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Detection of Cytomegalovirus and *Helicobacter pylori* DNA in Arterial Walls with Grade III Atherosclerosis by PCR

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Abstract

It has been suggested that some microorganisms may play a role in the etiology or progression of atherosclerotic plaques. The purpose of this study was to assess for the presence of *Helicobacter pylori* and cytomegalovirus (CMV) DNA using polymerase chain reaction (PCR) technique in vascular-wall specimens obtained during autopsy. Four to 5 mm long samples from 3 different vascular wall specimens (coronary, carotid and abdominal aortas) of 30 patients (23 male, 7 female) were taken for pathologic and microbiologic investigations during autopsy. *H. pylori* DNA was found in 48.2% atherosclerotic and 19.6% non-atherosclerotic vascular wall specimens, whereas CMV DNA was found in 37.9% atherosclerotic and 32.7% non-atherosclerotic vascular wall specimens. In terms of CMV DNA detection, no statistically significant differences between the atherosclerotic and non-atherosclerotic groups were present ($P > 0.05$). However, there was a statistically significant difference between the atherosclerosis and non-atherosclerotic groups in terms of *H. pylori* DNA in coronary and abdominal aorta arteries ($p = 0.016$ and $p = 0.0029$ respectively) but not in carotid arteries ($p = 1.00$). In conclusion, the correlation between *H. pylori* and atherosclerosis could be suggested. These finding warrant further investigation regarding the role of *H. pylori* in atherosclerosis.

Key words: atherosclerosis, cytomegalovirus, *Helicobacter pylori*

Introduction

Conventional risk factors, including hyperlipidemia, hypertension, diabetes, tobacco use, sex, and family history of premature vascular diseases are not enough to explain the occurrence of atherosclerosis. There are additional, not yet fully defined, risk factors attributing to the development of atherosclerosis (Bloemenkamp *et al*., 2002).

In recent years, an association between infectious diseases and occurrence of atherosclerosis have been reported by numerous investigations (Roivainen *et al*., 2000; Ameriso *et al*., 2001). There is some evidence that chronic infections might contribute to the progression of atherosclerosis (Grahame-Clarke *et al*., 2003). *Helicobacter pylori* is an important pathogen involved in several gastroduodenal diseases. Also, there is a potential role of *H. pylori* infection in several extradigestive diseases like atherosclerosis (Takashima *et al*., 2002). Some investigators have demonstrated a positive relationship between *H. pylori* infection and atherosclerosis (Pasceri *et al*., 1998), but others have not found such a relationship (Regnstrom *et al*., 1998; Ridker *et al*., 1999).

It has also been reported that there is a relation between cytomegalovirus (CMV) infections and development of atherosclerosis (Grahame-Clarke *et al*., 2003). CMV DNA has been detected in arterial walls of patients with grade III atherosclerosis by PCR (Hendrix *et al*., 1990). It has been suggested that infection with *H. pylori* or CMV might be independent risk factors for atherothrombotic diseases (Bloemenkamp *et al*., 2002).

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The aim of this study was to assess for the presence of *H. pylori* and/or CMV DNA in vascular-wall specimens obtained during autopsy, using PCR technique in order to include them (or not) into factors contributing to atheriosclerosis.

Experimental

Materials and Methods

Specimen. All specimens were obtained during autopsy under sterile conditions from the Council of Forensic Medicine Presidency of Ankara Group. Autopsy was performed within 12 h of death. For microbiologic and pathogenic examinations, arterial segments approximately 4 to 5 mm long samples were placed in microcentrifuge tubes containing Tris-EDTA buffer. Transport vials were opened only in the laminar airflow safety cabinet at the microbiology laboratory. The specimens for description were kept at –20°C until microbiologic processing.

Pathologic examination. The severity of atherosclerotic lesions was graded according to the Stary classification (Stary *et al*., 1995). The specimens for pathologic examination were fixed in 10% buffered formalin, routinely processed and embedded in paraffin. Sections were prepared and stained with haematoxylin and eosin for light microscopic examination.

