The Potential Roles of Cyclooxygenase-2 and Matrix Metalloproteinase-9 in Cytomegalovirus-Infected Atherosclerotic Aorta and Coronary Artery

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The Graduate School

Yonsei University

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To

the grace of God.

Dedicated to

My family, Miju, Dayoung, and my parents,

whose sacrifice and love has made it possible to complete

this dissertation.

And to

My mentor, Jong-Bae Kim,

who was an invaluable source of strength and inspiration during

the most difficult hours, who instilled in me a love for

Biomedical Laboratory Science

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And to

all the support from colleagues, friends.

CONTENTS

LIST OF FIGURES AND TABLES

ABBREVIATION

AEC	:	3-amino-9-ethylcarbazole
AP	:	alkaline phosphatase
APS	:	ammonium persulfate
ATCC	:	American type culture collection
BCIP	:	5-bromo-4-chloro-3-indolyl phosphate
CHD	:	coronary heart disease
CMV	:	cytomegalovirus
COX-2	:	cyclooxygenase-2
DNA	:	deoxyribonucleic acid
dNTP	:	deoxyribonucleotide triphosphate
EBV	:	Epstein-Barr virus
ECM	:	extracellular matrix
EDTA	:	ethylenediaminetetraacetic acid
FITC	:	fluorescein isothiocyanate
G-6-PD	:	glucose-6-phosphate dehydrogenase
H&E	:	hematoxylin-eosin
HHV6	:	human herpes virus 6
HSV-1	:	herpes simplex virus type 1
ICAM-1	:	intercellular adhesion molecule-1

ISH	:	*in situ* hybridization
LDL	:	low density lipoprotein
MDV	:	Marek's disease virus
MI	:	myocardial infarction
MMP-9	:	matrix metalloproteinase-9
NBT	:	nitro blue tetrazolium
PCR	:	polymerase chain reaction
PBS	:	phosphate-buffered saline
PGE_2	:	prostaglandin E_2
SMCs	:	smooth muscle cells
TBE	:	tris-borate EDTA
TBS	:	tris-buffered saline
$TCID_{50}$	:	50% tissue culture infectious dose
TXA	:	thromboxane
VEGF	:	vascular endothelial growth factor
VLDL	:	very low density lipoprotein

ABSTRACT

The Potential Roles of Cyclooxygenase-2 and Matrix Metalloproteinase-9 in Cytomegalovirus-Infected Atherosclerotic Aorta and Coronary Artery

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*. This study hypothesized that a vascular infection with CMV may induce a chronic inflammatory reaction and activated inflammatory cells may express inflammatory mediators such as cyclooxygenase-2 (COX-2) and matrix metalloproteinases-9 (MMP-9). At first, the relationship between cytomegalovirus infection and atherosclerosis was evaluated by *in situ* hybridization and polymerase chain reaction (PCR) study, and

then, to confirm the hypothesis, the immunohistochemical study for the inflammatory mediators of atherosclerotic plaques was performed. This study 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.

Subsequently, the immunohistochemical stains for CMV late antigen, COX-2, MMP-9, macrophage, and T-lymphocyte were performed in the cases from which CMV was detected by *in situ* hybridization and PCR. The immunoreactivity for COX-2 and MMP-9 increased in the atherosclerotic plaques itself, predominantly around CMV detected area. These findings seem to support the hypothesis and CMV may play a pathogenetic role in atherogenesis or progression of atherosclerosis.

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.

Therefore, these data seem to support the hypothesis that CMV may potentially play a direct or indirect role in the pathogenesis of human atherosclerosis.

Key words: cytomegalovirus (CMV), atherosclerosis, cyclooxygenase-2 (COX-2), matrix metalloproteinase-9 (MMP-9), *in situ* hybridization, polymerase chain reaction, immunohistochemical staining

CHAPTER I

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

I. 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 (Figure I-1) (Hruban *et al*., 1990).

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 (Nieminen *et al*., 1993). As many as 50% of patients with atherosclerosis lack currently identified risk factors, an observation indicating that additional factors predisposing to atherosclerosis are as yet undetected. The pathogenesis of atherosclerosis is a subject of much debate, covered by theories such as the monoclonal hypothesis (Benditt *et al*., 1973) and the response to injury hypothesis (Ross *et al*., 1993). The former is based on the observation that 75% of atherosclerotic plaques from human tissues that were removed at surgery or at autopsy showed a

monoclonal phenotype of the glucose-6-phosphate dehydrogenase (G-6-PD) enzyme as identified by electrophoretic mobility. Monoclonal hypothesis proponents argue that the smooth muscle cell proliferation seen in the atherosclerotic lesion is similar to that in a benign tumor (Linder *et al*., 1965). On the other hand, the response to injury hypothesis reviewed by Ross *et al*. (1993) has gained widespread acceptance as a conceptual framework for understanding the pathogenesis of atheroslcerosis. But the clinically important 'injurious' agents that are responsible for initiating atherosclerosis remain elusive. Recently, a potential link between infectious agents and atherosclerosis has been suggested. Data obtained from several seroepidemiological studies have given rise to the hypothesis that an infection can initiate or maintain the atherosclerotic process (Danesh *et al*., 1997).

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*. (1978; 1981; 1983) 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 (Fabricant *et al*., 1981; Hajjar *et al*., 1986). These findings have stimulated interest in the possible role of herpesviruses as initiating or accelerating factors in human atherogenesis.

In fact, clinical, epidemiological, *in vitro* and *in vivo* studies has also implicated a relationship between herpesviruses and atherosclerosis in human (Morre *et al*., 2000). Human herpesviruses have been proposed as potential initiators of arterial injury. This theory was based on the results of studies using animal model (Minick *et al*., 1979), the epidemiological association between herpes viral infection and accelerated arteriosclerosis in heart transplant patients and in restenosis after angioplasty (Cunningham *et al*., 1988; Melnick *et al*., 1995; Hajjar *et al*., 1991; Visser *et al*., 1993).

Eight members of the family Herpesviridae are now known to infect humans (Frenkel *et al*., 1990). 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 viruses can be primary candidates related to atherosclerosis for investigations. In 1983, Benditt *et al*. reported the

presence of herpes simplex virus (HSV) mRNA in specimens of aortic wall removed from patients undergoing coronary bypass surgery. Presence of HSV genome was demonstrated by *in situ* hybridization in 13 aortic specimens, some of which appeared to represent early stages of atherosclerosis. Cytomegalovirus (CMV) or Epstein-Barr virus (EBV) genome was not detected in any of the specimens examined. It was also demonstrated that HSV type 1 (HSV-1) is capable of proliferating in cultured human fetal aortic smooth muscle cells. Benditt *et al*. (1983) postulated that expression of at least a part of the herpesvirus genome in arterial smooth muscle cells may in some cases be instrumental in initiating or maintaining the enhanced cell proliferation observed in the pathogenesis of atherosclerosis.

Cytomegalovirus, a beta-herpesvirus, infection is 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 (Fryd *et al*., 1980; Peterson *et al*., 1980; Grattan *et al*., 1989; Light *et al*., 1979; Cameron *et al*., 1989). Both direct infection of the aorta and coronary arteries by CMV, and indirect activation of the host immune system by CMV have been proposed (Grattan *et al*.,

1989).

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. This study employed two methods, *in situ* hybridization and polymerase chain reaction (PCR). *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 (Petrie *et al*., 1987; Yamashiroya *et al*., 1988). In addition to CMV, the presence of *Chlamydia pneumoniae* and *Helicobacter pylori* was also investigated to test the possible relationship of these infectious microorganisms to the pathogenesis of atherosclerosis.

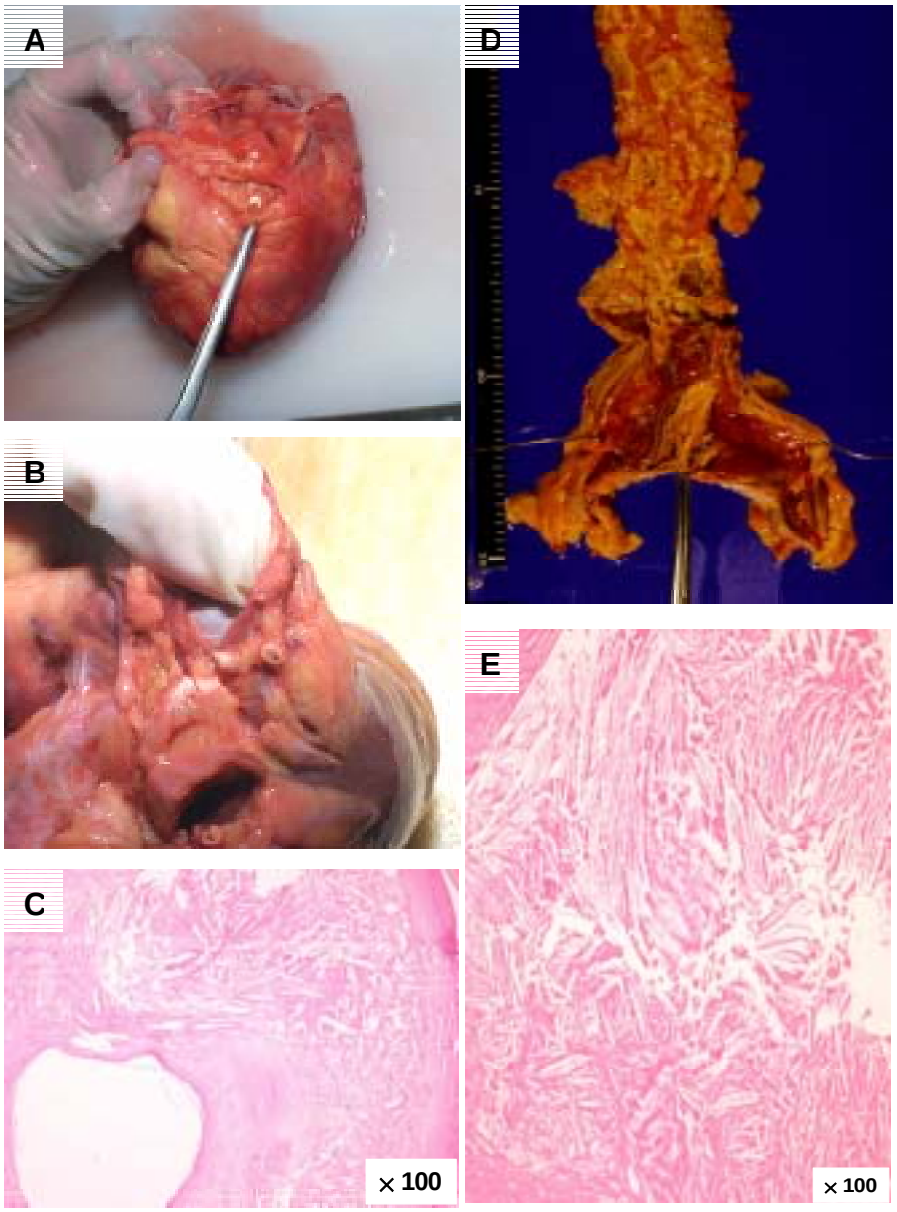

Figure I-1. Atherosclerosis of the coronary artery and aorta. Panel A, B, and D, gross pictures of coronary artery and aorta; Panel C and E, hematoxylin-eosin stain.

