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Detection of Cytomegalovirus in Atherosclerotic Plaques and Nonatherosclerotic Arteries

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Several studies have reported an association between infectious agents and atherosclerosis. Cytomegalovirus (CMV) is the most commonly implicated viral pathogen. However, the role of CMV in atherosclerosis remains obscure. The present study evaluated the presence of CMV DNA in atherosclerotic plaques and normal vessel walls. A total of 40 arterial specimens from coronary plaques and 27 samples from normal vessels were obtained from 26 patients who underwent aorto-coronary bypass surgery. The specimens were analyzed by polymerase chain reaction for the presence of the CMV immediate early genomic region. CMV DNA was detected in 9 out of 26 patients (34.6%). Viral DNA was detected in both nonatherosclerotic tissues and atherosclerotic plaques. No statistically significant differences were observed between normal and diseased vessels. Our findings, in accordance with previous studies, do not support a direct causative role of CMV in the development of atherosclerotic plaques.

Keywords: cytomegalovirus; atherosclerotic plaque; healthy vascular tissue; polymerase chain reaction

Cardiovascular disease (CVD) is a major cause of morbidity and mortality in Western societies. Atherosclerosis is the underlying disorder of the majority of aspects of CVD.[1]

Atherosclerosis is a complex, multifactorial disease associated with numerous environmental risk factors that interact with the genetic background of the individual.[2,3] Elevated plasma cholesterol levels, hypertension, diabetes mellitus, and smoking are recognized as major inducers of endothelial damage.[4] However, several studies have reported an association between certain persistent viral and bacterial pathogens and atherosclerosis. The potential involvement of adenovirus, herpes simplex virus (HSV), Epstein–Barr virus (EBV), and *Helicobacter pylori* has been assessed. However, *Chlamydia pneumoniae* and cytomegalovirus (CMV) are the most commonly implicated pathogens.[5-18]

Cytomegalovirus belongs to the subfamily of β-herpesvirinae of the family Herpesviridae. In developing countries, the majority of people are infected during early adulthood, whereas in developed countries, more than 70% of humans are infected by the age of 65.[19] The virus causes latent infections and periodically reactivates. In the majority of immunocompetent patients, CMV infection is asymptomatic. In contrast, the virus results in adverse clinical outcome when immunodeficient patients are infected.[20]

Previous studies have assessed the role of CMV infection in atherosclerosis; however, the results are quite contradictory. In the present study, we assessed the potential implication of CMV in atherogenesis by comparing the incidence of viral DNA detection in atherosclerotic lesions and normal vessels.

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Methods

Patients

The study population consisted of 21 male and 5 female patients with coronary artery disease (CAD) with a median age 66 years (range 43-78 years). All patients underwent coronary artery bypass grafting at the University Hospital of Crete between February 2005 and May 2005. Vascular segments were collected during surgery, from internal mammary artery grafts (peripheral part), saphenous veins, and coronary arteries (at the site of anastomosis). All samples were immediately separated into 2 parts. One part was collected and fixed immediately in 10% neutral-buffered solution containing about 4% formaldehyde for 24 hours and embedded in paraffin for conventional histology. The second part of all samples was immediately stored at 4°C for further DNA analysis. Histopathological examination classified vascular tissue into 2 categories: normal vessels or atheroscleorotic tissue (lesions II-VI in American Heart Association [AHA] classification).[21] The study protocol was approved by the institute's ethical committee. Informed consent was obtained from all the participants. Specimens were obtained from 39 noncalcified atherosclerotic plaques of coronary arteries and 1 plaque of mammary artery. As controls, nonatherosclerotic tissue from the respective bypass grafts was used (26 specimens from normal mammary arteries and 1 specimen from a normal saphenous vein). Clinical and epidemiological characteristics of the studied population—including age, sex, smoking habits, history of diabetes mellitus, dyslipidemia, and hypertension—were also recorded. Smoking was defined as a current or a prior history of tobacco use. Diabetes was defined as a fasting blood glucose level more than 126 mg/dL or treatment with dietary modification, oral hypoglycaemic agents, or insulin at the time of the study. Hypertension was defined as systolic blood pressure greater than 140 mm Hg and/or diastolic pressure more than 90 mm Hg on at least 3 occasions, or if such a diagnosis had been made in the past and the patient was being treated with medication or lifestyle modification. For patients with established CAD prior to the study, dyslipidemia was defined as treatment with lipid lowering medication or dietary modification or lipid levels greater than those recommended by the Third Joint Task Force of European and other Societies on Cardiovascular Disease Prevention in Clinical Practice[22] (Table 1).

