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Rapid Communication

High Prevalence of Latently Present Cytomegalovirus in Arterial Walls of Patients Suffering from Grade III Atherosclerosis

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The presence of cytomegalovirus (CMV) nucleic acids was demonstrated in arterial walls of patients with grade III and with maximally grade I atherosclerosis by dot blot and* in situ *DNA hybridization and by polymerase chain reaction (PCR) using probes and primers derived from immediate early (IE) and late (L) genomic regions. The presence of the complete viral genome could be demonstrated by both dot blot DNA hybridization and PCR. IE mRNA but not L mRNA could be demonstrated by* in situ *DNA hybridization, indicating the presence of latent CMV in the human arterial wall. By PCR 90% of the samples obtained from atherosclerotic patients were shown to contain viral nucleic acids as compared to 53% of patients with maximally grade I atherosclerosis, thus substantiating a role for this virus in the pathogenesis of atherosclerosis. (Am J Pathol 1990, 136:23–28)

Recently several investigators demonstrated the presence of cytomegalovirus (CMV) nucleic acids and/or antigens in the human arterial wall by using DNA hybridization techniques[1] or immunohistochemistry.[2] Most arterial specimen were taken from patients undergoing reconstructive vascular surgery for severe atherosclerotic disease, and so a role for the CMV in the pathogenesis of this disease was indicated.[3] On the basis of these observations it was suggested that the human arterial wall might be a site of latency for CMV.[1,2,4] Local reactivation of this virus could then lead to vascular injury and consequently contribute to the development of atherosclerotic lesions. However, the studies published did not include an age- and sex-matched control group of patients with minimal or no signs of atherosclerosis.

In our laboratory we demonstrated the presence of CMV nucleic acids in the human arterial wall[5] by dot blot and *in situ* DNA hybridization techniques. We didn't, however, observe any differences in the distribution of CMV nucleic acids in samples of patients with severe atherosclerosis or patients with maximally grade I atherosclerosis. A possible explanation for this observation is the relative insensitivity of the methods used. Only samples that were sufficiently rich in viral nucleic acids could be detected. More sensitive methods might still show a difference in the distribution of the virus among the patient groups investigated.

Using *in situ* DNA hybridization with a probe derived from immediate early (IE) genomic regions, we demonstrated that 50% of the samples tested contained CMV nucleic acids, which is considerably higher[5] than the percentages found by other researchers using various DNA probes.[1,3] We showed that mRNA-DNA hybrids contributed significantly to the hybridization reactivity observed. This finding that 55% of the arterial samples tested with the ES probe contained CMV nucleic acids shows that mRNA transcribed from IE genomic regions are not only present during reproductive viral cycles[6] but also during latent infections.

In this report we provide further evidence that the CMV is latently present in the human arterial wall by *in situ* DNA hybridization with a probe derived from genomic regions coding for structural proteins. We show also that the complete viral genome is conserved in the arterial wall by dot blot DNA hybridization and polymerase chain reaction (PCR). Using the PCR we found that the prevalence of CMV nucleic acids in patients suffering from severe ath-

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erosclerosis is significantly higher than in patients with maximally grade I atherosclerosis.

Materials and Methods

Patients and Controls

The subjects of this study were as described previously.[5] In brief, arterial biopsies were taken from CMV-seropositive patients undergoing reconstructive vascular surgery. Biopsies were taken from the abdominal aorta or from the femoral artery. CMV-seropositive controls consisted of age- and sex-matched patients with maximally grade I atherosclerosis as shown at autopsy. In this group biopsies were taken from the abdominal aorta. Autopsy was always performed within 12 hours after death. In addition to our previous study, we increased the number of patients entering the control group.

Processing of Tissue Specimens

Arterial tissues for *in situ* and dot blot hybridization and for polymerase-chain reaction were processed as reported previously.[5] For *in situ* DNA hybridization, tissue specimens were incubated overnight in a modified carnoy's solution, dehydrated by successive washes in graded alcohols, and embedded in paraffin, using routine histopathologic procedures. For dot blot hybridization and PCR, DNA was extracted from the arterial specimen by proteolytic digestion and phenol-chloroform extraction followed by ethanol precipitation. DNA was quantified by spectrophotometry at 260 and 280 nM.

Hybridization Probes

All enzymes were purchased from Boehringer-Mannheim (FRG). Enzyme reactions were performed under conditions recommended by the manufacturer. Recombinant plasmids were prepared by following published procedures[7] using vector pBR328.

