SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PH.D.)

PREVALENCE AND ACTIVITY OF HERPESVIRUSES IN APICAL PERIODONTITIS

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1. INTRODUCTION

Herpesviruses are supposed to be involved in several oral diseases in immunocompetent hosts, including mucosal inflammations, irreversible pulpitis, apical and marginal periodontitis. Apical periodontitis, the inflammation of the apical area of the tooth, is characterized by a polymicrobial infestation, with a dominance of opportunistic Gram-negative bacteria. Nevertheless, beside the bacterial flora, a pathogenic role of human herpesviruses such as Epstein-Barr virus (EBV) and human cytomegalovirus (HCMV) has been implicated recently.

Human herpesviruses are highly prevalent worldwide in the human population, more than 90% of the adults are infected with EBV and more than 50% are HCMV seropositive. HHV-6 is also frequent in European countries and the USA, the seropositivity rates range from 75% to 95% in both adults and children. Like other herpesviruses, these viruses are capable of persisting in a lifelong latency after primary infection. EBV initially causes lytic infection in oronasopharyngeal epithelial cells. Thereafter the infection spreads to B lymphocytes, the host cells that will serve as major site of latent EBV infection. In EBV infected B lymphocytes, the virus establishes type I latency, which does not influence host cell functions. The only viral antigen expressed at this stage is EBV nuclear antigen-1 (EBNA-1).

HHV-6 has two subtypes, A and B (HHV-6A and HHV-6B, respectively). The primary target cells are the CD4+ T lymphocytes for both subtypes, HHV-6A can replicate also in CD8+ T cells and natural killer (NK) cells. Monocytes, macrophages, dendritic cells (DC) and bone marrow progenitor cells (CD34+) serve as sites of latent infections for both subtypes.

HCMV establishes latent infection mainly in monocytes and also in CD34+ bone marrow myeloid progenitor cells and dendritic cell (DC) precursors in healthy seropositive carriers. A low level persistent viral replication can occur in salivary glands and the renal tubular epithelium.

Primary herpesviral infection is followed by the state of viral latency in the infected host. Reactivation, which can occur spontaneously, results in increased production of viral antigens, which are immediately targeted by cell mediated immunity in immunocompetent hosts. Nevertheless, cell mediated immunity can be impaired systemically or locally by several factors. General factors suppressing cell mediated responses include fever, drugs, tissue trauma, stress and infections. The anatomic situation can help locally to hide from immunity and concurrent microbial infections in infectious foci can also modulate locally the host defenses.
Although apical periodontitis is generally a chronic and asymptomatic inflammation, acute exacerbations may occur with acute pain, discomfort on biting and increased periapical bone resorption. Based on the hypothesized pathogenesis of acute exacerbations there is an influx of leukocytes during periapical inflammation and the mononuclear cells may carry latent herpesviruses. The inflammatory environment and the local endopathogenic bacteria may promote herpesviral activation from latency. The herpesviral infection may alter the production of a number of cytokines, e.g. tumor necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β), interleukin (IL)-1β, IL-8, IL-10, IL-15, which may cause local immunosuppression and immune-mediated tissue destruction. This may increase the virulence of the local endopathogenic bacteria. The cumulative effects of endopathogenic bacteria, herpesviruses and immune-mediated tissue destruction may lead to acute exacerbations of chronic apical periodontitis.

In EBV infected B-lymphocytes the virus can stay in a latent stage or a lytic reactivation can occur with new virion production and the lysis of the host cell. It is also noteworthy that EBV has a unique capability to exist in three different latency stages depending on the cellular environment. Based on preliminary literature data, we supposed rather changes in latency stage than reactivation in apical periodontitis. In type III latency all EBV nuclear antigens (EBNA-1, -2, -3A, -3B, -3C, -LP) and latent membrane proteins are expressed. Type III latency remarkably change the functions of the host cell through the alteration of cytokine and chemokine production. EBV infected B-lymphocytes in type III latency stage are known to produce TNF-α, TGF-β and IL-10. TNF-α has the ability to increase bone resorption and can induce powerful hyperalgesia. TGF-β is able to impair antiviral host defenses through repression of lymphocyte proliferation, cytotoxic T-cell functions, toll-like receptor (TLR) signaling, and the activation of macrophages and dendritic cells. IL-10 is also able to inhibit macrophage activation and the antigen presenting functions of macrophages and dendritic cells.

