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Role of herpes simplex-1, epstein barr and human cytomegalo viruses in aggressive periodontitis

Sneha Dani*, CD Dwarakanath, Ramesh Alampalli, Kishore Bhat, AN Savitha, Gayatri Gundannavar

MDS (Periodontics) K.L.E Institute of Dental Sciences and Hospital
MDS (Periodontics) Vishnu Dental College
MDS (Periodontics) Oxford Dental College, Hospital and Research Centre
MD (Microbiology)Maratha Mandal's Ngh Institute of Dental Sciences & Research Centre
MDS (Periodontics) Oxford Dental College, Hospital and Research Centre
MDS (Periodontics) Oxford Dental College, Hospital and Research Centre
**Corresponding author E-mail: drsnehadani@gmail.com*

Abstract

Objectives: Aggressive periodontitis is characterized rapid and severe destruction of the tooth supporting tissues with a complex and unclear etiopathogenesis. The present study was designed to identify the role of herpesviruses in pathogenesis of aggressive periodontitis.
Material and methods: Study included 15 subjects diagnosed with aggressive periodontitis (group A) and 15 periodontally healthy subjects (group B). Subgingival plaque was collected from the deepest periodontal pocket and gingival tissue biopsy from the adjacent interdental papilla. Results analyzed by polymerase chain reaction.
Results: EBV detected in 67% plaque and 73% tissue samples in group A, 7% plaque and none in the tissue samples in group B. HCMV identified in 53% plaque and 20% of tissue samples in group A, 20% plaque and 7% tissue samples in group B. HSV-1 found in 47% plaque and 13% tissue samples in group A, 13% plaque and 47% tissue samples in group B.
Conclusion: An increased prevalence of EBV, HCMV and HSV-1 in group A in comparison to healthy controls was observed. Only EBV showed significant difference between both groups. Despite similarity in pathogenic traits between herpesvirus diseases and Periodontitis, delineating the exact role that viruses play in the etiopathogenesis of aggressive periodontitis is difficult.

Keywords: *Aggressive periodontitis, Epstein barr virus (EBV), herpes simplex virus-1 (HSV), human cytomegalovirus (HCMV), polymerase chain reaction (PCR).*

1 Introduction

Aggressive periodontitis comprises of a group of rare, often severe, rapidly progressing form of periodontitis, characterized by early age of clinical manifestations and a tendency for cases to aggregate in families.[1] It generally affects 0.1–2% of adolescents and young adults, although prevalence as high as 6–7% has been reported.[2] It has a complex pathogenic microbiota.[3] Studies have focused principally upon the role of bacteria. Most researchers agree that gram negative anaerobes such as Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans, Fusobacterium nucleatum etc play an important role in its etiopathogenesis.[4] It is suggested that bacterial pathogens are necessary antecedents for the development of aggressive periodontitis. However, despite a long history of research into its pathobiology, probable cause about its etiology still remains elusive.[5]
Just the mere presence of these virulent bacteria does not provide sufficient explanation of several clinical features of aggressive periodontitis like early onset of disease, rapid destruction around teeth exhibiting scanty plaque, lack of overt gingival inflammation, propensity to proceed with periods of exacerbation and remission. The interplay between Aggregatibacter actinomycetemcomitans and antibodies seems even less capable of explaining other characteristics including the manifestation of cemental defects and the mirror like periodontal destruction on contralateral teeth. Often, some of these cases turn refractory defying all attempts to arrest the disease.[6]
It is unlikely that a single agent or even a small group of pathogens are the sole cause or modulators of this heterogeneous disease. This has led many investigators to look for a possible viral cause in the development of