Table 1. Clinical and Epidemiological Characteristics of Patients[a]

	Patients, *n* (%)	CMV Positive, *n* (%)	CMV Negative, *n* (%)
Median age (years)	66	61	68
Male gender	21 (81)	7 (78)	14 (82)
Hypertension	22 (85)	9 (100)	13 (76)
Diabetes mellitus	9 (35)	5 (56)	4 (24)
Smoking	16 (62)	6 (67)	10 (59)
Dyslipidemia	19 (73)	5 (56)	14 (82)
Total	26	9	17

NOTE: CMV = cytomegalovirus.
[a]Nonsignificant differences in all cases.

DNA Extraction and Detection of CMV

DNA extraction was carried out as previously described.[23] All specimens were examined for the presence of amplifiable DNA using *beta2-microglobulin* as a reference gene. In all specimens, a part of sequence of the major immediate early region of CMV was examined by polymerase chain reaction (PCR) using the primers forward 5′-GTGACCAAGGCCACGACGTT-3′ and reverse 5′-TCTGCCAGGACATCTTTCTC-3′. The reaction mix in all PCRs for CMV detection consisted of PCR buffer 1X, 0.5 mM $MgCl_2$, 0.05 mM of each dNTP, 0.3 mM of each primer, and 0.65 units Taq DNA polymerase. The cycling conditions for CMV consisted of an initial denaturation step at 94°C for 2.30 minutes; then 8 cycles of 94°C for 40 seconds, 57°C for 30 seconds, and 72°C for 30 seconds; and 27 cycles of 94°C for 40 seconds, 55°C for 30 seconds, and 72°C for 30 seconds; and a final extension step at 72°C for 10 minutes. PCR products were visualized on a 2% agarose gel. At least 3 repetitions of PCR reactions were carried out for each specimen with a high reproducibility score.

Statistical Analysis

Fisher's exact test was applied to estimate the association between the presence of viral DNA and atherosclerosis. To assess the independent effect of CMV on atherosclerosis, we included the presence of CMV and the conventional risk factors of CAD in a multiple logistic regression analysis. In all cases, *P* values less than .05 were considered to be significant. The analyses were performed using SPSSv10 (SPSS Inc, Chicago, IL).

Table 2. CMV DNA Detected in Atherosclerotic Lesions and Normal Vessels

	Atherosclerotic Lesions, n (%)	Normal Vessels, n (%)	*P* Value
CMV positive	9 (22.5)	4 (14.8)	0.4
CMV negative	31 (77.5)	23 (85.2)	
Total	40	27	

NOTE: CMV = cytomegalovirus.

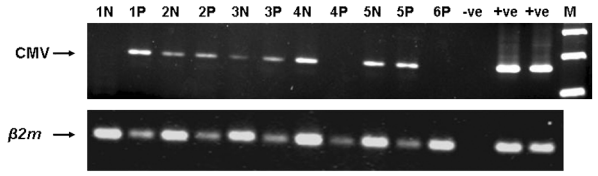

Figure 1. Representative examples of CMV positive atherosclerotic plaques (P) and normal vessel walls (N) in atherosclerotic patients. M, 100-bp ladder; –ve, negative control; +ve, positive control. The *beta2 microgloulin* (*?2m*) gene was amplified as a reference gene.

Results and Discussion

PCR analysis was employed both in atherosclerotic plaques and nonatherosclerotic arteries from 26 patients. CMV was detected in 9/26 patients (34.6%). A total of 3 male patients were positive for CMV in both their plaques and the normal mammary arteries. CMV DNA was found only in the atherosclerotic plaques in 3 male patients, whereas 1 female patient was CMV positive only in the plaque of her mammary artery. An additional male patient presented CMV in the normal mammary artery alone, whereas CMV was detected in only 1 out of 3 atherosclerotic plaques, but not in the mammary artery, of 1 female patient. Representative examples of CMV positive samples by PCR are shown in Figure 1.

CMV DNA was detected in 9/40 (22.5%) atherosclerotic plaques and 4/27 (14.81%) nonatherosclerotic vessels (Table 2). Statistical analysis showed no significant difference in CMV incidence between the plaques and the nonatherosclerotic tissues, indicating that normal mammary arteries were infected as often as atherosclerotic plaques. Furthermore, no significant difference was observed between the presence of CMV and clinical or epidemiological parameters, including age, gender, arterial hypertension, diabetes mellitus, smoking, or hypercholesterolemia.