HHV-6 can suppress the secretion of IL-12 a critical mediator of Th1 polarized antiviral responses. HHV-6 infection is also able to suppress interferon-γ and IL-2 production by the host cell and can induce the production of TNF-α, IL-1β, IL-8 and IL-15. In dendritic cells, HHV-6 reduced the expression of MHC class I molecules and the stimulation of allogenic T-cell proliferation. The major receptor molecule for both HHV-6A and B is the ubiquitous CD46 human glycoprotein, which has an important protective effect against autologous complement activation.
Since HHV6 down-regulates the expression of the receptor, it makes the infected tissues more susceptible to complement-mediated cellular damage.

Regarding HCMV, the differentiation of infected monocytes into tissue macrophages or the maturation of infected DCs is followed by reactivation of latent infection. The released infectious virions are capable of infecting further macrophages, T-lymphocytes, endothelial and connective tissue cells. The lytic infection of the cytopathic HCMV results in dysfunction of macrophages. HCMV is able to suppress chemokine-mediated migration of infected monocytes and inhibit their ability to recruit other immune cells. The downregulation of MHC molecules in HCMV-infected DCs and macrophages serves as a viral strategy to escape recognition by the immune system.

2. OBJECTIVES

1. To determine the prevalence of EBV, HHV-6 and HCMV by the detection of viral DNA in apical periodontitis samples and healthy controls.

2. To determine the activity of EBV, HHV-6 and HCMV by the detection of mRNA in apical periodontitis samples and healthy controls.

3. To analyze the association of herpesviral prevalence and activity with patient history, clinical and radiological symptoms.
3. METHODS

3.1. Collection and classification of specimens

A total of 40 apical periodontitis samples and 40 healthy pulp samples were collected. Patients were seeking dental care at the Department of Oral and Maxillofacial Surgery, Faculty of Dentistry, University of Debrecen. The Local Ethical Committee approved the study (approval number: 2885-2008). Patients filled informed consents and they were anonymously coded for identification. Patients with apical periodontitis also filled questionnaires, which contained questions about general diseases, medications, history of the involved tooth and related symptoms.

The inclusion criteria for patients were as follows: individuals in good health condition (American Society of Anaesthesia I or II) with no severe systemic disease, previous history of symptoms of the affected tooth and requirement for surgical apicoectomy because of the failure of conventional root canal therapy. The previous history of symptoms and the radiographs showing periapical radiolucent area indicated that the patients suffered from chronic apical periodontitis. Patients with either poor general status or systemic diseases or periodontally involved teeth (probing depth > 4 mm) were excluded.

Samples with apical periodontitis were divided into symptomatic and asymptomatic groups according to the symptoms of the involved teeth. Symptomatic lesions were characterized by acute pain, discomfort on biting, sensitivity by percussion or palpation at the apical region of mucosa. The asymptomatic lesions did not have any clinical symptoms with the exception of periapical radiolucent area on radiographs. Based on the radiographic size of the periapical lesion, samples were divided into two subgroups: more than or equal to 5 mm (≥5 mm, large) and less than 5 mm (<5 mm, small) diameter of lesions.

Samples with apical periodontitis were collected during apico-ectomy, while healthy pulp samples were originated from impacted third molars.