aggressive periodontitis.[7] Since the mid1990s viruses have emerged as putative pathogens in aggressive periodontitis, in particular Human cytomegalovirus (HCMV), Epstein-Barr virus (EBV) and Herpes simplex virus-1 (HSV-1).
Herpesviruses seem to be the most important DNA viruses in oral pathology. It has been shown that these viruses act synergistically with Aggregatibacter actinomycetemcomitans and Porphyromonas gingivalis and influence both occurrence and extent of aggressive periodontitis. The herpes viral–bacterial hypothesis of periodontitis proposes that an active herpesvirus infection initiates periodontal tissue breakdown and that host immune responses against the herpesvirus infection are an important component of the etiopathogenesis of the disease. Herpesviruses can exert direct cytopathic effects on fibroblasts, keratinocytes, inflammatory and bone cells. They trigger a release of pro-inflammatory cytokines that have the potential to activate osteoclasts and matrix metalloproteinases which plays a role in pathogenesis of periodontitis.[8].
Molecular based detection methods especially polymerase chain reaction (PCR), have greatly facilitated investigations of herpesviruses in oral diseases. It has therefore the highest sensitivity of any microbiological method.
In the light of the above facts, the present study is designed to elucidate the periodontopathic role of herpesviruses in aggressive periodontitis which may help our further understanding of the disease pathogenesis and thereby aid in the prevention and treatment of the disease.

2 Study population and methods

2.1 Patient selection

The present study included 30 subjects (range 18-30 years) of which 15 subjects (group A) were diagnosed with aggressive periodontitis, both localized and generalized (four women, eleven men; mean age 27.27±2.76 years) and 15 were healthy individuals (group B, six males, nine females; mean age 23.13±3.11 years). Aggressive periodontitis subjects were selected as per the criteria's laid down by AAP 1999 classification.[9] All patients were systemically healthy and had not undergone periodontal therapy in the previous 6 months and anti-viral therapy in the previous 3 months. Smokers and patients on corticosteroids and chemotherapeutic agents were excluded from the study. Ethical clearance was obtained and the study was conducted in accordance with the Helsinki Declaration of 1975, as revised in 2000. After thorough explanation of the procedure, a written informed consent was obtained from all patients.
Clinical parameters assessed for the study were plaque index (Silness and Loe) [10], gingival index (Loe and Silness) [11], gingival bleeding index (Ainamo and Bay) [12], probing pocket depth [13] and clinical attachment level.

2.2 Method of collecting the sample for viral assay

Prior to sampling, supra gingival plaque was gently removed with a sterile cotton pellet. The sample site was isolated with cotton rolls and dried. In subjects with aggressive Periodontitis, a sterile periodontal curette was inserted to the deepest periodontal pocket to remove sub gingival plaque sample and the tissue sample collected from the adjacent interdental papilla. In healthy subjects, plaque sample was collected from healthy gingival sulcus of upper first molar and tissue sample from the adjacent interdental papilla. The samples were stored in TRIS-EDTA BUFFER (T.E buffer) medium until DNA extraction.

2.3 DNA extraction procedure from tissue and plaque sample

The samples were crushed with a sterile blade. They were then transferred to a tube containing T.E. buffer and centrifuged at 50000 rpm for 2 min. The supernatant obtained was discarded. 200 µl of fresh T.E. buffer was added and centrifuged for 3-4 minutes. The above procedure was repeated 5 times with fresh T.E. buffer. The supernatant was discarded and 500 µl of lysis buffer 1 was added, centrifuged at 5000 rpm. The supernatant obtained was discarded and 50 micro liter of lysis buffer [2] was added and centrifuged. 5 µl of proteinase-K was added to the centrifuge, kept in water bath for 2 hours, then in boiling water bath for 10 minutes at 900C. The DNA extracted was stored at -200C till the amplification process.[14]

2.4 PCR amplification process

A multiplex PCR was performed on the entire collected sample for quantitative assessment of HSV-1, HCMV and EBV.
The HSV-1 primer sequences were 5'- CGTACCTGCGGCTCGTGAAGT-3' forward and 5'-AGCAGGGTGCTCGTGTATGGGC-3'reverse; for HCMV 5'-ACGTGTTACTGGCGGAGTCG-3'forward and 5'-TTGAGTGTGGCCAGACTGAG-3' reverse; EBV 5'-AGCACTGGCCAGCTCATATC-3' forward and 5'-

