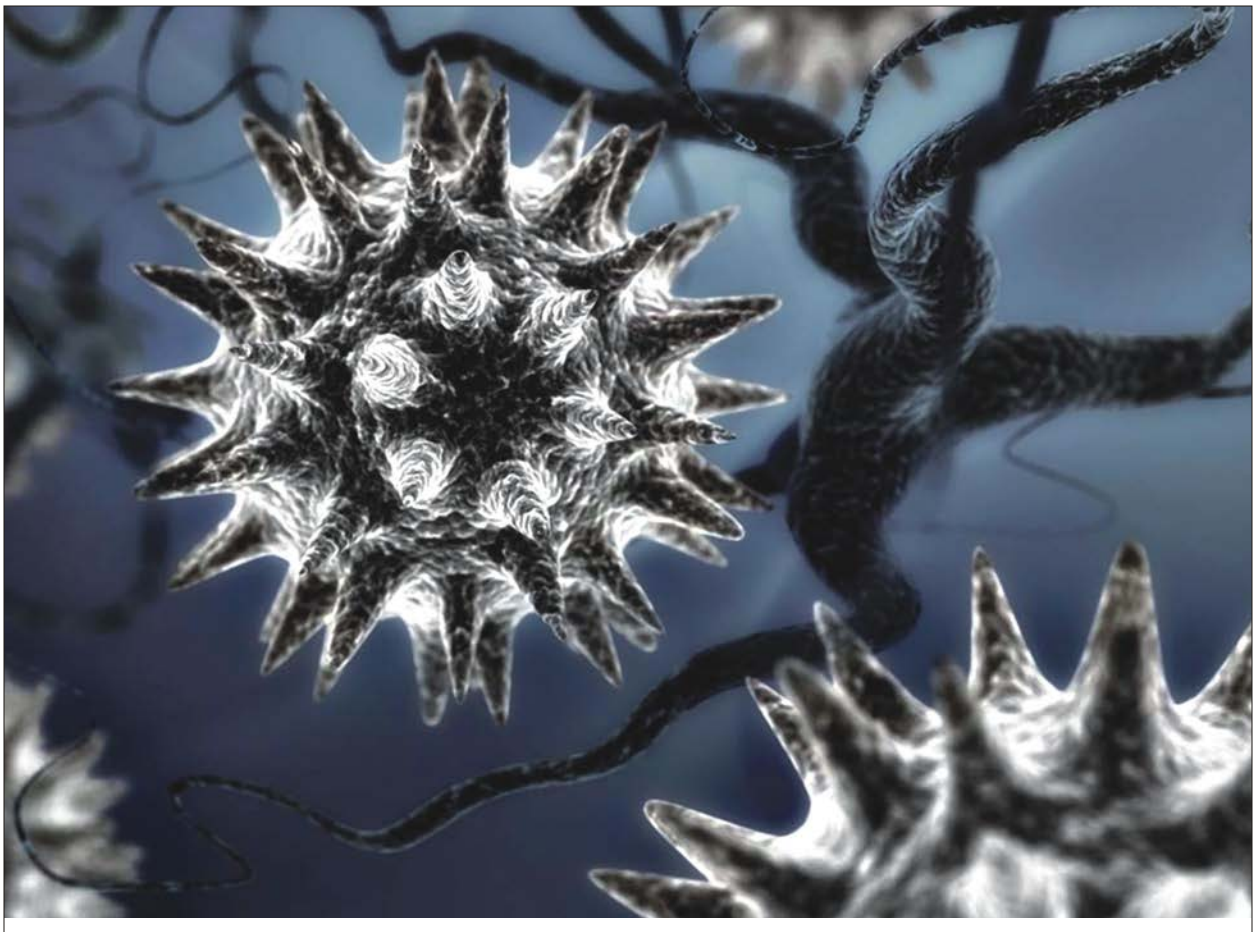

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Herpesviruses at Human Aggressive Periodontitis Sites*

