DETECTION OF HUMAN CYTOMEGALOVIRUS IN THE ATHEROSCLEROTIC CEREBRAL ARTERIES IN HAN POPULATION IN CHINA

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Summary. – The association of atherosclerosis (AS) and Human cytomegalovirus (HCMV) infection was studied. AS plays an important role in the brain stroke and HCMV infection is supposed to be involved in the process of atherosclerotic formation. The presence of HCMV DNA and antigens was examined in the internal carotid arteries collected from 35 patients with ischemic stroke and from 20 patients from the control population. All patients belonged to the ethnic Han population in China. Three methods, immunohistochemistry (IHC), hybridization *in situ* (HIS), and PCR were used to detect the HCMV immediate early (IE) and late (L) antigens as well as viral DNA in vessel walls. Levels of HCMV IE gene/protein were significantly higher in the stroke group than in control group detected by the three methods (IHC 34.3% vs. 10.0%; HIS 40.0% vs. 10.0; PCR 60.0% vs. 30.0%). However, there was no significant difference in the levels of HCMV L gene/protein between these two groups of patients (IHC 11.4% vs. 5.0%; HIS 11.4% vs. 10.0%; PCR 20.0% vs. 20.0%). We concluded that the presence of HCMV IE antigen and HCMV DNA in the vessel wall was associated with the pathological process of AS formation.

Key words: Human cytomegalovirus; atherosclerosis; internal carotid arteries; ischemic stroke

Introduction

AS plays an important role in the brain stroke. It is reported that about 68% of patients suffering of ischemic stroke developed carotid AS (Palak *et al*., 1994). Years of observational research have shown that well-known host factors such as family history, genetic susceptibility, and lifestyle affects the development of AS, but these risk factors do not represent the all preconditions for the development of the disease. Over the years, herpesviruses have increasingly attracted an attention regarding their potential role in the process of AS development. In particular, the supposed interrelation between HCMV and AS has been a favorite subject of investigation in both human and animal studies.

The existence of an association between HCMV infection and AS was originally based on a case-control study of cardiovascular patients undergoing surgery, who had high levels of HCMV antibodies associated with a clinical manifestation of AS (Adam *et al*., 1987). Likewise, the results from another cohort study suggested that HCMV infection represented a risk factor for AS (Nieto *et al*., 1996). The level of carotid intimal-medial thickening (IMT) was compared with the titer of antibodies against HCMV. The patients with higher levels of IMT had higher titers of HCMV antibody than control subjects suggesting that HCMV might play some role in the development of AS.

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Abbreviations: AS = atherosclerosis, atherosclerotic; HCMV = Human cytomegalovirus; E = early; HE = hematoxylin and eosin; IE = immediate early; IHC = immunohistochemistry; HIS = hybridization *in situ*; IMT = intimal-medial thickening; L = late; MAb(s) = monoclonal antibody(ies)

In addition to the data from seroepidemiological studies, the data from histopathological as well as *in vitro* studies supported the hypothesis that HCMV may be implicated in the pathogenesis of AS. In patients without clinical symptoms of HCMV infection, the virus is present in the arterial wall (Yamashiroya *et al*., 1988). HCMV DNA was present in 90% of the arterial walls of patients with severe AS compared to 50% of those in control subjects with no or only small AS changes detected by PCR (Hendrix *et al*., 1990). In addition, heart transplant recipients who were actively infected with HCMV were particularly prone to develop accelerated atherosclerosis in the transplanted organ (Fateh-Moghadam *et al*., 2003; Loebe *et al*., 1990; McDonald *et al*., 1989).

HCMV is an important human pathogen with an ability to cause a disease in immunocompromised subject. It is a complex virus and its genes are divided into three groups based on the time of appearance in permissively infected cells (DeMarchi *et al*., 1980). Gene expression in HCMV-infected cells occurs in an ordered sequential fashion. The viral ORFs are expressed in a highly organized and regulated cascade of IE, E, and L transcription. The first genes transcribed are IE genes, which are required for subsequent expression and regulation of E genes and may be required for L genes expression. L gene expression occurs after the initiation of viral DNA synthesis. Viral RNA at late time of infection originates from all regions of the genome. It has been postulated that all L genes are transcribed early, but the late mRNA is not maximally translated until the late time of infection as a result of posttranscriptional controls (Depto *et al*., 1989).