II. MATERIALS AND METHODS

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.

Tissue preparation

Immediately following removal of aorta and coronary artery segments, each segment was fixed with 10% formalin in order to maintain vascular morphological integrity. To preserve the integrity of the adventitia and perivascular tissues, aorta and coronary artery specimens were carefully removed in a segment along with adjacent tissues and rinsed with PBS (phosphate buffered saline). 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 from 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.

Verhoeff's Elastic Tissue Stain

The elastic lamina serves as a barrier for cells and macromolecules between the intima and media, media and adventitia in the vascular wall. The morphological changes of the elastic lamina architecture in the atherosclerotic aorta and coronary arteries were evaluated by Verhoeff's elastic tissue staining method.

The paraffin sections (5 μm) were made and transferred to glass slides, and then were deparaffinized and rehydrated through the following solution: xylene 3 times for 3 minutes in each, and serially 100%, 95%, 80%, 75%, and 70% ethyl alcohol for 3 minutes in each.

The sections were then treated with Verhoeff's elastic tissue staining solution (alcoholic hematoxylin 22 ml, 10% ferric chloride 8 ml, iodine solution 8 ml) for 10 minutes, followed by washing in distilled water. The sections were differentiated in 2% ferric chloride for only a few minutes, checked under microscope and if differentiated too far, restained. The sections were then treated with 5% sodium thiosulfate for 1 minute. Washed in tap water for 5 minutes, and then counterstained in van Gieson's staining solution (10% acid fuchsin 5 ml, saturated aqueous solution picric acid 100 ml) for 1 minute. The sections were differentiated in 95% ethyl alcohol, 2 changes of absolute ethyl alcohol, 2 changes of xylene, and mounted.

In Situ Hybridization

Five μm thick sections of formalin fixed paraffin-embedded tissue (aorta, coronary artery) were floated from a bath of distilled water onto acid cleaned 3-aminopropyltriethoxysilane coated slides (ProbeOn^{TM} 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°C. 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. An acid-washed, siliconized coverslip was placed over the section and sealed with rubber cement. The slides were incubated at 65°C for 10 minutes which improve results by a) inactivating endogenous alkaline phosphatase activity, thus reducing background and eliminating the need for levamisole, and b) removing any secondary structure present in the mRNA target, thus increasing hybridization efficiency. The slides were hybridized for 2 hours at 37°C. After hybridization the slides were submerged in post-hybridization solution (TBS containing 0.1% Triton X-100), allow coverslips to float off. The tissue sections were washed at room temperature with 3 changes of TBS/Triton for 3 minutes. The slides were placed on incubation tray and the tissue sections were covered

with 100 µl of blocking solution (normal rabbit serum diluted 1:5 in TBS containing 3% w/v bovine serum albumin and 0.1% v/v Triton X-100), and incubated at room temperature for 10 minutes. The blocking solution was tipped off and the sections were covered with detection antibody (rabbit F(ab') anti-FITC, conjugated to alkaline phosphatase, diluted 1:200 in TBS containing 3% w/v bovine serum albumin and 0.1% v/v Triton X-100), and incubated at room temperature for 30 minutes. Non-specific binding to the specimen was kept at a minimum due to the use of an antibody F(ab') fragment. The tissue sections were washed at room temperature with 2 changes of TBS for 3 minutes. The slides were immersed in alkaline phosphatase substrate buffer (100 mM Tris/HCl pH 9.0, 50 mM magnesium chloride, 100 mM sodium chloride) for 5 minutes. The slides were placed in incubation tray and the alkaline phosphatase activity was demonstrated by covering the sections with 100 µl of NBT/BCIP chromogen solution (80 µl nitro blue tetrazolium 75 mg/ml in 70% v/v dimethyl formamide, 80 µl 5-bromo-4-chloro-3-indolyl phosphate 50 mg/ml in dimethyl formamide, 10 µl 1 M levamisole, 10 ml alkaline phosphatase substrate buffer), and incubated at room temperature in the dark overnight. The slides were washed in running water for 5 minutes, counterstained lightly in Nuclear Fast Red (0.1%

nuclear fast red, 5.0% aluminum sulfate) for a maximum of 10 seconds, and washed in running water. Mounted with aqueous mountant.

Using this technique, only minimal background hybridization was produced. Positive staining was recognized under the microscope as a blue/black color at the site of hybridization. Hybridization was considered positively only in those cases in which the intensity of hybridization was above background, and the control probes did not hybridize.

DNA Extraction in Paraffin-Embedded Tissues

For PCR reaction, several of 5 μm-thick sections of each paraffin embedded sample were put in 2.0 ml microcentrifuge tube using clean forceps. To deparaffinize samples, 1 ml of xylene was added and then inverted several times, and incubated at room temperature for 10 minutes properly on a rocking platform. Centrifuged for 3 seconds at 15,000 rpm in microcentrifuge to pellet the tissue, carefully decanted and 1 ml of xylene was added, inverted several times and incubated at room temperature for 10 seconds and centrifuged for 3 seconds at 15,000 rpm. To remove remained xylene, the pellet was washed by adding 100% ethanol, inverted the tube two or three times, centrifuged

for 3 seconds at 15,000 rpm in microcentrifuge to pellet the tissue and decanted. The pellet was washed by adding 95% ethanol and 70% ethanol serially. To digest tissues, the pellet was resuspended in 600 μl of digestion buffer containing freshly thawed proteinase K (stock 500 μg/ml, 10 μg/20 μl), incubated at 60℃ in water bath with shaking for overnight.

To extract the DNA, the tubes were centrifuged lightly for 5 seconds, 200 μl of supernatant was put into new tube, 150 μl of saturated phenol and 150 μl of chloroform-isoamylalcohol (24:1) were added, vortexed vigorously for 15 seconds, and centrifuged for 5 minutes at 13,000 rpm. The upper phase was put into new tube, repeated upper process (150 μl of saturated phenol and 150 μl of chloroform-isoamylalcohol (24:1) were added, vortexed vigorously for 15 seconds, and centrifuged for 5 minutes at 13,000 rpm). The upper phase was put into new tube. To remove phenol, 150 μl of chloroform-isoamylalcohol (24:1) was added, vortexed vigorously for 15 seconds, and centrifuged for 5 minutes at 13,000 rpm. The upper phase was put into new tube. Two volume of absolute ethanol and 1/10 volume of 0.3 M sodium acetate were added to new tube, inverted several times, placed at –20℃ for 2 hours. Centrifuged at 4℃ in 13,000 rpm for 30 minutes, removed the supernatant carefully.

One ml of 70% ethanol was added for washing of DNA pellet, centrifuged in 13,000 rpm for 15 minutes at 4℃, twice. The pellet was dried at 56℃ for 10 minutes. 20 μl of distilled water was added and stored at -20℃ until used.

Oligonucleotide Synthesis

Oligonucleotides were purchased from the Bioneer corporation (Cheongwon, Korea) at a scale of 50 ~ 200 nmoles. Oligonucleotides were purified by denaturing polyacrylamide gel electrophoresis using 8 ~ 12% acrylamide/ 8 M urea gels and desalted by passage over a Waters Sep-Pack cartridge or Pharmacia NAP-25 column as described elsewhere (Table I-1) (Sambrook *et al*., 1989).

Table I-1. Sequences of primers for the detection of HSV-1, CMV, HHV6, EBV, *C. pneumoniae* and *H. pylori*

Sequence (5'→3')	Target gene (Location)	Amplicon length (bp)	Reference
HSV-1	**DNA Polymerase gene**		**McGeoch *et al.*,**
Sense: TCC TCG TAG ACG CCC ATC GGC G	**65884-65865**	**134**	**1986**
Antisense: CTT GTA ATA CAC CGT CAG GT	**65977-65958**		
CMV	**major immediately-early gene**		**Horvath *et al.*,**
Sense: CCT AGT GTG GAT GAC CTA CGG GCC A	**171362-171386**	**249**	**2000**
Antisense: CAG ACA CAG TGT CCT CCC GCT CCT C	**171137-171161**		
HHV6	**immediately-early gene**		**Horvath *et al.*,**
Sense: CCG CAA TCG AAT CCA CCT AGC GG	**47254-47277**	**435**	**2000**
Antisense:GTG AGA ACG GAT TCG AAC AGT GCT G	**47665-47689**		
EBV	***Bam*H-IW region gene**		**Saito *et al.*,**
Sense: CCT AGG GGA GAC CGA AGT AA	**14554-14573**	**234**	**1989**
Antisense: GAC CCT TCT ACG GAC TCG TCT G	**14787-14766**		
C. pneumoniae	**16S rRNA gene**		**Charlotte *et al.*,**
Sense: TGA CAA CTG TAG AAA TAC AGC	**8362-8382**	**463**	**1992**
Antisense: CGC CTC TCT CCT ATA AAT	**8809-8826**		
H. pylori	**urease gene**		**Clayton *et al.*,**
Sense: GCC AAT GGT AAA TTA GTT	**2236-2253**	**411**	**1992**
Antisense:CTC CTT AAT TGT TTT TAC	**1843-1860**		

HSV-1, herpes simplex virus type 1; CMV, cytomegalovirus; EBV, Epstein-Barr virus; HHV6, human herpes virus 6; *C. pneumoniae*, *Chlamydia pneumoniae*; *H. pylori*, *Helicobacter pylori*

Polymerase Chain Reaction (PCR)

According to the published DNA sequences, specific primers that amplify corresponding target gene regions were synthesized (HSV-1: DNA polymerase gene, McGeoch *et al*., 1986; EBV: *Bam*H-IW region gene, Saito *et al*., 1989; CMV: major immediately-early gene, Horvath *et al*., 2000; HHV6: immediately-early gene, Horvath *et al*., 2000; *C. pneumoniae*: 16S rRNA gene, Charlotte *et al*., 1992; *H. pylori*: urease gene Clayton *et al*., 1992), all of which were expected to be highly conserved (Table 1). 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 (Table 1) at 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, CT, 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).

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). For each reaction the following reagents were added to a separate tube: 8.0 μl of Terminator ready reaction mix, 5 μl of PCR products (200-500 ng), 4 μl of each of primer (3.2 pmol), and deionized water (3 μl) to total volume of 20 μl. Cycle sequencing on the GeneAmp 9600 (Perkin Elmer, Norwalk, CT, USA): the tube was placed in a thermal cycler and repeated the

following for 25 cycles; 96°C for 10 sec, 50°C for 5 sec, 60°C for 4 min. Rapid thermal ramp to 4°C and holded until ready to purify. The entire contents of each extension reaction was put into a 1.5 ml microcentrifuge tube and 60 μl of 100% isopropanol and 20 μl of distilled water were added. The final isopropanol concentration was about 60% (v/v). The tube was vortexed briefly and leaved at room temperature for 15 min to precipitate the extension products. Microcentrifuged for 20 min at maximum speed, the supernatants was aspirated carefully, to rinsing, 250 μl of 75% isopropanol was added and vortexd briefly. Microcentrifuged again and the samples were dried in a thermal cycler at 90°C for 1 min. Each pellet was resuspended in 6 μl of loading buffer (5 parts deionized formamide and 1 part 25 mM EDTA with blue dextran (50 mg/ml). The samples were vortexed and spun, then the samples were heated at 95°C for 2 min to denature. Placed on ice until ready to load. To prepare 5% polyacrylamide gel, 20 g of urea was dissolved in 13.5 ml distilled water at 56°C for 30 min. 4 ml of 50% Long Ranger acrylamide stock solution (FMC Bioproducts; Rockland, MA, USA) and 0.5 g of Amberlite were added, stirred gently for 5 min, then filtered and degassed by vacuum. Eight ml of 5× TBE was added and distilled water upto total 40 ml. Fifteen μl TEMED and 250 μl of 10%

ammonium persulfate (APS) were added. Gel solution was poured and polymerization for 2 hours. Samples were loaded in 1.5 μl per well and electrophored according to the manufacturer's instruction. The sequence was analyzed and determined.