TTGACGTCATGCCAAGGCAA-3' reverse. The expected sizes of the amplified sequences in the HSV-1, CMV and EBV were 271, 368 and 326 base pairs respectively.
A master Mix (25µl) containing all of the components necessary to make new strands of DNA in the PCR process was prepared. The Master Mix reagents included:
1) 1X Buffer: - Keeps the master mix at the proper pH to facilitate the PCR reaction.
2) 200 uM Deoxynucleotides:- Provides both energy and nucleosides for the synthesis of DNA. It is important to add equal amounts of each nucleotide (dATP, dTTP, dCTP, dGTP) to the master mix to prevent mismatches of bases.
3) 0.5 pmole Primers (Bioserve India Pvt ltd):- Short pieces of DNA (20-30 bases) that bind to the DNA template allowing Taq DNA polymerase enzyme to initiate incorporation of the deoxynucleotides.
4) 2.5U/100ul Ampli Taq polymerase:- A heat stable enzyme that adds the deoxynucleotides to the DNA template.
5) 0.05-1.0ug Template DNA:- The DNA which will be amplified by the PCR reaction.
6) Water
PCR amplification conditions were, pre-denaturation step at 95˚C for 3 minutes, followed by denaturation at 95˚C for 30 secs, annealing at 54˚C for 30 secs and primer extension at 72˚C for 30 secs, in an automated cycler. The final extension was performed for another 7 minutes, repeating 45 cycles of amplification.

2.5 Detection of the amplified products

The amplified PCR products were subjected to electrophoresis on 2% agarose gel solidified in a casting tray. A loading dye- Bromophenol blue and glycerol were added to the gel. The final amplified products were visualized with UV light after staining with ethidium bromide. Electrophoresis was performed with Genei machine electrophoresis apparatus.

3 Statistical tests

Mann Whitney U test was used to find the significance of periodontal parameters between the two groups. Z - Test for proportions has been used to find the significance of the proportion of EBV, HCMV and HSV-1 between the two groups. [15]

4 Results

Table 1 describes clinical characteristics of the study subjects having aggressive periodontitis or periodontal health. Except for age, the means of all clinical variables were significantly different between the two groups.
Table 2 compares the presence of all the three viruses between the two groups in plaque samples. Higher number of HCMV was seen in group a (53%) when compared to group B (20%). Presence of EBV was positive in greater number of aggressive periodontitis subjects (67%) than healthy controls (7%). HSV-1 virus was recorded more frequently in the test group (47%) than in the control group (13%). Out of the three viruses, the presence of EBV alone in plaque samples was statistically significant.
As with the plaque samples, HCMV was detected more often in tissue samples of group a (20%) when compared to the healthy group (7%). Similar to plaque samples, it was seen that EBV occurred more frequently in the tissue samples of group a subjects (73%) also. Healthy subjects showed no EBV in the tissues. Lower number of positive results for HSV-1 was obtained in the tissues of group a (13%) in comparison to healthy group (47%). The results between the two groups pertaining to the presence of EBV in the gingival tissues showed statistical significance. (Table 3)
When both plaque and tissue samples of group a subjects were compared, it was seen that HCMV and HSV-1 were detected more in plaque than in the tissues. Slightly reduced number of positive results for EBV was obtained in plaque than in the tissues. (Table 4)
Table 5 compares the results between plaque and tissue samples in healthy group. HCMV and EBV were seen more in plaque than in the tissues. However HSV-1 was obtained more in plaque than in the tissues. No statistically significant difference was noted

Table 1: Comparison of clinical parameters between the two groups

Parameter	Group	Mean	Std dev	Mean difference	t	P-Value
Plaque Index	Aggressive Periodontitis	1.20	0.56	1.165	8.125	<0.001*
	Healthy	0.03	0.02			
Gingival Index	Aggressive Periodontitis	1.66	0.49	1.628	12.940	<0.001*
	Healthy	0.03	0.04			
PPD (mm)	Aggressive Periodontitis	3.56	1.12	2.193	7.213	<0.001*
	Healthy	1.36	0.19			
Gingival Bleeding Index (%)	Aggressive Periodontitis	96.33	14.20	94.328	25.543	<0.001*
	Healthy	2.01	1.70			
CAL (mm)	Aggressive Periodontitis	2.33	1.40	2.330	6.444	<0.001*
	Healthy	0.00	0.00			

The difference between the two groups with respect to presence of EBV in plaque samples was statistically significant.

