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Herpes Simplex Virus as a Determinant Risk Factor for Coronary Artery Atherosclerosis and Myocardial Infarction

DIMOSTHENIS KOTRONIAS and NIKIFOROS KAPRANOS

MITERA Maternity and Surgical Center of Athens, Athens, Greece

Abstract. *Background: Viruses have been detected in atherosclerotic and non-atherosclerotic vascular tissues and may be involved in the mechanisms of atherogenesis. In the present study, we investigated the role of herpes simplex virus (HSV) in the early and late stages of coronary artery atherosclerosis. Methods and Results: HSV prevalence was investigated in coronary artery samples from 42 autopsy cases, in which death was related to myocardial infarction (MI), and 28 young age autopsy cases without heart disease, who had died from fatal injuries (young victim group), using nested polymerase chain reaction (nPCR) and the highly sensitive in situ hybridization with tyramide signal amplification (ISH-TSA). HSV was detected by nPCR in 18 out of 42 (43%) myocardial infarction cases and in 7 out of 28 (25%) young victim group cases, respectively. Using ISH-TSA, HSV DNA was detected in the coronary arteries of the MI group in 16 out of 42 (38%) of the cases; the hybridization signal was localized in the nuclei of endothelial cells, the nuclei of smooth muscle cells, the macrophages around the atheroma, and in the lymphocytes infiltrating the vascular wall. In the young victim group, HSV DNA was detected by ISH-TSA in 7 out of 28 (25%) autopsy cases; the signal was localized in the endothelial and the intimal spindle cells of the coronary arteries. Conclusion: The findings of this study suggest that HSV seems to play a significant role in the initiation and progression of coronary atherosclerosis, and may open new perspectives in preventing the development of vascular damage via an appropriate antiviral treatment.*

Coronary heart disease (CHD) is one of the most common life-threatening diseases in the Western industrialized countries, with severe social and economic consequences. CHD is the result of chronic myocardial ischemia, which is mostly caused by epicardial coronary arteries atherosclerosis. Myocardial infarction, a common fatal complication, generally occurs after a sudden decrease of blood flow because of a thrombotic occlusion of a coronary artery formerly narrowed by atherosclerosis (1). Although many risk factors, including dyslipidaemia, smoking, hypertension, glucose intolerance and obesity have been identified, the etiology and pathogenesis of this disease still remains obscure (2).

According to seroepidemiological data, bacterial and viral infections may be implicated in the pathogenesis of coronary artery disease and restenosis after angioplasty. Bacterial pathogens include *Chlamydia pneumoniae* and *Helicobacter pylori*, while the main viral pathogens are coxsackie B4 and herpes viruses (3-5). Virions of the herpes virus have been identified by electron microscopy in aortic tissue from patients with atherosclerosis undergoing cardiovascular surgery (6). Moreover, herpes viral antigens and nucleic acids have been detected in a few studies so far in atherosclerotic and normal vascular tissue (7-12) .

We studied the relationship between herpes simplex virus (HSV) and coronary artery atherosclerosis at early and late stages of the disease using very sensitive molecular techniques. More specifically, using the nested polymerase chain reaction (nPCR) and *in situ* hybridization with tyramide signal amplification (ISH-TSA) techniques, we examined and compared the presence of HSV nucleic acids in coronary artery tissues taken from two autopsy groups. The first autopsy group consisted of cases who had died from myocardial infarction, while the second autopsy group consisted of young age subjects without overt heart disease who had died from fatal injuries (young victim group).

Materials and Methods

Samples. Coronary artery segments were obtained during autopsy from 42 cases, who had died from myocardial infarction (MI), and from 28 young age cases, killed through vehicular or other accidents without overt heart disease.

In the first group (33 males and 15 females), the age at death ranged from 28 to 91 years (mean age: 62.4 years), whereas in the second group (21 males and 7 females) it ranged from 17 to 33 years (mean 24.5 years).

