

Detection of Enterovirus, Cytomegalovirus, and *Chlamydia pneumoniae* in Atheromas

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To investigate the presence of infectious agents in human atherosclerotic arterial tissues. Atherosclerotic plaques were removed from 128 patients undergoing carotid endarterectomy or other bypass procedures for occlusive disease, and from twenty normal arterial wall samples, obtained from transplant donors with no history of diabetes, hypertension, smoking, or hyperlipidemia. Using the polymerase chain reaction (PCR) or reverse transcription-PCR, these samples were analyzed for the presence of *Chlamydia pneumoniae*, cytomegalovirus, enterovirus, adenovirus, herpes simplex viruses types 1 and 2, and Epstein-Barr virus. The amplicons were then sequenced, and phylogenetic analyses were performed. Enteroviral RNA was found in 22 of 128 atherosclerotic vascular lesions (17.2%), and *C. pneumoniae* and cytomegalovirus were each found in 2 samples (1.6%). In contrast, adenovirus, herpes simplex viruses, and Epstein-Barr virus were not identified in any of the atherosclerotic samples. Enterovirus was detected in 6/24 (25.0%) aortas, 7/33 (21.2%) carotid arteries, 6/40 (15.0%) femoral arteries, and 3/31 (9.7%) radial arteries of patients with chronic renal failure. There were no infectious agents detected in any of the control specimens. Using phylogenetic analysis, the enterovirus isolates were clustered into 3 groups, arranged as echovirus 9 and coxsackieviruses B1 and B3. Enteroviral RNA was detected in 17.2% of atherosclerotic plaques, but was not observed in any of the control specimens. This suggests a connection between enteroviral infection and atherosclerosis. These findings differ from those of other studies, which found more frequent incidence of *C. pneumoniae* and cytomegalovirus infection in atherosclerotic plaques.

Key words: atheroma, PCR, enterovirus, cytomegalovirus, *Chlamydia pneumoniae*

Atheroma formation in humans seems to be of a multifaceted etiology. While some of the risk factors involved are already well established, such as smoking, hyperlipidemia, diabetes, and hypertension, accumulating but circumstantial evidence implicates certain pathogens to be associated with atheroma formation. Pathogens have been identified within atherosclerotic plaques or in the arterial walls of patients afflicted with atherosclerosis. The contributing role of bacterial or viral pathogens in coronary atheroma formation has been substantiated with indirect evidences. This evidence have been acquired through the study of animal models (Marek's disease) and by epidemiological surveys that have shown the presence of serum antibodies to infectious agents such as *Chlamydia pneumoniae* and CMV in atherosclerotic patients (Adam *et al.*, 1987; Hajjar, 1991; Saikku, 2000; Bloemenkamp *et al.*, 2003). *Chlamydia pneumoniae* has also been detected in atherosclerotic lesions using electron microscopy (EM)

techniques (Shor *et al.*, 1992). The role of these pathogens in the pathogenesis of atherosclerosis, however, is still controversial.

PCR is currently considered to be the most sensitive method for detection of infectious agents. In contrast to dot blot or in situ hybridization, PCR has been utilized in the identification of viruses in patients suffering from mild or severe atherosclerosis (Hendrix *et al.*, 1990). We, therefore, employed this technique to analyze the presence of infectious agents in human atherosclerotic tissue.

Materials and Methods

Patients and specimens

Between 2000 and 2002, we removed atherosclerotic plaques during bypass procedures which were performed on 128 patients undergoing treatment for occlusive lower limb ischemia, carotid artery stenosis, abdominal aortic aneurysm, and chronic renal failure. These patients ranged in age from 19 to 81 years. The sample included 92 males (mean age, 60.0 years) and 36 females (mean age, 57.2

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years).

The normal arterial control group was composed of twenty renal or hepatic arteries acquired from adult transplant donors who did not possess known risk factors for atherosclerosis, such as diabetes, hypertension, smoking, and hyperlipidemia.

Template preparation

DNA was extracted from tissues using the QIAamp DNA mini kit (Qiagen, UK), according to manufacturer's instructions. Tissues were quickly cut into an appropriate size and incubated with 180 µl ATL buffer and 20 µl Proteinase K (20 mg/ml) at 56°C until the tissues were completely lysed. To each sample was added 200 µl AL buffer, and the samples were then incubated at 70°C for 10 min. To each was added 200 µl absolute ethanol, and each solution was subsequently applied to a spin column. The column was washed with AW1 and AW2 buffers. RNA was extracted using the QIAamp Viral RNA extraction kit (Qiagen, UK), according to manufacturer's instructions. RNA was recovered in 150 µl nuclease-free water and stored at -80°C.