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Abstract: The aim of present study was to evaluate the frequency of 3 members of herpes virus family in the subgingival plaque samples from periodontal pockets and healthy sites of an aggressive periodontitis population. Fifteen subjects with aggressive periodontitis took part in this study. Subgingival plaque samples were harvested from their 3 deepest pockets and 3 healthy sites and subjected to a nested Polymerase Chain Reaction (PCR) technique to detect Human Cyto-megallo Virus (HCMV), Epstein Barr virus 1 (EBV-1) and Herpes Simplex Virus 1 (HSV-1). Seven individuals revealed HCMV in their diseased sites while only 2 of these individuals revealed HCMV at their healthy sites too. Three individuals showed EBV-1 only at their diseased sites, while the remaining diseased sites and all healthy sites were negative for this virus. Finally, HSV-1 was found only at one of the diseased samples, but none of the healthy samples. Members of the Herpesvirus family might be recovered at periodontal sites. The prevalence of these viruses seems to be greater at diseased sites than healthy sites. Herpesviruses might be involved in the pathogenesis of periodontal disease.

Key words: PCR, EBV-1, HSV-1, HCMV, aggressive periodontitis

Introduction

Periodontitis is an infection that is caused by certain pathogenic bacteria. Recently, herpes viruses has been purported to be playing roles in the pathogenesis of periodontitis (Contreras *et al*., 1999; Contreras *et al*., 2000). Theoretically, herpes viruses may contaminate cells and change their structural and defensive mechanisms within periodontal as well as other tissues, hence, altering the ability of these tissues to withstand the bacterial aggression.

Recent studies have revealed inter-relationship between 8 members of the herpes virus family and destructive periodontal disease. Genome from human cytomegallovirus (HCMV) and Epstein-Bar virus type 1 (EBV-1) have been frequently detected in lesions of advanced adult periodontitis (Contreras and Slots, 1996; Parra and Slots, 1996; Contreras *et al*., 1999), localized juvenile periodontitis (Michalowicz *et al*., 2000; Ting *et al*., 2000) periodontitis associated with Papillon-Lefevre syndrome (Velazco *et al*.,1999) and Down syndrome (Hanookai *et al*., 2000), HIV associated periodontitis (Mardirossian *et al*., 2000) and the ANUG (Contreras *et al*., 1997). Close correlation has been reported between HCMV and EBV-1 in one hand and periodontitis in another (Britt and Alford, 1996;

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Boeckh and Boivin,1998; Contreras *et al*., 2000). Other members of the herpes virus family might also be observed in periodontitis; however, their relationship with the disease causation has not been clarified yet (Contreras *et al*., 2000). Recently, herpesviruses have been associated with eusophagitis, pneumonia and some other diseases known to have only a bacterial origin (Slots and Contreras, 2000).

Most individuals experience a primary infection with HCMV and EBV during their early childhood. This usually lacks any clinical signs. When HCMV has clinical features, it usually resembles the infectious mononucleosis. The EBV has also been related to Burket lymphoma and eusophagal carcinoma (Purtilo, 1980). After primary infection with Herpes Simplex Virus (HSV), it remains in the patient's body in a latent and clinically undetectable form for long periods of time. The HSV, EBV and HCMV may occasionally exit their latent form resulting in a clinically evident infection. Reactivation of HSV often follows a change in systemic conditions or depletion of local immunity, particularly, cell mediated immunity. During its primary infection, the HCMV may be seen in epithelial cells of various tissues and in later phases it may be seen in monocytes and macrophages. DNA from HCMV may also be observed in the PMNs during the active infection. Other cells, which might be contaminated by MCHV, are T cells, endothelial cells, fibroblasts, salivary gland cells and the central nervous system cells. The HCMV is able to stimulate protein synthesis and cell proliferation in infected cells. Moreover, it may upregulate the expression of IL1-β, TNF-α and IFN-γ in mononuclear cells. EBV contaminates oropharynx epithelium during the primary infection and the B-lymphocytes during the later phases. The ability of herpes viruses to contaminate PMNs, macrophages and lymphocytes decreases the host resistance and hence, may favour the localization and growth of periodontopathic bacteria (Slots and Contreras, 2000).