III. RESULTS

Histopathologic Analysis

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

Compared to 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.

Verhoeff's Elastic Tissue Stain

Verhoeff's elastic tissue stain of the vessels from control group revealed smooth and regular pattern of elastic lamina without disruption of its structure (Figure I-2, A-C; Figure I-3, A-C). Conversely, the elastic lamina from atherosclerotic sections revealed an altered pattern characterized by fragmentation and disorganization of the elastic lamina associated with an increase in the thickness (Figure I-2, D-F; Figure I-3, D-F).

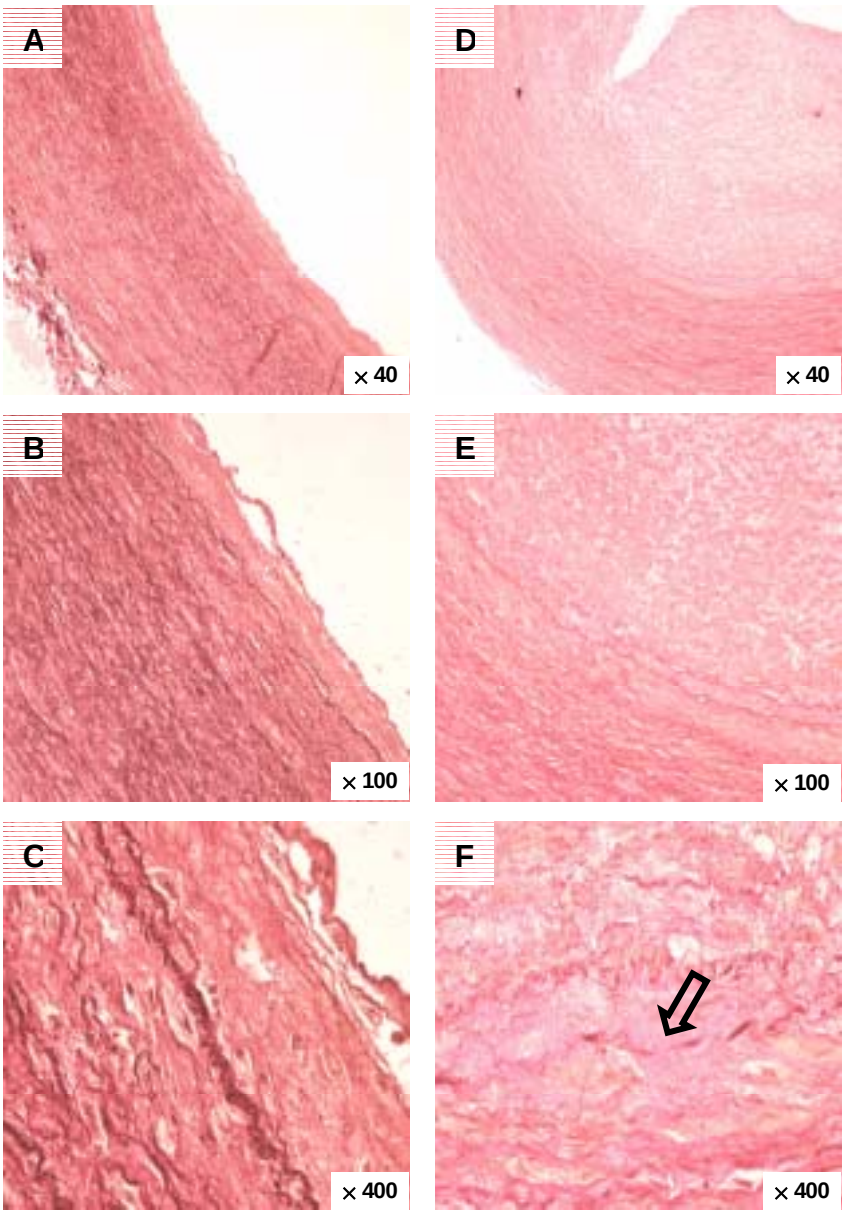

Figure I-2. Verhoeff's elastic fiber stain of the elastic lamina in aorta. The structure of the elastic lamina in normal aorta (panel A-C), and atherosclerotic aorta (panel D-F). Atherosclerotic aorta revealed the elastic lamina was fragmented and disorganized (arrow in panel F).

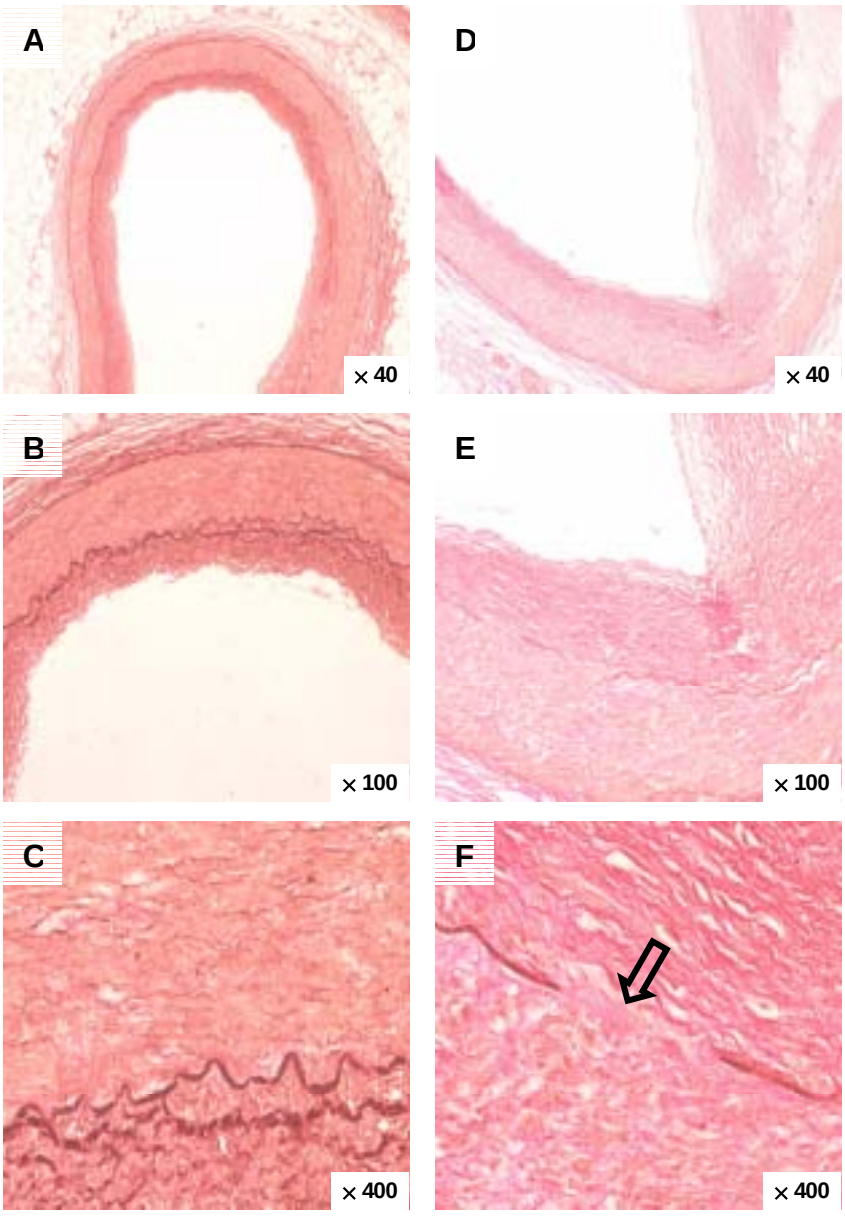

Figure I-3. Verhoeff's elastic fiber stain of the elastic lamina in coronary artery. The structure of the elastic lamina in normal coronary artery(panel A-C), and atherosclerotic coronary artery (panel D-F). Atherosclerotic coronary artery revealed the elastic lamina was fragmented and disorganized (arrow in panel F).

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 I-2).

By *in situ* hybridization, CMV was 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 (Figure I-4 and I-5).

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 I-3). T-lymphocytic infiltrates were evident in the intima of all 14 cases. CMV genome or antigen 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 I-4).

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

Table I-2. 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

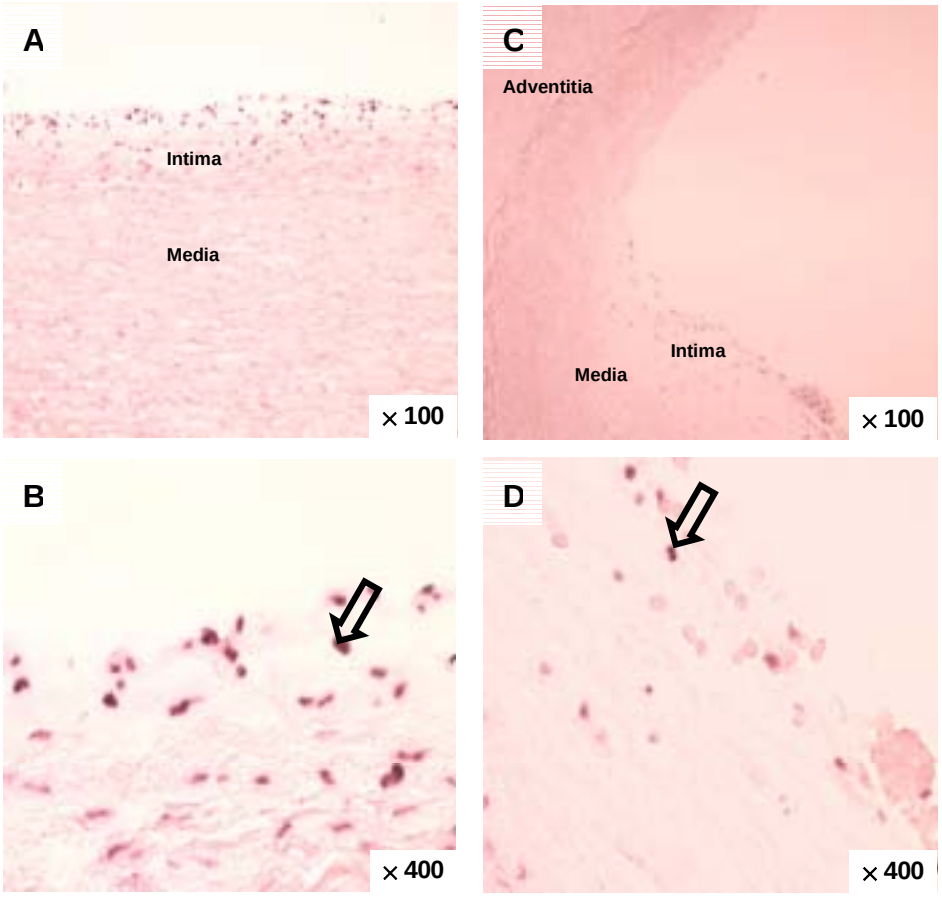

Figure I-4. Atherosclerotic lesions showing the presence of CMV in intimal cells by *in situ* hybridization. Panel A-B, hybridization was seen in aorta (arrow in panel B); Panel C-D, hybridization was seen in coronary artery (arrow in panel D).

