ORIGINAL ARTICLE

Increased incidence of cytomegalovirus but not *Chlamydia pneumoniae* in atherosclerotic lesions of arteries of lower extremities from patients with diabetes mellitus undergoing amputation

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Aims: To evaluate the association between cytomegalovirus (CMV) or *Chlamydia pneumoniae* infection and the development of accelerated atherosclerotic lesions in patients with diabetes who are known to have an impaired immune response to infection and a high incidence of atherosclerosis.
Methods: Two hundred arterial samples from patients with diabetes who had undergone surgical amputation for gangrenous lower limbs were selected to assess the presence of CMV or *C pneumoniae* nucleic acid by means of the polymerase chain reaction.
Results: CMV nucleic acid sequences were detected in 64 of 200 (32%) samples and *C pneumoniae* in seven of 200 (3.5%) arterial samples with severe atherosclerosis. Of those positive for *C pneumoniae*, six were also positive for CMV.
Conclusion: The significantly higher incidence of CMV nucleic acid sequences in the arterial samples of patients with diabetes supports the hypothesis that this organism is involved in the pathogenesis of atherosclerosis in patients with diabetic mellitus. It is possible that the potential role of different infectious agents in the pathogenesis of atherosclerosis might rely on their biological properties and their infectivity in hosts with varying immunological status.

Patients with diabetes are relatively immunocompromised, remain persistently at risk for infectious complications,[1][2] and are highly prone to develop accelerated atherosclerosis.[3][4] The widely accepted "response to injury" hypothesis of atherosclerosis proposed that an initial insult to the arterial wall triggers atherogenesis and plaque formation.[5] Although many factors may initiate atherogenesis, the process ultimately involves an inflammatory state in which macrophages and T cells play a major role.[6–8] Antigen within the plaque may trigger/perpetrate the immunological activity and therefore the atherogenic inflammation. The potential role of common infectious agents in the pathogenesis and progression of atherosclerosis has been studied increasingly over the past decade. Herpes simplex virus and cytomegalovirus (CMV) were first detected in human atheromatous tissue by the groups of Melnick and Benditt, respectively, in 1983.[9][10] Antigens and nucleic acid sequences of CMV have been found in arterial lesions in human atherosclerosis,[11] and several studies have also shown an association between atherosclerosis and antibodies against CMV.[12] However, the most convincing evidence for a role for herpes viruses in atherosclerosis comes mainly from the association of CMV with coronary artery disease in transplant accelerated atherosclerosis[13–15] and restenosis following angioplasty.[16] The evidence for *C pneumoniae* as a potential causative agent is based on the findings from numerous sero-epidemiological studies,[17] the examination of atheromatous plaque specimens,[18] animal models,[19] and recently pilot anti-chlamydial antibiotic intervention trials.[20][21] Despite a high frequency of antibody against *C pneumoniae* in the population, these studies have consistently demonstrated a significant difference between earlier *C pneumoniae* infection and atherosclerosis.

> "Several studies have also shown an association between atherosclerosis and antibodies against cytomegalovirus"

We hypothesise that the impaired immune response in patients with diabetes may cause the reactivation of persistent pathogens, such as CMV and *C pneumoniae*, resulting in damage to the vessel wall, and thus initiating atherosclerosis. There has been only one report evaluating the role of CMV infection in the development of atherosclerosis in patients with diabetes. In that study, Visseren and co-workers[22] detected anti-CMV antibodies more often in those patients with diabetes who had atherosclerosis compared with those without atherosclerosis (70.7% *v* 45.2%).

The purpose of our study was to evaluate the association between CMV or *C pneumoniae* infection and the development of accelerated atherosclerotic lesions in patients with diabetes by the detection of CMV and *C pneumoniae* nucleic acids by means of the polymerase chain reaction (PCR) in atherosclerotic tissues from arterial samples obtained from patients with diabetes.

MATERIALS AND METHODS

Patients

To study the presence of CMV and *C pneumoniae* in the arterial wall, archival, paraffin wax embedded arterial tissue samples (including those of the anterior and posterior tibial arteries, and the pedis dorsalis artery) of 200 patients with type II diabetes mellitus for more than five years, who had undergone surgical amputation of lower extremities between 1990 and 1996 in Kaohsiung Medical Center, Chang Gung Memorial Hospital, Taiwan, were selected. They included 119 men and 81 women, with a mean age of 65 years and 69 years, respectively. In addition, 24 non-diabetic patients who had undergone surgical amputation of a lower extremity as a result of other

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Table 1 Sequences of primers used