Sabiston (1986) proposed an association of HCMV and ANUG. Contreras *et al*. (1997). demonstrated that the GCF from black children with ANUG and malnutrition showed a greater prevalence of HCMV, EBV and HSV. Rones *et al*. (1983) showed that epithelial and fibroblast cells were highly susceptible to HSV infection. Parra and Slots (1996) demonstrated the presence of HCMV, EBV, HSV, HPV and HIV in gingival crevicular fluids of advanced periodontitis patients and concluded that human viruses might be recovered at a rather large frequency from periodontitis lesions. Contreras and Slots (1998) studied the incidence of HCMV, EBV1, EBV2 and HSV in subgingival samples of 27 adult periodontitis and gingivitis patients using PCR technique and demonstrated that 89% of the test group have one to five of the eight members of herpesvirus family in their deep pockets. Mandirossan *et al*. (2000) evaluated periodontal pockets and gingival tissues of periodontal lesions and reported the presence of HCMV and EBV1 in these samples. They suggested that the HHV6, HHV7 and HHV8 might also be recovered from gingival tissues.

So far, few studies have reported the incidence of herpes virus family in healthy sulci and diseased pockets of the same individuals. One possibility is that when herpes viruses are activated and exit from regional neural ganglions, some of the gingival pockets might become contaminated by these viruses and host defence cells in gingival crevice become invaded by the viruses, enhancing the virulence of invading bacteria, or inducing self-destructive host-mediated hyper-inflammatory reactions which results in destruction of periodontal tissues. The aim of this study is to evaluate the presence of HCMV, EBV1 and HSV1 in periodontal pockets from subjects with aggressive periodontitis patients as compared to healthy gingival crevices of the same subjects.

Materials and Methods

Fifteen subjects with aggressive periodontitis took part in the study. The patients were selected from subjects referred to the Periodontology Department of Mashhad Dental School, Iran. The

patients had an age range of 17-33 years with little plaque and calculus, which did not commensurate with their extensive periodontal destruction. The patients were systemically healthy and had not been using antibiotics within the past 6 months prior to the study. An informed consent was taken from all patients. From every patient, the 3 deepest pockets, with ≥6 mm and Bleeding On Probing (BOP) were selected using sterile paper points. Within the same patient, 3 healthy gingival sulci (≤3 mm and no BOP) were also selected and sampled. For sampling, first the supragingival plaque was removed by a curette and the region was dried using a gentle blast of air. One paper point was gently inserted into the bottom of pocket and kept in place for 30 sec. The 3 diseased sites samples were pooled and transferred into a sterile plastic microtube. The healthy sites were also sampled accordingly. In the lab, the paper points were transferred into micro centrifuge vials containing 10 mL TE buffer, (Tris HCl, 1000 M EDTA, pH = 7.5) until homogenized. A nested Polymerase Chain Reaction (PCR) technique, as described by Contreras *et al*. (2000) was used to detect herpes virus genomes. DNA extraction was performed using the preferential binding of DNA to the silica particles at high concentration of guanidine thiocyanite. Optimised condition for the PCR was obtained using the negative and positive controls. Each PCR tube contained 2-5 μL of the sample DNA, 20-30 pmol of primer, 0.2 mM dNTP (dATP, dCTP, dTTP and dGTP), 1.25 U *Taq* polymerase enzyme (Promeger Co) and various concentration of $MgCl_2$. A nested PCR amplification was accomplished using a DNA thermal cycler. The 1st round included an initial denaturation at 95°C for 1 min. The following cycles included of 30 cycles of denaturation at 94°C for 1 min, annealing at 50-60°C for 1 min and extension at 72°C for 1 min. The final extension was carried out for 1 min at 72°C. For the 2nd amplification round the following materials were used: 2-5 μ mL of the 1st PCR product, 40-100 pmol of each of the inner primers, dNTP, *Taq* polymerase, *Taq* polymerase buffer and $MgCl_2$. It was performed at 35 cycles each including of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min. A final extension was carried out at 72°C fot 1 min. PCR products were subjected to electrophoresis using 1.5% agarose gel and EDTA buffer. The gels were stained using 0.5 μg mL^{-1} of ethidium bromide for DNA visualization. The final product was visualized under 300 nm UV light. A 1 kb DNA ladder was used as a marker of molecular size.