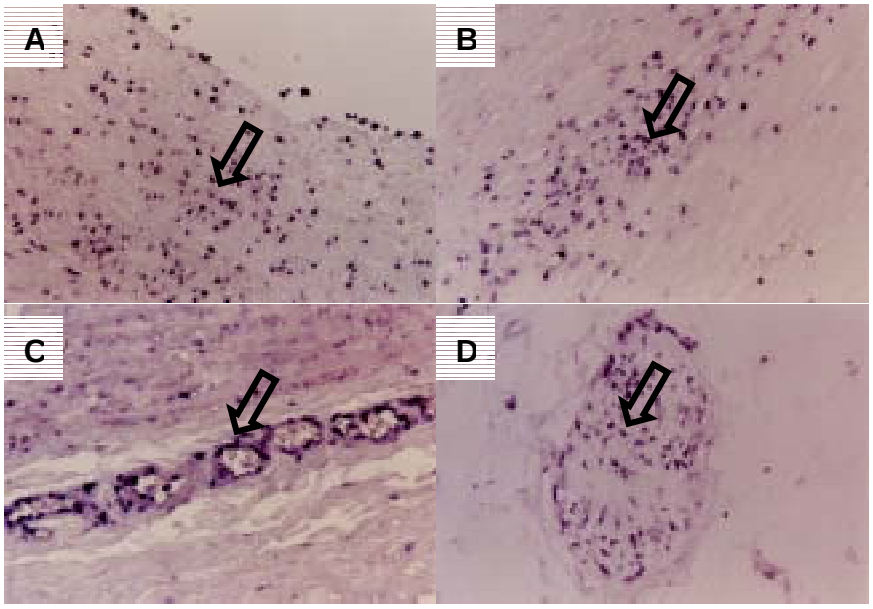

Figure I-5. Atherosclerotic lesions showing the presence of CMV by *in situ* hybridization (×200). Panel A, hybridization with CMV probe was seen in intimal cells; panel B, smooth muscle cells (SMCs); panel C, vascular endothelial cells in adventitia; panel D, Schwann cells (arrow in panels).

Table I-3. 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 I-4. 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

PCR amplification of CMV in aorta and coronary artery

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 (Figure I-6 and I-7) (Table I-5).

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. 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*. (1983) and Melnick *et al*. (1995) 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.

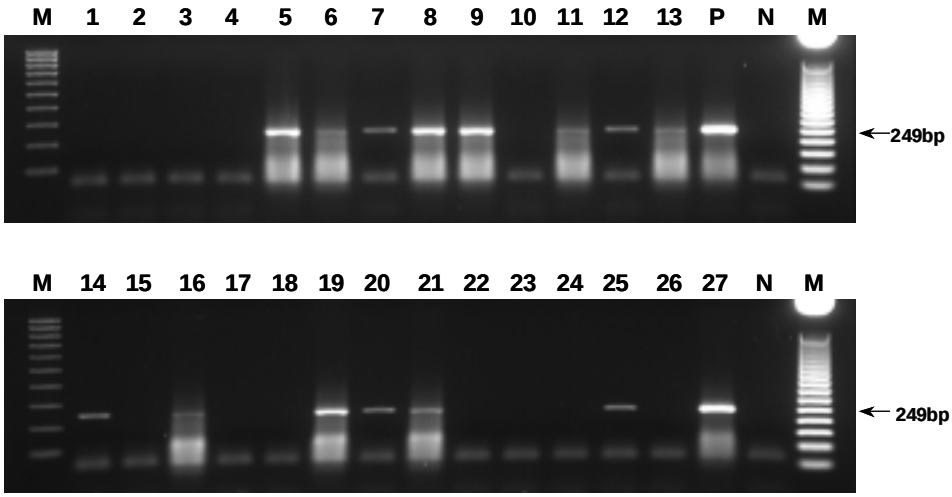

Figure I-6. Amplification of major immediately early (IE) gene of CMV by PCR in aorta specimens. Lane M, 100 bp and 50 bp ladder marker; lanes 1-4, control aorta; lanes 5-27, atherosclerotic aorta specimens from autopsy; P, positive control (AD-169); N, negative control.

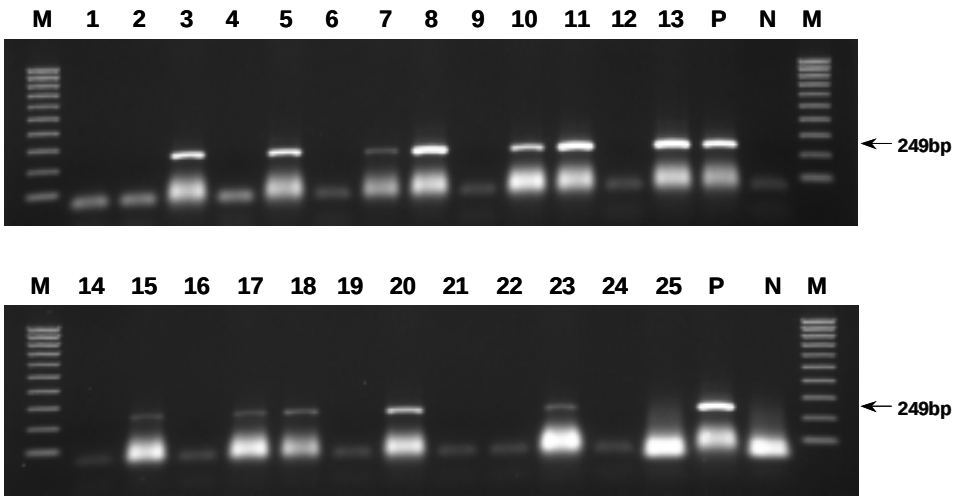

Figure I-7. Amplification of major immediately early (IE) gene of CMV by PCR in coronary artery specimens. Lane M, 100 bp ladder marker; lanes 1-4, control coronary artery; lanes 5-25, atherosclerotic coronary artery specimens from autopsy; P, positive control (AD-169); N, negative control.

Table I-5. 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

Specificity and Sensitivity of PCR

The specificity of the six primer sets to amplify specific target gene was tested by amplification of DNA from herpes simplex virus type 1 (HSV-1), cytomegalovirus (CMV), human herpes virus 6 (HHV6), Epstein-Barr virus (EBV), *Chlamydia pneumoniae*, *Helicobacter pylori*. Nonspecific amplifications were not obtained, nor was any interassay cross amplification observed. Assay sensitivity was determined by amplification of extracted DNA from duplicates of serial 10-fold dilutions of CMV (AD-169) 50% tissue culture infectious dose ($TCID_{50}$) stock (AD-169 stock titer: $5\times10^{5.75}$ $TCID_{50}$/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 (Figure I-8).

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).

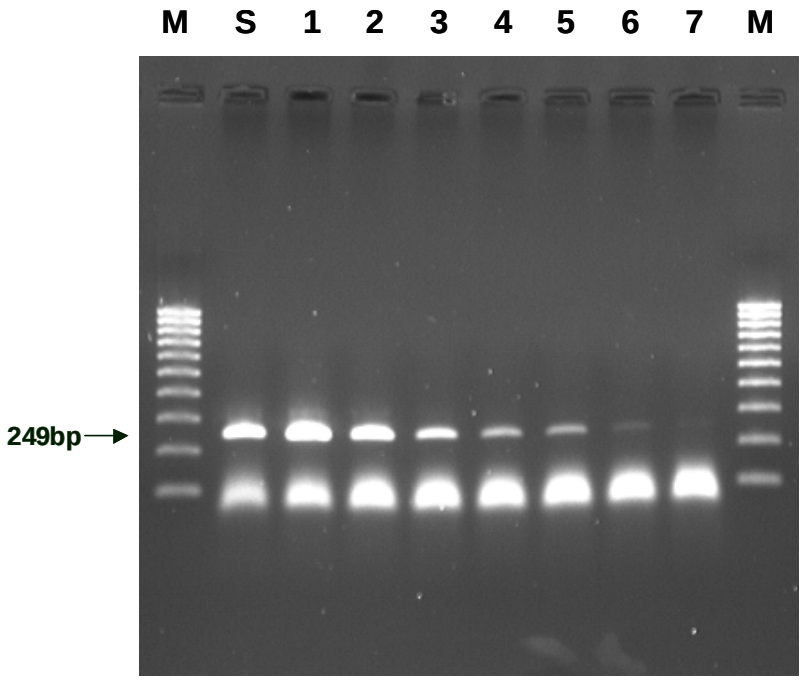

Figure I-8. Sensitivity of CMV PCR.

Lane M, 100 bp ladder marker; lane S, CMV (AD-169) stock (starting titer: $5\times10^{5.75}$ $TCID_{50}$/ml); lanes 1-7, serial 10-fold dilutions of CMV 50% tissue culture infectious dose ($TCID_{50}$) stock.

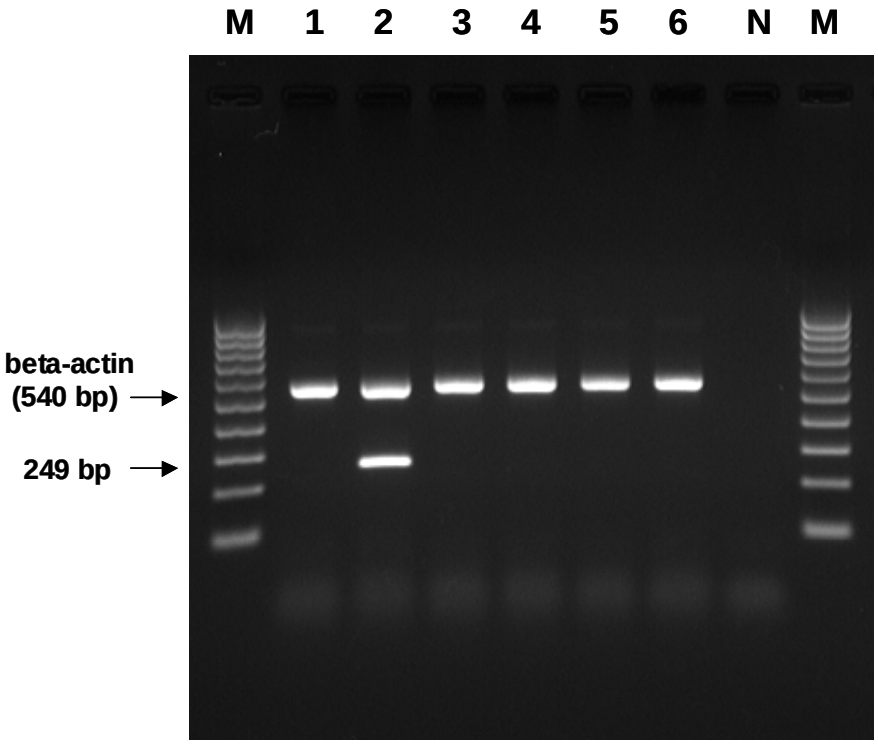

Figure I-9. Amplification of HSV-1, CMV, HHV6, EBV, *C. pneumoniae*, and *H. pylori* by PCR. Lane M, 100 bp ladder marker; lane 1, herpes simplex virus type 1 (HSV-1); lane 2, cytomegalovirus (CMV); lane 3, human herpes virus 6 (HHV6); lane 4, Epstein-Barr virus (EBV); lane 5, *Chlamydia pneumoniae*; lane 6, *Helicobacter pylori*.