A prominent characteristic feature of this virus is the persistence in the host after the primary infection (Hendrix *et al*., 1990). Several reports suggest that the arterial wall might be a site of latency for HCMV and a frequent reactivation from this latent state might contribute to the pathogenesis of AS (Melnick *et al*., 1990). However, these findings were concerning only Caucasians and focused on the cardiovascular and peripheral arteries. Some reports revealed that the locations of cerebral AS vary with different ethnics. In Caucasians, there were more AS plaques in external carotid arteries, while in Asian and African population in internal carotid arteries (Sacco *et al*., 1995; Inzitari *et al*., 1990). In this respect, a choice of the internal carotid arteries of Han ethnic population for examination is more appropriate.

This study explores an association between HCMV infection and carotid AS in the ethnic Han Chinese. We investigated the presence of IE and L genes as well as corresponding antigens of HCMV in the internal carotid arteries of patients suffering from stroke by the 3 methods IHC, PCR, and HIS.

Materials and Methods

Patients. All patients were ethnic Han people from China. The stroke group included 35 patients that died of ischemic stroke, 25 males and 10 females aged 50–76 years. The 20 patients of control group included 13 healthy males and 7 females matched for age and sex with the patients in the stroke group. The patients in the control group did not suffer of cerebral vascular disease, HCMV related diseases, or immunocompromised status. The presence of specific IgG antibody against HCMV in blood samples of 55 examined patients were tested by ELISA (Virion-Serion, German). The seropositive patients were removed from the control group. The internal carotid arteries from all patients were collected by autopsy within 24 hrs after death.

Histology. The type of AS was scored according to the guidelines of the American Heart Association classification (Stary *et al*., 1995) with type I-III being classified as early lesions, and type IV-VI as advanced lesions. Type I lesions were defined as lesions containing isolated macrophage foam cells in the media. Type II lesions (fatty streaks) were defined as lesions consisting primarily of layers of macrophage foam cells. Type III lesion was the lesion type placed between types II and IV and called "preatheroma". Type IV lesion called "atheroma" aggregated extracellular lipid pools and a large confluent lipid core. In type V lesion, the lipid core expanded covering the entire lesion. Lesions with fissures and/or ulcerations and/or thrombotic deposits were defined as type VI lesions.

For every patient the mean lesion area and number of lesions were calculated and the numbers were compared between stroke and control group. The arteries were isolated and fixed in 3.7% PBS-buffered formaldehyde solution for histological examination and determination of HCMV antigens and DNA.

PCR. To detect the HCMV DNA in intracranial arteries, two pairs of primers were used. One pair of oligonucleotide primers specific for the HCMV, strain AD169, major IE exon 4 region, produced a 248 bp product. The second pair was specific for the HCMV L gene region (Gozlan *et al*., 1993). The L gene encoding the major capsid protein produced a 263 bp amplicon. The ß-actin primers produced a 354 bp product (Table 1).

Sections of the carotid (5 μm) were cut with sterile blade and DNA was extracted by the mixture of phenol, chloroform, and isoamyl alcohol (Yi *et al*., 2004). The PCR conditions were as follows: 94°C/5 mins, 35 cycles of 94°C/40 secs, 59°C/60 secs (IE gene), 60°C/60 sec (L gene), 72°C/60 secs, and 72°C/10 mins.

Each PCR assay was run with at least two negative (H_2O instead of the template or primers) and two positive controls (HCMV DNA instead of the template) to prevent false-negative and false-positive results. PCR products were analyzed by agarose gel electrophoresis and photographed under UV light. The pBR322/*Hinf*I was used as DNA size markers (1631, 517, 506, 396, 344, 298, 221, 220, 154, and 75 bp).