Correspondence to: Dimosthenis Kotronias, MD, MITERA Maternity and Surgical Center, 6 Erythrou Stavrou Str., 151 23 Athens, Greece. Tel: (+30) 210-6869134, Fax: (+30) 210-6462686, e-mail: nkapranos@mitera.gr

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Table I. *List of primers used in the detection of HSV in the coronary samples.*

Amplified locus	Sequence	PCR product
HSV-1 DNA pol gene (UL30 gene)	Outer primers 5-CCC GTG GTG GTG TTC GAC TTT GCC-3 5-GCA CAA AGA TGG AGT CCG TGT CCC-3	544 bp
	Inner primers 5-TTC TTC GTC AAG GCT CAC GTG CG-3 5-CCG AGT TAC ACA CGA CCT TGA TGG C-3	154 bp

A transverse section of the coronary artery near the atherosclerotic plaque and a small cuff of surrounding fibrofatty and muscular tissue was snap-frozen and stored at -70˚C until the time of molecular analysis, while a cross section of coronary artery from the young injury cases was also snap-frozen and stored at -70˚C. A similar adjacent section from all the above cases was fixed in 10% neutral buffered formalin for 24 hours and embedded in paraffin for routine histological examination and ISH-TSA.

nPCR. Nested-PCR was applied to DNA extracted from frozen tissue using the protocol of the EPICENTRE DNA extraction kit (MasterPure DNA Purification kit, Madison, USA). All the samples were examined for the presence of HSV DNA by the nPCR technique using one set of outer primers for primary PCR and one set of inner primers for nested PCR (Table I). The nPCR technique used was described in detail in a previous publication (13). The PCR products were examined by electrophoresis in a 2% agarose gel and photographed on a UV light transilluminator. DNA extracted from HSV-1- and HSV-2-infected Vero cells was used as positive control, and a reaction mixture devoid of template DNA as negative control.

ISH-TSA. The ISH-TSA technique was applied on 4-μm formalin-fixed paraffin-embedded tissue sections from coronary arteries. The sections were deparafinized in xylene and rehydrated in graded ethanols. Target retrieval of histological specimens was performed using Tris-EDTA buffer (TE) pH 7 at 95˚C for 40 min, followed by digestion with 0.01% pepsin in 0.2 M HCl for 15 min at room temperature (RT). Quenching of endogenous peroxidase was performed by 0.3% H_2O_2 for 20 min. Twenty μl DNA probe labelled with biotin containing different fragments of HSV-1 and HSV-2 genome 100-1000 base pairs long (ENZO Diagnostics, NY, USA) was applied on each section and the coverslip was sealed with rubber cement. Probe and target DNA were simultaneously denaturated on a 95˚C hot plate for 10 min.

The hybridization reaction was performed by incubating the slides in a humid chamber at 37˚C for 16 h. Post hybridization washing of the specimens was performed in 0.5XSSC at 75˚C for 10 min.

After washing the slides twice in TBST (Tris buffer saline, 0.025% Tween 20), tyramide signal amplification was performed according to the manufacturer's instruction (Genepoint Kit, DAKO, Glostrup, Denmark). Primary streptavidin- HRP at a dilution of 1:150 was applied for 15 min at RT. After washing, the sections were incubated with biotinyltyramide solution for 15 min at RT, followed by the secondary streptavidin-HRP for 15 min at RT. Finally, the slides were incubated with 0.6 mg/ml 3'-3'-diaminobenzidine tetrahydrochloride in TBS containing 0.03% hydrogen peroxide for 5 min at room temperature, counterstained with hematoxylin, dehydrated in graded ethanols, cleared in xylene and coverslipped with DPX.

The statistical significance between the HSV incidence in the MI group and the young victim group was analyzed with Fisher's exact probability test. A p value of > 0.05 was considered insignificant.

