

Relationship between *Porphyromonas gingivalis*, Epstein-Barr virus infection and reactivation in periodontitis

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Abstract: The aim of this study was to assess the relationship between *Porphyromonas gingivalis*, Epstein-Barr virus (EBV) infection and reactivation in periodontitis using real-time PCR. The mean proportion of *P. gingivalis* cells to total bacterial cells in the saliva from EBV-positive periodontitis patients was significantly higher than that in saliva from EBV-negative patients. An EBV-positive B-cell line was used to determine whether *P. gingivalis* sonicate induced reactivation of EBV, using real-time PCR to measure the virus genome in the culture medium. A significant increase in EBV numbers was observed after the stimulation with *P. gingivalis* sonicate. These findings suggest that the interaction between EBV and *P. gingivalis* is bi-directional, with EBV reactivation suppressing host defenses and permitting overgrowth of *P. gingivalis*, and *P. gingivalis* having the potential to induce EBV reactivation. (J. Oral Sci. 46, 203-206, 2004)

Key words: Epstein-Barr virus; *Porphyromonas gingivalis*; reactivation; real-time PCR.

Introduction

Epstein-Barr virus (EBV), a human gamma-herpes virus, is present in approximately 90% of the population worldwide. EBV is the causative agent in infectious mononucleosis and is associated with Burkitt's lymphoma, nasopharyngeal carcinoma, chronic fatigue syndromes and immunoblastic lymphomas in immunosuppressed individuals, particularly in transplant and HIV patients (1). Results of recent studies have implicated EBV in the pathogenesis of periodontal diseases such as chronic periodontitis, juvenile periodontitis, Down's syndrome periodontitis, HIV-associated periodontitis and acute necrotizing ulcerative gingivitis (2-9). There have also been several reports of EBV in subgingival plaque being associated with periodontal parameters and the presence of periodontopathic bacteria such as *Porphyromonas gingivalis* (10-13). However the relationship between EBV, *P. gingivalis* and periodontal disease remains to be determined.

The aim of the present study was to further delineate the possible relationship between EBV and *P. gingivalis*, by measuring the EBV genome and determining *P. gingivalis* cell numbers in saliva from periodontitis patients, and assessing the potential of *P. gingivalis* to induce EBV reactivation.

Materials and Methods

Subjects and saliva samples

This study was approved by the Nihon University School of Dentistry Institutional Review Board and all subjects

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gave written informed consent for study participation. The study group comprised 33 systemically-healthy subjects with periodontitis, and mean age 49.9 years (range 25 - 68 years). Periodontitis was defined as the presence of at least two sites with probing depth greater than 4 mm. Paraffin wax-stimulated whole saliva was collected from each subject, and the samples were stored at -80°C until analyzed.

Real-time PCR

Saliva samples were boiled for 10 min, centrifuged at $10,000 \times g$ for 5 min, and $5\mu\text{l}$ of supernatant was used as a template for PCR. Real-time PCR was performed using the ABI PRISM 7700 Sequence Detection System (ABI, Foster City, CA). Each reaction tube contained $50\mu\text{l}$ of reaction mixture, including $5\mu\text{l}$ of sample, $1 \times$ Universal PCR Master Mix (ABI), 900 nM of each primer and 250 nM of probe.

The primer and probe sets for EBV and *P. gingivalis* are listed in Table 1 (14,15). In order to quantify total bacteria, conserved sequences were selected from the 16S rRNA gene sequence reported previously (16,17). Total bacterial numbers were determined using total bacteria primers and probes that hybridized to all bacterial 16S rRNA. Amplification of total bacterial 16S rRNA was carried out in a separate reaction but at the same time and under the same conditions as those used for the EBV- and *P. gingivalis*-specific amplification. The probes were labeled at the 5' end with the reporter dye 6-carboxyfluorescein (6-FAM) and at the 3' end with the quencher dye 6-carboxytetramethylrhodamine (TAMRA). Data were

analyzed using the Sequence Detection System software from ABI. The number of EBV was determined using synthesized nucleotides of the amplified region (98 mer), and the proportion of EBV to total bacterial cells was calculated. The number of *P. gingivalis* cells was determined using DNA from known amounts of *P. gingivalis*, and the proportion of *P. gingivalis* to total cell number was calculated. The number of bacterial cells was determined using DNA from known numbers of bacteria.

P. gingivalis sonicate

P. gingivalis strain 381, ATCC 33277 and ATCC 49417 were harvested by centrifugation $10,000 \times g$ for 15 min at 4°C . Bacterial sonicate was prepared by sonication at 20 W for 5 min, and insoluble debris was removed by centrifugation at $10,000 \times g$ for 30 min at 4°C . The supernatant was filter sterilized and stored at -80°C until used.

EBV reactivation

An EBV-positive B-lymphoid cell line (Raji; Japanese Collection of Research Bioresources) was used in this study. Cells were maintained in RPMI 1640 medium supplemented with heat-inactivated fetal bovine serum, penicillin (100 U/ml), streptomycin (0.1 $\mu\text{g}/\text{ml}$) and L-glutamine (2 mM) in 75 cm^2 tissue culture flasks at 37°C in 5% CO_2 in air high humidity. Raji cells in log phase of growth were resuspended at 1×10^6 cells/ml in serum-free culture medium in the presence of PMA (phorbol 12-myristate 13-acetate; 10 nM) or *P. gingivalis* sonicate (3 $\mu\text{g}/\text{ml}$). Culture supernatants were harvested 48 hours later, and lytic cycle induction was assessed by measuring the virus genome in culture medium using real-time PCR.

