.9% of the chronic periodontitis patients (Table 1). The prevalence of infectious agents studied was similar in the shallow sites of periodontally healthy subjects and chronic periodontitis subjects (Table 2). However, *P. gingivalis*, *T. forsythia*, EBV-1 and CMV occurred at significantly higher frequencies in deeper sites than in shallow periodontal sites of patients with chronic periodontitis (Table 2). The detection frequency of *A. actinomycetemcomitans* and EBV-2 did not differ significantly between periodontally healthy subjects and patients with chronic periodontitis.

Logistic regression analysis found that *P. gingivalis*, *T. forsythia*, EBV-1 and CMV were statistically associated with chronic periodontitis lesions with pocket depths ≥6 mm, but not with periodontal sites with depths ≤3 mm (Table 3). Logistic regression analysis was also used to determine whether any bacterial pathogen singly or in combination with the herpesviruses had coincident effects on chronic periodontitis. *T. forsythia* and EBV-1 demonstrated coincident effects in probing depths ≥6 mm (*T. forsythia:* $P < 0.001$, odds ratio 57.00; EBV-1: $P < 0.001$, odds ratio 102.82) (Table 3).

Table 1 Description of the study subjects

	Healthy controls ($n = 40$)	Chronic periodontitis ($n = 40$)
Age (years)	42.0 ± 12.0	40.9 ± 12.2
Males	16 (40%)	19 (47.5%)
Females	24 (60%)	21 (52.5%)
Bleeding on probing (%)	0	36.9

DISCUSSION

The results of the present study, showing statistically significant associations between *P. gingivalis*, *T. forsythia*, EBV-1 and CMV, and chronic periodontitis, are in agreement with previous findings (Ezzo & Cutler,

Table 2 Occurrence of bacterial pathogens and herpesviruses in healthy controls (HC) and in patients with chronic periodontitis (CP)

	HC ($n = 40$)	CP ($n = 40$)		Comparison of HC with CP (*P*-value)		Comparison of CP in both ≤3 mm and ≥6 mm (*P*-value)
Infectious agents	≤3 mm	≤3 mm	≥6 mm	≤3 mm	≥ 6 mm	
Porphyromonas gingivalis	26 (65%)	31 (77.5%)	38 (95%)	0.22	0.001	0.04
Tannerella forsythia	2 (5%)	6 (15%)	30 (75%)	0.26	0.001	0.001
Aggregatibacter actinomycetemcomitans	3 (7.5%)	6 (15%)	5 (12.5%)	0.48	0.71	1
Epstein–Barr virus type 1	1 (2.5%)	4 (10%)	29 (72.5%)	0.40	0.001	0.001
Epstein–Barr virus type 2	0 (0%)	–	4 (10%)	–	0.12	–
Cytomegalovirus	2 (5%)	–	20 (50%)	–	0.001	–

Table 3 Statistical association between periodontal pathogens and chronic periodontitis

	Logistic regression for probing depth ≤3 mm		Logistic regression for probing depth ≥6 mm	
Infectious agents	Odds ratio	*P*-value	Odds ratio	*P*-value
Porphyromonas gingivalis	1.86	0.22	10.23	0.004
Tannerella forsythia	3.35	0.16	57.00	0.001
Aggregatibacter actinomycetemcomitans	2.18	0.30	1.76	0.460
Epstein–Barr virus type 1	4.33	0.20	102.82	0.001
Epstein–Barr virus type 2	–	–	1.79	0.999
Cytomegalovirus	–	–	19.00	0.001

2003; Slots, 2010). *P. gingivalis* and *T. forsythia* are capable of elaborating an array of virulence factors that enable them to invade host cells and cause periodontal tissue damage (Lamont & Yilmaz, 2002). Herpesvirus infection can also facilitate bacterial colonization of epithelial and connective tissue cells (Bakaletz, 1995). Taken together, the combined action bacterial pathogens and herpesviruses may increase the risk of periodontal tissue breakdown.

Aggregatibacter actinomycetemcomitans was not associated with chronic periodontitis. Other studies have also reported no relationship between the presence of *A. actinomycetemcomitans* and herpesviruses in chronic or aggressive periodontitis (Contreras *et al.*, 1999; Saygun *et al.*, 2004; Imbronito *et al.*, 2008**)**. Also, EBV-2 was detected at low frequency in all test samples. EBV-2 can occur in both aggressive and chronic periodontitis, but with a lower prevalence in chronic periodontitis (Wu *et al.*, 2005). EBV-2 has deletions in the EBNA-2 gene, which lead to a decreased transformation ability of B lymphocytes, but the decreased transformation may be compensated for by an increased lytic potential of EBV-2 (Klemenc *et al.*, 2005). As virus-induced lysis of periodontal cells may constitute an important pathogenetic factor of periodontal disease, EBV-2 may preferentially be found in sites of aggressive periodontitis.

In conclusion, the present study identified a high prevalence of periodontopathic bacteria and herpesviruses in chronic periodontitis sites of Iranian subjects. These findings support an association between periodontopathic bacteria and herpesviruses, and this co-infection may constitute a factor in periodontal tissue destruction. Several mechanisms have been suggested by which herpesviruses may contribute to chronic periodontitis (Slots, 2010). Herpesviruses show tropism for cells of the immune system, possibly suppressing immune responses in the periodontium, and hence permitting overgrowth of pathogenic bacteria (Slots, 2010). Bacteriological and virological studies are warranted into the mechanisms by which a combined bacterial–herpesviral periodontal infection may lead to the destruction of human periodontal tissue.

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