Sequencing of PCR products

Sequence analysis revealed that PCR products extracted from atherosclerotic lesions using CMV-specific primer sets were indeed cytomegalovirus DNA of family Herpesviridae (Figure I-10).

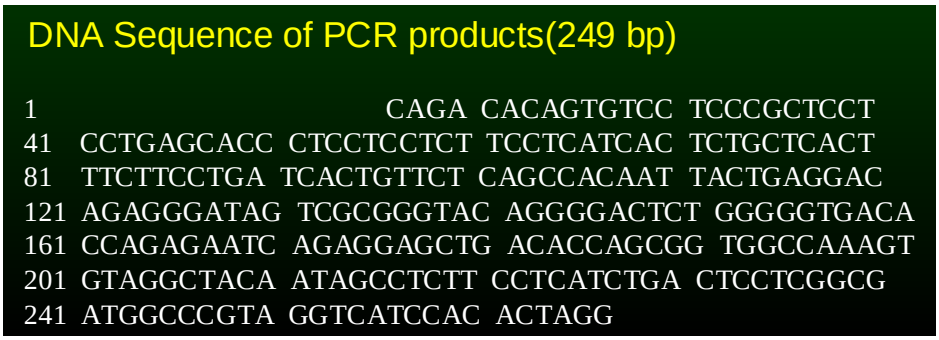

Figure I-10. DNA sequencing result of PCR product (249 bp).

IV. DISCUSSION

The possible role of herpesviruses in human atherosclerosis has been implicated in several studies. Because vascular smooth muscle cells can be transformed *in vitro* by HSV-2 (Nachtigal *et al*., 1990), it was hypothesized that herpesvirus-induced transformation may contribute to periods of uncontrolled growth in the atherosclerotic vessel wall. Whether the vessel wall actually contains transformed cells that are part of the foam cell population in atherosclerotic plaque regions remains unproven, however. Cunningham and Pasternak (1998) stressed the potential role of viruses in the pathogenesis of atherosclerosis and proposed a viral cause hypothesis. Extensive studies have revealed the importance of viruses, particularly herpesviruses, in atherosclerosis. In another PCR study, 90% of the samples obtained from patients with grade III atherosclerosis contained CMV viral nucleic acids, whereas only 53% of patients with grade I atherosclerosis contained these CMV nucleic acids (Hendrix *et al*., 1990). CMV DNA was apparently widely distributed throughout the arterial trees (Hendrix *et al*., 1991). The above findings support the thesis of Melnick *et al.* (1983) that the artery wall may be a site of latent CMV and possibly HSV.

In this study, *in situ* hybridization was chosen to investigate the

possible presence of CMV in human aorta and coronary artery. *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 (Ambinder *et al*., 1987), and it has been suggested that the IE gene is expressed in latent infections (Rice *et al*., 1984). 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 present study, the elastic lamina from atherosclerotic sections revealed an altered pattern characterized by fragmentation and disorganization of the elastic lamina associated with an increase in thickness. Several studies have demonstrated discontinuities and abnormalities in the elastic lamina structure of elastic and muscular arteries during advanced atherosclerosis (Nakatake *et al*., 1985; Sims *et al*., 1985; Nakatake *et al*., 1987; Roach *et al*., 1988; Martin *et al*., 1989; Sims *et al*., 1989; Rekhter *et al*., 1991). It has been

hypothesized that structural defects in the elastic lamina may play an important role during the formation of an atherosclerotic lesion, allowing cell migration and macromolecule transfer between the intima and different layers of the vascular wall (Sims *et al*., 1985; Sims *et al*., 1989). This elastic lamina changes may be associated with the neoadventitial formation and phenotypic change of myofibroblasts. The arterial wall is an integrated functional component of the circulatory system that is continually remodeling in response to hemodynamic conditions and disease states. The disruption of elastin is enough to facilitate the migration of myofibroblasts and the proliferation of smooth muscle. Thus, elastin is a molecular determinant of arterial morphogenesis such as vascular remodeling, stabilizing arterial structure by regulating proliferation and reorganization of vascular smooth muscle (Li *et al*., 1998). The decrease in the percentage of elastin content in the elastic lamina was associated elastolytic activity. Contrary to collagen, elastin has an extremely long half-life (40-70 years). Loss of elastin is almost certainly a manifestation of excessive elastolysis rather than insufficient synthesis. This study suggests that structural changes of elastic lamina may play an important role in the development of atherosclerotic matrix reorganization, resulting in vascular remodeling.

In animal models of virus-induced atherosclerosis, herpesviruses have been demonstrated in endothelial cells, smooth muscle cells and lymphocytes (Gyorkey *et al*., 1984; Hendrix *et al*., 1991; Gnann *et al*., 1988). 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*. (1978, 1981) and Minick *et al*. (1979) 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 (Grattan *et al*., 1989). 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*. (1990). More subtle forms of viral-induced endothelial injury could also promote arteriosclerosis

by reducing the anticoagulant properties of endothelial cells (Visser *et al*., 1988; Etingin *et al*., 1990).

CMV infection has also been associated with an upregulation of major histocompatibility (MHC) antigen expression by endothelial and smooth muscle cells (van Esa *et al*., 1984; von Willebrand *et al*., 1986; Hosenpud *et al*., 1991). Upregulation of MHC antigen expression is presumably mediated by the release of γ-interferon by activated T-lymphocytes responding to the CMV infection. The induced MHC antigen expression is itself associated with allograft rejection and the development of accelerated arteriosclerosis (Hruban *et al*., 1990). 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 (Fujinami *et al*., 1988).

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 (Fabricant *et al*., 1981; Minick *et al*., 1979; Hajjar *et al*., 1986). CMV may promote atherosclerosis by inducing the proliferation of smooth muscle cells (Benditt *et al*., 1976). In addition to smooth muscle and endothelial

cells, CMV infection of lymphocytes has also been demonstrated (Rice *et al*., 1984; Schrier *et al*., 1985). CMV infection of lymphocytes may activate lymphocytes and this activation of lymphocytes may promote graft rejection and the development of accelerated arteriosclerosis (van Esa *et al*., 1984).

Melnick *et al*. (1983) 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, and it was suggested that the artery wall may be a site of CMV latency. In 1984, Gyorkey *et al*. 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. Petrie *et al*. (1987) reported the presence of nucleic acid sequences of CMV in cells cultured from human arterial tissue, thus extending the earlier

findings by Melnick *et al*. (1983) of CMV antigen detection in cultured arterial cells.

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 (Myerson *et al*., 1984). 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 nonpermissive. Productive infection in permissive cells may result in alterations of aortic lipid metabolism and/or direct injury of endothelial or intimal cells. In Marek's disease virus infection of chicken, lipid metabolism has been shown to be altered with significant accumulation of cholesterol and cholesteryl esters in infected aortic smooth muscle cells (Fabricant *et al*., 1981; Hajjar *et*

al., 1986). CMV-infected human fetal aortic cells have also been reported to proliferate and accumulate cholesterol *in vitro* (Hajjar *et al*., 1986). CMV has also been shown to replicate in smooth muscle cell cultures derived from human umbilical arteries (Tumilowicz *et al*., 1985). 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*. 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 (Westmoreland *et al*., 1976; Sakuma *et al*., 1977). 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 (Tevethia *et al*., 1985). Benditt *et al*. (1983) 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*. (1986) 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. Of obvious interest in this regard is CMV infection of arterial tissues that induce somatic cell gene alterations leading to smooth muscle cell proliferation and atherosclerotic plaque development.

Many of the important cellular effects of CMV were derived from expression of its immediate-early (IE) gene products in the absence of early and late gene expression and of viral replication. In this type of infection, called an abortive infection, death of the host cell does not occur. It is likely that abortive infections are of biological relevance, providing a mechanism whereby intracellular pathogens contribute to disease progression. One molecular mechanism by which CMV may increase cellular proliferation is through inhibition of the tumor suppressor gene, p53; the p53 gene product inhibits cell cycle

progression (Levine *et al*., 1997) and therefore cell proliferation (Yonemitsu *et al*., 1998). IE2-94, 1 of the IE gene products of CMV, is capable of binding to p53 and inhibiting its transcriptional activity (Speir *et al*., 1994; Tsai *et al*., 1996). Another mechanism by which CMV infection could lead to cellular proliferation is stimulating the secretion of growth factors or increasing the expression of growth factor receptors. CMV infection of smooth muscle cells has recently been shown to increase PDGF receptor expression (Zhou *et al*., 1999). CMV infection inhibits apoptosis in human endothelial cells (Kovacs *et al*., 1996), an effect caused at least partly by the IE gene products of the virus (Zhu *et al*., 1995). One of the mechanisms by which this occurs is probably inhibition of the p53-modulated apoptotic program (Levine *et al*., 1997).

The histopathologic findings in this study may indicate that CMV infections are associated mainly with areas showing early or advanced atheromatous changes in the coronary arteries and with lesion areas in the aorta. Microscopically, lymphocytic infiltrates were evident in both sites in the majority of virus-positive cases with early or advanced atheromatous changes. Emeson *et al*. (1986) have demonstrated mononuclear cell infiltrates in the coronary arteries and aorta, and have identified some of the inflammatory cells as T-cell

subsets and monocytes/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 (Rice *et al*., 1984). Macrophages may be recruited to the plaque causing weakening of the fibrous cap and subsequent plaque rupture (Kol *et al*., 1998).

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 (Zhu *et al*., 2000; Rupprecht *et al*., 2001).

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 (Grundy *et al*., 1993; Span *et al*., 1991), procoagulant effects (Etingin

et al., 1990), increased scavenger receptor expression and activity (Zhou *et al*., 1996), enhanced uptake of cholesterol and of modified low-density lipoprotein (Hajjar *et al*., 1986), increased smooth muscle cell migration and proliferation (Zhou *et al*., 1999), anti-apoptotic effects (Tanaka *et al*., 1999) and autoimmune response to infection (Epstein *et al*., 2000).

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 (Epstein *et al*., 1999).