Table 2: comparison of results (for each type of virus) between the two groups in plaque samples

Virus	Aggressive Periodontitis (n=15)		Healthy (n=15)		Z	P-Value
	n	%	N	%		
HCMV	8	53%	3	20%	1.89	0.058
EBV	10	67%	1	7%	3.41	0.001*
HSV-1	7	47%	2	13%	1.99	0.050

The difference between the two groups with respect to presence of EBV in plaque samples was statistically significant.

Table 3: Comparison of results (for each type of virus) between the two groups in tissue samples

Virus	Aggressive Periodontitis (n=15)		Healthy (n=15)		Z	P-Value
	N	%	n	%		
HCMV	3	20%	1	7%	1.07	0.283
EBV	11	73%	0	0%	4.17	<0.001*
HSV-1	2	13%	7	47%	-1.99	0.050

The difference between the two groups with respect to presence of EBV in tissue samples was statistically significant.

Table 4: Comparison of the results between plaque and tissue sample in aggressive periodontitis group

Virus	Plaque (n=15)		Tissue (n=15)		Z	P-Value
	n	%	n	%		
HCMV	8	53%	3	20%	1.89	0.058
EBV	10	67%	11	73%	-0.40	0.690
HSV-1	7	47%	2	13%	1.99	0.050

Table 5: Comparison of the results between plaque and tissue sample in healthy group

Virus	Plaque (n=15)		Tissue (n=15)		Z	P-Value
	n	%	n	%		
HCMV	3	20%	1	7%	1.07	0.283
EBV	1	7%	0	0%	1.02	0.309
HSV-1	2	13%	7	47%	-1.99	0.050

5 Discussion:

Aggressive periodontitis is a disease attributable to multiple infectious agents and interconnected cellular and humoral host immune responses. However, it has been difficult to unravel the precise role of various putative pathogens and host responses in its pathogenesis. These uncertainties galvanized efforts to find additional etiologic factors. Recent studies have revealed an association between herpesvirus (HCMV, HSV-1, and EBV) and aggressive periodontitis.[16] It was perceived that identification of herpesvirus factor in development of aggressive periodontitis may help clarify hitherto unexplained clinical and pathologic characteristics of the disease.[5]

Kornman stated that paper points do not yield enough material for microbial analysis. Also studies have shown that paper points collect plaque only from the outer layer of the pocket wall and are less from the apical part of the pocket,

where more viruses could be expected. In comparison to paper points, curettes collect plaque from the entire pocket lining.[17]

Polymerase chain reaction (PCR) based methods are more specific and sensitive as they are based on detection of gene specific DNA sequences. Hence, it represents a valuable method to determine the viral load in diseased and healthy sites.

In group A, higher prevalence of EBV was found followed by HCMV and HSV-1. However in group B, HCMV was more frequently identified than HSV-1 and EBV. Increased presence of viruses in group a subjects when compared to group B is in accordance with the previous studies.[18], [19], [20] Aggressive periodontitis patients' exhibit variable amount of plaque and gingival inflammation. Also, there is an increase in the number of certain periodontopathic bacteria such as Porphyromonas gingivalis, a key periodontal pathogen. Previous studies have revealed a bi-directional interaction between herpesviruses and Porphyromonas gingivalis.[21].

Highest genomic copies of EBV (2.6x105counts/ml), when compared to HCMV (8x104counts/ml) and HSV-1(3.9x104counts/ml) were seen in the plaque samples of group a subjects. The corresponding values obtained in group B for EBV, HCMV and HSV-1 was 4.2x103, 2.6x104 and 4.6x105counts/ml respectively.

Highest DNA copies of EBV found in plaque samples is consistent with the literature data.[22, 23] However studies by Slots and Contreras have found more of HCMV than EBV and HSV-1 in aggressive periodontitis.[24] These differences could be attributed to the sampling technique. Most of the previous studies have collected pooled plaque sample from two or more periodontal sites.[21] As periodontitis is a site specific disease, the method of sample collection could be an important determinant of the type of herpesvirus recovered. Another possible explanation could be due to the ability of herpesviruses to establish lifelong persistent infections that involves an asymptomatic phase interrupted by periods of recrudescence where viral replication and possibly clinical disease manifests. It could be assumed that at the time of sampling, these viruses would have been in inactive phase or the sample might have been collected from stable periodontal sites.

