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Detection of Cytomegalovirus in Atherosclerotic Aorta and Coronary Artery by *In Situ* Hybridization and PCR

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Chronic infection and inflammation have recently been implicated as important etiologic agents of atherosclerosis. Several agents have been suggested as possible candidates including cytomegalovirus (CMV), herpes simplex virus type 1 (HSV-1), Epstein-Barr virus (EBV), *Chlamydia pneumoniae*, and *Helicobacter pylori*. We evaluated the relationship between cytomegalovirus infection and atherosclerosis by *in situ* hybridization and polymerase chain reaction (PCR). We examined 23 subjects with atherosclerosis and 10 matched control subjects without atherosclerosis. CMV was detected by *in situ* hybridization in 60.9% (14/23) of aorta and 42.9% (9/21) of coronary arteries in subjects with atherosclerosis. It was also detected by PCR in 65.2% (15/23) of aorta and 52.4% (11/21) of coronary arteries. CMV was detected on areas showing early or advanced atheromatous changes. Cells morphologically identical to smooth muscle cells, endothelial cells, lymphocytes, fibroblasts, and Schwann cells were positively reacted with the CMV probe. However, none of the cells to which the probe hybridized contained inclusion bodies, thus strongly suggesting that the arterial wall may be a site of CMV latency. This result indicates that CMV may potentially play a direct or indirect role in the pathogenesis of human atherosclerosis.

Key Words: Cytomegalovirus (CMV), Atherosclerosis, *In situ* hybridization, Polymerase chain reaction

INTRODUCTION

More than half of the population of developed countries is dying as a result of cardiovascular disease or stroke primarily, although not exclusively, related to atherosclerosis. Atherosclerosis is characterized grossly by diffuse narrowing of the aorta or coronary arteries and microscopically by a concentric fibrointimal thickening with a proliferation of smooth muscle cells and infiltration of lipid-laden macrophages and T-lymphocytes[32].

Conventional risk factors including hyperlipidemia, hypertension, diabetes, cigarette smoking, sex, and family history of premature vascular disease account only for approximately half of the patients with clinically apparent atherosclerosis[43]. Recently, a potential link between infectious agents and atherosclerosis has been suggested. Data obtained from several seroepidemiological studies has given rise to the hypothesis that an infection can initiate or maintain the atherosclerotic process[9].

Viruses have long been suspected of playing a role in cardiovascular diseases, particularly the cardiomyopathies and certain chronic valvular diseases of unknown etiology. Experimental studies by Fabricant *et al.*[15~17] clearly established that infection of normocholesterolemic chickens with Marek's disease virus (MDV), a herpesvirus, led to an arterial disease that closely resembled chronic human atherosclerosis. Marek's herpesvirus infection was also shown to alter aortic cholesterol metabolism and enhance cholesterol and cholesteryl ester accumulation in infected chickens and in cultured arterial smooth muscle cells[16,26]. These findings have stimulated interest in the possible role of herpesviruses as initiating or accelerating factors in human atherogenesis.

Cytomegalovirus (CMV), a beta-herpesvirus, infection is

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characterized by a primary infection leading to a lifelong persistence of the viral genome. Periodically, the virus reactivates from latency and recovers its ability to multiply. It has been associated with the development of accelerated arteriosclerosis and increased graft rejection in heart transplant recipients[5,19,22,35,46]. Both direct infection of the aorta and coronary arteries by CMV, and indirect activation of the host immune system by CMV have been proposed[22].

In humans, clinical, epidemiological, *in vitro in vivo*, and studies has also implicated a relationship between herpesviruses and atherosclerosis[41]. Human herpesviruses have been proposed as potential initiators of arterial injury. This theory was based on the results of animal model studies[40], the epidemiological association between herpes viral infection and accelerated arteriosclerosis in heart transplant patients and in restenosis after angioplasty[8,27,39,61]. Eight members of the family Herpesviridae are now known to infect humans[18]. Herpes simplex virus type 1 (HSV-1), herpes simplex virus type 2 (HSV-2), Epstein-Barr virus (EBV) and cytomegalovirus (CMV) are widespread in the general population. Therefore, these virus can be primary candidates related to atherosclerosis for investigations. This study used *in situ* hybridization and polymerase chain reaction (PCR) to provide further evidence that the existence of the herpesviruses are related to atherosclerosis. *In situ* hybridization was employed because this technique has been shown to be sensitive in detecting CMV in vessels and it provides topological information as to the distribution of viral nucleic acids[47,64].

The purpose of this study was to examine the aorta and coronary arteries of subjects with atherosclerosis for the presence of CMV, and to evaluate the relationship between cytomegalovirus and atherosclerosis.

MATERIALS AND METHODS

1. Materials

The study population consisted of 23 persons (15 men and 8 women; range of age, 21~65 years; mean age, 43 years) with atherosclerotic aorta and coronary artery, who were all referred to National Institute of Scientific Investigation (NISI; Seoul, Korea) for autopsy between April and October 2001. For control studies, aorta and coronary artery specimens, from which atherosclerotic lesions including fatty streak and plaque were excluded, were obtained from 10 persons (6 men and 4 women; range of ages, 18~54 years; mean age, 36 years) who were died from traffic accidents.