Table 1 Sequences of Oligonucleotide Primers and Probes (14,15)

Sequence (5'→3')	
EBV	
Forward	AGT CCT TCT TGG CTA CTC TGT TGA C
Reverse	CTT TGG CGC GGA TCC TC
Probe	CAT CAA GAA GCT GCT GCGGC C
<i>P. gingivalis</i>	
Forward	TACCCATCGTCGCCTTGGT
Reverse	CGGACTAAAACCGCATACTTGT
Probe	GCTAATGGGACGCATGCCTATCTTACAGCT
Total bacteria	
Forward	GGATTAGATACCCTGGTAGTC
Reverse	TACCTTGTTACGACTT
Probe	TGACGGGCGGTGTGTACAAGGC

Statistical analyses

Differences in the proportion of *P. gingivalis* between EBV-positive and negative patients were analyzed using the Mann-Whitney's *U*-test. Correlation between the proportion of *P. gingivalis* and EBV was analyzed by the Spearman rank correlation test. Differences in the EBV genome numbers in culture medium were analyzed using Student's *t*-test. All statistical analyses were performed using SPSS[®] software (SPSS, Chicago, IL, USA).

Results

Table 2 summarizes the results of EBV and *P. gingivalis* detection in saliva from periodontitis patients. Using PCR analysis, EBV was detected in 16 out of 33 (48.5%) subjects with periodontitis. The mean proportion of *P.*

Table 2 Levels of Salivary EBV and *P. gingivalis* in Periodontitis Patients

Variable	Positive (n =16)	Negative (n =17)
EBV proportions (%)	$7.8 \pm 3.7 \times 10^{-2}$	0*
Number of EBV/ml	$7.11 \pm 3.80 \times 10^5$	0*
<i>P. gingivalis</i> proportions (%)	0.25 ± 0.42	$0.02 \pm 0.03^*$
Total number of bacteria/ml	$9.02 \pm 0.28 \times 10^8$	$9.26 \pm 0.47 \times 10^8$
Age	46.4 ± 3.7	53.2 ± 3.1

All values are expressed as mean \pm SE. *Statistically significant differences between EBV-positive and EBV-negative periodontitis patients, $P < 0.01$ (Mann-Whitney's *U*-test).

Table 3 Effects of sonicate from three strains of *P. gingivalis* and PMA on EBV reactivation

Variable	Induction levels (fold)
Control	1
ATCC 49417	$7.06 \pm 1.19^*$
381	$5.76 \pm 2.50^*$
ATCC 33277	$9.14 \pm 3.09^*$
PMA	$57.50 \pm 23.10^*$

All values are expressed as mean \pm SE. *Statistically significant difference between control and experimental groups, $P < 0.01$ (Student's *t*-test).

gingivalis in saliva from EBV-positive patients was significantly higher than that in saliva from EBV-negative patients. There was significant relation between the proportions of *P. gingivalis* and EBV ($n = 33$, $r = 0.57$, $P < 0.01$).

An EBV-positive B-cell line was used to determine whether *P. gingivalis* sonicate induces reactivation of EBV, using real-time PCR to measure the virus genome in culture medium. A significant increase in EBV numbers was observed after the stimulation with *P. gingivalis* sonicate (Table 3). No statistically significant associations were found among the three bacterial strains studied.

Discussion

Using conventional PCR, an association between the presence of subgingival EBV and elevated levels of periodontal pathogens, such as *P. gingivalis*, has been reported in adults with periodontitis (10). Our results confirm these findings, with a higher proportion of *P. gingivalis* detected in EBV-positive subjects using real-time PCR. Several mechanisms have been suggested by which

EBV may contribute to overgrowth of periodontal pathogens. For example, gingival EBV infection may impair the local host defense via viral interleukin-10 mediated immunosuppression, polyclonal B lymphocyte activation or induction of anti-neutrophil antibodies (7), so permitting overgrowth of *P. gingivalis* in EBV positive subjects.

The present study also demonstrated that stimulation with *P. gingivalis* sonicate results in EBV reactivation. Disruption of EBV latency is mediated by ZEBRA, the protein product of the immediate-early EBV gene, BZLF1 (18). EBV reactivation may occur as a result of concurrent infection, fever, drug usage, tissue trauma, emotional stress, exposure to UV light or a compromised host immune defense (19). In vitro, PMA, a potent activator of protein kinase C (PKC), induces reactivation of EBV (20), and it is known that *P. gingivalis* has potential to activate PKC signaling (21). These results suggest that the interaction between EBV and *P. gingivalis* is bi-directional, with EBV reactivation suppressing host defenses and permitting overgrowth of *P. gingivalis*, and with *P. gingivalis* having the potential to induce EBV reactivation.

In conclusion, the present findings support a positive association between EBV and *P. gingivalis*. We suggest that EBV in periodontal tissue may facilitate overgrowth of *P. gingivalis* which, in turn, induces EBV reactivation. Further study is needed to clarify the relationship between EBV and *P. gingivalis*.

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