RT-PCR for enterovirus

Enteroviral RNA was amplified by reverse transcription-nested PCR using primers of 5' nontranslated regions (Table 1). This region is highly conserved among the enterovirus serotypes and was selected in order to maximize detection rate. First-strand cDNA, used in the detection of the enteroviral genome, was generated from 10 µl

of extracted total RNA by incubation for 1 h at 37°C with 1 unit RNase inhibitor, first strand buffer (75 mM KCl, 50 mM Tris HCl, pH 8.3), 10 mM DTT, 0.2 mM of each dNTP, 15 pmol outer downstream primer, and 200 U MMLV reverse transcriptase (Gibco BRL, Germany). The enzyme was inactivated at 90°C for 5 min. Primary amplification of nested PCR was achieved with the addition of 1 µl cDNA to a tube containing 20 pmol U1 and D1 primers, amplification buffer (1.8 mM MgCl₂, 10 mM Tris HCl, pH 8.3), 0.2 mM of each dNTP, and 1 U Taq polymerase (AmpliTaq, Perkin Elmer Cetus, USA), followed by amplification on a DNA thermal cycler (Perkin Elmer 9600, USA). The amplification protocol consisted of 1 cycle of denaturation at 94°C for 5 min, annealing at 52°C for 15 sec, and extension at 72°C for 15 sec; 30 cycles at 94°C for 15 sec, 52°C for 15 sec, and 72°C for 15 sec; and one cycle at 94°C for 15 sec, 52°C for 15 sec, and 72°C for 5 min. A second enzymatic amplification was performed with the addition of 3 µl of the first PCR product to a tube containing 25 pmol of U2 and D2 primers, amplification buffer, 0.2 mM of each dNTP, and 1.25 U Taq polymerase. The protocol for amplification consisted of an initial denaturation at 94°C for 4 min; followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min 30 sec, and extension at 72°C for 20 sec (first cycle, with addition of 1 sec for each subsequent cycle). This process was followed by a final extension for 5 min at 72°C. Amplified products were then electrophoresed on 2% agarose gels, which were stained with ethidium bromide for visualization under UV.

Table 1. Primer sequences used and PCR product sizes

Virus/Bacteria	Primer name	Target site	Primer sequence (5' → 3')	PCR product size (bp)
K-ras		12th codon	5'-TATTATAAGGCCTGCTGAAAATGACTGAAT-3' 5'-TTACCTCTATTGTTGGATCATATTTCGTCCA-3'	135
<i>Chlamydia pneumoniae</i>	HL-1(S) HM-1(S) HR-1(A)	474bp <i>Pst</i> I fragment	5'-GTTGTTTCATGAAGGCCTACT-3' 5'-GTGTCATTCGCCAAGGTTAA-3' 5'-TGCATAACCTACGGTGTGTT-3'	437 229
Cytomegalovirus	QT65F QT65R	pp65 region	5'-AAAGAGCCCCGACGTCTACTACACGT-3' 5'-CCAGGTACACCTTGACGTACTGGTC-3'	169
Enterovirus	EntU1 EntD1 EntU2 EntD2	5' conserved region	5'-GGTGCGAAGAGCCTACTGAG-3' 5'-CACCGGATGGCCAATCCA-3' 5'-CCCCTCAATGCGGCTAAT-3' 5'-ATTGTCACCATAAGCAGCCA-3'	228 155
Epstein-Barr virus		EcoRI B fragment	5'-GTGTGCGTCGTGCCGGGGCAGCCAC-3' 5'-ACCTGGGAGGGCCATCGCAAGCTCC-3'	375
Herpes simplex virus 1,2		DNA pol gene region	5'-GGAGGCGCCCAAGCGTCCGGCCG-3' 5'-TGGGGTACAGGCTGGCAAAGT-3'	229(HSV1) 241(HSV2)
Adenovirus		Hexon region	5'-AGCACGCCGCGGATGTCAAAG-3' 5'-GCCGCAGTGGTCTTACATGC-3' 5'-TGAGCCCCGGGCTGGTGCAGTTTGC-3'	308 250

