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Herpesviral–bacterial interrelationships in aggressive periodontitis

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Background: Recent findings have begun to provide a basis for a causal link between herpesviruses and aggressive periodontitis. One theory is that herpesviruses cooperate with specific bacteria in the etiopathogenesis of the disease. This study examined whether the presence of herpesviruses [human cytomegalovirus (HCMV), Epstein–Barr virus (EBV) type 1, herpes simplex virus (HSV) type 1 and 2] is associated with the presence of putative pathogenic bacteria (*Porphyromonas gingivalis*, *Prevotella intermedia*, *Tannerella forsythia*, *Campylobacter rectus, Actinobacillus actinomycetemcomitans*) in aggressive periodontitis lesions.

Methods: The study included 18 young adults with advanced periodontitis and 16 periodontally healthy subjects from Ankara, Turkey. Subgingival specimens pooled from two sites in each subject were collected by a periodontal curette. Qualitative polymerase chain reaction methodology was used to identify herpesviruses and bacteria. Chi-square tests were employed to determine statistical associations among herpesviruses, bacteria and periodontal disease.

Results: HCMV, EBV-1 and HSV-1 were each detected in 72–78% of the aggressive periodontitis patients. HSV-2 occurred in 17% of the periodontitis patients. EBV-1 was detected in one periodontally healthy subject. The study bacteria occurred in 78–83% (*P. gingivalis*, *T. forsythia*, *C. rectus*) and in 44% (*P. intermedia*, *A. actinomycetemcomitans*) of the periodontitis samples, and in 0–19% of the samples from healthy periodontal sites. HCMV, EBV-1 and HSV-1 were positively associated with *P. gingivalis*, *P. intermedia*, *T. forsythia* and *C. rectus*, but not with *A. actinomycetemcomitans*. HSV-2 was not associated with any test bacteria.

Conclusions: These results support the notion that the clinical outcome of some types of severe periodontal infection depends on the presence of specific herpesviruses and bacterial pathogens. Our findings open the door to testing a variety of hypotheses regarding the deleterious aspects of combined herpesviral-bacterial infections in periodontal sites.

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The course of periodontitis can vary considerably between young and older individuals (1, 2). The disease course in adolescents and young adults is typically aggressive and relatively brief. In older individuals, the course is more often slow and frequently associated with pronounced gingival inflammation and heavy accumulation of dental plaque and calculus. These observations may suggest that aggressive periodontitis in young patients requires less infectious agent stimulus to trigger a progressive disease response than the more chronic type of the disease in older individuals. Periodontitis may debut and progress rapidly in immunologically immature young people, whereas the disease may tend to stabilize in adults having protective immunity from past infection. Similarly, rapidly advancing periodontitis may occur in immunocompromised adults unable to control the pathogenic

infection (3). It may be that some types of aggressive and chronic periodontitis do not constitute fundamentally different disease entities but a continuous spectrum of diseases, whose clinical expression depends on the presence of specific infectious agents and the immune status of the host.

However, the pathogenic events that initiate aggressive periodontitis have been difficult to delineate because of the complexity of the pathogenic microbiota (4) and the multiple pathophysiologic effects of several pro- and anti-inflammatory mediators (5). A generally accepted theory states that, in a susceptible host, destructive disease occurs when an agent or event produces an inflammatory reaction capable of disrupting and overwhelming the periodontal defense. A number of bacterial species have been incriminated in the etiopathogenesis of periodontitis (6), but a bacteriologic cause alone seems insufficient in explaining several clinical features of the disease (7). Recent studies suggest that periodontal herpesviruses comprise an important source for triggering periodontal tissue destruction (8, 9). In particular, human cytomegalovirus (HCMV) and Epstein–Barr virus (EBV) occur frequently in aggressive periodontitis sites (10, 11). Clinical disease from herpesviral infections is generally restricted to hosts with inadequate or altered immune functions (12). Herpesvirus productive infection may initiate or accelerate periodontal tissue destruction due to a virally mediated release of cytokines and chemokines from inflammatory and non-inflammatory host cells, or a virally induced impairment of the periodontal defense resulting in a heightened virulence of resident pathogenic bacteria (13).