In this study gingival tissue biopsy samples were taken along with plaque samples. In group a, 73% of the samples were positive for EBV, 20% for HCMV and 13% for HSV-1. In group B, HCMV was present in 7% and HSV-1 in 47%. None of the tissue samples in the healthy group demonstrated presence of EBV. Herpesviruses show tropism for cells of the immune system such as macrophages and lymphocytes, which are increased substantially in aggressive periodontitis.[19] Thus, increase in the number of these inflammatory cells could be responsible for more viruses detected in group A.

Genomic copies of EBV, HCMV and HSV-1 found in the tissue samples of group a subjects were 4.9x105, 7.9x103 and 1.8x103 counts/ml respectively. Group B subjects showed more of HSV-1 (2.1x104counts/ml) than HCMV (1.8x103counts/ml).

When plaque and tissues samples collected from group A were compared, EBV was more prevalent in the tissues than in the plaque. This is in agreement with the previous reports.[19] B lymphocytes which are the main reservoir of EBV are seen abundantly in the gingival tissues of aggressive periodontitis lesions.

HCMV and HSV-1 were more prevalent in the plaque than in the tissue samples of aggressive periodontitis subjects. These results are not in harmony with the earlier studies. HCMV mainly infects periodontal macrophages and lymphocytes, which predominate in gingival tissue, and may only sporadically; infect polymorphonuclear leukocytes, which predominate in periodontal pockets. HSV-1 mainly resides in T- lymphocytes that are present more in the gingival tissues. The variation reported could be attributed to the uneven distribution of T-lymphocytes and macrophages in the gingival tissues of aggressive periodontitis lesions.[25] Some specimens may have been harvested inadvertently from gingival tissue areas with little or no HCMV and HSV-1 present, and missed gingival domains with significant occurrence of these viruses.

Prevalence of herpesvirus carriage varies by age, country, and region and population subgroup. The genotype distribution and seroprevalence of these viruses also differs among population subgroups.[26] It is impossible to ascertain whether viruses were active in the diseased individuals or merely present during the earlier stage of periodontal destruction. Despite a large body of evidence supporting the fact that herpesviruses play an important role in the pathogenesis of aggressive periodontitis along with the periodontal pathogens, well designed longitudinal studies in diverse populations are lacking to corroborate these findings.

6 Conclusion

Based on the findings from this study it could be concluded that herpesvirus may be associated with aggressive periodontitis but whether they take active participation in the destruction process still remains to be elucidated. Despite intriguing similarity in pathogenic traits between classic herpesvirus diseases and periodontitis, the body of data pertinent to the herpesvirus hypothesis of aggressive periodontitis is still small. It is not known whether viruses play an active role in periodontal tissue destruction or they are secondary invaders in response to reduced host immunity

following periodontal infection. Also the prevalence of herpesviruses in periodontal sites could be dependent upon a number of factors such as the type of disease, method of detection, ethnicity and genetic predisposition of the individual. Thus, delineating the exact role that viruses play in the etiopathogenesis of aggressive periodontitis is difficult.