Results

The history of the examined autopsy cases with myocardial infarction revealed that conventional risk factors were as follows: hypertension (blood pressure > 140/90 mm Hg), 5 out of 42 cases; smoking, 37 out of 42 cases; diabetes, 6 out of 42 cases; and hypercholesterolemia (cholesterol level >240 mg/dL), 13 out of 42 cases.

Histological examination of the coronary artery samples in the MI group showed typical advanced atherosclerotic and thrombotic lesions in all cases. In the MI group, HSV DNA was detected by nPCR in 18 out of 42 (43%) autopsy cases (Figure 1). In the same group, HSV was detected by ISH-TSA in 16 out of 42 (38%) cases (Table II). The 2 nPCR-positive cases that were negative by ISH-TSA showed extensive thrombosis and calcification. The hybridization signal in the HSV-positive cases was mostly localized in the spindle cells of the intima and media in the area of atherosclerosis (Figure 2) as well as in monocytes/macrophages around the atheroma (Figure 3). HSV-positive lymphocytes infiltrating the vascular wall were also observed in 3 cases. HSV-positive endothelial cells were rarely identified in the luminal surface of atheromatous coronaries, but they were easily found in 2 cases with recanalization of the thrombotic vessel (Figure 4). Interestingly, HSV was also observed in the nerve bundles around the atherosclerotic arteries in 3 cases.

In the young victim group, HSV DNA was detected by both nPCR and ISH-TSA in 7 out of 28 (25%) autopsy cases. The difference between the rate of HSV incidence in the MI group and the young victim group (43% and 25%, respectively) was not statistically significant ($p=0.10$). The coronaries in 6 out of 7 HSV-positive cases revealed atheromatous changes characterized by intimal thickening, focal intimal clusters of spindle or foam cells and duplication of internal elastica lamina. HSV DNA was localized in the

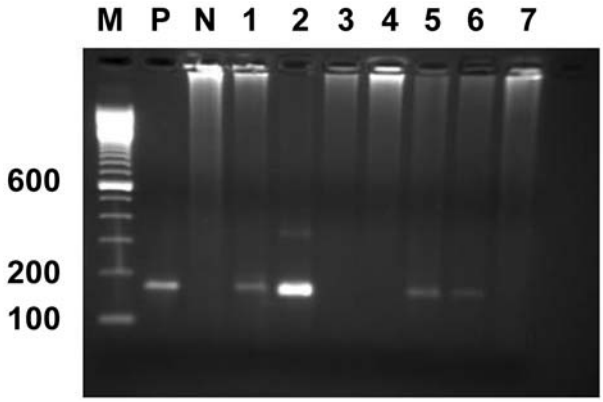

Figure 1. *HSV detection by nPCR in DNA extracted from atherosclerotic coronary arteries of the MI group cases. M: Molecular weight markers. P: PCR product from positive control. N: Negative control (No DNA). 1,2,5,6: HSV-positive cases. 3,4,7: HSV-negative cases.*

foci of the intimal spindle or macrophage cells and in the luminal endothelial cells (Figure 5). The 7th HSV-positive case showed no histologically obvious atheromatous changes and the hybridization signal was localized in the nuclei of endothelial cells and spindle subendoendothelial cells.

Discussion

The first experimental link between herpesvirus and atherosclerosis was established 25 years ago from animal models, in which chicken infected with Marek's disease virus –an avian herpes virus– disclosed arterial changes that closely resembled human atherosclerosis (14). Subsequent *in vitro* studies revealed that infection of smooth muscle cells with this virus resulted in intracellular and extracellular cholesterol accumulation (15). The mechanism responsible for this accumulation was clarified some years later by Hajjar *et al.,* who found that HSV infection of human smooth muscle cells decreases lysosomal and cytoplasmic cholesterol ester hydrolytic activity (16).