Statistical Analysis

A 2×2 contingency table was created for the absence or presence of each viruses at either healthy or control sites. McNemar test would be suitable for this data type. If the assumptions of the McNemar test did not hold true, then a Fisher's exact probability was calculated. The analysis was carried out using SPSS software (version 10).

Results

Table 1 shows the clinical and viral profiles of study sites. Of the 15 aggressive periodontitis patients, 14 had at least 3 deep pockets and 3 healthy sites. One patient (case No.14) had no healthy site. Therefore, only gingivitis sites from this patient were sampled as non-periodontitis sites. Seven individuals revealed HCMV in their diseased sites while only 2 of these individuals revealed HCMV at their healthy sites too (Table 2). The remaining 8 persons were negative for this virus at both diseased and healthy sites. The difference between healthy and diseased sites was marginally significant ($p = 0.063$, McNemar test). Three individuals showed EBV-1 only at their diseased sites,

Table 1: Clinical and viral profiles of study sites

				Periodontitis sites				Healthy sites			
No	Initials	Sex	Age (years)	Mean PD (mm)	HCMV	EBV1	HSV	Mean PD (mm)	HCMV	EBV1	HSV
1	MA	Female	33	6.7	-	-	-	2.7	-	-	-
2	MR	Male	28	7	-	-	-	2.3	-	-	-
3	SY	Female	30	7.7	+	-	-	2.3	-	-	-
4	AS	Female	17	6.7	+	-	-	3	+	-	-
5	ZM	Female	24	6.3	+	+	-	3	-	-	-
6	IS	Male	34	7	+	+	-	3	-	-	-
7	MG	Female	28	6.3	-	-	+	3	-	-	-
8	AM	Male	30	6.7	+	-	-	3	-	-	-
9	AH	Female	19	7	-	-	-	3	-	-	-
10	TF	Female	35	6.7	-	-	-	2.3	-	-	-
11	MA	Female	35	7	+	+	-	2.3	-	-	-
12	HS	Male	34	6.7	+	-	-	3	+	-	-
13	AT	Male	25	7.3	-	-	-	2	-	-	-
14	NA	Female	31	6.3	-	-	-	1.3	-	-	-
15	FS	Female	32	7	-	-	-	2.3	-	-	-

+: Positive for virus, -: Negative for virus

Table 2: Cross tabulation table showing the association between periodontal status and HCMV presence

		Non-periodontitis sites		
		HCMV absent	HCMV present	Total
Periodontitis Sites	HCMV absent	8 (53.33%)	0 (0%)	8 (53.33%)
	HCMV present	5 (33.33%)	2 (13.33%)	7 (46.66%)
Total		13 (86.66%)	2 (13.33%)	15 (100%)

McNemar Test exact significance p = 0.063

Table 3: Cross tabulation table showing the association between periodontal status and EBV-1 presence

		Non-periodontitis sites		
		EBV1 absent	EBV1 present	Total
Periodontitis sites	EBV1 absent	12 (80%)	0	12 (80%)
	EBV1 present	3 (20%)	0	3 (20%)
Total		15 (100%)	0	15 (100%)

McNemar Test exact significance p = 0.000

while the remaining diseased sites and all healthy sites were negative for this virus (Table 3). The difference was significant (p = 0.001). Finally, HSV-1 was found only at one of the diseased samples, but none of the healthy samples.