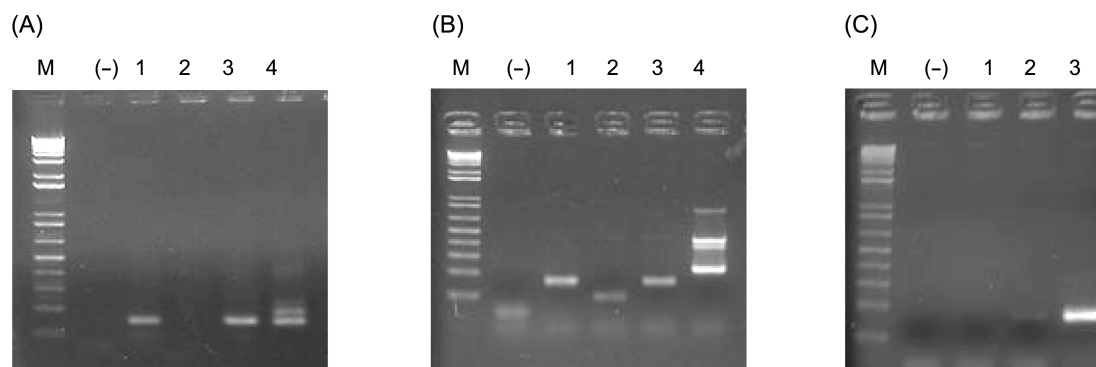


Fig. 1. Representative gel electrophoresis demonstrating amplification of (A) enterovirus, (B) *C. pneumoniae*, and (C) CMV from clinical specimens. (A) Lane M, 1 kb marker; (-), negative control; Lanes 1-3, patient nos. 70, 90, and 121, respectively; Lane 4, enterovirus positive control. (B) Lane M, 1 kb marker; (-), negative control; Lanes 1-3, patient nos. 61, 69, and 79, respectively; Lane 4, *Chlamydia pneumoniae* positive control. (C) Lane M, 1 kb marker; (-), negative control; Lane 1 and 2, patient nos. 100 and 106, respectively; Lane 3, CMV positive control.

PCR for DNA virus and *Chlamydia pneumoniae*

All amplifications were performed in a PCR reaction mixture consisting of 1x buffer, 4 µl DNA, 0.2 mM of each dNTP, 25 pmol of each primer, and 1.25 U Taq polymerase. The PCR reactions of each of the selected DNA viruses were performed in different conditions. In the case of cytomegalovirus (CMV), the amplification protocol was 94°C for 5 min; followed by 35 cycles of 94°C for 30 sec, 68°C for 30 sec, and 72°C for 30 sec. For adenovirus, the amplification protocol was 95°C for 5 min; followed by 35 cycles of 95°C for 1 min, 55°C for 1 min, 72°C and 1 min. In the case of HSV1 and HSV2, the amplification protocol was 95°C for 5 min, followed by 35 cycles of 95°C for 1 min, 61°C for 40 sec, and 72°C for 1 min. For parvovirus, the amplification protocol was 94°C for 3 min, followed by 40 cycles of 94°C for 2 min, 37°C for 2 min, and 72°C for 3 min. For K-ras, the amplification protocol was 98°C for 6 min, followed by 40 cycles of 94°C for 2 min, 60°C for 1 min 30 sec, and 72°C for 3 min.

Nucleotide sequence analysis

The enteroviral-specific amplification product was analyzed using direct nucleotide sequencing with a T7 Sequenase v2.0 Kit (Amersham Bioscience, Sweden). The reaction products were then labeled with ³⁵S dATP and separated on an 8% denaturing polyacrylamide gel.

Phylogenetic study

Multiple sequences were aligned using the CLUSTAL W (Chenna *et al.*, 2003) method with full alignment and a DNA identity matrix. The gap penalties were 10 for open and end, 0.05 for extension and 8 for distances. Gblocks v 0.91h was used to eliminate inadequately aligned positions and divergent regions in order to make this technique more suitable for phylogenetic analysis (Castresana, 2000) with less stringent options. Phylogenetic analyses were completed using the PHYLIP v 3.6a3 package (Felsenstein, 1985). The distance matrices were generated

using the F84 model in the DNADIST program. A tree was then constructed by the neighbor-joining clustering method present in the NEIGHBOR program. The tree was plotted using the program Tree view v 1.6.6 (Page, 1996) and statistically evaluated using 1000 bootstrap samples.

Results

Detection of infectious agents

Atherosclerotic plaques were analyzed for the presence of cytomegalovirus, enterovirus, herpes simplex virus types 1 and 2, Epstein-Barr virus, and *Chlamydia pneumoniae* (Fig. 1).