Polymerase chain reaction (PCR). The specimens and controls (*H. pylori* NCTC 11637 and CMV AD 169) were pre-treated with a solution containing 10 mM Tris-HCl (pH 8.3), 1 mM EDTA, and 100 mg of proteinase K per ml and incubated at 60°C for 1 h and heated at 96°C for 10 min, then nucleic acids were extracted with phenol:chloroform:isoamyl alcohol (25:24:1) method. Extracted nucleic acids were dissolved in 50 µl DNase- and RNase-free deionized distilled water and subjected to PCR reaction.

For the detection of *H. pylori* DNA we used UreC primers (5'- AAG CTT TTA GGG GTG TTA GGG GTT T -3 and 5'- AAG CTT ACT TTC TAA CAC TAA CGC-3) as described by Ho *et al*., 2004. DNA was amplified in 50 µl volumes containing 200 mM each deoxynucleoside triphosphate, 1 mM each primer, 2 U of Taq polymerase (Promega Corporation, USA), 10 mM Tris-HCl, 1.5 mM $MgCl_2$, and 50 mM KCl. The amplification was performed in an automated thermocycler for 40 cycles at 94°C for 30 s (5 min for the first cycle), 54°C for 30 s, and 72°C for 60 s (10 min for the last cycle).

For the detection of CMV DNA we used P1 (5'- AAG CTT TTA GGG GTG TTA GGG GTT T-3) and P2 (5'-AAG CTT ACT TTC TAA CAC TAA CGC-3) primers as described by Saygun *et al*., 2004. DNA was amplified in 50 µl volumes containing 200 mM each deoxynucleoside triphosphate, 1 mM each primer, 2 U of *Taq* polymerase, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, and 50 mM KCl. The amplification was performed in an automated thermocycler for 35 cycles at 94°C for 30 s (5 min for the first cycle), 56°C for 30 s, and 72°C for 60 s (10 min for the last cycle).

Amplification products, 294 and 264 bpDNA fragments for *H. pylori* and CMV respectively, were visualized by electrophoresis in 2% agarose gel containing ethidium bromide at 0.5 µg/ml under UV light box (Gel Doc 2000, BIO-RAD, USA). *H. pylori* NCTC 11637 and CMV (AD 169) were used as a positive control in each run of PCR assays. PCR assay was performed at least twice for each agent.

Statistical analysis. Statistical analysis was performed using SPSS for Windows version 7.5 (SPSS Inc., Birmingham, UK). Data are expressed as proportions or means ± SD. Statistical comparisons were performed using χ^2 test for categorical variables and Mann-Whitney U test for continuous variables. $P < 0.05$ was considered statistically significant.

Results

A total of 90 artery specimens including 30 coronary, 30 carotid and 30 abdominal aorta arteries were obtained from 30 patients comprised of 23 men and 7 women (median age, 37.6). Twenty nine of them had grade III atherosclerosis. The demographic data of coronary and carotid artery and abdominal aorta artery in both atherosclerotic and non-atherosclerotic lesion groups were listed in Table I. No significant differences were found between the two groups with respect to age, sex and smoking history.

As shown in Table II, *H. pylori* DNA (Fig. 1.A) was found in 14 atherosclerotic and 12 non-atherosclerotic vascular wall specimens; CMV DNA (Fig. 1B) was found in 11 atherosclerotic and 20 non-atherosclerotic vascular wall specimens. Five of atherosclerotic lesions were positive for both *H. pylori* and CMV DNA.

Table I
The demographic data in non- and atherosclerotic groups in the patients

Parameter	Coronary artery			Carotid artery			Abdominal aorta artery		
	Grade III atherosclerotic group (n = 6)	Non-atherosclerotic group (n = 24)	*p*	Grade III atherosclerotic group (n = 12)	Non-atherosclerotic group (n = 18)	*p*	Grade III atherosclerotic group (n = 11)	Non-atherosclerotic group (n = 19)	*p*
Mean age ±	37.8 ± 18.7	37.2 ± 16.8	0.959	43.2 ± 20.3	33.9 ± 16.0	0.161	41.6 ± 16.7	35.3 ± 18.9	0.35
Sex (no. male/no female)	5/1	20/4	1.000	10/2	15/3	1.000	9/2	16/3	1.00
Smoking history (%)	4 (66.7)	18 (75.0)	0.645	9 (75.0)	13 (72.2)	1.000	8 (72.7)	14 (73.7)	1.00