Other microorganisms, such as *Chlamydia pneumoniae* (Davidson *et al*., 1998; Yamashita *et al*., 1998; Kuo *et al*., 1997; Grayston *et al*., 1995) and *Helicobacter pylori* (Ossei-Gerning *et al*., 1997; Birmie *et al*., 1998; Hoffmeister *et al*., 2001; Markus *et al*., 1998), 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.

CHAPTER II

Coexpression of Cyclooxygenase-2 and Matrix Metalloproteinase-9 in Human Atherosclerotic Lesions Infected by Cytomegalovirus

I. INTRODUCTION

Atherosclerosis is currently considered to be an exaggerated response of the vessel wall to injury characterized by inflammation and fibrocellular proliferation rather than a degenerative disease due to hemodynamic loading (Ross *et al*., 1993; Alexander *et al*., 1994). This view is supported by the demonstration of abundant macrophages and T-lymphocytes in atherosclerotic plaque, as well as studies examining markers of inflammation such as C-reactive protein (Jonasson *et al*., 1986; Ridker *et al*., 1997; Liuzzo *et al*., 1994). Plaque rupture leading to thrombosis is the key event in myocardial infarction and it has been shown to be related to increased inflammation within the plaque, rather than plaque morphology or degree of vessel stenosis (van der Wah *et al*., 1994). Chronic inflammatory lesions are often associated with a significant destruction of connective tissue. The association of the monocytes/macrophages with lesions where there is the destruction of connective tissue in atherosclerotic tissue favors their participation in the breakdown of collagen.

In addition, chronic infection and inflammation have recently been implicated as important etiologic agents for atherosclerosis. Several agents have been suggested as being responsible for chronic

inflammation including cytomegalovirus, *Helicobacter pylori*, and *Chlamydia pneumoniae* (Ridker *et al*., 1998; Mattila *et al*., 1998; Kuo *et al*., 1995).

The production of matrix metalloproteinases (MMPs) by human monocytes has been shown to occur through a prostaglandin (PG) E_2-cAMPs dependent pathway (Wahl *et al*., 1977; McCarthy *et al*., 1980; Wahl *et al*., 1987). Signaling through this pathway involves the modulation of prostaglandin H synthase, that is, cyclooxygenase (COX) (Corcoran *et al*., 1994). Two COX isoforms have been identified, referred to as COX-1 and COX-2. In contrast to COX-1, which is a constitutively expressed enzyme involved in maintaining low levels of PG, COX-2 is induced in response to cell activations such as growth factors, cytokines, and phorbol esters, suggesting that this enzyme is involved in the generation of PG in inflammation. The induction of COX-2 in monocytes and the resulting production of PGE_2 have been shown to be involved in the signal transduction pathway leading to the production of MMPs by these cells (Mertz *et al*., 1994).

In view of the interactions of COX-2 and MMPs, this study hypothesized that the inflammation via COX-2 and the production of MMPs might be involved in the development of atherosclerosis in

human aorta and coronary arteries. The aim of this study was to determine the cellular location of COX-2, and to investigate for codistribution of COX-2 and MMP-9. This study performed immunohistochemical staining on the atherosclerotic tissue sections obtained from paraffin embedded blocks that were positive to cytomegalovirus by *in situ* hybridization and polymerase chain reaction, by using antibodies to CMV late antigen, COX-2, MMP-9, macrophage and T-lymphocyte.

The purpose of this study was to determine the possible pathogenetic role of COX-2 and MMP-9 in cytomegalovirus-infected atherosclerotic aorta and coronary artery by histopathologic observation.

II. MATERIALS AND METHODS

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.

Tissue preparation

Immediately following removal of aortic and coronary artery segments, each segment was fixed with 10% formalin in order to maintain vascular morphological integrity. To preserve the integrity of the adventitia and perivascular tissues, aorta and coronary artery specimens were carefully removed in a segment along with adjacent tissues and rinsed with PBS (phosphate buffered saline). Each segment was embedded in paraffin and cut in 5 μm sections, which

were stained with hematoxylin-eosin (H&E). Sections of these tissues were also used for immunohistochemical staining. One lesion from each section, which had morphological characteristics of atherosclerosis ranging from fatty streak to complicated atherosclerosis lesion, was assigned for histopathologic analysis.

Immunohistochemical stain for CMV late antigen, COX-2, MMP-9, Macrophage, T-lymphocyte

Mouse anti-cytomegalovirus (CMV) late antigen monoclonal antibody (Chemicon International, Temecula, CA, USA), mouse anti-human cyclooxygenase-2 (COX-2) monoclonal antibody (BD Biosciences, San Jose, CA, USA), and rabbit anti-human matrix metalloproteinase-9 (MMP-9) polyclonal antibody (Chemicon International, Temecula, CA, USA) were used as the primary antibodies for immunohistochemical staining. To characterize the type of inflammatory cells, rabbit anti-human macrophage (CD68) monoclonal antibody (Lipshaw Immunon, Pittsburgh, PA, USA) and rabbit anti-human T-lymphocyte (CD45RO) monoclonal antibody (Lipshaw Immunon, Pittsburgh, PA, USA) were used for the immunohistochemical staining. Peroxidase-conjugated secondary antibodies were used with these primary antibodies.

The paraffin sections (5 μm) were made and transferred to poly-L-lysine coated glass slides (Polysine^{TM}, Portsmouth, NH, USA). The paraffin sections were deparaffinized and rehydrated through the following solution: xylene, 3 times for 3 minutes in each, and serially 100%, 95%, 80%, 75%, and 70% ethyl alcohol for 3 minutes in each. The sections were then treated with 3% H_2O_2 for 10 minutes to suppress endogenous peroxidase activity. The sections were boiled in plastic container which was filled with 10 mM sodium citrate, pH 6.0 for 5 minutes with microwave oven (800W) to retrieve antigen sites, and cooled at room temperature for 20 minutes, followed by washing in TBS (Tris buffered saline; pH 7.2) for 5 minutes. Nonspecific binding was blocked by incubation with 10% normal goat serum for polyclonal antibodies and 10% normal horse serum for monoclonal antibodies. The sections were incubated at room temperature in moist chamber for 1 hour with primary antibodies (1:40 diluted anti-CMV late antigen, 1:50 diluted anti-MMP-9, 1:100 diluted anti-COX-2, 1:200 diluted anti-macrophage, 1:200 diluted anti-T-lymphocyte). After washing and bathing in TBS (Tris buffered saline; pH 7.2) for 5 minutes, the biotinylated secondary antisera cocktail including goat anti-mouse and anti-rabbit IgG diluted 1:400 was incubated on the slides for 20 minutes at room temperature in a moist chamber. The

sections were then processed by the streptoavidin-biotin-peroxidase complex method using the LSAB plus kit (DAKO Inc, Carpinteria, CA, USA), and stable AEC substrate solution (3-amino-9-ethylcarbazole) was used as a chromogen. Gill's hematoxylin was used as a counterstain, and sections were dehydrated, cleared, and mounted.

III. RESULTS

Histopathologic Analysis

The sections of aorta and coronary artery taken from control groups showed no histological evidence of atherosclerosis, except minimal intimal thickening, and normal patterns of elastic media (Figure II-1, panel A; Figure II-2, panel A). In contrast to the control group, lesions that ranged from fatty streak to complicated atherosclerotic plaque were noted in all persons with atherosclerosis, and showed a thickened intima and fragmented elastic lamina. There was also prominent inflammatory infiltration with mononuclear cells and foam cells in atherosclerotic plaque, especially in the central core, and to a lesser extent in the inner media and adventitia.

Immunohistochemical Stain

Serial sections of each tissue samples (aorta and coronary artery) were examined sequentially for viral antigen, and for correlation of virologic results with histopathologic findings. Positive staining reactions observed in the aorta and coronary artery tissue sections with immunohistochemical stain are illustrated in Figures II-1 through II-3. CMV was demonstrated in occasional cells in the luminal surface and

in focal clusters of spindle-shaped or ‘foamy’ cells in the subendothelium as well as deeper intimal layers. Although the histogenesis of these cells has not been definitely established, they corresponded to endothelial cells, smooth muscle cells, or monocytes/macrophages on the basis of morphology and tissue location.

Sections from control specimens showed little immunoreactivity for CMV late antigen, COX-2, MMP-9, macrophage, and T-lymphocyte in the minimal thickened intima, and no immunoreactivity in the media (Figure II-1, panel B & C; Figure II-2, panel B & C). In atherosclerotic sections (plaque with central core), however, CMV late antigen was stained within the plaque macrophages/mononuclear cells (Figure II-3, panel A & D). The immunoreactivity for COX-2 and MMP-9 was evident in all cases of atherosclerosis along with plaques, mainly in macrophages/foamy cells, intimal and medial smooth muscle cells, and endothelial cells of the intima (Figure II-1, panel E & F; Figure II-2, panel E & F). Within the intima, the increased immunoreactivity for COX-2, MMP-9 was colocalized to the area stained with CMV late antigen. Immunoreactive patterns of COX-2 and MMP-9 were similar. Immunoreactivity for macrophage and T-lymphocyte were found in a

similar pattern and distribution with COX-2, MMP-9 (Figure III-3, panel B, C, E, and F). In fatty streak, immunoreactive patterns for COX-2, MMP-9, macrophage, and T-lymphocyte were similar, although immunoreactivity was less prominent.

The immunoreactivity for COX-2 and MMP-9 increased in the atherosclerotic plaques itself, predominantly in the surrounding area of immunoreactive cytomegalovirus. These finding support that cytomegalovirus may participate in a pathogenetic mechanism for atherogenesis or progression of atherosclerosis.

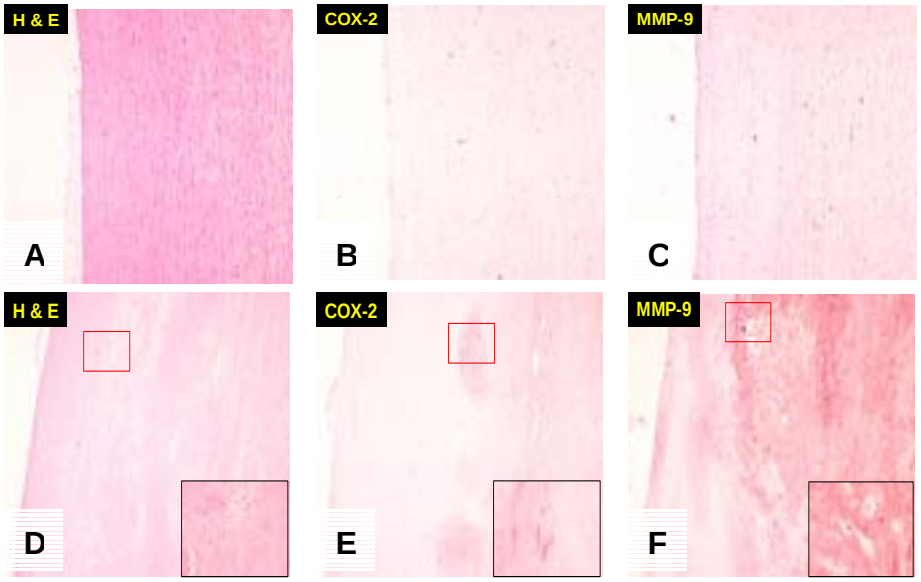

Figure II-1. Hematoxylin-eosin stain, immunohistochemical stain for COX-2 and MMP-9 of control (panel A-C, respectively, ×100) and atherosclerotic aortas (panel D-F, respectively, ×40; small box in panel, ×400). Normal aorta (panel A) showed normal pattern of elastic media. Compared with controls, there was prominent inflammatory infiltration with mononuclear cells and foam cells in the atherosclerotic plaque (panel D). In nonatherosclerotic section (panel B & C), immunoreactivity for COX-2 and MMP-9 were not shown. Atherosclerotic lesion (panel E & F) demonstrated strong immunoreactivity for COX-2 and MMP-9.