Immunohistochemistry. For immunohistochemical analysis, the mouse monoclonal antibodies (MAbs) anti-IE antigen (Chemicon) and anti-L antigen (Novacastra) were used. The sections were incubated overnight at 4°C with a 1:200 dilution of MAbs anti-IE and a 1:50 dilution of MAbs anti-L. Then, the biotinylated affinity purified horse anti-mouse IgG (Santa Cruz) and biotin-strepta-

Table 1. Primers used in PCR and HIS

Genes	Forward primer (5'-3')	Reverse primer (5'-3')
HCMV IE	GCGCCTTTAATA/TGATGGGA	TTCATCCTTTTTAGCACGGG
HCMV L	GTGATCCGACTGGGCGAAAA	GAGCGCGTCCACAAAGTCTA
β-actin	AGAGCTACGAGCTGCCTGAC	ACGTCTGCTGGAAGGTGGAC

vidin-HRP complex were added to develop the reaction (Sambiase *et al*., 2000). The positive cells were counted under 5 high power fields in 5 continuous sections.

As a positive control, human mucous membrane of intestine that contains the inclusion body of HCMV was used. In the negative controls, PBS was used instead the MAbs.

Hybridization in situ. The DNA probes of HCMV IE and L genes were obtained with the same primers used in PCR. These probes were directly labeled by adding DIG Labeling Mix (Roche) instead of the dNTP to the PCR reaction mixture. The predicted DNA probe sizes were 248 bp and 263 bp, respectively. The HIS protocol was described previously (Hendrix *et al*., 1989). Specifically hybridized DNA probe was visualized by diaminobenzidine-4HCl and counterstained with hematoxylin-eosin (HE). Five high power fields in 5 continuous sections were selected to count the number of positive cells. Human mucous membrane of intestine was used as a positive control. The hybridization solution lacking of HCMV DNA probe was used as a negative control.

Statistic analysis. Statistical software SPSS 10.0 was used for management and analysis of data. Chi-square test was used for non-parameter data and Fisher exact test was used, when Chi-square test was not suitable. Significant difference was taken into account, if significance level $p < 0.05$.

Results

Histology of arteries

The arteries of 35 patients from stroke group were stained by HE and the type of AS was determined. From 35 samples, 18 patients were of type I-III and 17 were type IV-VI. The arteries of control group were determined as type I-III.

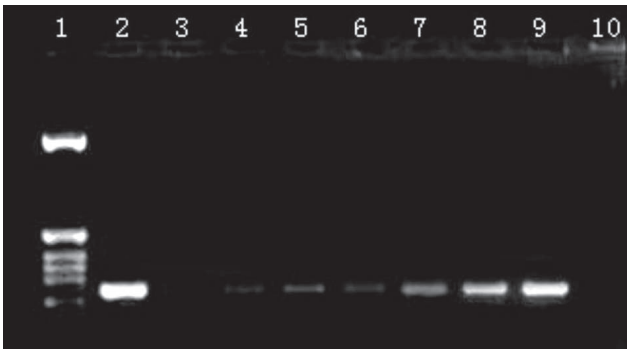

Fig. 1

PCR of amplified HCMV IE gene fragment of 248 bp detected by agarose gel electrophoresis

DNA marker pBR322/Hinf I (lane 1), positive control (lane 2), negative control (lane 3), AS patients (lanes 4–10).

Detection of HCMV genes in arteries by PCR

The specific band of HCMV IE gene was detected in 21 of 35 patients (60%) from stroke group (Fig. 1), what is significantly more than the number of positive patients (30%) from the control group ($p = 0.032$, Table 2). The specific band was positive in 7 patients of 18 (38.9%) with type I-III, what was significantly less than in type IV-VI (82.4%) ($p = 0.009$, Table 3).

For the L gene, there was no significant difference between the stroke and control groups of patients ($p = 1.000$, Table 2), as well as between type I-III and type IV-VI ($p = 0.228$, Table 3).

Table 2. Detection of HCMV genes/proteins in the cerebral arteries by PCR, IHC, and HIS

Method	HCMV IE genes/protein			HCMV L genes/protein		
	Stroke patients (%) of positive	Control patients (%) of positive	p*	Stroke patients (%) of positive	Control patients (%) of positive	p*
PCR	21 (60.0)	6 (30.0)	0.032	7 (20.0)	4 (20.0)	1.000
IHC	12 (34.3)	2 (10.0)	0.047	4 (11.4)	1 (5.0)	0.643
HIS	14 (40.0)	2 (10.0)	0.018	4 (11.4)	2 (10.0)	1.000

*Probability.