The detection of HSV DNA in human vascular tissue from the ascending aorta in patients undergoing coronary bypass surgery was initially reported by Benditt *et al.* (17) in 11 out of 160 tissue samples. According to this study, the positive tissues were in most cases non-atherosclerotic, although in 2 cases an intimal thickening of the vascular wall was present. In addition, an electron microscopy study confirmed the presence of herpes viral particles in various stages of replication in aortic tissue from 10 out of 60 patients with atherosclerosis (6). In another study, herpes viruses were detected by *in situ* hybridization in both atherosclerotic and non-atherosclerotic aortic tissue obtained during autopsy (11). According to this study, HSV-1, EBV and CMV were detected in atherosclerotic tissue in 80%, 80% and 40% of the cases, respectively, whereas in non-atherosclerotic tissues the same viruses were detected in 13%, 13% and 4% of the cases, respectively.

Table II. *HSV detection in coronary arteries from myocardial infarction group and young victim group with and without atheromatous changes.*

	No. of cases	nPCR pos cases (%)	ISH-TSA pos cases (%)
Myocardial infarction	42	18 (43)	16 (38)
Young victim group	28	7 (25)	7 (25)
Atheromatous changes	23	6 (26)	6(26)
No atheromatous changes	5	1(20)	1(20)

This is the first study, according to our knowledge, in which HSV has been detected in the coronary artery of patients, who died from myocardial infarction, using the very sensitive nPCR and ISH-TSA techniques. In a previous study, HSV antigens were detected by immunohistochemistry in 45% of the biopsies obtained from atherosclerotic coronary arteries in patients undergoing coronary artery bypass grafting (12). In the present study, 43% of the cases who died from myocardial infarction harbored HSV DNA in atherosclerotic coronary tissues mainly localized in the smooth muscle and endothelial cells. In addition, HSV DNA was also localized in the macrophages and lymphocytes infiltrating the atherosclerotic area, which probably means that infected cells can be detected in the blood circulation at least in an active phase of the disease. We have already confirmed this by detecting HSV DNA in the peripheral blood of patients with coronary atherosclerosis using the nPCR technique (unpublished data). An interesting point for clinical practice would be the correlation of the circulating viral copies number –viral load– at several time intervals with the clinical course of the patient. Although HSV was found to be implicated in 43% of the cases, we assume that the percentage of positive cases may be even higher as the virus may not be detectable in coronaries with extensive atherothrombotic alterations.

In the young victim group, HSV DNA was found in 25% of autopsy cases, which is comparable to the findings of a previous study in which HSV was detected in 6 out of 20 (30%) young trauma autopsy cases (18). In the coronaries with minimal coronary atherosclerosis, HSV DNA was localized in the endothelial cells as well as in the subendothelial intimal cells. Consequently, it is reasonable to suppose that the earliest functional damage in the atherosclerotic process is manifested in the HSV-infected endothelium. This dysfunction, according to *in vitro* studies, is evidenced by the acquisition of procoagulant properties of HSV-infected endothelial cells, which is accomplished in 3 ways (19,20): first, by the reduction of synthesis and surface expression of heparan sulfate proteoglycan and thrombomodulin by the endothelial cells