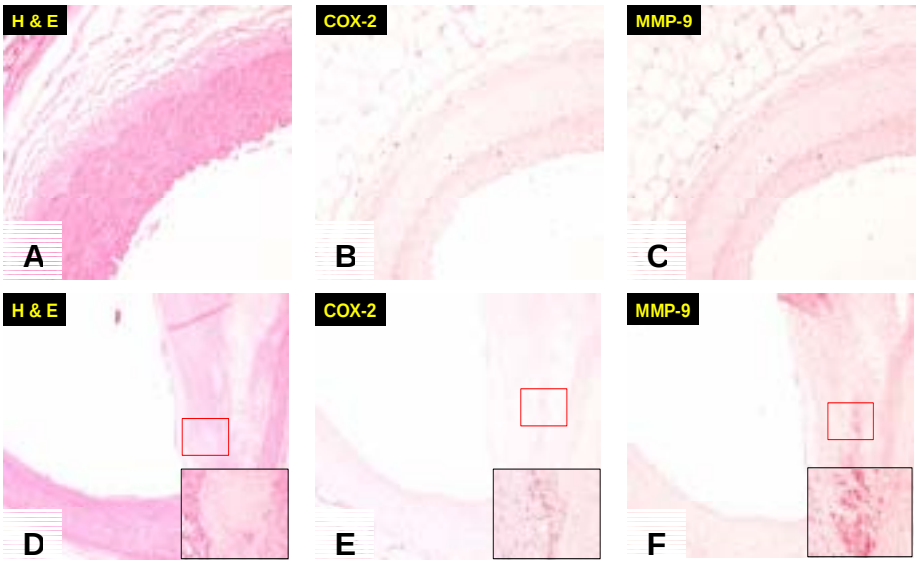

Figure II-2. Hematoxylin-eosin stain, immunohistochemical stain for COX-2 and MMP-9 of control (panel A-C, respectively, ×100) and atherosclerotic coronary artery (panel D-F, respectively, ×40; small box in panel, ×400). Normal coronary artery (panel A) showed normal pattern of elastic media. Compared with controls, there was prominent inflammatory infiltration with mononuclear cells and foam cells in the atherosclerotic plaque (panel D). In nonatherosclerotic section (panel B & C), immunoreactivity for COX-2 and MMP-9 were not shown. Atherosclerotic lesion (panel E & F) demonstrated strong immunoreactivity for COX-2 and MMP-9.

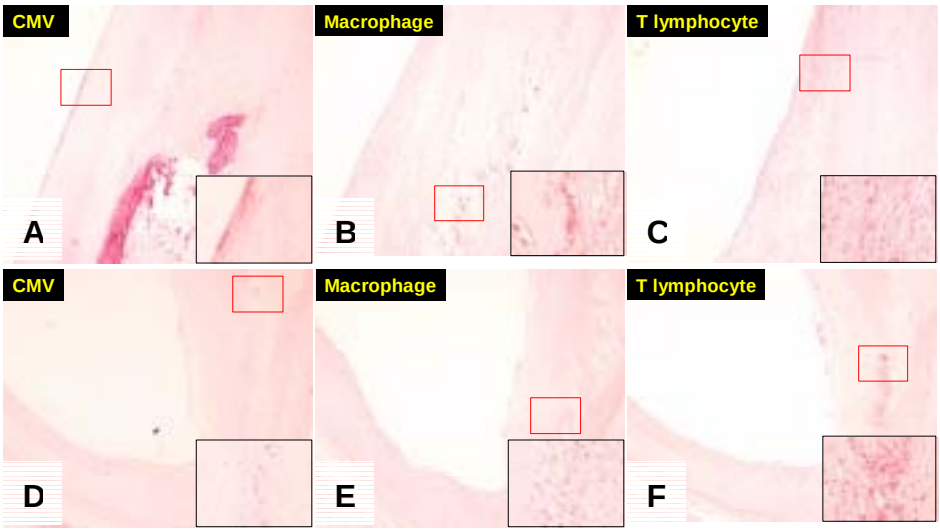

Figure II-3. Immunohistochemical stain for CMV late antigen, macrophage, T-lymphocyte of atherosclerotic aorta (panel A-C, respectively, ×40; small box in panel ×400) and coronary artery (panel D-F, respectively, ×40; small box in panel ×400). The immunoreactivity to CMV was primarily located in endothelial lining of the vascular wall (panel A & D). The immunoreactivity for macrophages (panel B & E) and for T-lymphocytes (panel C & F) was shown. Note the similar distribution of macrophages and T-lymphocytes in the coronary artery (panel E & F).

IV. DISCUSSION

Atherosclerosis and its related diseases, in particular myocardial infarction (MI) and coronary heart disease (CHD), are a major cause of the morbidity and mortality worldwide. The differences in the prevalence of conventional atherosclerotic risk factors (such as smoking, hypertension, diabetes mellitus, and hypercholesterolemia) do not fully account for the variation in the prevalence or severity of atherosclerosis. Consequently, there is intense research interest focused on seeking other atherogenic risk factors.

Current data supports the hypothesis that atherosclerosis is an inflammatory disease rather than a degenerative disease owing to hemodynamic loading (Ross *et al*., 1993; Alexander *et al*., 1994) and studies examining markers of inflammation (e.g., C-reactive protein) demonstrate a relationship between an increasing inflammation and the risk of a vascular event. It is also recognized that plaque activity and the function of the cellular components can be a more important determinant of the clinical manifestations of atheroslcerosis than the degree of stenosis of the arterial lesions. Although many factors may initiate atherosclerosis, the process ultimately involves an inflammatory state in which macrophages and T-lymphocytes play a

major role (Jonasson *et al*., 1986; Ridker *et al*., 1997; Liuzzo *et al*., 1994; van der Wah *et al*., 1994).

Inflammation appears to have a major role in the development of atherosclerosis. Cyclooxygenase-2 (COX-2) is involved in the inflammatory response via the generation of prostanoids that, in turn, are involved in the production of matrix metalloproteinase-9 (MMP-9). This study aimed to investigate atherosclerosis in human aorta and coronary arteries for *in situ* tissue distribution of COX-2, MMP-9, macrophage, and T-lymphocyte. Immunohistochemical studies were performed on atherosclerotic lesions of aorta and coronary arteries from persons who were all referred to National Institute of Scientific Investigation (NISI; Seoul, Korea) for autopsy, by using antibodies to CMV late antigen, COX-2, MMP-9, macrophage, and T-lymphocyte. All specimens from diseased aorta and coronary arteries had atherosclerotic lesions ranging from fatty streak to atherosclerotic plaques. In control, there was no expression of COX-2, MMP-9, macrophage, and T-lymphocyte. Immunoreactivity for COX-2 was predominantly noted in macrophages and smooth muscle cells (SMCs) of the intima including atherosclerotic plaque itself and the medial layer of the plaque base. Immunoreactivity for MMP-9 was found in the same distribution as that of COX-2. This study demonstrates that

COX-2 is coexpressed with MMP-9 by macrophages and SMCs in atherosclerotic lesions. Thus, vascular inflammatory reactions may influence extracellular matrix remodeling by coactivation of MMP-9 in the development of atherosclerosis and, in turn, the progression of disease.

There is a growing amount of evidence to support an association between infection and atherosclerosis, with the first suggestion of a link being made by Osler early in this century. Since Fabricant *et al*. (1978) first reported that herpesviruses infection is as a risk factor for atherosclerosis, a number of seroepidemiological studies have shown a positive relationship between cytomegalovirus infection and atherosclerosis (Peterson *et al*., 1980; Benditt *et al*., 1983; Grattan *et al*., 1989). The results of *in situ* hybridization and PCR study also demonstrated a positive association between cytomegalovirus infection and atherosclerosis, so the present study suggest that cytomegalovirus may play a pathogenetic role in atherosclerosis.

As mentioned above, vascular inflammation and a chronic degenerative process are prerequisite for atherosclerosis. NF-kB, a ubiquitous transcription factor of particular importance in immune and inflammatory responses, increases the expression of the genes for many cytokines, enzymes, and adhesion molecules in chronic

inflammatory diseases. This increased expression is reflected by an increased amount of nitric oxide, which has a cytotoxic effect on vascular tissue. Cyclooxygenase-2 (COX-2) another inducible enzyme regulated by NF-kB, is responsible for the increased production of prostaglandins (PG) and thromboxane (TXA) in inflammatory diseases (Mertz *et al*., 1994). Two types of COX isoform have been identified, referred to as COX-1 and COX-2. In contrast of COX-1, which is a constitutively expressed enzyme involved in maintaining low levels of PG, COX-2 is induced in response to cell activations such as growth factors, cytokines, and phorbol esters, suggesting that this enzyme is involved in the generation of PG in inflammation. The induction of COX-2 in monocytes and the resulting production of PGE_2 have been shown to be involved in the signal transduction pathway leading to MMPs production in those cells (Wahl *et al*., 1977; Won *et al*., 1998). Induced COX-2 expression in monocytes-macrophages and fibroblasts results in increased synthesis of PGE_2 and TXA_2, which mediate inflammatory change, vasoconstriction, and platelet aggregation. The increased synthesis of COX-2 is considered to play an important role in inflammation and tissue injury. COX-2, which is induced in many cell types in response to cytokines, metabolizes membrane phospholipid arachidonic acid and plays a role

in the expression of an inflammatory mediator in heart failure (Won *et al*., 1998). Gelatinase-B also known as 92-kDa gelatinase or MMP-9, may contribute importantly to the instability of human atherosclerotic plaques. The regulation of transcription of MMP-9 depends in part on a NF-kB element in its promoter sequence. This transcription factor is known to be regulated by oxidative stress and may link the accumulation of oxidized lipoproteins in the intima to expression of this particular protease (Aikawa *et al*., 1998). This study was designed to test the hypothesis that the immunoreactivity for CMV late antigen and inflammatory mediators such as COX-2 and MMP-9 will be colocalized to inflammatory cells in atheromatous plaque if cytomegalovirus has some pathogenetic role on atherosclerotic diseases. That is, if the vascular infection of cytomegalovirus could induce a chronic inflammatory reaction in host vascular tissue and activate inflammatory cells, some inflammatory mediators such as COX-2 and MMP-9 may exhibit increased expression surrounding macrophages, which have key role in atherosclerosis, infected with cytomegalovirus. The results of this study demonstrated that COX-2 and MMP-9 were colocalized to the inflammatory infiltrates of diseased tissues, particularly within cytomegalovirus-stained macrophages/monocytes.