The periodontal presence of HCMV has been linked to a high occurrence of subgingival *Porphyromonas gingivalis* (14), *Dialister pneumosintes* (15) and other bacterial pathogens (16, 17). Periodontal EBV and herpes simplex virus (HSV) have also been associated with elevated presence of certain putative periodontopathic bacteria (16). Conceivably, herpesviruses rely on coinfection with periodontal bacteria to produce periodontitis and, conversely, periodontopathic bacteria may depend on viral presence for the initiation and progression of some types of periodontitis. Understanding the interdependent roles that herpesviruses and bacteria may play in the pathogenesis of periodontitis may help explain clinical characteristics of the disease and identify individuals at high risk of periodontal breakdown.

To expand our knowledge of periodontal infections, the present study was conducted to evaluate the interrelationship between herpesviruses and putative bacterial pathogens in aggressive periodontitis sites in patients from Ankara, Turkey. Polymerase chain reaction (PCR) was used to identify the herpesviruses HCMV, EBV-1, HSV-1 and HSV-2 and the bacteria *P. gingivalis*, *Prevotella intermedia*, *Tannerella forsythia*, *Campylobacter rectus* and *Actinobacillus actinomycetemcomitans*.

Material and methods

Subjects and clinical procedures

Eighteen patients (nine women and nine men, ages 17–31 years) with aggressive periodontitis and 16 periodontally healthy individuals (eight women and eight men, ages 20–30 years) participated in the study. The study subjects were selected at random from individuals scheduled for a routine dental check-up. Patients diagnosed as having aggressive periodontitis exhibited probing attachment loss in the excess of 5 mm on more than 14 teeth, at least three of which were teeth other than incisors or first molars. The periodontally healthy subjects showed little or no signs of gingival inflammation and little or no attachment loss in any tooth. All study subjects were systemically healthy and had not received periodontal treatment or antibiotics for at least 6 months prior to the study. The Ethical Committee of the Gülhane Military Medical Academy, Sciences of Dentistry, Ankara, Turkey approved the experimental protocol. Written informed consent was obtained from each study subject after all procedures had been fully explained.

The clinical periodontal evaluation was based on the Plaque Index (18), the Gingival Index (19), probing pocket depth, and probing attachment loss. Clinical variables were assessed at six sites per tooth, mesio-facial, mid-facial, disto-facial, mesio-lingual, mid-lingual, and disto-lingual. Probings were carried out using a Williams probe calibrated in millimeters.

Subgingival sample collection

Prior to sampling, supragingival plaque was gently removed with sterile cotton pellets and sample sites were isolated with cotton rolls and air-dried. Subgingival specimens were collected by means of a sterile curette. After gently inserting the curette to the bottom of a sample site, subgingival plaque was removed by a single stroke. In each periodontitis patient, a pooled specimen was obtained from the two deepest pockets of the dentition (7–10 mm probing depth). In each periodontally healthy subject, a pooled specimen was obtained from two random sites of 2–3 mm in probing depth. The specimens were suspended in 500 μl of TE buffer (10 mM Tris-hydrochloride, 1 mM EDTA, pH 8) and homogenized by vigorous mixing on a vortex.

DNA extraction and PCR procedures

DNA was extracted from subgingival specimens using an alkali phenol–chloroform–isoamyl alcohol procedure (20). Briefly, 100 μl of specimen was placed in 10 μl of protease solution (65 mg/ml) (Sigma-Aldrich Corp, St. Louis, MO, USA) and 250 μl of K buffer for 60 min at 42°C. Following centrifugation at 10,000 *g* for 10 min at 12°C, DNA was extracted from the supernatant using a mixture of 250 μl alkali phenol and 250 μl chloroform-isoamyl alcohol (25 : 24 : 1), and then pelleted using 500 μl isopropyl alcohol. DNA was washed in 75% ethyl alcohol at 10,000 *g* for 5 min at 4°C, air-dried at 37°C, and dissolved in 100 μl distilled water.

