

DETECTION OF HUMAN CYTOMEGALOVIRUS AND EPSTEIN-BARR VIRUS IN CORONARY ATHEROSCLEROTIC TISSUE

Ana Vitória Imbronito¹, Silvia Linardi Marcelino¹, Sabrina Rosa Grande¹, Fabio Daumas Nunes², Giuseppe Alexandre Romito¹

¹Departamento de Estomatologia, Faculdade de Odontologia, Universidade de São Paulo, São Paulo, SP, Brasil; ²Departamento de Patologia Bucal, Faculdade de Odontologia - Universidade de São Paulo, São Paulo, SP, Brasil.

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ABSTRACT

Previous studies indicated that patients with atherosclerosis are predominantly infected by human cytomegalovirus (HCMV), but rarely infected by type 1 Epstein-Barr virus (EBV-1). In this study, atheromas of 30 patients who underwent aortocoronary bypass surgery with coronary endarterectomy were tested for the presence of these two viruses. HCMV occurred in 93.3% of the samples and EBV-1 was present in 50% of them. Concurrent presence of both pathogens was detected in 43.3% of the samples.

Key words: atherosclerosis; cytomegalovirus; Epstein-Barr virus.

Atherosclerosis, considered the main underlying disease responsible for cardiovascular and cerebrovascular events, has multifactor etiology. Obesity, hyperlipidemia, diabetes, hypertension and cigarette smoking account for 50 to 60 percent of cases of cardiovascular disease (10). Atherosclerosis essentially develops from a mild chronic inflammatory reaction to an injury and there is evidence that infectious microorganisms, such as *Chlamydia pneumonia*, *Helicobacter pylori*, periodontal pathogens, human cytomegalovirus (HCMV), hepatitis A virus, enterovirus and Epstein-Barr virus (EBV) may play a pathogenic role (4,15,20).

The latent state of viral infections might differ from that of bacterial infections, and Herpesviridae in particular exert direct proatherogenic effects on injured vessel walls (5). Viruses may directly infect arterial intima, inducing or accelerating atherosclerosis. Moreover, viral infections may promote atherosclerosis by increasing the expression of adhesion

molecules and inflammatory cytokines (13) and procoagulant effects (8), and enhancing cholesterol uptake (14) as well as auto-immune response to infection (6). There is experimental evidence that HCMV can infect human coronary smooth muscle cells and initiate viral replication (12), while EBV can have a lifelong persistence in B cells (17). HCMV has been implicated in atherogenesis, and restenosis after coronary angioplasty and transplant vasculopathy (9). Seroepidemiological studies have implicated EBV in atherosclerosis (19) although only one study detected EBV in atherosclerotic aortic tissue (18).

The objective of the present study was to detect HCMV and EBV-1 in atherosclerotic plaques from patients who underwent coronary endarterectomy.

This study was approved by the Ethical Committee of the Evangélico Hospital (Londrina – Paraná) and all patients signed the informed consent form. Thirty patients (mean age

*Corresponding Author. Mailing address: Disciplina de Periodontia – FOU SP Av Prof Lineu Prestes, 2227 CEP 05508-000 Cidade Universitária São Paulo-Brasil.; Tel/Fax.: 55-11-3091-7833.; E-mail: anaimbronito@uol.com.br

61+11.5 years, 76.6% male) with symptomatic coronary artery disease were enrolled. All patients underwent aortocoronary bypass surgery with coronary endarterectomy. An atheroma was taken from each patient's infarct-related artery and the tissue was stored at -70°C with RNAlater® (Applied Biosystems) until DNA extraction. DNA was extracted from each atheroma using Chargeswitch® Gdna Tissue Kits (Invitrogen, São Paulo, Brazil) according to the manufacturer

instructions.

Nested-PCR was used to detect viral DNA using specific oligonucleotides for EBV-1 and HCMV. EBV-1 primers amplified a fragment of EBV nuclear antigens EBNA 2 and HCMV primers amplified a fragment of glycoprotein B gene. The outer and inner oligonucleotides primers used in the tests, the temperature of melting and the product length are described in Table 1.