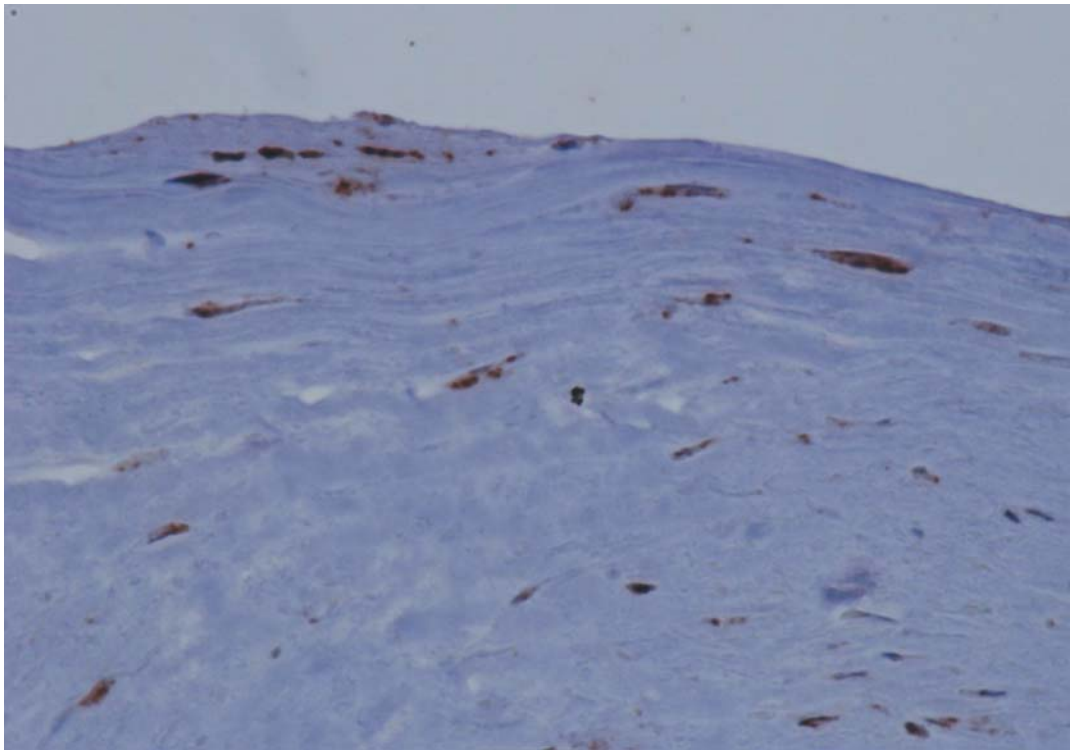

Figure 2. *HSV detection by ISH-TSA in a thrombotic coronary artery. Residual spindle cells show a strong nuclear hybridization signal for HSV DNA probe (X60).*