2. Tissue Preparations

Immediately following removal of aorta and coronary artery segments, each segment was fixed with 10% formalin in order to maintain vascular morphological integrity. Each segment was overnight processed through the following solutions: serially 70%, 75%, 80%, 90%, 95% ethyl alcohol, absolute ethyl alcohol, acetone, 2 changes of xylene, 2 changes of paraffin by Hypercenter XP Tissue Processing System (Shandon® Scientific Limited, Cheshire, England). The processed segment was embedded in paraffin and cut in 5 μm sections, which were stained with hematoxylin-eosin (H&E). One lesion from each segment, which had morphological characteristics of atherosclerosis ranging fatty streak to complicated atherosclerosis lesion was assigned for histopathologic analysis and matched with the corresponding lesions for *in situ* hybridization and polymerase chain reaction.

3. *In Situ* Hybridization

5 μm thick sections of formalin fixed paraffin-embedded tissue (aorta, coronary artery) were floated from a bath of distilled water onto ProbeOn™ plus slides (Fisher Scientific, Pittsburgh, PA, USA), and heated in a 60℃ incubator. The sections were then dewaxed in xylene 2 times for 3 minutes and rehydrated in serial-graded ethanol washes (100%, 95%, 80%, 70%), and immersed in water for 3 minutes. The slides were placed on an incubation tray and digested with 100 μl of proteinase K (20 μg/ml, diluted in 50 mM Tris/HCl buffer, pH 7.6), and incubated for 20 minutes at 37℃. The slides were immersed in water 2 times for 3 minutes and dehydrated again in serial-graded ethanol (70%, 80%, 95%, 100%) and air-dried.

The fluorescein-labeled oligonucleotide probes hybridization solution for detection of human cytomegalovirus early gene mRNA sequences (Hyb-probe™, Shandon® Scientific Limited, Cheshire, England) was applied in formamide solution (50% formamide, 0.1 M Pipes, pH 7.8 and 0.01 M EDTA) in a volume, usually 20 μl, sufficient to cover the section. Hybridization was performed according to the manufacturer's instruction.

4. DNA Extraction in Paraffin-Embedded Tissues

For PCR reaction, several of 5 μm-thick section of each paraffin embedded sample were deparaffinized by xylene. To digest tissues, the proteinase K (10 μg/20 μl) was used. To extract the DNA, the phenol-chloroform-isoamylalcohol (25:24:1) method was used.

5. Polymerase Chain Reaction (PCR)

Specific primers were synthesized according to the published DNA sequences corresponding to the target gene regions, all of which were expected to be highly conserved. The following primers were used: HSV-1[37], DNA polymerase gene (134 bp), sense (5' TCC TCG TAG ACG CCC ATC GGC G 3'), antisense (5' CTT GTA ATA CAC CGT CAG GT 3'); EBV[51], *Bam*H-IW region gene (234 bp), sense (5' CCT AGG GGA GAC CGA AGT AA 3'), antisense (5' GAC CCT TCT ACG GAC TCG TCT G 3'); CMV[30], major immediately-early gene (249 bp), sense (5' CCT AGT GTG GAT GAC CTA CGG GCC A 3'), antisense (5' CAG ACA CAG TGT CCT CCC GCT CCT C 3'); HHV6[30], immediately-early gene (435 bp), sense (5' CCG CAA TCG AAT CCA CCT AGC GG 3'), antisense (5' GTG AGA ACG GAT TCG AAC AGT GCT G 3'); *C. pneumoniae*[6], 16S rRNA gene (463 bp), sense (5' TGA CAA CTG TAG AAA TAC AGC 3'), antisense (5' CGC CTC TCT CCT ATA AAT 3'); *H. pylori*[7], urease gene (411 bp), sense (5' GCC AAT GGT AAA TTA GTT 3') antisense (5' CTC CTT AAT TGT TTT TAC 3'). All DNA isolates were tested for the presence of PCR inhibitors and for the human DNA isolation efficiency by the control amplification of the 540 bp long target of human beta-actin gene with primers, sense (5' GTG GGG CGC CCC AGG CAC CAG GGC 3') and antisense (5' CTC CTT AAT GTC ACG CAC GAT TTC 3'). The specificity of the oligonucleotide sequence was initially determined by a GenEMBL database search with the FastA algorithm, which showed 100% homology with the target gene and minimal homology with non-specific mammalian gene sequences.