The potential pathogenic role of infectious agents in the initiation and progression of atherosclerosis remains obscure. Adenovirus, enterovirus, herpes simplex viruses, and Epstein–Barr virus have been assessed as potential inducers of endothelial damage. However, CMV is the most commonly implicated viral pathogen in the literature.[5-18]

In the present case–control study, we tested both atherosclerotic arterial specimens and healthy vascular tissue for the presence of CMV DNA, applying a well-established and sensitive technique. We did not observe any positive association between CMV and atherosclerosis. However, our study had limited statistical power and we were therefore unable to establish a definite negative association. In fact, small sample size has been the main limitation of every similar study reported in the scientific literature. Thus, to arrive at a plausible conclusion regarding the implication of CMV in atherosclerosis, we performed a mini meta-analysis by reviewing similar studies in the literature from 1989 to the present, including articles assessing the presence of CMV DNA in human atherosclerotic vascular tissue. We ended up with 13 studies assessing the subject (Table 3). The majority—9 out of 14 studies—were observational. They lacked control samples and reported the incidence of CMV DNA in diseased vascular tissue.[8-15] A total of 5 studies applied a case–control methodology using healthy vascular tissue of variable origin as control samples.[5-7,16,17] In all, 3 out of 5 case–control studies applied PCR to detect CMV DNA in vascular tissue.[5-7] Taking into consideration the present study along with the 3 studies that used the same design and methodology, we found that, of 249 atherosclerotic samples tested, CMV DNA was detected in only 16 samples. Additionally, in 173 samples of normal vascular tissue, CMV DNA was detected in 4 specimens (6.4% vs 2.3%; odds ratio = 2.9; 95% confidence interval, 0.95 to 8.8; $P = .062$). The latter observation remains under the cut off point of statistical significance. Nevertheless, because of the low overall rate of CMV detection in vascular specimens, the power of the given sample size remains low. We therefore cannot definitively conclude that there is a negative association between the detection of CMV DNA in vascular tissue and atherosclerosis.

The detection of viral DNA in the vascular wall per se cannot be considered an independent predisposing factor of CAD. Perhaps CMV infection needs the presence of another triggering factor to initiate athrogenesis. However, such a hypothesis would be

Table 3. Incidence of CMV Detection in Atherosclerotic Vascular Tissue[a]

Patients		Controls				
CMV Positive	CMV Negative	CMV Positive	CMV Negative	Type of Specimen	Method of Detection	Authors
9	31	4	23	Coronary arteries, mammary arteries, saphenous veins	PCR	Xenaki et al (2008), this study
5	43	0	66	Coronary arteries, carotid arteries, saphenous veins	Real time PCR	Ibrahim et al (2005)[5]
2	126	0	20	Carotid arteries	PCR	Kwon et al (2004)[6]
0	33	0	60	Coronary arteries, mammary arteries, aortas	PCR	Pinar et al (2004)[7]
0	50	—	—	Carotid arteries	PCR	Hagiwara et al (2007)[8]
0	83	—	—	Carotid arteries	PCR	Latsios et al (2004)[9]
3	15	—	—	Basilary artery, coronary artery, thoracic aorta, abdominal aorta, renal arteries	Nested PCR	Rassu et al (2001)[10]
0	40	—	—	Carotid arteries	Immunohisto chemistry PCR	Saetta et al (2000)[11]
9	8	—	—	Carotid arteries	PCR	Qavi et al (2000)[12]
2	21	—	—	Carotid arteries	PCR	Hunter et al (1999)[13]
0	29	—	—	Coronary arteries	PCR	Daus et al (1998)[14]
27	49	—	—	Carotid arteries	Immunostaining	Chiu et al (1997)[15]
27	3	18	16	Abdominal aorta, femoral artery	Dot blot and in situ DNA hybridization	Hendrix et al (1990)[16]
19	25	22	8	Abdominal aorta, femoral artery	In situ hydridization	Hendrix et al (1989)[17]

NOTES: CMV = cytomegalovirus; PCR = polymerase chain reaction.
[a]Figures represent the number of specimens tested.

difficult to prove or disprove by independent studies conducted with small sample sizes. Large-scale clinical trials need to be conducted for a possible interaction of CMV infection and other predisposing factors of CAD to be revealed.

In our opinion, the most important finding remains the low overall rate of detection of CMV DNA in atherosclerotic vascular tissue. Thus, even if there were an association between the presence of CMV in the vascular wall and atherosclerosis, the clinical impact of such an association would be limited to a small number of CAD patients. Furthermore, the etiopathogenesis of atherosclerotic cardiovascular disease is pretty much clear. Nine well-established and modifiable risk factors, account for almost 90% of the total risk of acute myocardial infarction.[24] Thus, there is little room for alternative theories implicating viral or bacterial infectious agents in atherogenesis.

However, why the vascular wall can host CMV still remains to be explained. CMV has the ability to remain latent in the human body, with the vascular wall probably serving as a site of CMV latency. Furthermore, other members of herpes viruses—such as HSV-1, HSV-2, and EBV—have also been detected in the vascular wall, without any obvious clinical implications.

We conclude that the CMV detected in the vascular wall might reflect the increased rate of CMV seropositivity in adults. Moreover, till date there is no convincing evidence to suggest a causative role for the virus in the development of atherosclerotic plaques.

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