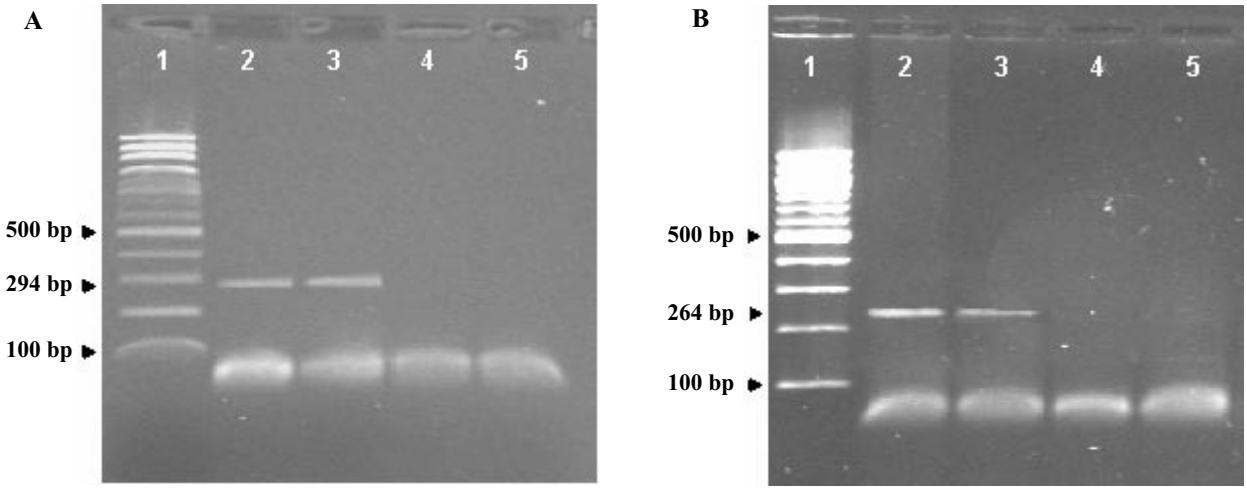

Fig. 1. Amplification of DNAs (294 and 264 bp) from *H. pylori* (GEL A) and CMV (GEL B).

PCR products were electrophoresed in 2% agarose gel. (GEL A) Lanes: 1, size marker; 2, positive control (*H. pylori*, NCTC 11637); 3, positive tissue sample; 4, negative tissue sample and 5, negative control. (GEL B) Lanes: 1, size marker; 2, positive control (CMV AD 169); 3, positive tissue sample; 4, negative tissue sample and 5, negative control.

The detection rate of *H. pylori* DNA in atherosclerotic specimens obtained from coronary and abdominal aorta arteries was significantly higher compared with non-atherosclerotic vascular wall specimens ($p<0.05$) but that obtained from carotid arteries was not significant ($p>0.05$). Detection rate of CMV DNA was not significantly different between atherosclerotic and non-atherosclerotic vascular wall specimens ($p>0.05$).

Table II
The presence of *H. pylori* and CMV DNA in the arteries of non- and atherosclerotic groups

Parameter	Coronary artery			Carotid artery			Abdominal aorta artery		
	Grade III atherosclerotic group (n = 6)	Non-atherosclerotic group (n=24)	*p*	Grade III atherosclerotic group (n = 12)	Non-atherosclerotic group (n = 18)	*p*	Grade III atherosclerotic group (n = 11)	Non-atherosclerotic group (n = 19)	*p*
H. pylori (%)	4 (66.7)	3 (12.5)	0.016	2 (16.7)	3 (16.7)	1.000	8 (72.7)	6 (31.6)	0.029
CMV (%)	3 (50)	4 (16.7)	0.120	5 (41.7)	7 (38.9)	1.000	3 (27.3)	9 (47.4)	0.442