PCR reaction mixtures contained 2 μl template DNA, 2 μl 10× buffer, 1.2 μl $MgCl_2$ (at a final concentration of 1.5 mM), 1.6 μl dNTPs (0.2 mM), 2 μl each primer (2 pmol/μl), and 1.25 U of thermostable *Taq* polymerase (Takara shuzo, Kyoto, Japan). The final volume was filled to 20 μl with sterile distilled water. The amplification reaction was performed on a thermal cycler (Perkin Elmer 9600, Norwalk, USA). The cycle parameters were as follows: first cycle of 94℃ for 3 minutes to initial denaturation; 34 subsequent cycles of 95℃ for 1 minute, 58℃ for 2 minutes, 72℃ for 1 minutes; final cycle of 72℃ for 7 minutes to enough extension. Five microliters of the amplification products were subjected to electrophoresis on 1.5% agarose gels (NuSieve GTG agarose; BMA, Rockland, ME, USA) at 100 V for 20 minutes. The gels were stained with ethidium bromide for 30 minutes. Gels were observed, photographed and data stored in a computer by Gel Documentation Systems Gel Doc2002 (Bio-Rad Laboratories, Hercules, CA, USA).

6. Sequencing of PCR products

The PCR product was sequenced using ABI 377 DNA sequencer with ABI PRISM BigDye Terminator Cycle Sequencing Kit (PE Applied Biosystems, Foster, CA, USA). Cycle sequencing was performed on the GeneAmp 9600 (Perkin Elmer, Norwalk, USA) according to the manufacturer's instruction.

RESULTS

1. Histopathologic Analysis

Sections of aorta and coronary artery from control groups showed no histopathologic evidence of atherosclerosis except minimal intimal thickening.

Compared with controls, all atherosclerotic aorta and coronary artery showed lesions that ranged from fatty streak to complicated atherosclerotic plaque. There was also prominent inflammatory infiltration of mononuclear cells and foam cells in atherosclerotic plaque, and to a lesser extent in the inner media and adventitia.

The relationship between the virologic findings and histopathologic observations in serial H&E stained sections of the aorta and coronary arteries were summarized in Table 2 and Table 3.

2. *In Situ* Hybridization

Of 23 subjects with atherosclerosis, CMV was detected by *in situ* hybridization in 60.9% (14/23) of aorta and 42.9% (9/21) of coronary arteries. Of 10 control subjects, CMV was detected in only one coronary artery (Table 1).

By *in situ* hybridization, CMV were localized in cells

morphologically consistent with endothelial cells, smooth muscle cells, lymphocytes, fibroblasts, and Schwann cells. CMV was sometimes detected in spindle-shaped cells located in discrete foci of increased cellularity (shoulder region) within the intima. CMV was detected more frequently in atherosclerotic areas than in non-atherosclerotic areas (Fig. 1 and Fig. 2).

Atheromatous changes consisted of focal or eccentric intimal thickening with smooth muscle cell proliferation and the presence of lymphocytic infiltrates and/or scattered foam cells. CMV was found in the aorta in 14 of 23 subjects. Of 14 positive subjects, 9 showed mild atheromatous changes, and 5 showed moderate atheromatous changes (Table 2). T-lymphocytic infiltrates were evident in the intima of all 14 cases. CMV was found in the coronary arteries in 9 of 21 subjects. Of 9 positive subjects, 4 showed mild atheromatous changes and 5 showed moderate atheromatous changes (Table 3).

This study shows evidence for the presence of CMV in the coronary arteries and aorta of Korean by *in situ* hybridization method.

3. Specificity and Sensitivity of PCR

The specificity of the six primer sets was tested by amplification of DNA from herpes simplex virus type 1 (HSV-1), cytomegalovirus (CMV), human herpes virus 6

Table 1. Detection of CMV in aorta and coronary artery by *in situ* hybridization

Sample site	Control		Atherosclerosis	
	Cases	Positive cases	Cases	Positive cases
Aorta	10	0	23	14
Coronary artery	10	1	21	9

