Original Article

Detection of *Chlamydia pneumoniae* in blood of stroke patients

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It has been increasingly recognized that *Chlamydia pneumoniae* may be involved in the pathogenesis of atherosclerosis; accordingly, detection of this bacterium in blood from atherosclerotic patients may play an important role in diagnosis of possible complications of atherosclerosis including stroke. However, there is little understanding about the detection incidence of *C. pneumoniae* in the blood of stroke patients. In the present study, we performed the PCR specific for *C. pneumoniae* 16S rRNA to determine the *C. pneumoniae* DNA in peripheral blood mononuclear cells (PBMCs) obtained from patients with atherothrombotic infarction. *C. pneumoniae* DNA was detected in 5 out of 13 blood samples from the patients with atherothrombotic infarction. These results indicate a possible correlation between *C. pneumoniae* infection and atherothrombotic infarction attributable to atherosclerosis.

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Introduction

Recent studies have indicated possible involvement of microbes in the pathogenesis of atherosclerosis, e.g., *C. pneumoniae*¹⁻⁵⁾, Herpes simplex virus^{1,5)}, *Helicobacter pylori*^{7,5)}, Cytomegalovirus^{1,5)} and *Mycoplasma pneumoniae*⁶⁾. Among these microbes, more attention has been focused currently on *Chlamydia* (*Chlamydophila*) *pneumoniae*, an obligate intracellular bacterium, because of the recent corroboration that *C. pneumoniae* is associated with not only respiratory diseases⁷⁾ but also chronic inflammatory diseases, such as atherosclerosis, endocarditis, asthma, and arthritis⁸⁻¹¹⁾.

Seroepidemiological findings revealed that 50 to 80 percent of the adult population had previous exposure to this pathogen¹²⁾. The results of these studies obviously bear out the findings that the carriers of *C. pneumoniae* DNA in their peripheral blood occupy significantly higher percentages among the patients with cardiovascular diseases as well as healthy subjects¹³⁾.

It is well accepted that atheromatous inflammation is an important factor leading to plaque vulnerability¹⁵⁾. The actual role of *C. pneumoniae* in the development of atherosclerosis and plaque rupture is a controversial issue¹⁶⁾. The diversity of

microorganisms found in atherosclerotic lesions precluded such an assessment. In our previous study, however, we conducted quantitative determination of *C. pneumoniae* in different kinds of atherosclerotic coronary lesions, thereby successfully demonstrating presence of larger numbers of this microorganism in vulnerable atheromatous plaques¹⁷⁾. In this connection, presence of *C. pneumoniae* in peripheral blood mononuclear cells (PBMCs) from patients with coronary atherosclerosis was confirmed by staining and detection of DNA; however, we have not yet performed demonstration of *C. pneumoniae* in PBMCs of stroke patients with atherosclerosis. In the present study, we investigated whether *C. pneumoniae* might exist in PBMCs obtained from patients with atherothrombotic infarction (advanced atherosclerotic lesion) as well as from stroke patients with lacunar, using a highly sensitive Polymerase Chain Reaction (PCR).

Materials and Methods

Blood specimens:

Twenty blood samples from 20 patients with stroke were collected at the University Hospital of St. Marianna University School of Medicine, Kawasaki, Japan. All stroke patients were diagnosed clinically, with 13 patients having atherothrombotic infarction (atherosclerosis) with/without ischemic coronary disease (ICD) history and 7 patients having lacunar with/without ICD history. Table summarizes the data on the patients' background characteristics. The 5.0-mL aliquot of the whole blood specimens, which had been collected in tubes containing EDTA, was subjected to density gradient centrifugation with Histopaque (SIGMA). The resulting PBMCs were then washed twice with Hanks' balanced salt solution and transferred to a new tube for DNA extraction. The study protocol was approved by the University Ethics Committee.

Bacterial DNA:

C. pneumoniae (TWAR strain) whole antigen was obtained from the Washington Research Foundation, Seattle, Wash. The chlamydia antigen was spiked into phosphate-buffered saline (PBS) with 2% fetal calf serum (FCS) at concentrations from 10^4 to 1 bacterial particles/mL.