Plasmid pES (Figure 1) containing the 7.0 Kb ES fragment, an EcoRI-Sal1 subdigestion of the EcoRI-J fragment of CMV strain Ad 169, coding for the major immediate early (IE) protein[8] was kindly provided by Dr. J. Geelen (University of Amsterdam, The Netherlands).

Plasmid pBH (Figure 1) containing the 2.9 Kb BH fragment, coding for the late nucleocapsid protein GP 64,[9,10] was constructed from a HindIII-Bam HI subdigestion of cosmid PCM 1075. This cosmid was kindly provided by Prof. Dr. A. J. van der Eb (University of Leiden, The Netherlands). Both plasmids were transfected into *Escherichia coli* mutant JM 109 and by standard procedures[7,11] pure ES and BH DNA was recovered. Controls consisted of total CMV strain AD 169 DNA, total RCMV-DNA,[12] and plasmid pBR 328 DNA without inserts. In all DNA biotin was inserted by incorporation of biotin-11-dUTP (Bethesda Research Laboratories) using a random primed DNA labeling kit (Boehringer–Mannheim).

Dot Blot Hybridization

Spotting of cellular DNA on nitrocellulose paper, hybridization conditions, and visualization of successful hybridization were carried out as described previously.[5]

The specificity of the biotinylated probes was evaluated with DNA extracted from CMV strain AD 169 infected human embryonic fibroblasts (HEF) and uninfected HEF spotted onto nitrocellulose paper. Only ES probe, BH probe, and total CMV probe showed hybridization with DNA extracted from infected HEF but not with DNA extracted from uninfected HEF. RCMV and plasmid probes never demonstrated hybridization.

In Situ *Hybridization*

The *in situ* DNA hybridization protocol performed on tissue sections of arterial specimens has been described previously.[5] The specificity of the biotinylated probes was evaluated on CMV strain AD 169 infected HEF monolayers and on uninfected HEF monolayers. Only ES probe, BH probe, and total CMV probe showed hybridization with infected HEF monolayers not with uninfected HEF monolayers. RCMV and plasmid probes never showed hybridization.

Oligomer Synthesis

The oligomers used as primers in the polymerase chain reaction (PCR) were synthesized by Dr. H. Meijer (Department of Genetics and Cell Biology, University of Limburg, Maastricht, The Netherlands) on a DNA synthesizer (cyclone DNA synthesizer, Biosearch Inc., New Brunswick, NJ) by the fosfamidid method. The sequences for the oligomer set complementary to the major IE gene were based on the sequence described by Akrigg et al[13] and located in the insert of plasmid pES. The sequences are 5-GGA-GAT-GTG-GAT-GGC-TT-G-TA-3 (IE_1 upstream primer) and 5-GAA-GGC-TGA-GTT-CTT-GGT-AA-3 (IE_2 downstream primer). The sequences for the oligomer set complementary to the nucleocapsid GP 64 gene correspond to published sequences[14] and are located in the insert of plasmid pBH. The sequences are 5-CCG-CAA-