Table 1 shows the PCR primers used in the identification of herpesviruses and bacteria. PCR was performed with a final volume of 50 μl mixture con-

Table 1. PCR primers for identifying infectious agents

Infectious agents	Primers	References
Human cytomegalovirus	5′-GGATCCGCATGGCATTCACGTATGT-3′ 5′-GAATTCAGTGGATAACCTGCGGCGA-3′	21
Epstein–Barr virus type 1	5′-GCCAGAGGTAAGTGGACTTTAATTT-3′ 5′-TGGAGAGGTCAGGTTACTTACC-3′	Designed and validated for this study
Herpes simplex virus type 1	5′-CGGCCGTGTGACACTATCG-3′ 5′-CTCGTAAAATGGCCCCTCC-3′	22
Herpes simplex virus type 2	5′-CGCTCTBCGTBAAATGCTTCCCT-3′ 5′-TCTACCCACAACAGACCCACG-3′	22
Porphyromonas gingivalis	5′-AGGCAGCTTGCCATACTGCG-3′ 5′-ACTGTTAGCAACTACCGATGT-3′	23
Prevotella intermedia	5′-TTTGTTGGGGAGTAAAGCGGG-3′ 5′-TCAACATCTCTGTATCCTGCG-T-3′	23
Tannerella forsythia	5′-GCGTATGTAACCTGCCCGCA-3′ 5′-TGCTTCAGTGTCAGTTATACCT-3′	23
Campylobacter rectus	5′-TTTCGGAGCGTAAACTCCTTTTC-3′ 5′-TTTCTGCAAGCAGACACTCTT-3′	23
Actinobacillus actinomycetemcomitans	5′-AAACCCATCTCTGAGTTCTTCTTC-3′ 5′-ATGCCAACTTGACGTTAAAT-3′	23

taining 25 pmol of each primer (MWG-Biotech, Ebersberg bei München, Germany), 1 U *Taq* DNA polymerase (Bioron GmbH, Ludwigshafen, Germany), 2 mM $MgCl_2$, 0.05 mM dNTP mix (8 mM each), 5 µl of 10 × Reaction Buffer (Bioron), and 10 µl of extracted DNA sample. PCR procedures included a 40-round amplification process and were performed in three steps covering denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 40 s.

Detection of PCR products was performed by electrophoresis in a 2% agarose gel containing 0.5 µg/ml ethidium bromide. Gels were analyzed by using Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical tests

Statistical testing was performed with Chi-square analysis (SPSS 10.0 statistical package; SPSS Inc, Chicago, IL, USA). *p*-values equal to or less than 0.05 were considered statistically significant.

Results

Table 2 shows that, in addition to more periodontal attachment loss, the aggressive periodontitis patients studied exhibited significantly more dental plaque accumulation and gingival inflammation than the periodontally healthy subject group. The average age of the participants in the two study groups were similar, approximately 24 years.

Table 3 reveals that HCMV, EBV-1 and HSV-1 were detected in 72–78%, *P. gingivalis*, *T. forsythia* and *C. rectus* in 78–83%, and *P. intermedia* and *A. actinomycetemcomitans* each in 44% of aggressive periodontitis samples. HSV-2 occurred in 17% of the periodontitis patients. All periodontitis patients showed at least one of the test herpesviruses, with seven subjects revealing two, six subjects three, and two subjects four types of herpesviruses. In the periodontally healthy group, one individual only showed EBV-1. *P. intermedia* and *T. forsythia* were not identified in any healthy subject, *P. gingivalis* and *A. actinomycetemcomitans* were each detected in one healthy subject, and *C. rectus* was demonstrated in three healthy subjects. Except for HSV-2, the test herpesviruses and bacteria occurred with statistically higher frequency in samples from periodontitis lesions than from periodontally healthy sites.

Table 4 shows the occurrence of strong statistically significant bivariate associations between most test viruses and bacteria. However, HCMV and HSV-1 were not associated with *A. actinomycetemcomitans*, EBV-1 was not associated with *P. intermedia* or *A. actinomycetemcomitans*, and HSV-2 was not associated with any of the bacteria studied. A multivariate logistic regression analysis showed similar results, but because of the relatively small study size, only data from Chi-square analyses were considered reliable.