DNA extraction:

Bacterial DNA was extracted from 200 μ L of PBS spiked with *C. pneumoniae* or PBMCs from stroke patients using a QIAmp DNA Mini Kit with a bacterial DNA extraction protocol (QIAGEN Inc.). Briefly, 200 μ L of sample was centrifuged for 30 min at 20,000 xg. The pellet was resuspended in 180 μ L

of buffer ATL with 20 μ L of proteinase K and incubated at 56 with occasional vortexing until the pellet was completely lysed, which usually took 30 min. After lysis of the sample, 200 μ L of buffer AL was added to the sample, and the mixture was incubated for 10 min at 70 . The mixture was then combined with 200 μ L of absolute ethanol and mixed by pulse-vortexing for 15 seconds. The mixture was applied to a spin column, which holds a silica-gel membrane, and spun for 1 min at 6,000 xg. The spin column was washed with 500 μ L of buffer AW1 by centrifugation at 10,000 xg for 1 min. The spin column was washed with 500 μ L of buffer AW2 by centrifugation at 20,000 xg for 3 min. The DNA bound on the membrane was eluted by centrifugation with 50 μ L of buffer AE after 5-min incubation at room temperature. The resulting DNA extracts were stored at -20 until PCR assessment. All reagents and spin columns were included in the kit (QIAGEN).

PCR:

The touchdown PCR (TETR) targeting *C. pneumoniae*-specific 16S rRNA gene was performed according to the published protocol¹⁸⁾ with minor modification. TETR with primer set were CPN 90 (5'-GGT CTC AAC CCC ATC CGT GTC GG-3') and CPN 91 (5'-TGC GGA AAG CTG TAT TTC TAC AGT T-3'). In brief, 0.2 μ g of extracted DNA was added to 25 μ L reaction volume containing PCR buffer (10 mM Tris [pH 9.0], 50 mM KCl, 0.01% gelatin), 200 μ M dNTP, 2.5 mM MgCl₂, 0.25 μ M of each primer and 0.5 unit of *Taq* polymerase (AmpliTaqGold, Perkin Elmer). The PCR cycle consisted of an initial denaturation for 5 minutes at 95 , which was followed by 60 cycles of denaturation at 94 for 45 seconds, annealing beginning at 62 and ending at 52 for 45 seconds, and extension at 72 for 1 minute. The annealing temperature was reduced by 1 every four cycles until it reached 52 ; this annealing temperature was kept until the end of the cycling process. The PCR products were visualized in 2% agarose gels containing 0.5 μ g/mL ethidium bromide. To prevent carryover contamination, an aerosol resistant tip was used in all steps. Preparation of the PCR mixture and running of the PCR were performed in separate rooms.

Results

Clinical profiles of stroke patients:

The data are shown in Table. Twenty stroke patients were recruited in total. Thirteen patients with atherothrombotic infarction were enrolled (6 men, 7 women), with 7 patients with lacunar (5 men, 2 women) being also recruited. Significant stroke

Table Clinical Profiles

	Total	Gender	Age	Category	ICD	Chlamydia DNA *
Stroke		M/F		Acute/Subacute	Yes/No	Positive/Negative
Atherothrombotic infarction	13	6/7	63.5 ± 7.4	9/4	1/12	5/8
Lacunar Infarction	7	5/2	62.7 ± 6.9	6/1	4/3	0/7

* *C. pneumoniae* DNA in PBMCs was detected by PCR that was specific for *C. pneumoniae* 16S rRNA gene.

Fig. Detection of *C. pneumoniae* DNA by PCR in PBMCs obtained from patients with stroke and detection of human β -actine gene by PCR. Five mL of whole blood obtained from stroke patients was processed for extraction of DNA utilizing the QIAmp DNA Mini Kit, and 2 μ L of the resulting 50 μ L DNA solution was subjected to PCR with primers either for 16S rRNA gene. The data are representative of three PCR experiments. Each number shows the sample number. M; molecular marker, P; positive control for PCR, N; negative control for PCR, IC; negative control for DNA extraction. Sample number 1, 4, 5, 6, 9, 10 and 11 were obtained from patient of atherothrombotic infarction, sample numbers 2, 3, 7, 8 were obtained from patient of lacunar. Sample numbers 1, 4, 5, 6 and 11 were positive for *C. pneumoniae* 16S rRNA gene.

was diagnosed based on clinical findings while significant ICD was diagnosed according to the medical history of the patients. Twenty percent (20%) of the patients had stroke in combination with ICD.