3.2. Nucleic acid extraction and PCR reactions

Homogenized tissue samples were divided into two portions: one for RNA and the other for DNA isolation. DNA was extracted by High Pure Viral Nucleic Acid Kit (High Pure Nucleic Acid Kit, Roche) according to the manufacturer’s instructions. Total RNA was extracted with TRI Reagent (Sigma) according to the manufacturer’s protocol. The extracted RNA was turned to
complementary DNA (cDNA) by using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) with random hexamers. The effectiveness of DNA isolation was controlled with PCR detection of human β-globin housekeeping gene. For the verification of RNA isolation PCR detection of a constitutively expressed acidic ribosomal phosphoprotein PO (h36B4) messenger RNA was used. Nested PCR amplification were used to increase the sensitivity of virus detection. Five microliters of the extracted DNA or cDNA was used as template in the first PCR round and 3 μl PCR product of the first round was used as template in the second round. The final PCR volume was 20 μl using 1 U Red Taq Ready Mix (Sigma) according to the manufacturer’s instructions.

Primers for EBV mRNA and DNA were amplified the EBNA-2 and BamH1-W fragments of the EBV genome, respectively. Primers for HHV-6 DNA amplification were designed to amplify sequences from the IE fragment, which is a conservative regulator region in the HHV-6 genome. Primers for HCMV mRNA and DNA were designed to amplify sequences from the pp65 gene, which is a lower matrix phosphoprotein and transcribed late during the infection cycle. PCR products were electrophoresed in 1.5% agarose gels containing ethidium bromide (0.5 μg/ml).

3.3. Statistical analysis
In most cases the association between apical periodontitis lesion and the presence of herpesviral infection was tested with Yates corrected khi-square statistical test. If an expected cell value was less than five in a 2x2 contingency table, Fisher exact test was used. For multivariate analysis, logistic regression statistics were used, where 95% confidence intervals and significance of odds ratios were calculated. In all statistical tests, significance was accepted on 5% level.
4. RESULTS

A total of 40 apical periodontitis samples were collected from 36 patients (age 18-80 years, mean age 49 years) and 40 healthy pulp control samples from 25 patients (age 17-29 years, mean age 23 years).

4.1. EBV prevalence and activity

EBV DNA was detected in apical periodontitis lesions at significantly higher frequency than in healthy pulp controls (72.5% vs. 2.5%, p<0.0001). Also the local activation of EBV infection as detected by EBNA-2 mRNA expression was significantly more frequent in apical periodontitis lesions (50% vs. 2.5%, p<0.0001).

Diseased samples were divided into symptomatic (n=17) and asymptomatic (n=23) subgroups according to the symptoms of the involved teeth. Both subgroups contained EBV DNA and EBNA-2 mRNA at significantly higher rates compared to healthy controls (p<0.0001). Although the occurrence of the EBV DNA was higher in symptomatic (82%) than in asymptomatic (65%) patient samples, the difference was not significant (p=0.30). Nevertheless, EBNA-2 RNA expression data indicated that symptomatic lesions more likely had active EBV infections than asymptomatic lesions (71% vs. 35%, p<0.055).

Lesions with apical periodontitis were classified according to the radiological size of periapical bone destruction: 19 lesions were less than 5 mm (small) and 21 were more than or equal to 5 mm (large). EBV DNA (91% vs. 53%, p=0.02) and EBNA-2 mRNA (76% vs. 21%, p=0.002) were significantly more frequent in large lesions compared to small lesions. Both EBV DNA (p=0.02) and EBNA-2 mRNA (p=0.002) were significantly associated with large lesion size.

4.2. Determinants of symptomatic apical periodontitis

Our results suggest that symptomatic manifestation and large lesion size tend to coexist and therefore their association with EBV infection was evaluated in uni- and multivariate analysis. In the univariate analysis large lesion size and EBNA-2 mRNA appeared to be significant determinants for symptomatic apical periodontitis. However, the adjusted odds ratios of the multivariate analysis indicated that the strength of association has been reduced to a tendency level (p=0.08) for lesion size and the association between EBNA-2 mRNA and symptomatic
manifestation has been eliminated. Since these parameters mutually weakened each other’s effect, we hypothesized a complementary effect of large lesion size and EBNA-2 mRNA expression upon symptomatic manifestation. Therefore, the involved parameters were combined and the odds ratios were calculated separately in subgroups with none, any and both of the supposed disease determinants. Indeed, the co-occurrence of large lesion size and EBNA-2 mRNA was strongly associated (OR=8.80) with symptomatic manifestation.