Discussion

This study supports the concept that HCMV, EBV-1 and HSV-1 periodontal infections contribute to the etiopathogenesis of aggressive periodontitis. The three herpesviruses were

Table 2. Clinical variables of aggressive periodontitis and periodontally healthy study subjects

Clinical variables	Aggressive periodontitis ($n = 18$)	Healthy periodontium ($n = 16$)	*p*-values
Age (years)	24.1 ± 3.2*	24.1 ± 3.4	Not significant
Plaque Index (whole mouth)	1.7 ± 0.5	0.7 ± 0.3	< 0.001
Gingival Index (whole mouth)	2.0 ± 0.1	0.4 ± 0.4	< 0.001
Probing pocket depth (sample sites)	7.8 ± 1.5	1.7 ± 0.4	< 0.001
Probing pocket depth (whole mouth)	5.0 ± 1.1	2.1 ± 0.6	< 0.001
Probing attachment loss (sample sites)	7.9 ± 1.6	1.7 ± 0.4	< 0.001
Probing attachment loss (whole mouth)	5.1 ± 1.1	2.1 ± 0.6	< 0.001

*Mean ± standard deviation.

Table 3. Herpesviruses and bacterial pathogens in aggressive periodontitis and periodontally healthy subjects

Periodontal diagnosis	HCMV	EBV-1	HSV-1	HSV-2	*Porphyromonas gingivalis*	*Prevotella intermedia*	*Tannerella forsythia*	*Campylobacter rectus*	*Actinobacillus actinomycetemcomitans*
Aggressive periodontitis ($n = 18$)	13* 72.2%	13 72.2%	14 77.8%	3 16.7%	14 77.8%	8 44.4%	14 77.8%	15 83.3%	8 44.4%
Healthy periodontium ($n = 16$)	0	1 6.3%	0	0	1 6.3%	0	0	3 18.8%	1 6.3%
p-values	< 0.001	< 0.001	< 0.001	0.092	< 0.001	< 0.003	< 0.001	< 0.001	0.013

*Number and percentage positive samples.
HCMV, human cytomegalovirus; EBV-1, Epstein–Barr virus type 1; HSV-1, herpes simplex virus type 1; HSV-2, herpes simplex virus type 2.

Table 4. Relationship among periodontal herpesviruses and bacteria

	Porphyromonas gingivalis		*Prevotella intermedia*		*Tannerella forsythia*		*Campylobacter rectus*		*Actinobacillus actinomycetemcomitans*	
Viruses	Chi-square	*p*	Chi-square	*p*	Chi-square	*p*	Chi-square	*p*	Chi-square	*p*
HCMV	9.188	0.004	5.988	0.033	6.839	0.014	4.859	0.039	1.555	0.254
EBV-1	7.20	0.013	1.964	0.228	8.993	0.005	10.261	0.002	1.045	0.264
HSV-1	7.201	0.013	4.941	0.042	13.741	< 0.001	6.275	0.017	3.283	0.116
HSV-2	0.155	1	1.012	1	0.084	1	0.508	0.591	0.080	1

HCMV, human cytomegalovirus; EBV-1, Epstein–Barr virus type 1; HSV-1, herpes simplex virus type 1; HSV-2, herpes simplex virus type 2.

each detected in approximately 75% of aggressive periodontitis lesions but were virtually absent in healthy periodontal sites. Given the young age and the severe periodontal destruction of the study patients, it is likely that several periodontitis lesions were in a state of progression at the time of sampling, pointing to an association between herpesviruses and active periodontal disease. In a similar way, Kamma *et al*. (24) detected herpesviruses at higher frequency in active than in stable periodontitis sites of young individuals. In localized aggressive periodontitis, Ting *et al*. (17) reported a close relationship between HCMV active infection and advancing disease. Since the prevalence of herpesvirus carriage varies by age, and by country, region within country and population subgroup (25), the high rate of herpesvirus detection consistently found in several periodontitis studies attests to the strength of the herpesvirus–periodontitis association. Furthermore, the temporal relationship between the presence of periodontal herpesvirus and early clinical appearance of periodontal breakdown is consistent with causation. However, the establishment of a herpesvirus infection as a cause of periodontitis will require, in a large sample of patients, the detection of the herpesvirus in the periodontium before or at the time of disease initiation, and the demonstration that eradication of the viral infection prevents or arrests the progression of periodontal breakdown.

In contrast, HSV-2 was rarely detected in the aggressive periodontitis lesions and was not statistically associated with the disease. Contreras and Slots (26) also described a low prevalence of HSV-2 in adult periodontitis lesions. Our data are in agreement with a strong predominance of HSV-1 in orofacial infections and a preference of HSV-2 for anogenital infections (25).