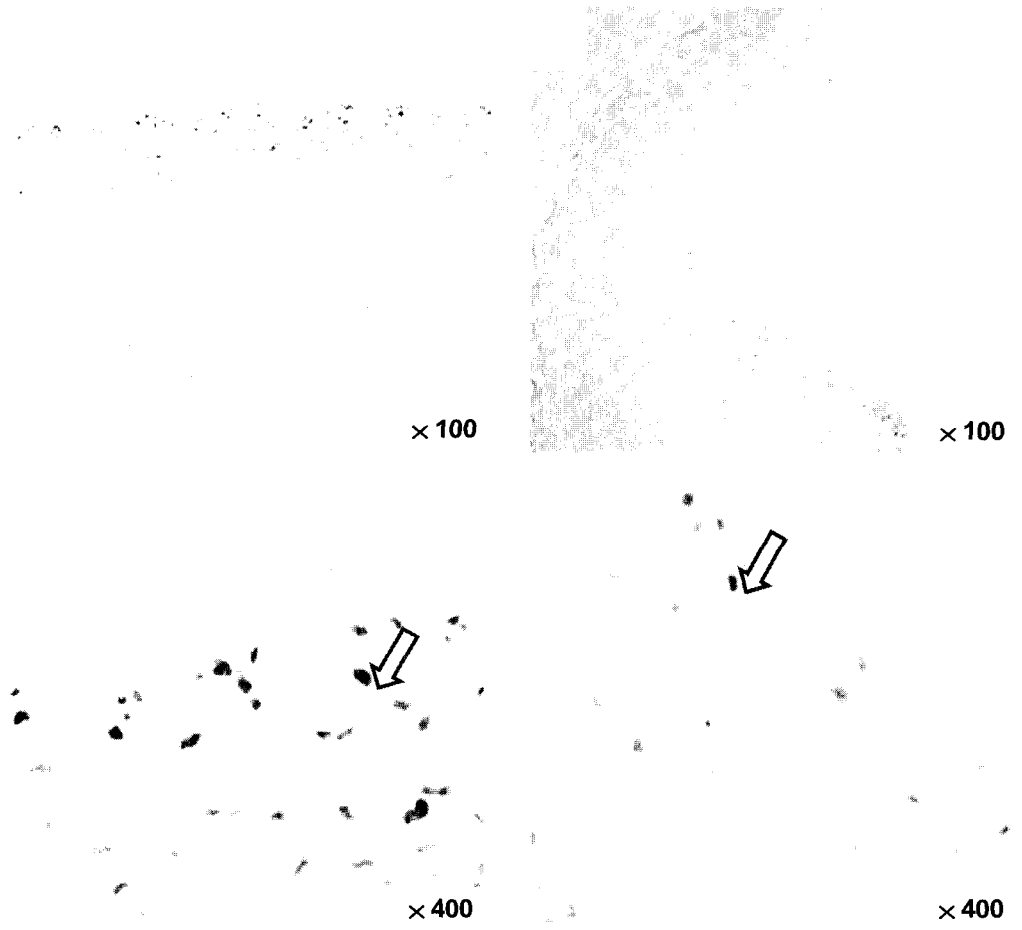

Fig. 1. Atherosclerotic lesions showing the presence of CMV in superficial and deep intimal cells by *in situ* hybridization. Panel **A-B**, Hybridization was seen in intimal cells of aorta (arrow in panel B). Panel **C-D**, Hybridization in coronary artery (arrow in panel D).