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References

[1] MS Tonetti, A Mombelli, Aggressive periodontitis, Clinical Periodontology and Implant dentistry - Vol 1, 5th edn, Blackwell publishing Ltd, 2008; 19-28.
[2] JM Albandar, EM Tinoco, Global epidemiology of periodontal diseases in children and young persons, Periodontol 2000 29 (2002) 153–176.
[3] JJ Kamma, J Slots, Herpesviral–bacterial interactions in aggressive periodontitis, J Clin Periodontol 30 (2003) 420–426.
[4] J Slots, M Ting, Actinobacillus actinomycetemcomitans and Porphyromonas gingivalis in human periodontal disease: Occurrence and treatment, Periodontol 2000 20 (1999) 82-121.
[5] A Kubar, I Saygun, A Ozdemir, M Yapar, Slots J, Real-time polymerase chain reaction quantification of human cytomegalovirus and Epstein–Barr virus in periodontal pockets and the adjacent gingiva of periodontitis lesions, J Periodont Res 40 (2005) 97–104.
[6] SS Socransky, AD Haffajee, JM Goodson, J Lindhe, New concepts of destructive periodontal disease, J Clin Periodontol 11 (1984) 21–32.
[7] M Yapar, I Saygun, A Ozdemir, A Kubar, S Sahin, Prevalence of human herpesviruses in patients with aggressive periodontitis, J Periodontol 74 (2003) 1634–1640.
[8] J Slots, Oral viral infections of adults, Periodontol 2000 49 (2009) 60-86.
[9] Consensus report on aggressive periodontitis, Ann periodontal 4 (1999) 53.
[10] P Silness, H Loe, Periodontal disease in pregnancy, Correlation between oral hygiene and periodontal conditions, Acta Odontol Scand 22 (1964) 121 – 35.
[11] H Loe, J Silness Periodontal disease in pregnancy. Prevalence and severity, Acta Odontol Scand 21 (1963) 533 – 51.
[12] J Ainamo, I Bay, Problems and proposals for recording gingivitis and plaque, Int Dent J 25 (1975) 229 – 235.
[13] L Nibali, D Pometti, Y-K Tu, N Donos, Clinical and radiographic outcomes following non-surgical therapy of periodontal intrabony defects, J Clin Periodontol 38 (2011) 50-57.
[14] CH Shin, GS Park, KM Hong, MK Paik, Detection and typing of HSV-1, HSV-2, CMV and EBV by quadruplex PCR, Yonsei Medical J 44 (2003) 1001-1007.
[15] B Rosner, Fundamentals of Biostatistics, 5th edn, Duxbury 2000; 80.
[16] 16. G Kaarthikeyan, ND Jayakumar, O Padmalatha, S Varghese, K Sheriff, Prevalence of TT Virus in patients with chronic periodontitis, patients with aggressive periodontitis and healthy controls, Braz J Oral Sci 9 (2010) 94-97.
[17] PM Jervoe-Storm, H Alahdab, M Koltzscher, R Fimmers R, S Jepsen, Comparison of curette and paper point sampling of subgingival bacteria as analysed by real-time polymerase chain reaction, J Periodontol 78 (2007) 909-917.
[18] M Idesawa, N Sugano, K Ikeda, M Oshikawa, M Takane, K Seki, Ito K, Detection of Epstein Barr virus in saliva by real-time PCR, Oral Microbiol Immunol 19 (2004) 230–232.
[19] J Slots, JJ Kamma, C Sugar, The herpesvirus–Porphyromonas gingivalis–periodontitis axis. J Periodont Res 38 (2003) 318–323.
[20] Rees TD. A profile of the patient with periodontal disease? Periodontol 2000 2003;32:9–10.
[21] Saygun I, Kubar A, Ozdemir A, Yapar M, Slots J. Herpesviral–bacterial interrelationships in aggressive periodontitis. J Periodont Res 2004; 39:207–212.
[22] A Rotola, E Cassai, R Farina et al, Human herpesvirus 7, Epstein Barr virus and Human cytomegalo virus in periodontal tissues and periodontally diseased and healthy subjects. J Clin Periodontol 35 (2008) 831-837.
[23] SB Mangalekar, DC Sharma, SB Reddy, SR Patil, K Bhat, Herpesviruses in chronic and aggressive periodontitis patients in an Indian population, J Oral Sci 51 (2009) 79-86.
[24] M Radvar, HR Arab, Herpesviruses at human aggressive periodontitis sites, Intl J Virol 6 (2010) 13-19.
[25] AC Johannessen, R Nilsen, T Kristoffersen, GE Knudsen, Variation in the composition of gingival inflammatory cell infiltrates, J Clin Periodontol 17 (1990) 298–305.
[26] J Slots, Herpesviral–bacterial interactions in periodontal diseases, Periodontol 2000 52 (2010) 117–140.