The normal arterial control group was composed of 20 renal or hepatic arteries obtained from adult transplant donors who did not possess risk factors for atherosclerosis, such as diabetes, hypertension, smoking, and hyperlipidemia. Ten of these individuals were men and ten were women. The average age of the control group was 33.5 years. Each of these individuals was proven to be free of risk factors associated with atherosclerosis, including diabetes, hypertension, hyperlipidemia, and smoking. Enterovirus was detected in one of these 20 controls. Sequence analysis showed that this was probably due to contamination. No other aberrant viral or bacterial sequences were observed in any of the control samples. In contrast, enterovirus sequences were detected in 22/128 (17.2%) of the atherosclerotic plaques when nested RT-PCR was used (Table 2). *C. pneumoniae* and cytomegalovirus sequences were also identified in two cases each (Table 2). No correlation was observed between enterovirus sequences and plaque location. In detail, enterovirus was detected in 6/24 (25.0%) aortas, 7/33 (21.2%) carotid arteries, 6/40 (15%) femoral arteries, and 3/31 (9.7%) radial arteries from patients with chronic renal failure ($p > 0.05$ for each pairwise comparison). No sequences corresponding to adenovirus, herpes simplex viruses types 1 and 2 or Epstein-Barr virus were detected

Table 2. Detection of infectious agents in atherosclerotic specimens II

Group	No. positive/No. tested samples (%)		
	<i>C. pneumoniae</i>	CMV	Enterovirus
Normal	0/20(0)	0/20(0)	0/20(0)
Atherosclerosis	2/128(1.6)	2/128(1.6)	22/128(17.2)
Carotid	—	—	7/33(21.2)
Aorta	—	1	6/24(25.0)
Femoral	1	1	6/40(15.0)
Radial	1	—	3/31(9.7)

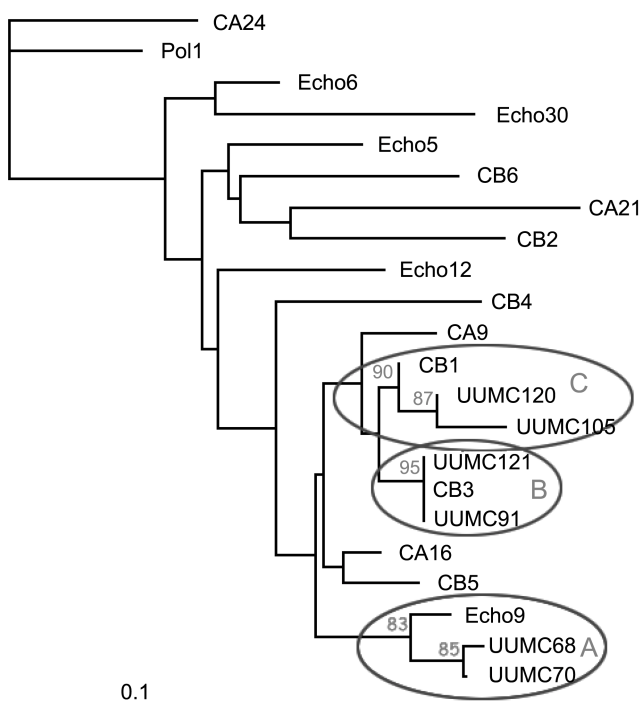


Fig. 2. Phylogenetic tree showing the relationships between the clinical isolates and standard strains. Numbers near the node indicate bootstrap values (values > 80% are relative to 1000 replicates). The scale bar represents the nucleotide changes. Standard strain sequences used, with their GenBank accession numbers are: Pol1, Poliovirus strain Sabin (V01150); CA9, Coxsackievirus A9 strain Griggs (D00627); CA16, Coxsackievirus A16 G-10 (U05876); CA21, Coxsackievirus A21 strain Coe (D00538); CA24, Coxsackievirus A24 (D90457); CB1, Coxsackievirus B1 (M16560); CB2, Coxsackievirus B2 strain Ohio (AF081485); CB3, Coxsackievirus B3 strain PD (AF231765); CB4, Coxsackievirus B4 (D00149); CB5, Coxsackievirus B5 (X67706); CB6, Coxsackievirus B6 strain Schmitt (AF114384); Echo5, Echovirus 5 (AF083069); Echo6, Echovirus 6 (U16283); Echo9, Echovirus 9 (X92886); Echo12, Echovirus 12 strain Travis (X77708); and Echo30, Echovirus 30 (AF162711).

in any of the control or atherosclerotic plaque samples.