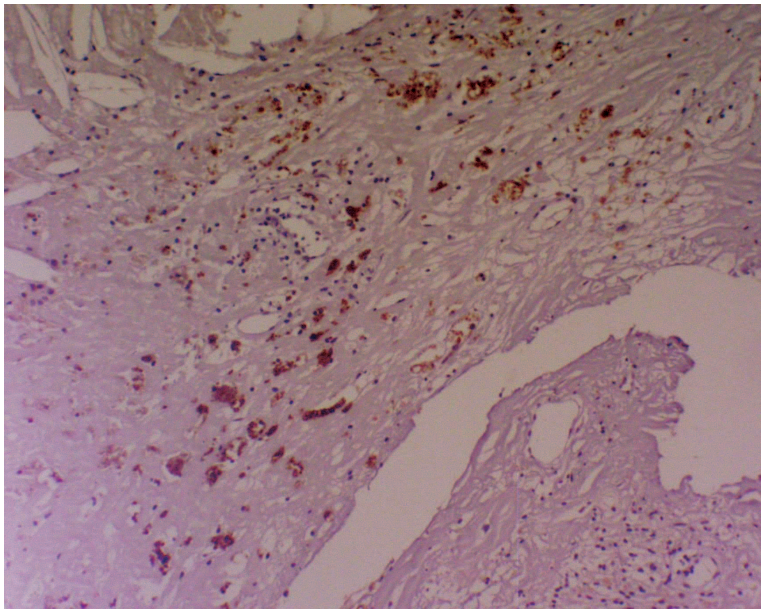

Fig. 2

IHC detection of HCMV IE antigen in the cerebral artery wall of AS patient

Magnification 200x.

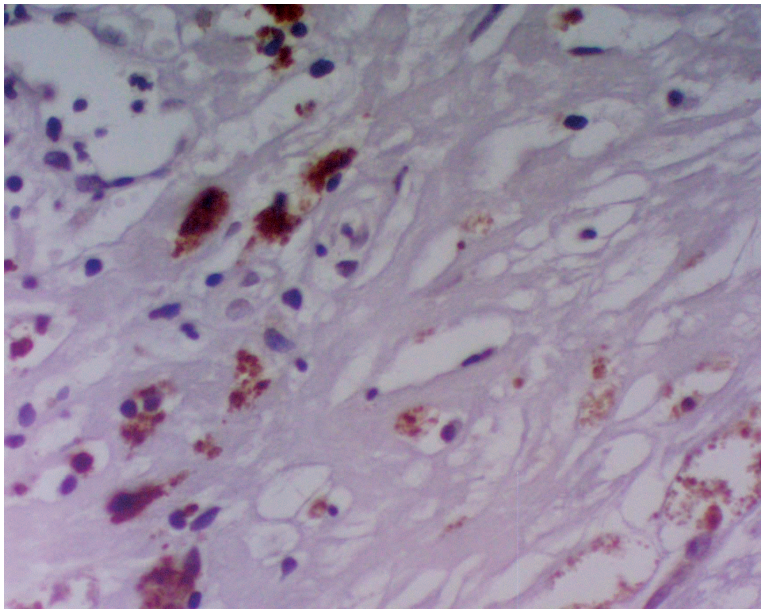

Fig. 3

HIS detection of HCMV IE gene in the cerebral artery wall of AS patient

Magnification 400x.

Table 3. Detection of HCMV genes/protein in the cerebral arteries of stroke group patients with different types of AS by PCR, IHC, and HIS

Method	HCMV IE genes/protein			HCMV L genes/protein		
	Type I-III No. of positive/total (%)	Type IV-VI No. of positive/total (%)	p*	Type I-III No. of positive/total (%)	Type IV-VI No. of positive/total (%)	p*
PCR	7/18 (38.9)	14/17 (82.4)	0.009	2/18 (11.1)	5/17 (29.4)	0.228
IHC	2/18 (11.1)	10/17 (58.8)	0.003	1/18 (5.56)	3/17 (17.6)	0.117
HIS	4/18 (22.2)	10/17 (58.8)	0.027	1/18 (5.56)	3/17 (17.6)	0.117

*Probability.