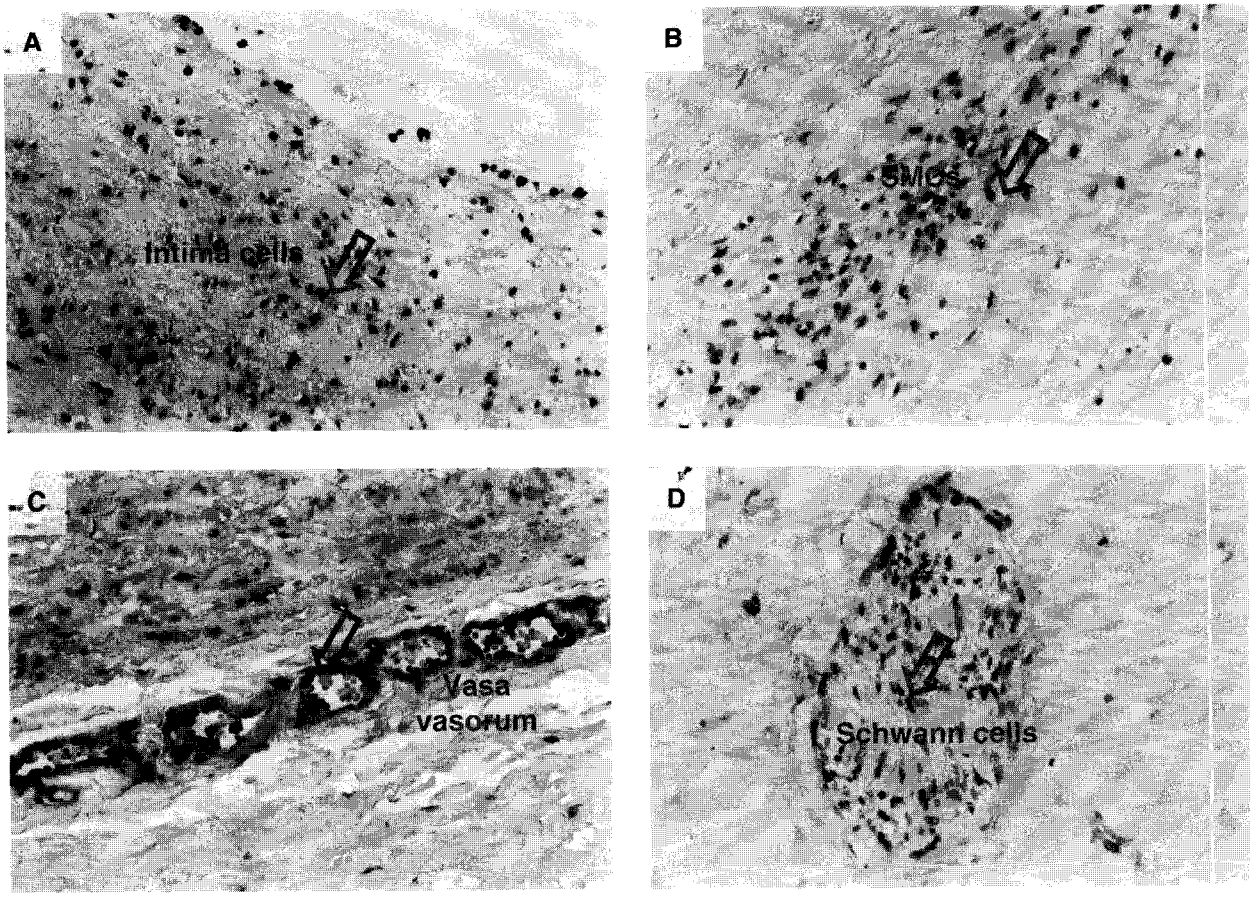

Fig. 2. Atherosclerotic lesions showing the presence of CMV by *in situ* hybridization (×200). Panel **A,** hybridization with CMV probe was seen in intimal cells; panel **B,** smooth muscle cells (SMCs); panel **C,** vascular endothelial cells in adventitia; panel **D,** Schwann cells.

Table 2. Relationship between degree of atherosclerosis and CMV detection in the aorta

Degree of Atherosclerosis	Cases	Positive cases
Mild	15	9
Moderate	7	5
Severe	1	0
Total	23	14

Table 3. Relationship between degree of atherosclerosis and CMV detection in the coronary arteries

Degree of Atherosclerosis	Cases	Positive cases
Mild	8	4
Moderate	12	5
Severe	1	0
Total	21	9

(HHV6), Epstein-Barr virus (EBV), *Chlamydia pneumoniae*, *Helicobacter pylori*. Nonspecific amplification was not obtained, nor was any interassay cross amplification observed (data not shown). Assay sensitivity was determined by amplification of extracted DNA from duplicate serial 10-fold dilutions of CMV (AD-169) 50% tissue culture infectious dose ($TCID_{50}$) stock (AD-169 stock titer: $10^{5.75}$ $TCID_{50}$/0.2 ml). Amplified products detected by agarose gel electrophoresis were observed at various dilutions and corresponded to a calculated minimal amount of detectable virus DNA of 2.8 $TCID_{50}$/ml for CMV (Fig. 3).

Positive control DNA was extracted from MRC-5 human embryonic lung monolayer cultures infected with American Type Culture Collection (ATCC; VR-538) reference strains of CMV (AD-169).

4. Polymerase Chain Reaction (PCR)

The viral nucleic acid of herpes simplex virus type 1 (HSV-1), cytomegalovirus (CMV), human herpes virus 6 (HHV6), Epstein-Barr virus (EBV), *Chlamydia pneumoniae*

Helicobacter pylori was studied by polymerase chain reaction (PCR) in aorta and coronary arteries from 23 autopsy cases.

Of 23 subjects with atherosclerosis, CMV was detected by PCR in 65.2% (15/23) of aorta and 52.4% (11/21) of coronary arteries, and in 3 of aorta and coronary arteries in 10 control subjects (Fig. 4 and 5) (Table 4).

Positive results were detected in 8.7% (2/23) for HSV-1, 4.3% (1/23) for EBV, 43.5% (10/23) for *Chlamydia pneumoniae*, but HHV6 and *Helicobacter pylori* were not detected (data not shown). Despite of the high genetic and biological similarity between CMV and HHV6, no substantial relation of HHV6 in atherosclerosis has been proved. In the atherosclerotic group, DNA of two or three viruses was noted several times in a single sample, whereas neither double nor triple infections occurred in the control group. These finding confirm and extend those of Benditt *et al.*[3] and Melnick *et al.*[39] of the presence of herpesviruses not only in the proximal aorta but also in the coronary arteries of human subjects. CMV and *C. pneumoniae* were encountered more frequently than HSV-1, EBV in both sample sites.

CMV was detected more frequently by PCR, compared with direct antigen detection with fluorescein-labeled oligonucleotide probes by the *in situ* hybridization method. The lower prevalence of viruses detected in the same tissues by *in situ* hybridization (ISH) may be in part due to low virus levels in the infected cells and the relative insensitivity of ISH compared with the very sensitive method of PCR.

In conclusion, this study suggests that the high incidences and kinds of herpes viruses are related to atherosclerosis.

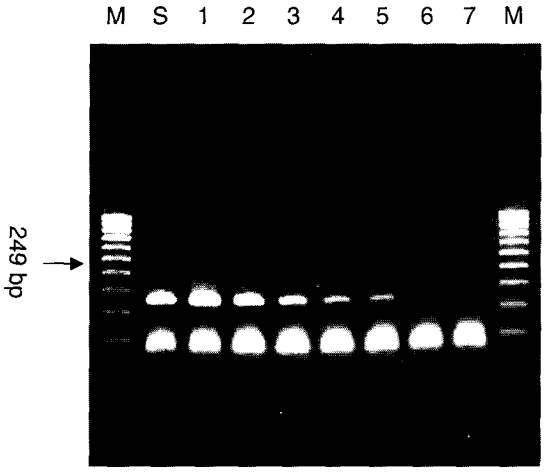

Fig. 3. Sensitivity of CMV PCR. Lane M, 100 bp ladder marker; lane S, CMV (AD-169) stock (starting titer: $10^{5.75}$ $TCID_{50}$/0.2 ml); lanes 1-7, serial 10-fold dilutions of CMV 50% tissue culture infectious dose ($TCID_{50}$) stock.