Neither the age nor the gender of the patients, nor tooth localization (left-right, upper-lower), nor type of teeth (incisors, canines, premolars) influenced the lesion size, the symptoms, the incidence of EBV DNA and EBNA-2 mRNA, respectively.

4.3. Prevalence and activity of HHV-6

HHV-6 DNA was observed in significantly higher frequency in apical periodontitis than in healthy pulp (20% vs. 2.5%, p=0.03). HHV-6 subtype A and subtype B were present at equal number in apical periodontitis lesions: 4 of the 40 samples harboured HHV-6A and 4 other samples contained HHV-6B, none of the samples had dual infection with both subtypes. There was only one HHV-6 positive control sample, which was infected by subtype A. We could not detect HHV-6 mRNA expression in either pathological or control samples.

Both symptomatic and asymptomatic lesions contained HHV-6 DNA at increased frequency (29% and 13%, respectively) compared to healthy controls (2.5%). Logistic regression analysis revealed a significant correlation between HHV-6 infection and the severity of the symptoms (p=0.008), i.e. progressing severity of the disease was associated with an increasing frequency of HHV-6 occurrence. A characteristic subtype distribution was observed: HHV-6B infections were significantly associated (p<0.01) with symptomatic large sized lesions.

There was an increased occurrence of HHV-6 in both large sized (24%) and small sized (16%) lesions compared to healthy controls (2.5%). Logistic regression analysis revealed a significant correlation between the lesion size and HHV-6 infection (p=0.018), i.e. increasing lesion size was associated with an increasing frequency of HHV-6 occurrence. Again a characteristic subtype distribution was observed: all four HHV-6B infections were detected in large sized lesions, while three of the four HHV-6A infections were present in small sized lesions.
4.4. Prevalence and activity of HCMV

Only four apical periodontitis lesions carried HCMV DNA and although HCMV was not found in healthy pulp controls the difference was not significant (10% vs. 0%, p=0.12). HCMV DNA was more frequent in asymptomatic lesions than in symptomatic ones (13% vs. 6%) and in large sized lesions compared to small ones (14% vs. 5%). We not could detect HCMV mRNA in either pathological or healthy samples.

4.5. Viral coinfections

Altogether 31 (77%) apical periodontitis samples harboured at least one of the tested herpesviruses. Single infection by HHV-6 was observed in one apical periodontitis sample harbouring subtype A and the remaining seven HHV-6 infections were found together with EBV. A similar distribution was found for HCMV, one single infection and three coinfections were found. One lesion harboured triple EBV-HHV-6-HCMV infection, which was an asymptomatic and small sized lesion. EBNA-2 mRNA was present in every multiple infected lesion. A similar rate of symptomatic manifestation was detected in lesions with single and multiple herpesviral infections (42% vs. 55%, p=0.73).

4.6. Patients with multiple lesions

Four patients provided multiple apical periodontitis samples. Both clinical and virological analysis of these samples revealed that separate lesions of a patient can differ from each other. The multiple lesions had different HHV-6 and EBV status in one and two patients, respectively. Ten persons from the control group provided multiple samples. Even in the control group, one person had one HHV-6 subtype A infected lesion and another lesion uninfected by the investigated herpesviruses.
5. DISCUSSION

5.1. EBV prevalence and activity
In this study not only the activity of EBV infection was analyzed but also the occurrence of EBV DNA was measured to detect the virus prevalence regardless of the infectious stage. The results showed that approximately two thirds of the EBV DNA positive periapical lesions had EBNA-2 mRNA expression. We pointed out that symptomatic manifestation and large lesion size tended to coexist and therefore their association with EBV infection was evaluated in multivariate statistical analysis. According to the results of the multivariate analysis, we hypothesized a complementary effect of large lesion size and EBNA-2 mRNA expression upon symptomatic manifestation. Subgrouping of the lesions according to this hypothesis allowed us to prove that large sized apical periodontitis lesions aggravated with EBV infection at type III latency stage will most likely be symptomatic.