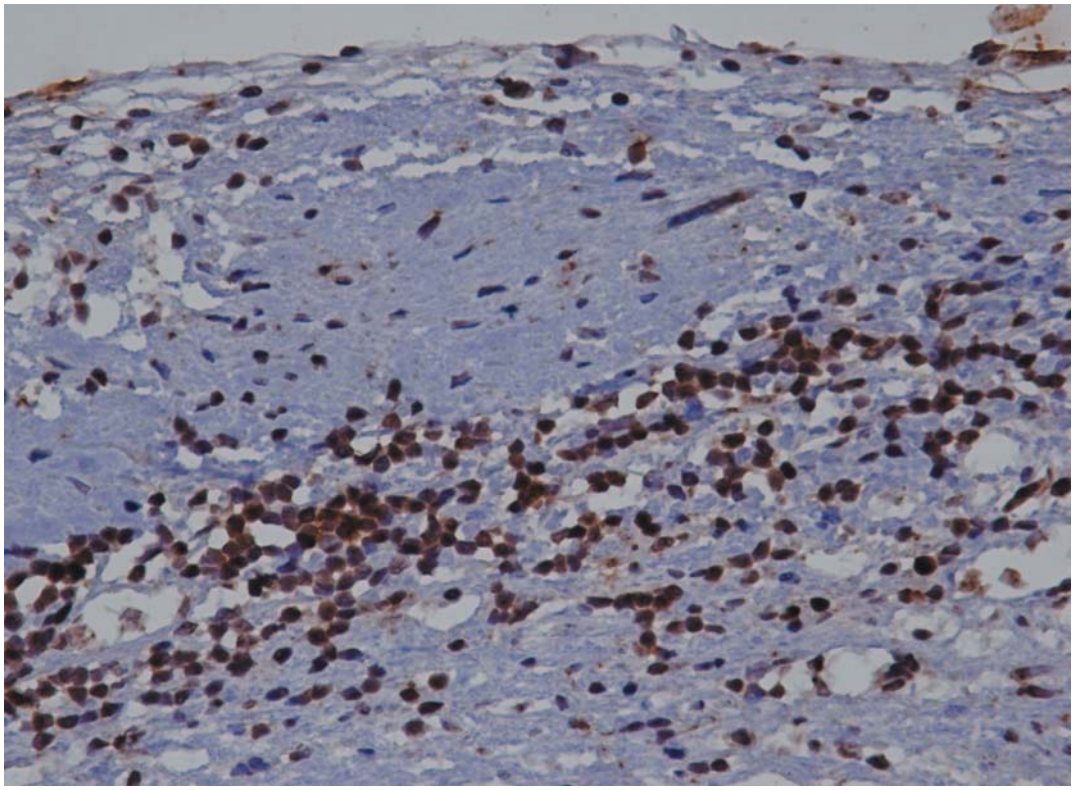

Figure 3. *ISH-TSA for HSV DNA in an atherosclerotic coronary artery. Lymphocytes, macrophages and rare spindle cells show intense nuclear labelling (40X).*

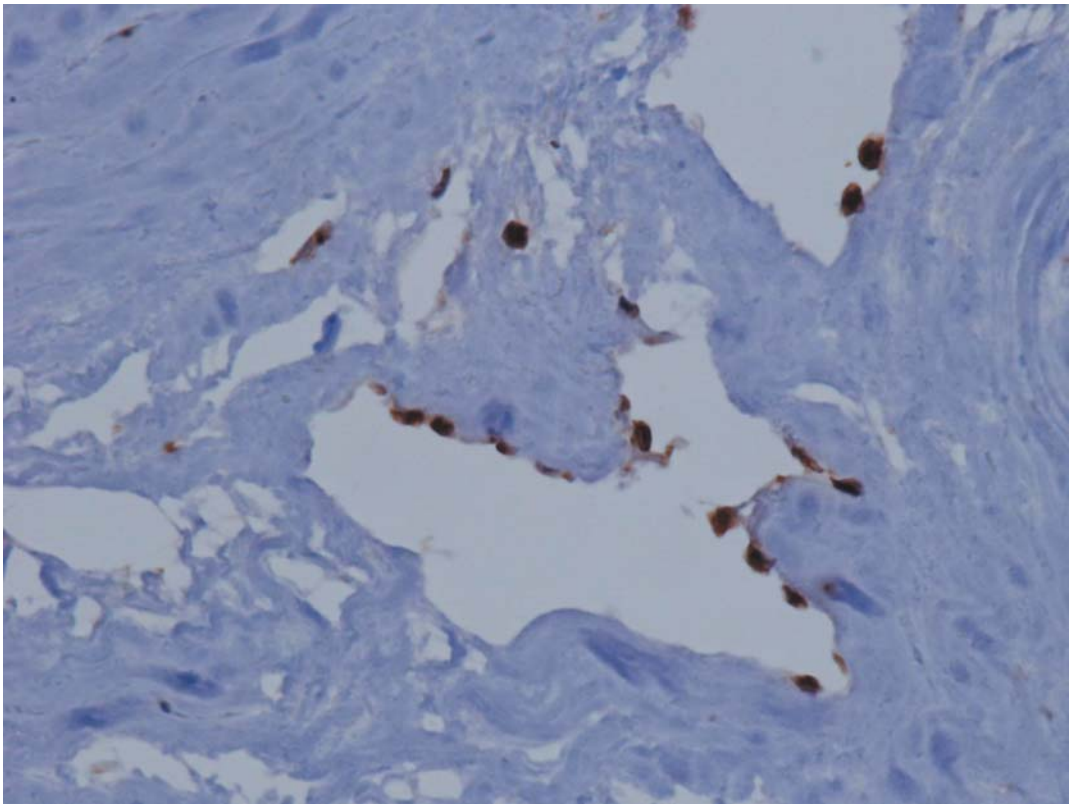

Figure 4. *HSV detection by ISH-TSA in the endothelial cells of a recanalized thrombotic coronary artery (60X).*