Table 4. Detection of CMV in aorta and coronary artery by polymerase chain reaction (PCR)

Sample site	Control		Atherosclerosis	
	Cases	Positive cases	Cases	Positive cases
Aorta	10	2	23	15
Coronary artery	10	1	21	11

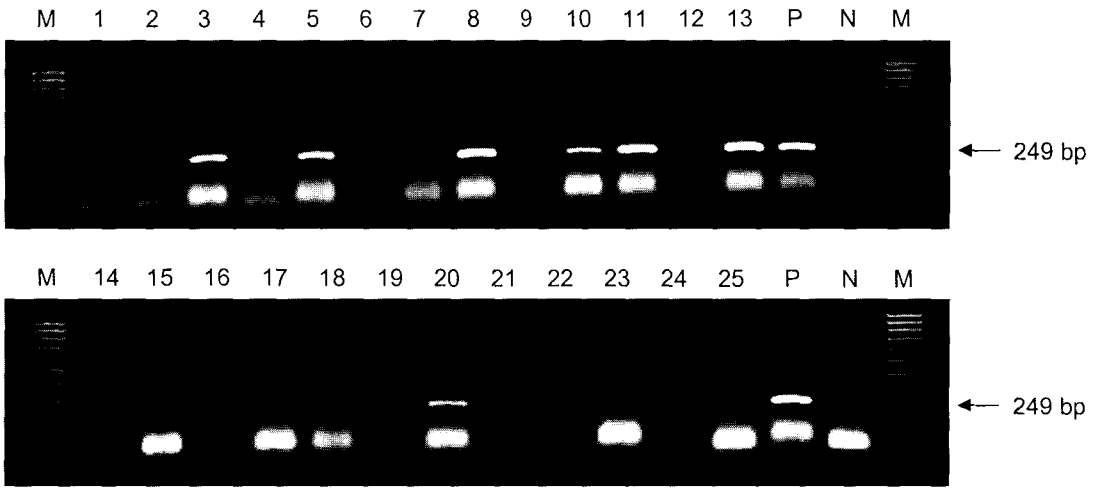

Fig. 4. Amplification of major immediately early (IE) gene of CMV by PCR in coronary artery specimens. Lane M, 100 bp ladder marker; lanes 1-4, control coronary artery; lanes 5-25, atherosclerotic coronary artery specimens from autopsy; P, positive control (AD-169); N, negative control.

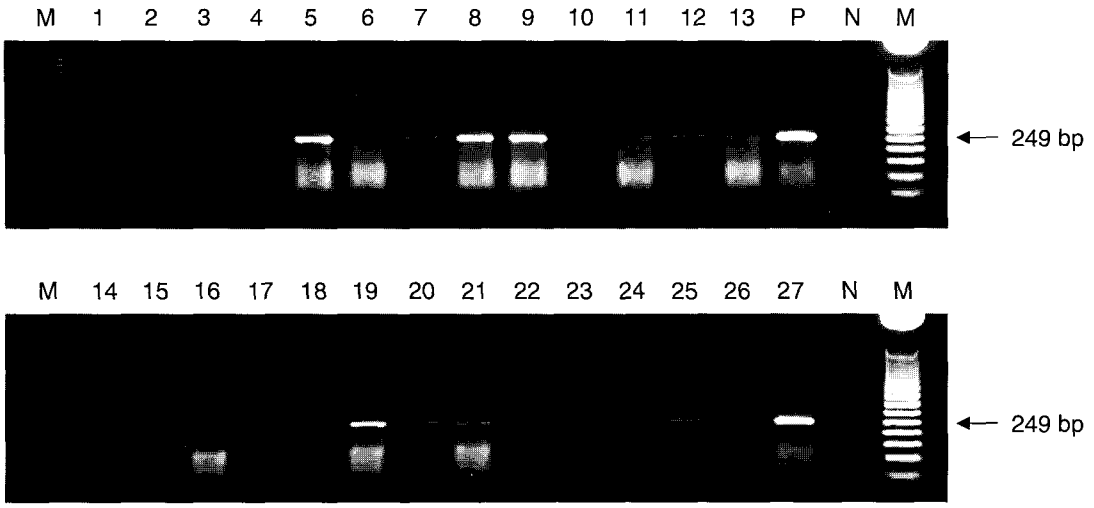

Fig. 5. Amplification of major immediately early (IE) gene of CMV by PCR in aorta specimens. Lane M, 100 bp and 50 bp ladder marker; lanes 1-4, control aorta; lanes 5-27, atherosclerotic aorta specimens from autopsy; P, positive control (AD-169); N, negative control.