Discussion

It has been proposed that atherosclerosis is caused by inflammatory process and microorganisms could be the prime initiators of this process. Most authors investigating the endovascular presence of infectious agents used directional coronary atherectomy. Therefore, they were limited to small samples, possibly underestimating the true incidence of pathogens, so they could not identify co-infections within the coronary arteries. In terms of potential pathogenic effects, these agents may act involving both direct effects on arterial walls and indirect effects mediated *via* the circulation (Libby *et al*., 1997). Results obtained from animal studies (Fabricant *et al*., 1978), sero-epidemiologic observations (Patel *et al*., 1995; Rechciński *et al*., 2005), pathologic-based investigations (Maass *et al*., 1998), and data from small-randomised clinical trials (Gupta *et al*., 1997) have provided evidence of direct pathogenic involvement of infectious agents in the process of atherogenesis.

Several microorganisms including CMV and *H. pylori* have recently been related to the pathogenesis of atherosclerosis (Carlsson *et al*., 2000). The CMV, a herpesvirus restricted exclusively to the human host, may predispose to the development of atherosclerosis (Reinhardt *et al*., 2003; Horne *et al*., 2003). Moreover, it can be inferred from serological studies that there is an epidemiological link between CMV infection and atherosclerosis (Nieto *et al*., 1996; Reinhardt *et al*., 2002), but the data remain controversial (Siscovick *et al*., 2000). In atherosclerotic vascular walls, the presence of CMV DNA has been demonstrated by the detection of viral antigen and nucleic acids in the cultured smooth muscle cells from coronary

artery plaque material (Melnick *et al*., 1994). CMV nucleic acid was extracted from atherosclerotic femoral arteries and abdominal aortas in one study (Hendrix *et al*., 1991). It has been shown that 90% of advanced atherosclerotic lesions (Grade III) obtained at surgery were positive for CMV nucleic acids, whereas 50% of Grade I lesions obtained at autopsy from age- and sex-matched patients were positive (Hendrix *et al*., 1990). In our study, CMV DNA was found in both non-atherosclerotic and atherosclerotic artery tissue, but no significant association between the atherosclerotic groups and non-atherosclerotic groups were observed ($P>0.05$). It can be inferred from this result that CMV could be in vessels, but it may not be a significant factor for atherosclerotic process.

The Gram-negative curved bacillus, *H. pylori*, occurs endemically in worldwide, and it is estimated that up to 50% of the world's population is infected (Sinha *et al*., 2002). *H. pylori* colonizes the gastrointestinal tract and is associated with chronic gastritis, peptic ulcer disease, and gastric cancer (Takashima *et al*., 2002). *H. pylori* has also been sero-epidemiologically linked to coronary heart disease and atherosclerosis by causing chronic infections (Heuschmann *et al*., 2001). *H. pylori* genome has been shown in diseased arterial segments (Danesh *et al*., 1999). However, two studies failed to demonstrate any evidence of *H. pylori* in the atherosclerotic plaques of abdominal aortic aneurysms (Blasi *et al*., 1996) and carotid arteries (Chiu *et al*., 1997) of patients being sero-positive for *H. pylori*. It has been shown that *H. pylori* has an important role in the pathogenesis of atherosclerosis, especially in Turkey, where infection is prevalent and conventional risk factors fail to explain the high prevalence of atherosclerotic vascular disease (Farsak *et al.*, 2000). *H. pylori* DNA has also been found in 53% of carotid atherosclerotic plaques by demonstrating the microorganism in lesions by specific immunohistochemistry (Ameriso *et al*., 2001). In our study, the *H. pylori* DNA has been found in both non-atherosclerotic and atherosclerotic artery tissues. However, there was a significant association between *H. pylori* infection and atherosclerosis for coronary and abdominal aorta arteries ($P<0.05$), whereas no significant association was found for carotid arteries ($P>0.05$). *H. pylori* infection rates change markedly among distinct populations. Consistent with previous findings of high seroprevalences in less developed countries, Turkish people have been reported to be a high-risk population (Porsch-Ozcurumez *et al*., 2003). In highly risky countries like Turkey, *H. pylori* may play a role in the development of atherosclerosis.

In conclusion, in this study, a relation between *H. pylori* and atherosclerosis but not CMV was shown. However, further large-scale studies are needed in order to confirm the significance of this finding.

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