

Detection of Human Herpesvirus 6 DNA but not Human Herpesvirus 7 or 8 DNA in Atherosclerotic and Nonatherosclerotic Vascular Tissues

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ABSTRACT

Introduction: Various viral infections are thought to play a role in the development of atherosclerosis. A number of studies suggest that certain viruses from the Herpesviridae family in particular may lead to atherosclerosis.

Methods: We investigated the presence of human herpesvirus 6 (HHV-6), human herpesvirus 7 (HHV-7), and human herpesvirus 8 (HHV-8) DNA in carotid, iliac, and coronary artery specimens obtained from a group of adult autopsy cases by means of polymerase chain reaction (PCR) analysis and nested PCR techniques. A 28-subject study group with at least type IV atherosclerosis and a 25-subject control group with no visible atherosclerosis were enrolled.

Results: HHV-6 DNA was found in the carotid artery specimen of 1 subject with atherosclerosis, in an iliac artery specimen of another subject, and in the iliac artery specimen of one of the control subjects. HHV-7 or HHV-8 DNA was not found in either the atherosclerosis or control cases.

Conclusions: This study is the first to demonstrate the presence of HHV-6 in atherosclerotic vascular tissues. HHV-7 and HHV-8 were not found in atherosclerotic tissues; however, further research on broader study groups and with different protocols is needed to determine whether these viruses play a role in the formation of atherosclerosis.

INTRODUCTION

The etiology of atherosclerosis is still uncertain but is thought to be multifactorial. Studies have suggested that in addition to such factors as smoking, poor nutrition, hypercholesterolemia, diabetes, hypertension, and various genetic characteristics, certain bacteria, particularly *Chlamydia pneumoniae*, may play a role in the formation of atherosclerosis [Ross 1992; O'Connor 2001; Kaklikkaya 2006]. Various viral agents have also been suggested to initiate vascular damage and trigger atherosclerosis, and atherosclerosis has even been established in chickens with Marek disease herpesvirus [Hajjar 1986; Ibrahim 2005].

Studies that have investigated the relationship between atherosclerosis and viral infections have mainly concentrated on viruses of the Herpesviridae family. The most frequently studied herpesviruses are cytomegalovirus, herpes simplex virus types 1 and 2, and Epstein-Barr virus [Ibrahim 2005]. These viruses are thought to possibly contribute to the atherosclerosis process by altering lipid metabolism in vascular cells, with the release of cytokines and growth factors, or by establishing prothrombotic effects in the vascular endothelium [Hajjar 1986; Nicholson 1998].

Human herpesvirus 6 (HHV-6), an agent in exanthem subitum (sixth disease) in children, remains latent in monocytes and macrophages following primary infection and exhibits reactivation, particularly in low-immunity patients. This virus is thought to be related to several pathologic conditions, such as meningoencephalitis, infectious mononucleosis, persistent lymphadenopathy, and fulminant hepatitis [Mendel 1995; Sumiyoshi 1995; Fujimaki 2006]. HHV-6 is capable of infecting endothelial cells and leading to the release of proinflammatory cytokines from these cells. It is likely that this virus can initiate atherosclerosis in this way; however, very few studies on the subject have been described in the literature [Rotola 2000; Caruso 2002; Chidiac 2002].

Human herpesvirus 7 (HHV-7) was first isolated in 1990 from the T-lymphocytes of an AIDS patient also infected with HHV-6 [Wyatt 1991]. HHV-7 was subsequently also identified in Kaposi sarcoma tissues [Kempf 1994]. The findings of primary HHV-7 infection resemble those of HHV-6 infection but are observed somewhat later. HHV-7 infection has been shown to cause reactivation of HHV-6 infection [Miyagawa 1999].

Human herpesvirus 8 (HHV-8) was first reported by Chang et al in 1994 in Kaposi sarcoma lesions of a patient with acquired immunodeficiency syndrome (AIDS) [Miyagawa 1999]. This virus is also related to the AIDS-related lymphoproliferative diseases body cavity-based lymphoma and multicentric Castleman disease [Maric 2002; Just 2009]. Various studies have shown that murid herpesvirus 68 (MHV-68), which like HHV-8 is from the Gammaherpesvirinae subfamily, can accelerate atherogenesis in experimental animal models [Alber 2000; Lin 2000]. One postmortem study of AIDS cases found a strong correlation between Kaposi sarcoma and the presence of atheroma [Grahame-Clark 2001].