Detection of *C. pneumoniae* DNA in whole blood specimens:

The detection sensitivity of the touchdown PCR targeting the *C. pneumoniae* 16SrRNA gene employed was examined by using the DNAs isolated from the PBMCs spiked with defined numbers of *C. pneumoniae* Elementary bodys(EBs). The results showed that the lower detection limit of the PCR was 10 organisms (IFUs) of *C. pneumoniae* DNA per PCR assay (data not shown). With this PCR protocol, assessment was made to detect presence of *C. pneumoniae* DNA in the 20 clinical samples obtained from stroke patients. Five (39 %) of the patients with atherothrombotic infarction showed detectable *C. pneumoniae* DNA in repeated and separate PCR experiments (representative PCR results are illustrated in Fig.); in sharp contrast, however, *C. pneumoniae* DNA was not detected in the PBMCs obtained from the patients presenting with lacunar. One patient of stroke in combination with ICD was positive *C. pneumoniae* DNA.

Discussion

Atherosclerosis and related diseases (stroke and coronary diseases, etc) are one of the major causes of morbidity and mortality throughout the world. Variable onsets of these diseases cannot be fully explained by known risk factors such as smoking, hypertension, and dyslipidemia. In recent years, of interest to note is the inference drawn from clinical experiences that chronic infection might be implicated as one of the putative causal factors in the occurrence of atherosclerosis; however, most of the relevant studies have evaluated the association between current or previous infections with *C. pneumoniae* and onset of atherosclerosis³⁾. For example, Wong *et al.* investigated the presence of *C. pneumoniae* DNA by PCR in PBMCs obtained from 1,205 patients who were subjected to evaluation by interventional coronary arteriography for diagnostic purposes. They found that 8.5 % of patients with coronary artery diseases (CAD) were positive for *C. pneumoniae* DNA¹⁹⁾. In contrast, however, the positive rate of the bacterial DNA in their PBMCs accounted for only 2.9% in the patients without CAD. Several reports have been documented following studies to pursue the potential mechanisms of this fortuitous correlation, thereby

reaching the assumption that *C. pneumoniae* could be involved in development of atherosclerosis development. Kalaglu and Byrne¹⁾ showed that human macrophages in cell culture with added lipid (HDL) in the media would ingest lipid to some extent; however, with the cells being infected with *C. pneumoniae*, greater amounts of cholesterol esters could be produced in these cells. Furthermore, it was evidenced that these infected cells augmented appearance of foam cells conducive to atheroma²⁰⁾.

Both the atherothrombotic infarction and lacunar are pathomorphologically classified as thrombosis from their clinical characteristics, besides representing a temporal course leading to ischemic stroke (thrombosis, vasoconstriction and embolism). Nevertheless, atherothrombotic infarction is different from lacunar in terms of respective target vessels. The former is associated with the subsequent infarction formation, resulting in complicating atherosclerosis with large plaque in major artery, while the latter shows a characteristic vascular pathology in the serial sections of the penetrating arteries (branches of the circle of willis, middle cerebral artery stem, or vertebral and basilar arteries) nourishing the territory of lacunar infarcts. These tiny vessels in lacunar are often accompanied with focal enlargements and small hemorrhagic extravasations through the walls of the arteries.

In our study, the positive rate of *C. pneumonia* DNA in PBMCs samples from stroke patients with atherothrombotic infarction reached 39% (5 out of 13 samples); in sharp contrast, however, the counterpart in PBMCs samples obtained from stroke patients with lacunar accounted for 0% (0 out of 7 samples), indicating that *C. pneumoniae* DNA was detected in only stroke patients with atherothrombotic infarction. *C. pneumoniae* is harbored in human monocytes, and also, harbored in human monocytes in atheromatous plaque. Blasi *et al.* reported, the rate of *C. pneumoniae* DNA detection in artery specimens and PBMCs was similar. Thus, PCR performed on peripheral blood is good marker for the identification of subjects carrying *C. pneumoniae* in the vascular wall¹⁴⁾. In atherothrombotic infarction, atherosclerosis lesion is widely enlarged, while the term lacunar is characteristic of its focal features. It is postulated that such morphological properties might permit easier detection of *C. pneumoniae* in patients with atherothrombotic infarction relative to those in patients with lacunar. These results may indicate a possible correlation between existence of *C. pneumoniae* and onset of stroke with atherothrombotic infarction.

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