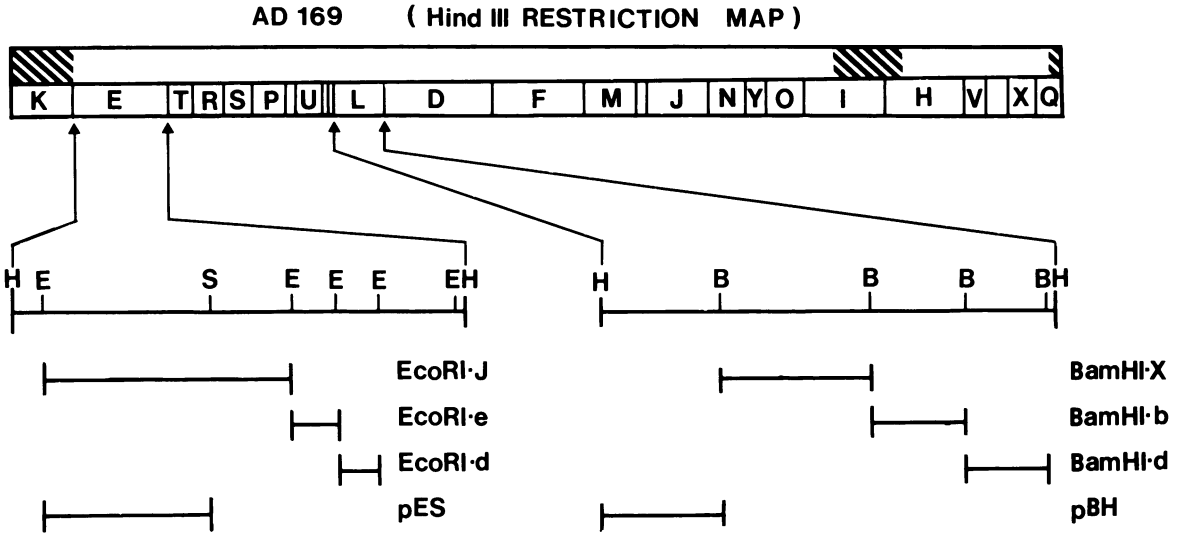

Figure 1. *Restriction endonuclease cleavage map of CMV strain AD 169. The topline represents the HindIII digestion map in prototype arrangement (8) showing the positions of the HindIII-E fragment containing IE sequences and the Hind III-L fragment containing late sequences.*

The left panel shows the position of the cloned ES fragment (7.0 KB) coding for the major IE protein (8) within the HindIII-E fragment relative to the EcoRI-J, c and d fragments. The right panel shows the position of the cloned BH fragment (2.9 KB) coding for the late nucleocapsid protein GP 64 (9-8) within the HindIII-L fragment relative to the BamHI-X, b and d fragments. H(HindIII), E(EcoRI), S(SalI), and B(BamHI) represent restriction endonuclease cleavage sites.

CCT-GGT-GCC-CAT-GG-3 (LA_1 upstream primer) and 5-CGT-TTG-GGT-TGC-GC-A-GCG-GG-3 (LA_2 downstream primer). The sizes of the PCR amplification products of the IE and GP 64 targets are 170 and 140 base pairs, respectively.

Polymerase Chain Reaction

Amplification of extracted cellular DNA was performed in a total volume of 30 μl. The reaction mixture contained 10 mM Tris-HCl (pH 9.6), 10 mM $MgCl_2$, 0.2 mg/ml BSA Boseral (Organon), 50 mM NaCl, 1 mM dATP, 1 mM dCTP, 1 mM dTTP, 1 mM dGTP (all nucleosides purchased from Promega), 0.5 μg of each primer, 1 U cloned thermostable DNA polymerase from Thermus Aquaticus (Ampli-Taq, Perkin-Elmer-Cetus) and 2.5 to 5.0 μg of sample DNA. The reaction mixture was overlayed with 20 μl paraffin oil to prevent evaporation. The amplification reaction was performed on a DNA thermal cycler (Cetus). Each primer set was evaluated with regard to annealing temperature and the number of cycles required to achieve the highest signal-to-noise ratio when the products were evaluated by direct staining of 2% agarose (Seakem) gels. The reaction conditions that follow were found to be optimal. After initial heating of the samples for 5 minutes at 94 C, 37 cycles were carried out. Each cycle was built up as follows: heating for 30 seconds at 94 C to denature the target DNA, then cooling to 59 C for 45 seconds to allow the primers and DNA to reanneal, and finally heating to 72 C for 120 seconds for primer extension. Fifteen μl of the reaction mixture was subjected to electrophoresis on 2% agarose (Seakem) gels containing 0.5 μg/ml EtBr. The DNA was visualized by UV fluorescence. The specificity and sensitivity of both primer sets have been tested extensively by others.[13,14] No cross reactivity was observed between the IE primer set and plasmid pBH, nor between the LA primer set and plasmid pES.

Also no reactivity was observed when testing both primer sets on DNA obtained from HEF or from leucocytes from healthy individuals. DNA samples that did not show reactivity with the CMV primers used were further analyzed with a primer set reactive with the β-globine gene[15] to check on DNA preservation. All samples tested this way showed an 110-bp amplification product of the β-globine gene, thus proving that DNA preservation was sufficient for PCR analysis.

Statistical Analysis

Statistical analysis was carried out using Fisher's exact tests. Results were considered significant when $P < 0.01$.