Discussion

The results of our study demonstrated that the occurrence of HCMV and EBV1 was greater in periodontitis sites as compared to healthy sites. This corroborates with several previous reports (Slots and Contreras, 2000). Contreras *et al*. (2000) showed that biopsy specimens from periodontitis sites might be a reservoir for the Herpes virus family members. The exact role of herpes viruses in the pathogenesis of periodontitis remains to be established. However, a few theories could be put forward and tested in the future studies. One possibility is that PMNs are infected when the HCMV or EBV1 is reactivated. Anti-PMN antibodies have been recognized due to EBV infection (Purtilo, 1980; Khanna *et al*.,1995; Platcher *et al*., 1995) and cause dysfunction of PMNs. The evidence of impaired

PMN function in the pathogenesis of aggressive periodontitis is ample. The possibility exists that, upon reactivation of HCMV or EBV1, PMNs in the periodontal area are affected and show impaired cellular functions such as chemotaxis or phagocytosis. This may, in turn, give rise to the overgrowth of putative periodontal pathogens such as *Porphyromonas gingivalis, Prevotella intermedia* and *Actinobacillus actinomycetemcomitans*. In fact, Contreras *et al* demonstrated that the subgingival recovery of these micro organisms were in concordance with the presence of HCMV and EBV1 in human adult periodontitis (Contreras *et al*., 1999).

The other possibility is the infection of macrophages, lymphocytes and other major contributing cells in the releases of proinflammatory cytokines. It has been shown that the HCMV-infected monocytes/macrophages produce IL1-β, TNF-α and other cytokines (Kapasi and Rice, 1988; Geist *et al*., 1994). The proinflammatory cytokines are known to be involved in tissue destruction and bone loss during the active phase of periodontitis lesions. Furthermore, over-production of these cytokines may be responsible for abnormal tissue turn over. Another possibility, therefore, is that fibroblast cells within the periodontium might become infected with the viruses and lose the control over the tissue remodelling and turn over by over production of cytokines or under production of various growth factors such as transforming growth factor -beta or insulin like growth factor. The epithelial cells infection, which is common in the reactivation of HSV1 cold sore, is also likely to happen in the periodontium. The contamination of junctional epithelial cells of the periodontium may give rise to increased release of cytokines or adhesion molecule such as IL-8 or ICAM-1 from these cells. Any of these events, if happens individually, may have profound effect on the host resistance to the microorganisms. However, it should be pointed out that any dysfunction due to viral contamination of a single cell type within periodontal tissues most likely will affect the whole cellular function via the closely inter-related network of cytokines. It is also likely, that more than one cell is affected by these viruses. Finally, one could argue that the presence of viruses in periodontal pockets may have nothing to do with host cells. Since the viruses can only survive as intracellular parasites, they may not be able to stand-alone independently in the gingival tissues or crevice. They might, however, have infected some bacteria from subgingival dental plaque. This may theoretically result in the change in some of the bacterial factors including their virulence factors favouring an increased pathogenecity of the bacteria. This possibility seems unlikely because the herpes viruses have also been detected in high proportions of gingival biopsies. The penetrance of bacteria into the tissues, although has been reported previously, does not look a common phenomenon and hence may be a poor explanation for frequent detection of viruses inside the tissues.

Although, previous reports (Contreras *et al*., 2000) indicated a rather high recovery of virus genomes from gingival biopsies, our results, consistent with the results of Saygun *et al*. (2002), demonstrated that GCF samples render a rather considerable recovery of herpes viruses from periodontitis sites (Saygun *et al*., 2002). Since, the PMNs are the major cellular element of the GCF and the viruses are intracellular parasites, one could speculate that it is most likely that it is the PMNs that are affected by the viruses.

Ting *et al*. (2000) reported that in an individual infected with the HCMV, not all the periodontal pockets might be positive for the virus. If the viruses have an important role in the etiopathogenesis of the periodontal lesions, this is consistent with the site-specific random burst hypothesis for the progression of periodontal disease. According to this paradigm, while the viruses are reactivated in some pockets initiating the rapid progression of disease in those sites, the others sites might not be affected by the virus and remain quiescent for some period of time.

Further studies are warranted. These studies will need focusing on the isolation of different cell types from periodontium and perform sensitive tests such as nested PCR for the detection of herpes virus genomes. Such investigations might provide greater insight as to which cellular component of the gingival tissues is affected by members of this family of viruses.

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