Primers	Region	Nucleotide sequence
CMV		
Outer PCR		
CMVR-1	IE exon 4	GTAATGAAGCGCCGCATTGAGGAGA
CMVL-1	IE exon 4	GGCAACTTCCTCTATCTCAGACAC
Inner PCR		
CMVR-2	IE exon 4	CCTAGTGTGGATGACCTACGGGCCA
CMVL-2	IE exon 4	CAGACACAGTGTCCTCCCGCTCCTC
Chlamydia pneumoniae		
HL-1		GTTGTTCATGAAGGCCTACT
HR-1		TGCATAACCTACGGTGTGTT
β actin		
β3		TGGACTTCGAGCAAGAGATG
β4		CTCCTTCTGCATCCTGTC

Primers for CMV are based on the sequence published by Kouzarades et al,[24] and for *C pneumoniae* on that by Campbell *et al.*[25]
CMV, cytomegalovirus; PCR, polymerase chain reaction.

causes, such as traumatic injury, were also collected as a control patient group. The histological slides were reviewed by a pathologist and appropriate paraffin wax embedded tissue blocks were sectioned for PCR analysis for both CMV and *C pneumoniae*.

Detection of CMV and *C pneumoniae* nucleic acid
Tissue preparation
Archival arterial wall tissue had been fixed in 10% buffered formalin and embedded in paraffin wax. Ten 5 µm thick paraffin wax embedded sections were cut using disposable knives to avoid contamination between blocks. DNA was purified from tissue sections using the Chelex boiling method. Briefly, sections were boiled in a 25% suspension of Chelex 100 chelation resin (Sigma Chemical Co, St Louis, Missouri, USA) in sterile water. The resulting supernatants were extracted with phenol/chloroform by standard methods and precipitated with ethanol. The DNA pellets were resuspended in 50 µl Tris/HCl buffer at pH 8.0. Mock extractions of buffer were done and amplified to ensure that no contamination occurred. DNA extraction and PCR amplification were performed in separate rooms with a different pipette with aerosol resistant tips.

Controls
The positive CMV control was prepared in human embryonic fibroblast (HEF) cells infected with CMV strain AD 169 in a 75 cm² flask. After a strong cytopathic effect had developed, the cell layer was washed with phosphate buffered saline. The cells were scraped into 5 ml PBS, and the suspension was frozen and thawed three times, followed by boiling for 10 minutes. The cellular debris was removed by centrifugation at 2000 ×*g*. The supernatant was stored at −70°C. Stock titres were determined by plaque assay.

The positive *C pneumoniae* control was prepared in HL cells infected with *C pneumoniae* strain AR-39 in a 75 cm² flask for 72 hours. The cells were scraped into 1.0 ml sucrose phosphate glutamic acid medium. The titres of these preparations were determined by the method of Furness *et al.*[23]

The CMV and *C pneumoniae* controls were added to lysis buffer (100mM Tris/HCl, pH 8.0, 25mM EDTA, 2% Triton X-100) with 50 µg/ml proteinase K. The lysate was incubated overnight at 50°C, and extracted sequentially with phenol, phenol/chloroform (1/1), and chloroform/isoamyl ethanol (24/1). The aqueous phase was precipitated with one tenth of 3M ammonium acetate (pH 5.2), one hundredth 10% glycogen, and two volumes of absolute ethanol. DNA pellets were air dried and resuspended in 50 µl Tris buffer.

Primers and probes
Table 1 lists the primers and probes. For the detection of CMV nucleic acid by nested PCR, primers derived from the immediate–early gene (IE) exon 4 were used.[24] For the detection of *C pneumoniae* nucleic acid, DNA samples were amplified using *C pneumoniae* specific primers HL-1 and HL-2.[25] In all experiments, the DNA extracts were also amplified with β actin primers β3 and β4 as controls for DNA preservation and the exclusion of false negative results.

PCR for CMV
A 50 µl volume of reaction mixture was prepared containing 1 mmol/litre each of dATP, dCTP, dGTP, and dTTP; 25 pmol of each oligonucleotide primer; 0.5 U of Taq polymerase (Perkin Elmer, Oak Brook, Illinois, USA); and 5 µl of specimen DNA in a buffer solution. The buffer solution consists of 50mM KCl, 3mM MgCl₂, 10mM Tris/HCl, pH 9.0, and 0.1% Triton X-100. Amplification of the target portion of the CMV IE gene was achieved by means of a nested PCR using a CMVR-1/CMVL-1 primer set in the first and a CMVR-2/CMVL-2 primer set in the second PCR. Samples were amplified using 35 cycles of denaturation at 94°C for one minute, primer annealing at 55°C for two minutes, and chain extension at 72°C for three minutes in a Perkin-Elmer Cetus thermocycler. PCR products (10 µl) were separated by electrophoresis using 1.5% agarose gel and visualised by ethidium bromide staining. To determine the sensitivity of the PCR procedures in detecting CMV DNA, varying amounts of DNA (10^2 to 10^3 plaque forming units) from the positive control DNA were amplified. Distilled water was used as a negative control in each set of PCR reactions.