Results

Patients and Controls

Tissue samples were obtained from 44 patients undergoing reconstructive vascular surgery (30 men, 14 women)

Table 1. *Results of Dot Blot and* In Situ *DNA Hybridization with Probes Derived from Immediate Early (ES Fragment) and Late (BH Fragment) Genomic Regions and of the Polymerase Chain Reaction with Primers Reactive with Immediate Early (IE Primers) and Late (LA Primers) Genomic Sequences*

Hybridization technique	Grade III atherosclerosis Nr. POS/ total nr. (%)	Max grade I atherosclerosis Nr. POS/ total nr. (%)
Dot blot hybridization		
ES fragment	11/44 (25%)	7/33 (21%)
BH fragment	4/14 (29%)	4/23 (18%)
In situ hybridization		
ES fragment	18/41 (44%)	19/33 (58%)
BH fragment	5/41 (12%)	5/33 (15%)
Polymerase chain reaction		
IE Primers	27/30 (90%)	18/34 (53%)
LA Primers	27/30 (90%)	18/34 (53%)

for obstructive atherosclerotic disease. Twenty-two samples were taken from the femoral artery (10 men, 12 women) and 22 samples were taken from the abdominal aorta (20 men, 2 women). The mean age of all patients was 66 years (range, 44 to 88 years).

The autopsy-control series contained 34 patients (16 men, 18 women) with a mean age of 69 years (range, 45 to 91 years). In this group samples were taken from the abdominal aorta within 12 hours after death.

Mean ages of atherosclerotic and autopsy-control group were in the same range as was the distribution among the sexes.

Dot Blot DNA Hybridization

Using the dot blot hybridization technique (Table 1) CMV nucleic acid sequences were detected in 11 of 44 (25%) samples obtained from patients with severe atherosclerosis using the ES probe and in 4 of 14 (29%) samples of the same patient group using the BH probe. Statistical analysis revealed that this difference between the two probes was not significant ($P = 0.5214$).

By evaluating the extracted DNA of the samples obtained from patients with maximally grade I atherosclerosis, CMV nucleic acids were detected in 7 of 33 (21%) cases using the ES probe and in 4 of 23 (18%) cases using the BH probe, which was not a statistically significant difference ($P = 0.5003$).

Also no statistical significant differences were observed in the distribution of CMV nucleic acids in samples of patients with or without atherosclerosis by using the ES probe ($P = 0.4565$) or by using the BH probe ($P = 0.343$).

Overall we could conclude that by using this technique 27% of the samples tested contained CMV nucleic acids, regardless of the patient group or the probe used.

In situ *DNA Hybridization*

In situ DNA hybridization (Table 1) of sections of arteries obtained from patients with severe atherosclerosis showed that 18 of 41 (44%) contained CMV nucleic acids by using the ES probe. However, by using the BH probe only 5 of 41 (12%) samples obtained from the same patient group were shown to contain CMV nucleic acids. The differences found between these two probes are statistically significant ($P = 0.002$).

Evaluation of the samples obtained from patients with maximally grade I atherosclerosis revealed that 19 of 33 (58%) contained CMV nucleic acids by using the ES probe and only 5 of 33 (15%) by using the BH probe. The difference between these two probes was also statistically significant ($P = 0.0004$).

There was no difference in the distribution of CMV nucleic acids as demonstrated by *in situ* hybridization between patients with or without atherosclerosis by using the ES probe ($P = 0.175$) or by using the BH probe ($P = 0.485$).

Overall we could conclude that 50% of the samples tested with the ES probe contained CMV nucleic acids, but by using the BH probe only 14% of these samples were shown to contain the cytomegalovirus.

Polymerase Chain Reaction

The polymerase chain reaction performed on pure DNA extracted from arteries of patients suffering from severe atherosclerosis showed that 27 of 30 (90%) contained CMV nucleic acids regardless of the primer set used (Table 1).

The samples obtained from patients without atherosclerosis showed exactly the same reactivity with regard to the primer set used. Eighteen of 34 (50%) samples were demonstrated to contain CMV nucleic acids by both primer sets, one directed against the DNA coding for the major IE antigen and one directed against the DNA coding for the nucleocapsid protein GP 64. Samples reactive with the IE primer set were also always reactive with the LA primer set. Negative samples did not react with the IE primer set or with the LA primer set.

We observed a great difference in the distribution of CMV nucleic acids obtained from the two patient groups under investigation. Far more samples, 27 of 30 (90%), obtained from patients with severe atherosclerosis contained CMV nucleic acids as compared to the samples obtained from patients with maximally grade I atherosclerosis, 18 of 34 (53%) ($P = 0.001$). Overall we could conclude that by using this technique 90% of the atherosclerotic patients contain CMV nucleic acids in their arterial

walls as compared to 53% of the patients with maximally grade I atherosclerosis, regardless of the primer set used.