Based on previous researches, we expected low viral load in the samples analyzed in this study. Therefore, we applied nested PCR to increase the specificity and sensitivity of the virus detection, and the cycle number in the primary PCR was limited to 30 to reduce the risk of in-procedure cross-contamination.

A further merit of this study is to provide data obtained on healthy tissue samples. We detected only a negligible occurrence of EBV DNA and EBNA-2 mRNA in healthy pulp tissues. Based on these results, the most probable source of EBV infection in apical periodontitis is the immigrating B-lymphocyte population.

5.2. HHV-6 prevalence and activity
Human herpesvirus 6 (HHV-6) infection was known to occur in marginal periodontitis, but no information has been available so far on the presence of this virus in chronic apical periodontitis. Our study was the first that determined the PCR prevalence of HHV-6 subtype A and B in chronic apical periodontitis and analyzed their relation with clinical symptoms. The prevalence rate of HHV-6 was sufficient to demonstrate a significant association between HHV-6 infection and apical periodontitis. On the other hand, like EBV and HCMV, HHV-6 had also a negligible occurrence in the healthy pulp samples of the study.
It is well documented that the two subtypes of HHV-6 exhibit different biological features and disease associations. Subtype B is more commonly associated with febrile illnesses during the first two years of age (e.g. exanthema subitum). In contrast, HHV-6A seems to play a role in neurologic and immunologic diseases. Our results also indicate that HHV-6 subtypes had a characteristic distribution: while subtype A was found in small or asymptomatic lesions and in a control pulp sample, subtype B was significantly associated with large sized symptomatic lesions, i.e. the biologic diversity of the two HHV-6 subtypes appeared also in apical periodontitis.

Our HHV-6 mRNA results indicated a latent stage of infection. Nevertheless, also the latent HHV-6 infection can cause phenotypic and functional changes in host cells, through the alteration of cytokine and chemokine signaling.

5.3. HCMV prevalence and activity
The literature on the prevalence of HCMV shows two peaks of distribution. Several studies found the frequencies of HCMV mRNA to be between 40-100% in apical periodontitis lesions. Our data are more consistent with the findings of other studies, which found HCMV occurrence between 0% and 15.9% in apical periodontitis. Since these investigations uniformly tested the pp65 matrix protein coding sequences on HCMV DNA or mRNA, the observed biological diversity is probably due to recent alteration of HCMV epidemiology at certain geographical regions.

5.4. Patients with multiple lesions
There were four patients with multiple lesions, each of them provided two samples. The results and observations on these lesions revealed that lesions in the same patient could differ in herpesviral infection, lesion size and symptomatic manifestation. This finding suggests that the local inflammatory environment is the major determinant of herpesviral involvement in apical periodontitis. Even if the host organism is infected by a herpesvirus, the healthy periapical tissues seem to be uninfected. Since the apical periodontitis lesions of separate teeth can develop independently, the characteristics of separate lesions of the same patient can also differ in local herpesviral infection.
5.5. Viral coinfections

Our results showed that EBV was the most frequent herpesvirus in apical periodontitis, followed by HHV-6 and HCMV. Both beta herpesviruses tended to occur in coinfection with EBV. All EBV/HHV-6 and EBV/HCMV coinfections were found together with type III EBV latency. Although HHV-6 infection itself had a significant trend to associate with increasing lesion size and progressing symptoms, further investigations are needed to clarify whether HHV-6 infection is an EBV dependent or an independent marker of symptomatic manifestation.