PCR for *C pneumoniae*
PCR was performed as described by Campbell and colleagues[25] to detect *C pneumoniae* specific DNA. Negative and positive controls consisting of PCR reagents but lacking the specimen, and varying dilutions of purified *C pneumoniae* DNA, respectively, were included in each PCR run. The amplification products, the expected 437 bp *C pneumoniae* specific DNA sequences, were analysed by gel electrophoresis through a 1.0% agarose gel according to standard protocols and transferred by Southern blotting to a nylon membrane (Amersham Life Science, Little Chalfont, Buckinghamshire, UK). The products were confirmed by hybridisation to a digoxigenin-dUTP labelled probe. DNA probes were labelled by means of the Genius DNA labelling and detection kit (Boehringer Mannheim Biochemicals, Indianapolis, Indiana, USA), and hybridisation was detected by immunochemiluminescence using Lumiphos 530, according to the manufacturer's directions.

Statistical analysis
Statistical analysis was carried out by Fisher's exact tests. Results were considered significant when $p < 0.05$.

Table 2 Cytomegalovirus (CMV) and *Chlamydia pneumoniae* DNA in vessel wall of patients with diabetes and non-diabetic controls

	Diabetic group (n=200)	Non-diabetic group (n=24)
CMV	64 (32.0%)*	2 (8.3%)
C pneumoniae	7 (3.5%)†	0 (0%)

*p<0.001 in comparison with *C pneumoniae*, and p<0.05 in comparison with non-diabetic group. †Six of the seven *C pneumoniae* positive cases were also also positive for CMV.

RESULTS

We have developed highly sensitive and specific PCR methods for the detection of the smallest traces of *C pneumoniae* and CMV in tissue samples. The sensitivity of the nested PCR for CMV was 0.1 plaque forming units. For *C pneumoniae*, as few as 0.4 inclusion forming units of *C pneumoniae* DNA could be detected by PCR and visualised by hybridisation of the product on a nylon membrane. There was no significant difference between the sensitivities of the two PCR protocols to detect CMV and *C pneumoniae* DNA in the samples.

Using PCR, CMV nucleic acid sequences were detected in 64 of 200 (32%) samples and *C pneumoniae* in seven of 200 (3.5%) samples of the anterior or posterior tibial artery of patients with diabetes who had undergone surgical amputation for gangrenous lower limbs with severe atherosclerosis. Of those positive for *C pneumoniae*, six (85.7 %) were also positive for CMV. In contrast, in the control group, CMV nucleic acid was detected in only two of the 24 arterial tissue samples, and none of them was positive for *C pneumoniae* (table 2). Statistical analysis revealed this difference between the incidences of these two pathogens to be highly significant (p < 0.05).

DISCUSSION

The higher incidence of atherosclerotic diseases in patients with diabetes than in those without diabetes cannot be explained only by the differences in known risk factors or hyperglycaemia, so that other potential risk factors might contribute to their development. There is an accumulating amount of data indicating that infections may be linked to atherosclerotic disease. There are also several potential underlying mechanisms of infection to augment the atherosclerotic process and contribute to later manifestations of overt clinical disease by facilitating plaque rupture and thrombosis.

CMV is ubiquitous, and the incidence of infection gradually increases with age. More than 90% of adults have experienced infection with CMV. As shown in a recent report from a blood centre of the Chinese Blood Foundation in southern Taiwan, of the 1800 consecutive sera from blood donors tested, only 150 (8.3%) were CMV seronegative.[26] CMV antigens in the arteries of young trauma victims were often present in the areas of the arterial wall showing early atheromatous change, which indicates that CMV is involved in the development of arterial wall damage rather than being the result of arterial wall damage.[11] A strong sero-epidemiological link between CMV infection and atherosclerosis has been suggested.[12–15] A case–control study in which patients who had undergone cardiovascular surgery were compared with a control group of subjects who had not undergone surgery but had similar cholesterol values and epidemiological factors was carried out. In approximately 160 pairs of patients, the prevalence of CMV antibodies was higher in the surgical group than in the control group, and a greater percentage of surgical cases than controls had high titres of anti-CMV antibodies.[27] In some prospective studies with immunosuppressed patients who had received a cardiac transplant, there was a high correlation between CMV positivity and the development of atherosclerosis in the transplanted heart and decreased survival after a five year follow up.[13–15] In contrast, several recent studies on CMV infection in non-immunocompromised hosts showed no significant correlation between the development of atherosclerosis and CMV infection.[28–30] This is an intriguing finding, considering that several previous studies have indicated an association between CMV seropositivity and atherosclerosis.[12] However, they were mostly defined on the basis of coronary restenosis after atherectomy[16] or the development of lesions in transplanted hearts when the patients were relatively immunocompromised.[13–15]