Discussion

In this study we demonstrated that the percentage of arterial specimens obtained from patients with or without atherosclerosis containing CMV nucleic acids depended highly on the DNA detection method and probes used. With a crude method like dot blot DNA hybridization only low percentages of samples (27%) containing the virus could be detected, regardless of the probes used. However, by using *in situ* DNA hybridization techniques, a higher percentage of samples could be shown to contain the viral nucleic acids, depending on the probes used. Probes directed against IE genomic sequences gave higher yields (50%) than probes directed against structural sequences (12%). The sensitivity of this method, however, wasn't sufficient to detect differences in the distribution of CMV nucleic acids between the patient groups under investigation. With a very sensitive method like the polymerase chain reaction we showed that a much higher percentage (90%) of atherosclerotic patients contained CMV DNA in their arterial walls as compared to patients with maximally grade I atherosclerosis (53%). In addition, with this method we demonstrated that both IE and late sequences were always simultaneously present in all the reactive samples.

As already stated,[5] the percentage of samples containing CMV nucleic acids as shown by dot blot hybridization with the ES probe (27%) equals the percentages found by others[1,4] using DNA probes derived from various genomic regions. This means that all samples containing sufficient viral DNA will show hybridization reactivity using this technique, regardless of the probe used. An important conclusion that may be drawn from these findings is that the whole viral genome is present in the human arterial wall. Our finding, using dot blot hybridization techniques, that arterial wall specimen showing hybridization reactivity with the ES probe almost always showed reactivity with the BH probe too, further substantiates this thesis.

Using *in situ* DNA hybridization with the ES probe, we found a considerably higher percentage (50%) of the samples investigated to contain CMV nucleic acids as compared to the dot blot technique (27%). Our explanation, confirmed with RNAse digestion,[5] was that not only DNA–DNA hybrids formed but that also mRNA–DNA hybrids contributed significantly to the hybridization reactivity observed. These mRNAs transcribed from IE genomic regions seem to be present not only during reproductive viral cycles[6] but also during latent infections. However, using *in situ* DNA hybridization with the BH probe only a small percentage (14%) of samples could be demonstrated to contain CMV nucleic, suggesting that no mRNA–DNA hybrids formed. The presence of IE mRNAs has been demonstrated using *in situ* DNA hybridization techniques with DNA probes derived from IE genomic regions for Herpes Simplex virus latently present in human trigeminal ganglia,[16–18] while no HSV nucleic acids could be detected when these ganglia were probed with DNA derived from genomic regions coding for structural proteins.[15] Our results with both DNA hybridization techniques therefore show a parallel situation for CMV, indicating that the human arterial wall is a site of latency for this virus. Histologically, the CMV nucleic acid sequences detected with both probes were mostly located in smooth muscle cells in the media of apparently normal arterial tissue.

Using the PCR we showed that all samples reactive with primers derived from IE genomic regions were also reactive with primers derived from late genomic regions, thus proving that probably the whole viral genome is conserved in the human arterial wall. The percentage of samples obtained from patients with maximally grade I atherosclerosis containing CMV nucleic acids as shown by *in situ* DNA hybridization with the ES probe (58%) equals the percentage found by PCR (53%). Both techniques seem to have equal sensitivity when studying apparently normal arterial walls using these probes and primers. However, when studying severely atherosclerotic arterial walls, PCR is superior. This can be explained by the localization of the CMV nucleic acids in the apparently normal smooth muscle cells in the arterial media. This arterial media has undergone massive atheromatous changes with greatly increased numbers of mononuclear leukocytes with macrophages and T lymphocytes[19] in patients suffering from severe atherosclerosis with only few smooth muscle cells left, reducing the chance of finding CMV nucleic acids by *in situ* DNA hybridization. The finding that 90% of the samples obtained from patients suffering from severe atherosclerosis contained CMV nucleic acids using PCR, as compared to 53% of the samples obtained from patients with maximally grade I atherosclerosis suggests a role for this virus in the pathogenesis of this disease. The reactivation of this latent virus to a local lytic infection in the vessel wall might result in repeated local inflammatory reactions that, in concert with other established risk factors, may lead to atheroma formation.

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