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The intent of the present study was to investigate the presence of HHV-6, HHV-7, and HHV-8 DNA in atherosclerotic plaques and nonatherosclerotic vascular tissue samples taken in cases in which autopsy had been performed for various reasons and to determine whether these viruses had contributed to the development of atherosclerosis.

METHODS AND MATERIALS

Subjects

The subjects were consecutive cases of adult fresh autopsies performed at the Forensic Medicine Institution, Trabzon, Turkey, between March and August 2008. Carotid artery, iliac artery, and coronary artery specimens were taken from each autopsy case and fixed in 10% formalin. The degree of atherosclerosis was scored according to American Heart Association classifications [Stary 1995]. Twenty-eight cases with advanced types of atherosclerotic lesions (at least type IV) served as subjects, and 25 cases with no visible atherosclerotic plaques were used as the control group. The direct cause of death, age at death, sex, height, weight, and histories of cigarette smoking, diabetes, and hypertension were all recorded. Body mass index was calculated from height and weight measurements. Cases of individuals less than 30 years of age were excluded from the study. Written informed consent for the use of postmortem tissues was obtained from the family of each patient prior to the autopsy examination, and the protocol was approved by the Justice Ministry Forensic Medicine Institution.

DNA Isolation

A sterile scalpel was used to take sections of 10 × 10 mm from the carotid artery and iliac artery and sections of 3 × 3 mm from the coronary artery of the individuals in the subject and control groups. Formalin was removed from the specimens by washing 3 times in distilled water and 3 times with 70% ethanol. Specimens were then homogenized with a sterile grinder in 1 mL Tris-EDTA buffer (10mM Tris-HCl, pH 7.5, 1mM EDTA, pH 8.0). Each homogenized specimen was mixed in a

1:1 ratio with lysis buffer (Tris-EDTA buffer containing 0.5% sodium dodecyl sulfate and 100 µg/mL proteinase K). The mixture was then incubated at 56°C for 2 hours. DNA was isolated with standard methods of phenol-chloroform extraction and ethanol precipitation, dissolved in a Tris-EDTA buffer (pH 8.0), and stored at -20°C until use [Fernandez 2002].

Polymerase Chain Reaction Analysis

The presence in specimens of HHV-6, HHV-7, and HHV-8 DNA was investigated by polymerase chain reaction (PCR) analysis and nested-PCR techniques. Table 1 presents the sequences of the primers used in this study and the respective amplicon lengths.

The master mixes prepared for PCR for HHV-6, HHV-7, and HHV-8 DNA contained PCR buffer (Promega, Madison, WI, USA), 0.2mM of each deoxynucleoside triphosphate, 1.5mM MgCl₂, 1 U *Taq* DNA polymerase (Promega), 0.2µM of each primer, 0.01% gelatin, and 10 µL DNA [Chapenko 2003]. Thermocycling conditions for HHV-6 DNA amplification were 5 minutes at 94°C; 40 cycles of 2 minutes at 94°C, 2 minutes at 57°C, and 2 minutes at 72°C; and a final extension of 5 minutes at 72°C. Similar conditions were employed for HHV-7 and HHV-8 DNA amplification, except that we used an annealing temperature of 60°C for HHV-7 DNA and 56°C for HHV-8 DNA. The first-round PCR products were then used as template for the second-round PCR, which was performed with the same thermocycling programs [Cermelli 1999; Pan 2001]. Tris-EDTA buffer (pH 8.0) was used as a negative control. To exclude negative results due to the presence of PCR inhibitors in the sample, we included control PCRs with primers for human β-actin DNA from peripheral blood mononuclear cells. A 320-base pair fragment of the human β-actin gene was amplified with the following primers: primer β-actin-1, 5'-ATCATGTTTTGAGACCTTCAA-3'; primer β-actin-2, 5'-CATCTCTTGCTCGAAGTCCA-3' [Meerbach 2001]. The PCR products obtained were electrophoresed along with PCR products of known molecular weight in 2% agarose gels containing 0.5 µg/mL ethidium