Elevated lipids may play a role in initiating atherosclerotic lesion development by stimulating inflammatory cytokine production, which may in turn initiate the inflammatory process. Inflammatory cytokines were localized to macrophages and endothelial cells overlying atherosclerotic lesions. Minimally oxidized LDL and beta-VLDL upregulate inflammatory cytokines, thereby attracting more leukocytes to develop atherosclerotic plaque, beginning the cycle of inflammation and intimal development (Berliner *et al*., 1990; Wilcox *et al*., 1995). As well, oxidized LDL and vascular endothelial growth factor (VEGF) accumulated in human atherosclerotic lesions induces the production of macrophage VEGF (Ramos *et al*., 1998). Also, VEGF upregulates the expression of matrix metalloproteinases in vascular smooth muscle cells (Wang *et al*., 1998). Similar events may occur in the adventitia during atherosclerotic development where the adventitial vasa vasorum endothelial cells may be affected by elevated lipids and express adhesion molecule early in the atherosclerotic process, thus contributing to the invasion of inflammatory cells into the adventitia as well (Berliner *et al*., 1990; Wilcox *et al*., 1995; Wood *et al*., 1993) Experimental hypercholesterolemia may induce the upregulation of specific cell-surface receptors in the adventitial vasa vasorum, which then predisposes them to proliferate in response to

growth factors and an increase in the vessel wall thickness (Edelman *et al*., 1992; Isner *et al*., 1996; Kwon *et al*., 1998). Jamieson and co-authors (1995) have demonstrated a correlation between apo(a) deposition within endothelial cells of vasa vasorum and the stage of atherosclerotic plaque, suggesting a specific link between apo(a) receptor and the pathogenesis of atherosclerosis. Several experimental studies have shown that in response to and increased presence of mildly oxidized lipoproteins in the outer media (Juul *et al*., 1996), which have been shown to be chemotactic for monocytes (Quinn *et al*., 1987), an increased number of monocytes can enter the intima and adventitia under hypercholesterolemic conditions (Lewis *et al*., 1985).

This study documents that there was significant expression of COX-2 and MMP-9 in atherosclerosis aorta and coronary artery, but very little in control tissues. Immunoreactivity for COX-2 and MMP-9 were colocalized to the inflammatory infiltrates, principally macrophages/foam cells in atherosclerotic intima, plaque itself, and vascular smooth muscle cells. In addition, these expressions were evident in the medial smooth muscle cells. These results suggest that COX-2 and MMP-9 have pathobiological roles in cytomegalovirus-infected atheroslcerosis as the inflammatory mediator or its product, which may regulate cellular activation and reorganize extracellular

matrix.

Macrophages are known to play an important role in regulating the turnover of extracellular matrix (ECM) in both normal and pathologic conditions through the secretion of proteases, including MMPs, protease inhibitors, and cytokines. MMP expression in macrophages is dependent on prostaglandin E_2 (PGE_2) (Corcoran *et al.*, 1992). PGE_2 synthesized from arachidonic acid, and cyclooxygenase (COX) is the rate-limiting enzyme in this pathway. The activation of macrophages has been previously correlated with the induction of COX-2 (Arias-Negrete *et al.*, 1995). Macrophages expressing COX-2 are known to produce eicosanoids that have proinflammatory effects, increasing vascular permeability, promoting chemotaxis, and favoring cell proliferation and cholesterol ester retention (Hajjar *et al.*, 1992; Herschman *et al.*, 1996).

Recent evidence is of particular relevance to the role of COX-2 in inflammation and atherosclerosis. COX-2 in activated human monocytes may be able to generate the prostaglandin, 8-epi-$PGF_{2\alpha}$, which is mitogenic leading to cellular proliferation, and vasoconstrictive leading to vasoconstriction, and thus may play a role in the genesis of atherosclerosis (Pratico *et al.*, 1996; Fukunaga *et al.*, 1993; Takabashi *et al.*, 1992). In this study, the immunolocalization

study confirmed the increased expression of COX-2 in atherosclerotic plaque, especially in macrophages.

Increased levels of several MMPs, including stromelysin, interstitial collagenase, and gelatinase A and B, show increased expression and/or activation in atherosclerotic plaques (Galis *et al*., 1994; Henney *et al*., 1991; Galis *et al*., 1995; Lee *et al*., 1996; Zaltsman *et al*., 1997). Furthermore, MMP-9 expression and MMP-2 expression and activation are positively correlated with lesion severity, consistent with a pathogenetic role in the late disease process (Zaltsman *et al*., 1997). The activation of MMPs and their proteolytic potential is tightly controlled by endogenous tissue inhibitors of matrix metalloproteinases (TIMPs) (Birkedal-Hansen *et al*., 1993; Cawston *et al*., 1996). This study showed coexpression of MMP-9 and COX-2 in macrophages and smooth muscle cells in intima and media, suggesting that inflammatory activation of MMP-9 as well as COX-2, may contribute to the enhanced local matrix degradation in atherosclerotic plaques.

The present study demonstrated that immunoreactivity for MMP-9 and COX-2 were noted in the intimal smooth muscle cells. Vascular adhesion molecules like intercellular adhesion molecule-1 (ICAM-1) have been characterized in the endothelial cells of the vasa vasorum

(Szekanecz *et al*., 1994). These molecules aid in the recruitment of inflammatory cells to the aorta and stabilize local T-cell receptor function (Holmes *et al*., 1995). This finding suggested that inflammatory reaction was evident coupling with intimal hyperplasia. Linton *et al*. (1999) demonstrated that COX-2 expression was also found in aortic lesions of apoE-deficient mice in fatty streaks and complex atherosclerotic lesion areas using paraffin sections. These results were concordant with this study.

In conclusion, former part of this study (chapter I) evaluated the association between cytomegalovirus and atherosclerosis indirectly by *in situ* hybridization and PCR study and suggested here a possible pathogenetic mechanism of cytomegalovirus infection on atherosclerosis by histopathologic method. Later part of this study (chapter II) demonstrated the presence of both COX-2 and MMP-9 in atherosclerotic lesions of aorta and coronary artery. These findings support the hypothesis that COX-2 and MMP-9 may interact and play a role in this disorder.

CONCLUSIONS

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

1. Verhoeff's elastic tissue stain of atherosclerotic sections revealed that the elastic lamina was fragmented and disorganized. The structural changes of elastic lamina may play an important role in the development of matrix reorganization, resulting in vascular geometric remodeling.

2. 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. In contrast, only one coronary artery of 10 control subjects revealed hybridization for CMV.

3. By *in situ* hybridization, CMV was localized in cells morphologically consistent with endothelial cells, smooth muscle cells, lymphocytes, fibroblasts and Schwann cells.

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

5. COX-2 and MMP-9 were colocalized to inflammatory infiltrates in atherosclerotic intima, plaque itself, and vascular smooth muscle cells, and coexpressed by macrophages and smooth muscle cells.

6. Inflammatory activation of MMP-9 as well as COX-2 may contribute to the enhanced local matrix degradation in atherosclerotic plaques.

7. The presence of COX-2 and MMP-9 were demonstrated in atherosclerotic lesions of aorta and coronary artery, and these findings support the hypothesis that COX-2 and MMP-9 may interact and play a role in atherosclerosis.

In conclusion, these results suggest that cytomegalovirus is present in the early or advanced atherosclerotic lesions of human aorta and coronary artery. CMV associated vascular inflammatory

reactions and extracellular matrix remodeling may influence in the development of atherosclerosis and, in turn, progression of disease. CMV may potentially play a direct or indirect role in the pathogenesis of human atheroslcerosis.

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국문 요약

Cytomegalovirus가 감염된 동맥경화성 대동맥과 관상동맥에서 Cyclooxygenase-2와 Matrix Metalloproteinase-9의 역할

동맥경화증의 병인론 중 고지혈증, 고혈압, 당뇨, 비만, 흡연, 고령, 남성, 가족력, 유전자변이 등은 위험인자로 이미 잘 알려져 있다. 그러나, 최근 감염체와 만성 염증이 동맥경화증의 중요한 원인으로 제시되고 있고, 이 중 특히, cytomegalovirus (CMV), herpes simplex virus type 1 (HSV-1), Epstein-Barr virus (EBV), *Chlamydia pneumoniae*, 그리고 *Helicobacter pylori* 등이 유력한 원인체로 보고되고 있다.

본 연구는 CMV에 의한 혈관내 감염이 만성 염증성 반응을 유도하고, 활성화된 염증세포들이 염증 매개물질인 cyclooxygenase-2 (COX-2)와 matrix metalloproteinase-9 (MMP-9) 등을 발현하여 동맥경화증을 유발하는지를 알아 보고자 하였다. 먼저 *in situ* hybridization 과 polymerase chain reaction 방법으로 동맥경화증 병변에서 CMV를 검출하여 상호 연관성을 평가하였고, 다음 CMV가 감염된 동맥경화증 병변에서 염증 매개물질인 COX-2와 MMP-9의 발현을 면역조직화학염색 방법

으로 확인하였다.

우선 동맥경화증 병변이 있는 23건과 대조군 10건에서 CMV 감염 정도를 평가한 결과 *in situ* hybridization 방법으로는 동맥경화성 대동맥 23건 중 14건 (60.9%), 관상동맥경화증 21건 중 9건 (42.9%)에서 CMV가 검출되었고, polymerase chain reaction 방법으로는 동맥경화성 대동맥 23건 중 15건 (65.2%), 관상동맥경화증 21건 중 11건 (52.4%)에서 CMV가 검출되었다. 대조군인 대동맥과 관상동맥 각 10건 (총 20건) 중에서는 3건만이 검출되었다.

이상과 같이 CMV의 존재가 확인된 동맥경화증 병변을 선정하여 CMV late antigen, COX-2, MMP-9, macrophage, T-lymphocyte에 대한 항체로 면역조직화학염색을 시행한 결과, CMV가 검출되었던 부위가 COX-2와 MMP-9에 대해 양성 반응을 보였다. 이러한 결과로 CMV는 동맥경화증 생성과정에 염증 반응을 일으키면서 병인론적으로 관여하여 동맥경화증을 악화시키는 것을 확인 하였다.

CMV probe는 smooth muscle cells, endothelial cells, lymphocytes, fibroblasts, Schwann cells 등의 세포들에서 hybridization 되었으나 특징적인 바이러스 봉입체를 포함한 세포에서는 hybridization이 관찰되지 않았는데, 이것은 동맥벽의 CMV 감염이 잠복감염 임을 시사한다.

결론적으로 이상의 실험 결과 CMV가 동맥경화증의 병인론에 직·간접적으로 관여함을 알 수 있었으며, 이러한 감염체의

항바이러스제제에 의한 치료가 동맥경화증 예방에 기여할 것으로 생각된다.

핵심어: cytomegalovirus (CMV), atherosclerosis, cyclooxygenase-2 (COX-2), matrix metalloproteinase-9 (MMP-9), *in situ* hybridization (ISH), polymerase chain reaction (PCR), immunohistochemical stain