Detection of HCMV antigens in arteries by IHC

We detected significant more positive findings of HCMV IE antigen (12 patients) in the stroke (34.3%) than in control group (10.0%) (p = 0.047, Table 2). From the group of patients positive for HCMV IE antigen, 2 of them were AS type I-III (11.1%) and 10 patients were AS type IV-VI (58.8%), what was a significant difference (p = 0.003, Table 3). Cells positive for IE antigens were found in intimal and smooth muscle region (Fig. 2). The number of cells positive for IE antigen present in the AS type I–III was significantly higher than that in type IV-VI (p = 0.015, Table 4).

Four of 35 patients belonging to the stroke group were positive for HCMV L antigen (11.4%), what is significantly less than for IE antigen. Moreover, no significant difference in comparison with controls was found (5.0%) (p = 0.643, Table 2). From 4 positive stroke patients 3 of them were type IV-VI and 1 patient was type I-III, but no significant difference was found between them (p = 0.117, Table 3).

Detection of HCMV genes in arteries by HIS

The level of HCMV IE gene in the stroke group and control group was significantly different (Table 2). In addition, significantly more positive findings of HCMV IE gene in the patients of stroke group with atherosclerotic type IV-VI than with type I-III (p = 0.027) was found (Table 3).

Histopathologically, the HCMV DNA sequences detected with HIS were frequently located in the apparently normal part as well as in the AS plaque of the arteries in stroke group. In a few occasions the viral DNA was located in the thickened intimal layer. Sequential sections stained with HE showed that the DNA hybridization activity was located mostly in the smooth muscle cells of the arteries (Fig. 3).

For the HCMV L gene, we did not find any significant difference between the stroke and control groups of patients (p = 1.000, Table 2), and also between type I-III and type IV-VI of AS (p = 0.117, Table 3).

Table 4. Average of HCMV IE-positive cells/field in the cerebral arteries of stroke group patients with different types of AS determined by IHC and HIS

Method	Type I-III	Type IV-VI	p*
IHC	7.50 ± 0.61	4.58 ± 0.59	0.015
HIS	5.93 ± 0.78	5.28 ± 1.04	0.072

*Probability.

Discussion

Several epidemiological studies have demonstrated an association between prior HCMV infection and the incidence of AS in humans. In the forth decade of life when AS becomes frequent, 50–80% of the general population in the industrialized world has already encountered the HCMV, as shown by the high percentage of seropositive cases (Krech, 1973). After the initial infection, most likely at young age, HCMV switches to a state of latency with intermittent periods of reactivation, when small amounts of infectious virus are shed (Roizman *et al.*, 1987). The periods of reactivation are common and may be induced by stress or other stimuli (Sarid *et al.*, 2002).

In the present study, we found that the proportion of cases that tested positive for HCMV IE antigen, but not L antigen and HCMV DNA were statistically greater in stroke patients compared to the control population. In addition, the positive findings detecting the presence of HCMV IE gene and antigen paralleled the severity of AS. This implied that IE antigen was not only involved in the AS formation, but also in its development. Among the three methods PCR, IHC, and HIS used to detect the IE gene, the PCR was the most sensitive. Although IHC and HIS were not as sensitive as PCR, they could localize the virus and count the number of positive cells. They were helpful to demonstrate a location and pathological features of virus infection. The HIS with its specific combination of probes to detect the virus gene was more sensitive and specific than IHC.

HCMV IE protein was found in cells with lipid deposit, while IE gene was found not only in AS plaques, but also in normal cells. It demonstrated wider distribution of IE gene than IE protein in cells. This might be the reason why HIS gave the higher positive detection of HCMV DNA (22.2%) in type I-III than IHC (11.1%), while the same percentage of patients was detected in type IV-VI (58.6%) by both methods. This outcome demonstrated that the HIS was more sensitive method than IHC in the early stage of AS, because it was able to detect the cells with no apparent HCMV infection.

HCMV DNA was located in the normal cells, but as the AS developed the cellular construction was destroyed and replaced by the deposits of calcium and lipids. This might be the main reason that we found fewer HCMV DNA positive cells in type IV-VI than in type I-III (Hansson *et al*., 1986).