Table 1. Primers Used and Predicted Amplicon Length

Primer	Sequence	Amplicon Length, bp*	Reference
HHV6-1	5'-GCG TTT TCA GTG TGT AGT TCG GCA G-3'	258	[Chapenko 2003]
HHV6-2	5'-TGG CCG CAT TCG TAC AGA TAC GGA GG-3'		
HHV6-3	5'-GCT AGA ACG TAT TTG CTG CAG AAC G-3'		
HHV6-4	5'-ATC CGA AAC AAC TGT CTG ACT GGC A-3'		
HHV7-1	5'-TAT CCC AGC TGT TTT CAT ATA GTA AC-3'	124	[Yadav 1997; Cermelli 1999]
HHV7-2	5'-GCC TTG CGG TAG CAC TAG ATT TTT TG-3'		
HHV7-3	5'-CAG AAA TGA TAG ACA GAT GTT GG-3'		
HHV7-4	5'-AGA TTT TTT GAA AAA GAT TTA ATA AC-3'		
HHV8-1	5'-CGCCTGTAGAACGAAACAT-3'	138	[Pan 2001]
HHV8-2	5'-TTGCCCGCCTCTATTATCAG-3'		

*bp indicates base pair.

bromide. The width of the band of the appropriate size was investigated with an ultraviolet illuminator.

Statistical Analyses

Measured data were compared with the Student *t* test, and data obtained by counting were compared with the chi-square test. The level of statistical significance was set at .05.

RESULTS

Twenty-eight autopsy cases (24 male and 4 female) were enrolled as the study group, and 25 other autopsy cases (19 male and 6 female) were enrolled as the control group. Table 2 presents the direct causes of death for the case and control subjects.

In the advanced-atherosclerosis case group, the incidence of death from cardiovascular causes was significantly higher than for deaths caused by suicide and trauma (*P* = .020).

Table 3 summarizes the characteristics of the subjects, including sex, age, body mass index, history of smoking, heart weight, and diabetes and hypertension findings.

The presence of HHV-6 DNA was determined by PCR analysis in atherosclerotic arterial tissue samples taken from 2 autopsy cases with at least type IV atherosclerosis. PCR analysis detected HHV-6 DNA in the carotid artery of 1 case and in the iliac artery of the other. HHV-6 DNA was found in the iliac artery of 1 case with no visible atherosclerosis. No HHV-7 or HHV-8 DNA was found in any of the specimens taken from the case and control subjects (Table 4).

DISCUSSION

Atherosclerosis-related diseases are among the main causes of morbidity and mortality. Levels of death from cardiovascular causes are high, especially among patients with advanced atherosclerosis [Ross 1992]. In our study, we also found the number of deaths from cardiovascular causes to be higher in the advanced-atherosclerosis group, compared with deaths due to suicide or trauma. Death from cardiovascular causes was determined in 2 cases of the control group. The autopsies established that the direct cause of death in these 2 cases was cerebral infarction but that this cerebrovascular event was not caused by atherosclerosis.

Table 2. Direct Causes of Death for the Case and Control Subjects

Direct Cause of Death	Case Subjects (n = 28)	Control Subjects (n = 25)
Suicide, n*	12 (42.85%)	10 (40%)
Trauma, n†	5 (17.85%)	13 (52%)
Cardiovascular disease, n‡	11 (39.28%)	2 (8%)

*Hanging, drugs.

†Traffic accident, wounding with a sharp implement, trapped under landslide, falling from a high altitude.

‡Heart failure, myocardial infarction, cerebral infarction.

Table 3. Details of Cases and Control Subjects*

	Case Subjects (n = 28)	Control Subjects (n = 25)	<i>P</i>
Male/female sex, n	24/4	19/6	.488
Age at death, y	69.0 ± 11.36	52.0 ± 11.99	<.0005†
Body mass index, kg/m ²	28.6 ± 3.90	27.2 ± 3.44	.184
History of smoking, n	20 (71.4%)	13 (52%)	.241
Heart weight, g	449.29 ± 91.36	377.20 ± 81.062	.004†
Diabetes, n	6 (21.42%)	3 (12%)	.474
Hypertension, n	12 (42.85%)	5 (20%)	.138

*Data are presented as the mean ± SD where indicated.