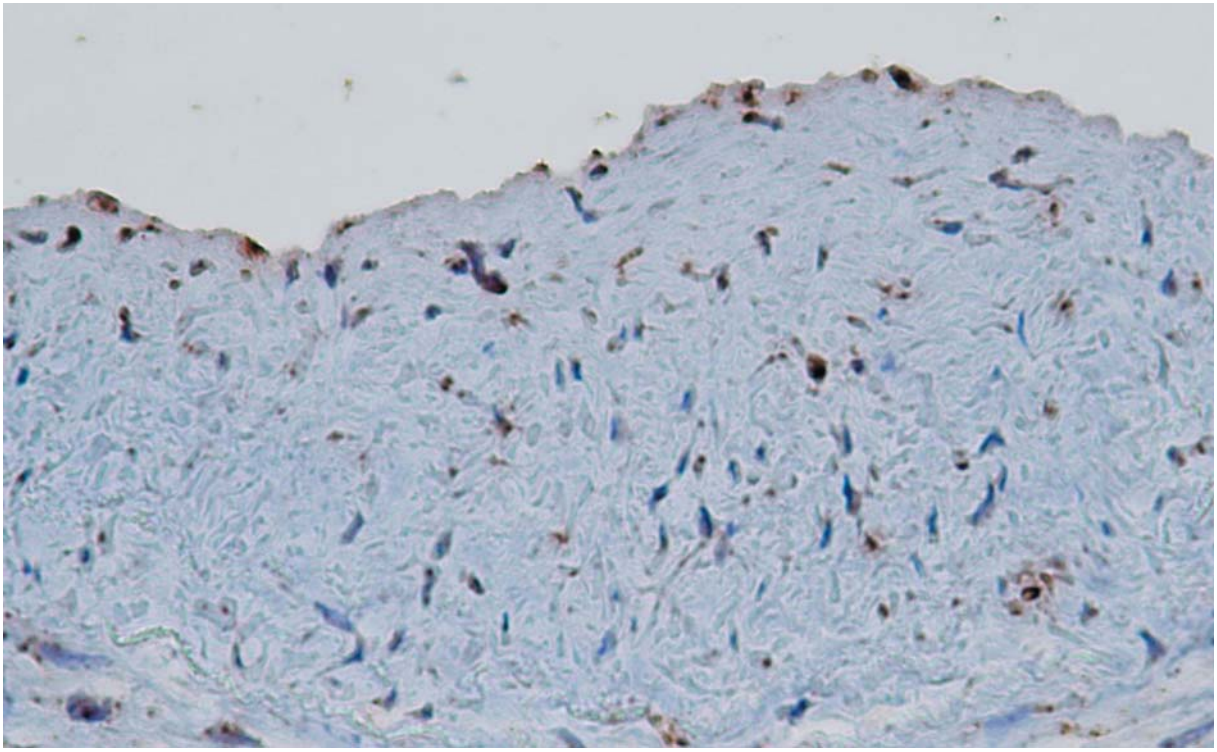

Figure 5. *Detection of HSV in a coronary artery showing intimal thickening. HSV-positive endothelial and intimal spindle cells are seen (40X).*

resulting in diminished inactivation of several coagulation proteases and higher thrombin generation; second, by alterations in the phospholipid topography of the endothelial cell membrane after HSV infection, which results in enhanced thrombin production and consequently increased platelet binding to the endothelium; third, by increased attraction to the infected endothelium of the inflammatory cells and platelets. This, in turn, is accomplished by endothelial cell expression of P-selectin and von Willebrand factor, which act as monocyte and platelet receptors, respectively.

Moreover the expression of Fc, C3b and viral glycoprotein E by HSV-infected endothelial cells results in the increased adhesion of granulocytes. The increased attraction of inflammatory cells to the HSV-infected endothelium may further amplify the prothrombotic effects on the endothelium through the release of inflammatory cytokines.