AS is a chronic inflammatory disorder of the large and medium size arteries (Ross, 1999). The primary event in the atherosclerotic process is the injury or dysfunction of endothelial cells. Subsequently, the permeability of the vascular wall of affected endothelium increases leading to the adhesion of platelets and monocytes/macrophages to the endothelium. Concomitantly, smooth muscle cells migrate, proliferate, and secrete extracellular matrix proteins causing intimal thickening ultimately contributing to the aggravation of the AS process.

In the development of AS, the key step is the recruitment and accumulation of leukocytes, specifically monocytes, and T lymphocytes to the intimal space. The migration of these leukocytes is regulated by chemoattractant cytokines or chemokines, what is leading to an increase in the monocytes and T lymphocytes present in the vessel walls (Burnett *et al*., 2004). When epithelial cells are infected, the increase of cytokines will enhance the adherence of white cells to epithelium and the monocytes would transfer latent HCMV to the arterial wall. The immunological and inflammation reactions activated by the virus worsen the injury of the intima and formation of neointima even when a tiny amount of virus starts this process (Kloppenburg *et al*., 2005; Rott *et al*., 2003; Altannavch *et al*., 2002).

HCMV was reported to induce smooth muscle cells migration and proliferation, increases a modified low-density lipoprotein uptake in vascular smooth muscle cells (Zhou *et al*., 1996), a promoter of leukocyte influx, and inductor of an increased expression of adhesion molecules (e.g. ICAM-1), inflammatory cytokines (e.g. IL-6), and chemokines (e.g. MCP-1), (Reinhardt *et al*., 2005; Burnett *et al*., 2004; Rott *et al*., 2003; Zhou *et al*., 1999). Additionally, one of the IE gene products of HCMV, IE2-84, binds to and inhibits p53 transcriptional activity and protects smooth muscle cells from p53-mediated apoptosis. The inhibitory effect of IE2-84 on p53 could increase the smooth muscle cells. HCMV infection predisposes to the smooth muscle cells accumulation, thereby contributing to the stenosis and AS (Tanaka *et al*., 1999).

In the present study, both IHC and HIS detected the virus IE gene and IE protein in intima and media layer of the arteries, not only in cells with lipid deposit but also in plaques and normal cells. The presence of HCMV IE gene positive cells in normal cells raised a possibility that infection of HCMV occurred before formation of AS lesions began to appear. Several reports suggested that the arterial wall might be a site of latency for HCMV and a frequent reactivation of virus from the latent state might significantly contribute to the pathogenesis of AS (Melnick *et al*., 1990). The endothelial and smooth muscle cells are supposed to be latently infected with HCMV (Shin *et al*., 2002).

In the control group, all three methods were used to detect the presence of IE and L genes and their proteins. PCR demonstrated 30% of positive findings of IE gene. Worldwide, the prevalence of HCMV infection in population is high. In United States, the HCMV infection rate is 10–15% among young people rising to 40–50% in population older than 35 years and to 60–70% in population older than 65 years. This increasing tendency of incidence of anti-HCMV antibodies parallels the incidence of AS in mentioned age groups (Melnick *et al*., 1990). Therefore, we cannot exclude the possibility of infection of HCMV before the AS formation starts.

We did not find any significant difference in the detection of L gene between the stroke and control group by the methods used. Late HCMV genome expression is strictly regulated by the IE and E gene products. L gene expression occurs after the initiation of viral DNA synthesis. L antigen is an indicator of complete viral replication (Cruz *et al*., 2002). In immunocompetent host, infection with HCMV leads to latent infection that persists lifelong without formation and release of infectious virus particles. The absence of L gene and L antigen in the vascular walls raised the question, whether the plaque formation was triggered by the presence of HCMV. According *in vitro* studies, several experiments implied that HCMV infection activated a systemic inflammatory response, thereby exacerbating AS plaque formation in the long run (Vliegen *et al*., 2002). HCMV might also have an indirect effect on the AS process. A local effect of the virus on AS process seemed to be less important, but the indirect effect might be the dominant factor in aggravating the AS (Vliegen *et al*., 2005).

Our results showed that the HCMV IE gene/antigen but not L gene/antigen was present in the internal carotid arteries of Chinese stroke patients. The artery wall might be a dormant infection site and HCMV IE gene might be associated with the pathological process of AS formation and development.

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