†Statistically significant.

Table 4. Human Herpesvirus 6 (HHV-6), HHV-7, and HHV-8 DNA Positivity in the Cases and the Control Subjects

	Case Subjects (n = 28)	Control Subjects (n = 25)	<i>P</i>
HHV-6 DNA–positive specimens, n	2 (7.14%)	1 (4.0%)	1.000
HHV-7 DNA–positive specimens, n	0	0	–
HHV-8 DNA–positive specimens, n	0	0	–

Because our study was a case-control study, we wanted the ages of the advanced-atherosclerosis subjects and nonatherosclerosis control subjects to be similar. Cases with at least type IV atherosclerosis were included in the study group. Because advanced atherosclerosis increases with age, the mean age was higher than in the nonatherosclerosis group. These findings support the view that the prevalence of advanced atherosclerosis increases with age [Ross 1992].

Although the cardiovascular risk factors of male sex, body mass index, smoking, diabetes, and hypertension were higher in our cases than in the control group, the difference was not statistically significant because of our small number of cases. The mean heart weight for our cases was significantly higher than in the control group. This finding, as was shown in the Framingham study, reveals that increased heart weight is an indicator of coronary diseases of atherosclerotic origin [Kannel 1970].

Our report is the first to describe the presence of HHV-6 DNA in atherosclerotic tissues. HHV-6 DNA was found in the carotid artery of one of our cases and in the iliac artery in another. In a previous study, in which specimens from the aorta, vasa vasorum, and heart microvessels were taken from an immunocompetent patient with aortic insufficiency and aortic aneurysm and cultured *in vitro*, HHV-6 DNA was found in cultures of endothelial cells obtained from the aortic tissues but not in cultures of cells from the other vascular tissues [Rotola 2000]. The same report also showed that this virus could replicate at low levels in aortic endothelial tissues. Another study reported that in addition to the aorta, the umbilical vein and heart microvascular endothelium could also be infected with HHV-6, after which monocyte chemoattractant protein 1 and interleukin 8 production by the aorta and microvascular endothelium was up-regulated [Caruso 2002]. Our detection of HHV-6 DNA in arterial tissues from both nonatherosclerotic and advanced-atherosclerosis cases suggests that this virus may play a role in the commencement or progression of the atherosclerotic process. Another possibility is that because HHV-6 can remain latent or in a low replicative state in peripheral blood mononuclear cells, these cells can then settle in the tissues after they become atherosclerotic. Hence, the presence of HHV-6 DNA was shown in these tissues.

Host cells carrying HHV-7 are more restricted than those carrying HHV-6. HHV-7 has been grown in cord blood mononuclear cells and in the established T-cell line. There are insufficient studies, however, regarding whether HHV-7 infects arterial endothelial tissues [Cermelli 1999; Pan 2001]. No HHV-7 DNA was detected in any arterial tissue in our study. The reason for this finding may be that this virus is unable to infect the arterial endothelium and contribute to the atherosclerotic process.

No HHV-8 DNA was detected in any atherosclerotic or nonatherosclerotic arterial tissues in this study. Grahame-Clarke et al [2001] investigated postmortem AIDS cases to determine whether the occurrence of Kaposi sarcoma is an indicator of HHV-8 infection and found that atherosclerosis levels were higher in cases with Kaposi sarcoma. Researchers therefore predicted a powerful link between HHV-8 and

atherosclerosis. In addition, MHV-68, which like HHV-8 is a member of the Gammaherpesvirinae subfamily, has been shown to accelerate atherogenesis in apolipoprotein E-deficient mice [Alber 2000]. Another study showed that bovine herpesvirus 4 infection could be established experimentally in rabbits [Lin 2000]. The absence of any HHV-8 DNA in any atherosclerotic or nonatherosclerotic specimens in our study may be due to the low seroprevalence of this virus in Turkey and suggests that the virus does not play a role in the development of atherosclerosis in humans.

In conclusion, our study is the first to document the presence of HHV-6 in atherosclerotic arterial tissue. The presence of HHV-7 or HHV-8 was not demonstrated. There is now a need, however, for further research with wider study groups and with different protocols to establish whether these viruses play a role in the formation of atherosclerosis.

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