DNA Sequence of PCR products (249 bp)

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  1                          CAGA CACAGTGTCC TCCCGCTCCT
 41  CCTGAGCACC CTCCTCCTCT TCCTCATCAC TCTGCTCACT
 81  TTCTTCCTGA TCACTGTTCT CAGCCACAAT TACTGAGGAC
121  AGAGGGATAG TCGCGGGTAC AGGGGACTCT GGGGGTGACA
161  CCAGAGAATC AGAGGAGCTG ACACCAGCGG TGGCCAAAGT
201  GTAGGCTACA ATAGCCTCTT CCTCATCTGA CTCCTCGGCG
241  ATGGCCCGTA GGTCATCCAC ACTAGG
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Fig. 6. DNA sequencing result of PCR product (249 bp).

5. Sequencing of PCR products

Sequencing analysis revealed that PCR products extracted from atherosclerotic lesions using CMV-specific primer sets were indeed cytomegalovirus DNA of herpesviridae (Fig. 6).

DISCUSSION

In situ hybridization was used because this technique has been shown to be more sensitive than immunoperoxidase techniques in detecting herpesviruses in vessels. The fluorescein-labeled oligonucleotide probes for detection of human cytomegalovirus immediate early (IE) gene mRNA sequences was chosen because it has been shown to hybridize with the full spectrum of clinical isolates of CMV from humans, it lacks homology with other viruses, and it has minimal homology with human placental DNA[1], and it has been suggested that the IE gene is expressed in latent infections[48]. Although homology between human and herpesviral sequences has been described, the probes used here are not reactive with control human tissues by *in situ* hybridization.

In animal models of virus-induced atherosclerosis, herpesviruses have been demonstrated in endothelial cells, smooth muscle cells and lymphocytes[21,25,28]. These are the same cell types to which this study found hybridization of CMV probes. Hybridization to cells without inclusion bodies or cytopathic effects suggests that the CMV present in these vessels is in a latent state.

In addition, there is some suggestive evidence that CMV may be important as a cofactor in the development of atherosclerosis. This evidence comes from animal models and patient studies. In chickens, Fabricant *et al.*[15,16] and Minick *et al.*[40] demonstrated that infection with Marek's disease virus (MDV) leads to occlusive atherosclerosis in

large muscular arteries. The atherosclerosis induced by MDV closely resembles that in humans and occurs in both normocholesterolemic and hypercholesterolemic chickens.

CMV infection may promote the development of atherosclerosis in human through a number of direct and indirect mechanisms[22]. CMV infection could directly injure endothelial cells, and the response to this injury could promote the development of arteriosclerosis as described by Ross *et al.*[49] More subtle forms of viral-induced endothelial injury could also promote arteriosclerosis by reducing the anticoagulant properties of endothelial cells[14,60].

CMV infection has also been associated with an upregulation of major histocompatibility (MHC) antigen expression by endothelial and smooth muscle cells[31,59,62], which is presumably mediated by the release of γ-interferon by activated T-lymphocytes responding to the CMV infection. This induced MHC antigen expression is itself associated with allograft rejection and the development of accelerated arteriosclerosis[32]. An immune response generated by the recipient against viral proteins could lead to a heightened response to donor MHC antigens and thus further promote allograft rejection and the development of accelerated arteriosclerosis[20]. CMV infection of smooth muscle cells could also promote accelerated arteriosclerosis. For example, the infection of arterial smooth muscle cells with MDV alters lipid metabolism and results in enhanced lipid accumulation in these cells[16,26,40]. CMV may induce the proliferation of smooth muscle cells[2]. In addition to smooth muscle and endothelial cells, CMV infection of lymphocytes has also been demonstrated[48,53]. It may activate lymphocytes and this activation of lymphocytes may promote graft rejection and the development of accelerated arteriosclerosis[59].

Melnick *et al.*[38] demonstrated by immunofluorescence tests the presence of CMV antigen in smooth muscle cells cultured from arterial tissues of patients undergoing blood vessel surgery. More than 25% of the cell cultures of arterial tissues derived from both carotid artery plaques and punch biopsy samples of uninvolved areas of the aorta were reported to contain antigens of CMV but not of HSV-1 or HSV-2. Replicating CMV was not detected by electron microscopy in the antigen-positive cells. However, Gyorkey *et al.*[25] reported the presence of virions of the herpesviridae family on direct electron microscopic examination of punch biopsy specimens from the proximal aorta of patients with atherosclerosis. Herpes-type virions were detected in occasional smooth muscle and rare endothelial cells in uninvolved areas of the aorta in 10 of 60 patients examined.

The identification of CMV in the arterial tissues is of epidemiologic interest because of the high prevalence of primary infection with this virus in peoples. In disseminated CMV infection local viral spread is believed to occur via endothelial cell infection[42]. Whether the presence of CMV in the coronary arteries and aorta represents primary infection with hematogenous spread to lesion predilection sites or secondary seeding following reactivation of latent infection at a distant site remains to be established.