The main impetus for this study was the hypothesis that herpesvirus infections of the periodontium can lead to an increased occurrence of periodontopathic bacteria, probably through virally mediated impairment of local host defenses (8, 13). Significant associations were found between HCMV and HSV-1 and *P. gingivalis*, *P. intermedia*, *T. forsythia* and *C. rectus*, and between EBV-1 and *P. gingivalis*, *T. forsythia* and *C. rectus*. A relationship between periodontal herpesviruses and *C. rectus* has not been reported previously. HSV-2 showed no coupling to any of the test bacteria. In earlier studies of aggressive periodontitis, HCMV was found to be linked to *P. gingivalis* (14) and *D. pneumosintes* (15), and HSV-1 to *P. gingivalis* (14), and the herpesviral–bacterial associations were considered to play major roles in the pathogenesis rather than constituting merely secondary events to clinical manifestations of the disease. The present findings also agree with an adult periodontitis study, in which linkages were demonstrated between periodontal HCMV and *P. gingivalis*, *Prevotella nigrescens*, *T. forsythia* and *Treponema denticola,* and between EBV-1 and *P. gingivalis*, *P. intermedia*, *P. nigrescens*, *T. forsythia* and *T. denticola* (16). HCMV and EBV occur together with elevated levels of anaerobic bacteria in periapical pathosis of endodontic origin as well (27). In addition, herpesviruses have been identified in many medical diseases that have a bacterial component, including inflammatory bowel disease,

enterocolitis, esophagitis, pulmonary infections, sinusitis, acute otitis media, dermal abscesses and pelvic inflammatory disease (15). Other mammalian viruses may also work in a similar way and support bacterial pathogens in the causation of various human respiratory diseases, intestinal infections and otitis media (28). In periodontal disease, the ability of HCMV and lipopolysaccharide of gram-negative bacteria to act synergistically in stimulating interleukin-1β gene transcription may be an important pathogenic determinant (29). In sum, based on data from our studies of herpesviruses in periodontal disease and general knowledge about herpesviral infections, it seems reasonable to suggest that certain herpesviruses cooperate with specific pathogenic bacteria in the pathogenesis of various types of destructive human periodontal disease.

Noticeably, the herpesviruses studied here showed no statistical relationship with *A. actinomycetemcomitans*. A similar lack of association between herpesviruses and *A. actinomycetemcomitans* has been reported in chronic (adult) periodontitis patients (16). However, HCMV has been linked to an elevated occurrence of *A. actinomycetemcomitans* in localized aggressive periodontitis in the USA (17) and Jamaica (30). The predominance of generalized periodontitis patients in this study may explain the lack of association between periodontal herpesviruses and *A. actinomycetemcomitans*. It may be that herpesvirus periodontal infections require unique host immune conditions, as may exist in certain juveniles, to produce overgrowth of periodontal *A. actinomycetemcomitans* and localized disease (8).

The notion of cooperation between herpesviruses and bacteria in the development of periodontitis may help explain various clinical features of the disease. Espousing solely a bacterial cause for periodontitis has difficulty in explaining why dental plaque amount and level of dental care are often not commensurate with disease severity, why the disease tends to occur in a pattern of progression and remission, and why tissue breakdown often appears in a localized and bilaterally symmetrical pattern. On the other hand, various characteristics of herpesviruses, such as the ability to switch between latency and productive infection, and the tendency to exhibit tissue tropism, are in accordance with important clinicopathologic features of periodontitis (8, 10).

In summary, it is unlikely that a single class of infectious agents is the sole cause or modulator of the heterogeneous group of diseases collectively termed as periodontitis. Our present and previous results support the concept that the clinical outcome of an aggressive periodontal infection depends on interactions among herpesviruses, specific bacteria, and probably coordinated immune responses to viral and bacterial replication. The observation that herpesvirus periodontal infections tend to be associated with subgingival pathogenic bacteria offers testable hypotheses for explaining the periodontopathic effects of these viruses. It is hoped that such information will help bridge the gap between our clinical understanding of destructive periodontal disease, and our lack of knowledge, at the molecular level, of the mechanisms involved in periodontal tissue breakdown.

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