Chlamydia pneumoniae is an intracellular Gram negative bacterium that commonly causes respiratory infections in all age groups.[31] Specific antibodies to *C pneumoniae* have been found in more than half of the adult population.[32] Persistent infection is not uncommon after acute respiratory infection with *C pneumoniae*.[33] Several observations support the hypothesis that persistent *C pneumoniae* infection might be a risk factor for vascular disease, comparable in magnitude to the classic risk factors.[12 17 34] Seropositivity for *C pneumoniae*, but not CMV, was recently reported to be associated with atherosclerosis and increased risk for future cardiovascular disease.[35] Local *C pneumoniae* infection has subsequently been detected in 52% of atherosclerotic lesions, but in only 5% of control samples of the arterial tissue.[12] *Chlamydia pneumoniae* first establishes persistent infection in the lung. Infected lung macrophages might then serve as a vehicle to disseminate the infection and to establish persistent infection in the arterial wall.[36] Infected monocytes not only directly transmit infection to endothelial cells, but also independently enhance the infectivity of *C pneumoniae* for endothelial cells; the monocytic stimulation of infectivity with *C pneumoniae* has also been shown to be specific to endothelial cells.[37] Pre-existing injury or atheromatous lesions, initiated by other risk factors associated with atherosclerosis, in addition to the presence of monocytes, may be needed to establish infection of *C pneumoniae* in the endothelial cells. In contrast to CMV, active infection with *C pneumoniae* usually occurs in immunocompetent individuals.

> "The impaired immune response may lead to repeated or continuous CMV reactivation from its latent status in the vascular wall, resulting to endothelial damage, smooth muscle cell proliferation, and an inflammatory response, thus initiating a process of atherosclerosis"

The biological properties of both CMV and *C pneumoniae* are consistent with a potential role in the pathogenesis of atherosclerosis. However, the potential role of different infectious agents in the pathogenesis of atherosclerosis might rely on their biological properties and their infectivity to hosts with varying immunological status. As shown in our present study, a high incidence of CMV nucleic acid sequences, but not *C pneumoniae*, is found in the arterial samples of patients with diabetes and severe atherosclerosis. Most of the samples that were positive for *C pneumoniae* were also CMV DNA positive. Our data support the hypothesis that CMV, but not *C pneumoniae*, is involved in the pathogenesis of atherosclerosis in patients with diabetes mellitus. It has been shown that both cellular and humoral immune responses to viral antigens in patients with diabetes mellitus are impaired.[38–40] As already proposed by others,[10 11 41] the arterial wall may be a site of latency for CMV, and the impaired immune response may lead to repeated or continuous CMV reactivation from its latent status in the vascular wall, resulting in endothelial damage, smooth muscle cell proliferation, and an inflammatory response, thus initiating a process of atherosclerosis. It may act alone or in synergy with other recognised risk factors. This mechanism might contribute to the high incidence of atherosclerotic diseases in patients with diabetes mellitus.

Take home messages

- There was a significantly higher incidence of cytomegalovirus (CMV) nucleic acid in the arterial samples of patients with diabetes
- These results support the notion that CMV is involved in the pathogenesis of atherosclerosis in patients with diabetic mellitus
- *Chlamydia pneumoniae* did not appear to be involved in the pathogenesis of atherosclerosis in these patients
- The potential roles of different infectious agents in the pathogenesis of atherosclerosis might rely on their biological properties and their infectivity in hosts with varying immunological status

We conclude that latent CMV infection of the arterial wall may be a common occurrence in patients with diabetes mellitus. The ability to reactivate the virus from the latent status leading to a local lytic infection in the vessel wall might result in repeated local inflammatory reactions, eventually leading to vascular injury. This injury, together with other established risk factors, might contribute to the process of atherogenesis. Although our present results do not suggest a direct role for CMV in the pathogenesis of atherosclerosis, determining the presence of CMV infection may be a valuable parameter in the management of patients with diabetes. Further studies are needed to elucidate the factors leading to reactivation of CMV and the development of atherosclerosis as a consequence of a local productive infection.

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