Enteroviral sequence analysis

The relationship between the clinical isolates and the standard strain was phylogenetically assessed (Fig. 2). A

sequence length of 115 bp was utilized (95% of the original sequence) for phylogenetic analysis by Gblock using multiple sequence alignment of ClustalW. The isolates were then clustered into 3 groups which corresponded to echovirus 9 and coxsackieviruses B1 and B3. These clusterings were firmly supported by bootstrap analysis, despite the unreliable topology of the total tree with low bootstrap values (less than 50%) of other nodes. This result suggested that the region of analysis was adequate for phylogenetic strain typing, although it proved less informative for evolutionary tracking. Conclusively, UUMC 68 and 70 were typed to echovirus 9, UUMC 91 and 121 to coxsackievirus B3, and UUMC 105 and 120 to coxsackievirus B1.

Discussion

Historically, the pathogenesis of atherosclerosis has focused specifically on arterial wall injury and inflammation. In the mid 1800s, von Rokitansky developed an “incrustation hypothesis”, which proposed that atherosclerosis was caused by lipid accumulation following fibrin deposition. Virchow developed a “lipid hypothesis”, which suggested that lipid deposition is a result of the shearing forces of blood flow on endothelial cells (Virchow, 1999). Ross’s “response-injury” hypothesis suggested that focal inflammation, as a response to various metabolic, mechanical, chemical, or viral injuries, may be a cause of atherosclerosis (Ross, 1993). Furthermore, any endothelial injury would result in monocyte migration and the formation of fatty streaks and atherosclerotic plaques (Ross, 1993).

Infectious agents are increasingly thought to play an important role in the progress of atherosclerosis (Kuvn and Kimmelstiel, 1999). It has been suggested that the position or range of the infectious response is relevant to the cause and progress of atherosclerosis (Mendall *et al.*, 1996; Jander *et al.*, 1998; Libby and Ostergard, 1998). For example, infection with *C. pneumoniae* has been shown to accelerate intimal thickening and inflammatory atherosclerosis-like changes in the aorta (Laitinen *et al.*, 1997; Muhlestein *et al.*, 1998). This bacterium has been identified in smooth muscle cells, endothelial cells, macrophages and atherosclerotic plaques subsequent to endarterectomy (Grayston *et al.*, 1995; Jackson *et al.*, 1997; Maass *et al.*, 1997; Yamashita *et al.*, 1998). In contrast, other investigators were not able to detect *C. pneumoniae* in atherosclerotic carotid and coronary arteries (Paget *et al.*, 1997; Andreassen *et al.*, 1998; Lindholt *et al.*, 1998; Paterson *et al.*, 1998). In support of the latter, this study detected *C. pneumoniae* in only two of 128 patients (1.6%) showing signs of atherosclerotic plaque. Both of these cases were due to occlusive disease, not aortic aneurysms.

CMV has also been found in the aortas, femoral arteries and coronary arteries of patients with atherosclerosis through the use of PCR, in situ hybridization and immu-

nohistochemical staining (Hendrix et al., 1990; Adam et al., 1997; Yonemitsu et al., 1998). One previous report has claimed that CMV is present in 90% of samples obtained from atherosclerotic patients, which suggests a role for this virus in the pathologic mechanism of atherosclerosis (Hendrix et al., 1990). Other researchers, however, have not been able to isolate CMV in atherosclerotic samples, which suggests that the virus is not for a crucial factor in atheroma formation (Daus et al., 1998; Saetta et al., 2000). In support of the latter research, our study detected CMV in only two of 128 patients (1.6%).

Coxsackievirus B (CVB) is manifested clinically in varied forms. Some of these manifestations include the common cold, pneumonia, endocarditis, skin diseases, and aseptic meningitis. In rats, CVB has been shown to cause acute coronary artery infection, thus suggesting a relationship between CVB infection and coronary artery disease (Sohal et al., 1968). Of the 128 patients we assayed, enterovirus was isolated from 22 (17.2%) atherosclerotic plaques. It is not clear why this high prevalence of enterovirus has been observed in atheroma in Korea. One report has stated that enteroviruses, as well as adenovirus, were detected in tap water in Korea (Lee and Kim, 2002). To further subgroup the enteroviruses, we performed sequence analysis and phylogenetic analysis. Out of 22 amplicons, 5 were not sequenced and 11 were partially sequenced but were too short for performance of phylogenetic analysis. Although only 6 out of 22 amplicons were analyzed, phylogenetic analysis results confirmed that these amplicons were homologous to the 5'-NTR of CVB genomic RNA. However, since the 5'-NTR sequences are not necessarily correlated with serotypes, sequence analysis of VP1 regions is additionally required to confirm the association between atherosclerosis and CVB infection.

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