The mere presence of herpesviruses in the arterial wall does not necessarily signify a causal relationship to atherosclerosis. However, CMV could play a significant role in the initiation or progression of human atherosclerosis by several mechanisms, depending on the particular cell type involved and whether the infection is permissive or non-permissive. Productive infection in permissive cells may result in alterations of aortic lipid metabolism and/or direct injury of endothelial or intimal cells. CMV-infected human fetal aortic cells have also been reported to proliferate and accumulate cholesterol *in vitro*[26]. CMV has also been shown to replicate in smooth muscle cell cultures derived from human umbilical arteries[58]. CMV can infect endothelial cells *in vivo*, in support of the concept that endothelial cell integrity may play an important role in the initial stages of atherogenesis.

Immunologic injury as a consequence of productive or lytic infection with CMV may also play a role in atherogenesis. In 1974, Smith *et al.*[54] reported the presence of measles and herpesvirus antigen-antibody complexes in aorta from autopsy cases and postulated that a significant cause of vascular tissue injury in humans may be chronic viral infection of the blood vessels *per se* and/or the deposition of antigen-antibody complexes in the vascular tissues. Several human viruses, including CMV, have been shown to induce formation of Fc receptors in a variety of cultured cells[52,63]. Whether Fc and C3 receptor are induced in arterial endothelial and smooth muscle cells *in vivo* by CMV remains to be established.

In addition to lytic infection, latency or persistence, or less frequently, acquisition of a transformed phenotype are pathogenic features of human herpesvirus infection[57]. Benditt *et al.*[3] have suggested that expression of at least a part

of the herpesvirus genome in arterial smooth muscle cells may initiate or maintain enhanced smooth muscle cell proliferation leading to monotypic atheromatous plaque formation. Penn *et al.*[45] demonstrated that plaque cells exhibit molecular alterations similar to that observed in oncogenic transformation and have proposed that one or more unidentified transforming genes may play a role in the proliferation of smooth muscle cells in atherogenesis.

Microscopically, lymphocytic infiltrates were evident in both sites in the majority of virus-positive cases with early or advanced atheromatous changes. Emeson *et al.*[11] have demonstrated mononuclear cell infiltrates in the coronary arteries and aorta, and have identified some of the inflammatory cells as T-cell subsets and monocyte/macrophages. CMV has been shown to abortively infect peripheral human lymphocytes of T- and B-cells lineage and monocytes with virus expression limited to synthesis of immediate-early viral polypeptides[48].

This study found the presence of CMV genome significantly more often in the atherosclerotic group than in the non-atherosclerotic group. In the atherosclerotic group, the DNA of either two or three viruses was noted in a single sample. These present results indicate that only atherosclerotic tissues are likely to contain multiple infectious agents. More recent studies have suggested that the impact of infection on atherogenesis is related to the aggregate number of pathogens with which an individual is infected, a concept referred to as pathogen burden[50,68].

In the present study, the results may simply represent one potential means of arterial injury, and are compatible with the aforementioned response to injury hypothesis. At the least, this study data suggest that viral infections might promote atherosclerosis, perhaps by increased expression of adhesion molecules and inflammatory cytokines[24,55], procoagulant effects[14], increased scavenger receptor expression and activity[66], enhanced uptake of cholesterol and of modified low-density lipoprotein[26], increased smooth muscle cell migration and proliferation[67], anti-apoptotic effects[56], and autoimmune response to infection[13].

The pathway for the delivery of these viruses to the arterial wall has not yet been identified. It is possible that these and other pathogens directly infect the vessel wall and persist in a latent state, either producing an abortive infection or replicating at a low (and possibly intermittent) level. An alternative possibility focuses on the circulating monocyte as a 'Trojan horse', a vehicle for the delivery of the pathogen to the vessel wall[12].

Other microorganisms, such as *Chlamydia pneumoniae* [10,23,34,65] and *Helicobacter pylori*[4,29,36,44], are also related to atherosclerosis. The fact that multiple pathogens have been associated with atherosclerosis implies that many 'atherogenic' pathogens exist. It is also evident that variability exists in host susceptibility to the atherogenic effects of pathogens. The lack of unique or specific pathogens for atherosclerosis does not deny the causal role of infectious pathogens; this depends on the susceptibility of the host.

In conclusion, this study demonstrates that CMV genome and antigen is commonly present in the aorta and coronary arteries of Korean by *in situ* hybridization and PCR methods. The availability of fresh autopsy tissues from Korean provided a unique opportunity to determine the presence and expression of these ubiquitous viruses in human atherosclerotic lesions. The virologic and histopathologic findings presented in this study further substantiate the possible role of cytomegalovirus in the pathogenesis of human atherosclerosis. Because atherosclerosis is a multifactorial disease, the relationship of these findings to epidemiologic features such as age, sex, race, and other risk factors will be assessed in an expanded study population as part of a multicenter study.

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