The coinfection of HHV-6 with EBV is also known in other diseases, such as infectious mononucleosis and Hodgkin’s disease. In EBV infected B lymphocytes a phenotype change from type I latency to type III latency may occur. Type III latent EBV infection is transient and uncommon in lymphatic tissues of immunocompetent persons. The local environment of inflamed periapical tissues may facilitate the persistence of lymphatic cells with type III EBV latency, which may secrete inflammatory cytokines, i.e. TNF-α, TGF-β and IL-10. The Th1 suppressive cytokine secretion by EBV and HHV-6 infected cells might promote local escape from immune surveillance in a synergistic way. EBV and HHV-6 infections may contribute to the pathogenesis of periapical flare ups: the cumulative effects of local immunosuppression, immune-mediated tissue destruction and endopathogenic bacteria may result in increased periapical bone resorption and progressing clinical symptoms.
6. CONCLUSIONS

1. EBV was found to be the most frequent herpesvirus in apical periodontitis lesions, followed by HHV-6 and HCMV, while all three herpesviruses had a negligible occurrence in healthy pulp tissues.

2. Our results confirmed that approximately two thirds of the EBV DNA positive apical lesions had EBNA-2 mRNA expression, which is characteristic for type III latency stage. This suggests changes in latency stage in EBV infected B lymphocytes during periapical inflammation.

3. Large sized apical lesions aggravated with EBV infection at type III latency will most likely be symptomatic.

4. HHV-6 subtypes had a characteristic distribution: while subtype A was found in small or asymptomatic lesions, subtype B was significantly associated with large sized and symptomatic lesions.

5. HHV-6 and HCMV tend to occur in coinfection with EBV and all coinfections were found together with type III EBV latency.

6. Different periapical lesions of the same patient can differ in clinical and virological characteristics indicating that the lesions of separate teeth can develop independently of each other. This finding suggests that the local inflammatory environment is the major determinant of herpesviral involvement in apical periodontitis.
Apical periodontitis, the inflammation of the apical area of the tooth, is characterized by a polymicrobial infestation, with a dominance of opportunistic Gram-negative bacteria. Nevertheless, a pathogenic role of human herpesviruses such as Epstein-Barr virus (EBV) and human cytomegalovirus (HCMV) has been implicated recently. The aims of this study were to determine the prevalence, activity and disease association of EBV, HCMV and HHV-6 in apical periodontitis.

40 samples with apical periodontitis (17 symptomatic and 23 asymptomatic) and 40 healthy pulp controls were collected. EBV, HCMV and HHV-6 prevalences were measured by PCR detection of the viral DNA and viral activity was tested by reverse transcription PCR amplification of viral mRNA.

EBV DNA and EBNA-2 mRNA were found in apical periodontitis lesions at significantly (p<0.0001) higher frequencies (72.5% and 50%, respectively) than in controls (both 2.5%). Presence of EBV DNA in apical lesions was significantly associated with large (≥5mm) lesion size (p=0.02). Symptomatic manifestation was significantly associated with the co-occurrence (OR=8.80, CI95%: 1.69-45.76) of EBNA-2 mRNA and large lesion size. HHV-6 DNA was observed in significantly higher frequencies in apical periodontitis samples than in controls (20% vs. 2.5%, p=0.03). Further classification of apical lesions revealed that subtype B of HHV-6 was significantly associated with large sized and symptomatic lesions (p<0.01). Occurrence of HCMV infection was rare in both apical lesions (10%) and controls (0%). EBV (72.5%) was the most frequent herpesvirus in apical periodontitis, followed by HHV-6 (20%) and HCMV (10%).

Our findings suggest that EBV and HHV-6B infections are frequent events in apical periodontitis, especially in large sized and symptomatic lesions and symptomatic manifestation was likely to occur if a large sized apical periodontitis lesion is aggravated with active EBV infection.
8. PUBLICATIONS

8.1. Publications related to the dissertation


8.2. Posters and lectures related to the dissertation


Candidate: Katinka Hernádi
Neptun ID: TCWHEF
Doctoral School: Doctoral School of Pharmaceutical Sciences

List of publications related to the dissertation


Total IF: 4,708
Total IF (publications related to the dissertation): 4,708

The Candidate’s publication data submitted to the Publication Database of the University of Debrecen have been validated by Kenezy Life Sciences Library on the basis of Web of Science Scopus and Journal Citation Report (Impact Factor) databases.

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