The initial arterial injury caused by HSV infection may further be augmented by mechanical factors, such as endothelial abrasion at arterial bifurcation sites. This mechanical action may advance the atherogenic vascular changes and offer a rational explanation for the predilection for these sites in atheroma formation.

The detection of HSV DNA in the coronaries of subjects of both the MI as well the young victim group with early atherosclerotic changes suggests that the virus is involved in the early, as well as in the late stages of the atherosclerotic process, in a significant percentage of cases. HSV, as well as other herpes viruses, is capable of escaping immune response and establishing a long-term, persistent infection of the vascular wall with periods of latency and reactivation.

The fate of HSV vascular infection in an immunocompetent individual, according to our hypothesis, seems to be the result of the interaction of several factors, the most important of which are the duration of the viral infection and, especially, the rate of reactivation of the latent infection. According to the present as well as other studies (11,18), the detection of HSV DNA in the young victim group's coronary arteries without atherosclerotic changes indicates that the simple presence of the virus in the vascular wall may not be such an important factor for atherogenesis, as compared to the rate of virus reactivation. The rate of virus reactivation seems to be the most critical factor for the establishment and evolution of the atherosclerotic lesion.

The above behavior of viral infection is probably responsible for the wave-like nature of the disease, characterized by intermittent acute exacerbations (21), which we suggest may be aligned with the virus reactivation cycle. This is probably the reason why some researchers were unable to find any association between the simple presence of HSV or CMV IgG serum antibodies and the increased risk of coronary atherothrombosis (22).

HSV reactivation mainly depends on the immune system's response, which in turn also depends on psychological factors. Ishihara *et al.* investigated the correlation between psychological factors and immune response in coronary heart disease and reported the presence of NK cells activity inhibition, which is an integral part of the antiviral defense mechanism, in individuals with depressive or neurotic behaviour (23). According to the above, if the immune-psychological profile of the person permits a high reactivation rate of viral infection, this may result in the faster local expansion of the virus and hence greater atherosclerotic changes. In addition, the final course of vascular changes in an individual will also depend on the background of other risk factors, as well as additional infectious agents present.

This hypothesis is in agreement with recent studies that clearly show that the increased pathogen burden is positively-related to the faster progression of atherosclerosis (24,25), which may be the result of immunological overcharge and, consequently, inadequate immunological response to control the virus. This situation parallels the fast development of atherosclerosis in CMV-infected organ transplant recipients because of prolonged immunosuppression (26). Moreover, the entrapment of various infectious agents in a growing atherosclerotic plaque may aggravate the lesion through further increasing the local inflammatory process (27).

Further evidence supporting the significant role of herpes viruses in atherosclerosis derives from the study of Leeson *et al.*, who found that prolonged breast feeding (> 4 months) is related to sclerotic changes of the arterial walls (28). This association could very well be attributed to the fact that herpes viruses may be transmitted to the breast-feeding infants through the milk. According to our previous findings, HSV was present within breast milk in 47% of the examined women, while CMV and HHV7 were present in 10.3% of the cases (29,30). Consequently, the longer the duration of breast feeding, the greater the viral load transmitted to the infant and, hence, the greater the risk of vascular infection, particularly in a period in which infants are characterized by the immaturity of their immune system.

Finally, a significant association between spontaneous loss at early pregnancy and increased risk for development of maternal ischemic heart disease later in life has recently been reported (31). This association can also be explained by our HSV model, as it has been found that male sperm and the endometrium show a high frequency of HSV infection (13,32), which, according to some studies, is an important factor of first trimester pregnancy loss (33,34).

In conclusion, the findings of our study indicate a significant association between HSV infection and coronary atherosclerosis, since HSV infection seems to be involved in the initiation and progression of atherosclerosis. It is highly probable that an early and long-term treatment of the viral infection in HSV-positive cases may prevent the progression of vascular damage and reduce the risk of myocardial infarction.

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