Table 1. PCR primers, temperature of melting (TM) and amplicon length (base pairs) in the identification of the infectious agents

Infectious agent (blast accession)	Primer	TM °C	Amplicon length	Reference
Epstein Barr virus type I (S71027)	1° round 5'-AGGGATGCCTGGACACAAGA-3' 5'-TGTGCTGGTGTCTGCTGGTGG-3'	56	602	7
	2° round: 5'-AACTTCAACCCACACCATCA-3' 5'-TTCTGGACTATCTGGATCAT-3'	46	116	
Human Cytomegalovirus (X17403)	1° round 5' GAGGACAACGAAATCCTGTTGGGCA-3 5'-TCGACGGTGGAGATACTGCTGAGG-3'	56	150	3
	2° round 5'-ACCACCGCACTGAGGAATGTCAG-3' 5'-TCAATCATGCGTTTGAAGAGGTA-3'	50	100	

PCR was performed with a final volume of 25µl mixture containing 25pmol of each primer (Invitrogen, Carlsbad CA), 1U Taq platinum DNA polymerase (Invitrogen), 50 mM MgCl₂, 0.05mM dNTP mix and 1 to 10 µl of extracted DNA sample.

The PCR conditions were optimized for the different primers using as a basis one initial denaturation cycle at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min for the first round of EBV-1, at 55°C for 1 min for the second round of EBV-1, at 56°C for 50 s for the first round of HCMV, at 57°C for 50 s for the second round of HCMV, extension at 72°C for 1 min, and a final extension step at 72°C for 7 min.

All specimens were tested in duplicates to each of the target microorganisms. Negative and positive controls were included in every reaction: DNA free mix as negative control, and DNA from EBV positive cell B lymphoma diagnosed in

our laboratory as positive control for EBV. The Laboratory of Virology of University of São Paulo provided the control for HCMV. Detection of PCR products was performed by electrophoresis in a 2% agarose gel containing 0.5µg/ml ethidium bromide.

A total of 30 coronary atheroma specimens were investigated. PCR for HCMV yielded positive results for 28 samples (93.3%), whereas EBV-1 was detected in 15 samples (50%). At least one of the viruses was detected in every C. Concurrent infection with HCMV and EBV-1 was observed in 13 specimens (43.3%) (Table 2).

Table 2. Positivity for Epstein-Barr-1 virus (EBV-1) and human cytomegalovirus (HCMV) in 30 atheroma samples.

	viral detection
EBV-1+/ HCMV -	2
EBV-1 -/HCMV+	15
EBV-1 + /HCMV+	13

This study detected DNA from HCMV in most coronary atherosclerotic lesions and indicated that concurrent infection with EBV-1 is also frequent. The high incidence of HCMV in coronary arteries wall of patients suffering from atherosclerosis has also been reported by Hendrix *et al.* (15), although Shi & Tokunaga (18) detected HCMV in 40% of atherosclerotic aortic tissue. In our study, 50% of the coronary artery atheromas were positive to EBV-1. HCMV DNA in atherosclerotic tissue was reported for the first time by Shi & Tokunaga (18) in 8 out of 10 atherosclerotic aortic tissues from autopsies.

Most studies implicate HCMV infection with greater risk of accelerated atherosclerosis following heart transplantation (12) and restenosis following coronary atherectomy (22). Grahame-Clarke *et al.* (11) showed that relatively young asymptomatic individuals seropositive for HCMV have abnormal vascular reactivity consistent with endothelial dysfunction. Their results also suggest that previous HCMV infection might be associated with increased atherosclerotic burden, as assessed by coronary artery calcification. HCMV infection has been shown to upregulate IL-6 gene expression (16) and cytokine production itself (1). Mediated by this systemic inflammatory process, HCMV infection might contribute to the atherosclerotic and atherothrombotic processes, as reported by Blankenberg *et al.* (2). These authors concluded that HCMV seropositivity in patients with an inflammatory response is independently associated with future cardiac mortality. Since this study detected HCMV in 93.3% of coronary atheromas, this virus may be strongly implicated in the etiology of atherosclerosis.

Our results indicate that 43.3% of the samples contained both HCMV and EBV-1 DNA. It has been shown that the aggregate number of pathogens to which an individual had been exposed might be involved in the pathogenesis of atherosclerosis (infectious burden). This concept was demonstrated in cross-sectional as well as prospective studies (5, 23, 24). Rupprecht *et al.* (20) identified EBV as the strongest independently predictive of future death from cardiovascular causes and showed for the first time that prior

infection to EBV may influence the course of atherosclerosis. Herpesviridae in a latent state may contribute to the atherogenic process independently of an inflammatory response. A controlled trial using antiviral substances for the prevention of atherosclerosis and restenosis after coronary bypass surgery or angioplasty seems justifiable.

This study confirms that HCMV and/or EBV-1 DNA are present in coronary arteries atheromas and the association of both viruses is frequent. Clinical implications of this association and the pathogenic impact on atherosclerosis should be further investigated.

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