Chlamydia pneumoniae in Atherosclerotic Middle Cerebral Artery

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Background and Purpose—Atherosclerotic middle cerebral arteries are frequent sites of thrombosis, leading to stroke. Previous studies have suggested a role for Chlamydia pneumoniae in the pathogenesis of atherosclerosis. However, the presence of this pathogen in atherosclerotic middle cerebral arteries has heretofore not been documented. In the present study, we analyzed atheromatous plaques from middle cerebral arteries for the presence of C pneumoniae.

Methods—Atherosclerotic middle cerebral arteries from 15 cadavers who died of natural causes and corresponding nonatherosclerotic arteries from 4 otherwise healthy trauma victims were examined. Assays for C pneumoniae DNA were carried out by nested polymerase chain reaction (nPCR) specific for the C pneumoniae ompA gene. The presence of the bacterium was assessed by transmission electron microscopy.

Results—Five of the 15 atherosclerotic arterial samples and none of the control tissues were positive for C pneumoniae by nPCR. Particles similar in morphology and size to C pneumoniae elementary bodies were detected by transmission electron microscopy in 4 of the 5 nPCR-positive atherosclerotic samples.

Conclusions—The demonstration of C pneumoniae in atherosclerotic middle cerebral arteries is consistent with the hypothesis that this bacterium is involved in acute and chronic cerebrovascular diseases. (Stroke. 2001;32:1973-1978.)

Key Words: atherosclerosis ■ C pneumoniae ■ cerebrovascular disorders

Atherosclerosis is a multifactorial disease. The various explanations of the pathogenic process include chronic infection with certain pathogens. The microorganism most strongly implicated in the initiation/progression of atherosclerosis is the obligate intracellular bacterium Chlamydia pneumoniae, which commonly causes respiratory infections. Evidence for a possible link between C pneumoniae infection and atherosclerosis at different vascular sites has come from seroepidemiology, analysis by polymerase chain reaction (PCR), electron microscopy, in situ hybridization, immunohistochemistry, culturing, and animal models.1–4 However, the association of chronic C pneumoniae infection and cerebrovascular diseases has not been well investigated. Case-control studies revealed that specific anti–C pneumoniae antibody levels were significantly higher in patients with cerebrovascular disease than in control patients,5–7 and a follow-up study indicated that high antibody titers to C pneumoniae were associated with an increased risk of future stroke.8 Immunoreactivity to C pneumoniae–specific antigen was recently demonstrated in a low percentage of anterior and posterior cerebral arteries but not in middle cerebral arteries.9 The middle cerebral artery and internal carotid artery are frequent sites of thrombosis leading to stroke. One of the most important factors in the development of local thrombosis is the underlying atherosclerosis of the vessel wall. In the present study, we used nested PCR (nPCR) and transmission electron microscopy (TEM) to examine samples of middle cerebral arteries with atheromatous plaques and also samples of nondiseased vessels for the presence of C pneumoniae.

Subjects and Methods

Atherosclerotic samples of middle cerebral arteries were obtained from 15 consecutively autopsied subjects. Samples were collected within 24 hours after death with the use of sterile instruments. Half of each sample was frozen at −70°C for nPCR analysis; the other half was fixed in 3% glutaraldehyde for TEM and histology. For control tissues, samples of 4 nonatherosclerotic middle cerebral arteries were collected from trauma victims who died (at ages 31 to 40) during the study period. Histological assessment of the vessel samples from the 15 patients indicated moderate or severe atherosclerotic stenosis. The control samples from the trauma victims were assessed as histologically normal. The study was approved by an institutional review committee.

DNA was extracted from frozen samples with the High Pure PCR Template Preparation Kit (Boehringer-Roche) according to the manufacturer’s instructions. Samples from cases or controls were tested in a blinded fashion for C pneumoniae DNA with a GeneAmp 2400 PCR system (Perkin-Elmer) with the use of nPCR primer pairs.
**Results**

The patient characteristics reviewed from the autopsy records and the nPCR results are listed in the Table. *C pneumoniae* DNA was amplified in 5 of the 15 atherosclerotic samples, as demonstrated by a 206-bp DNA fragment visualized by agarose gel electrophoresis, whereas none of the 4 arterial samples from healthy trauma victims were nPCR positive (Figure 1). Sequencing of nPCR fragments from 2 of the 5 atherosclerotic samples revealed identity to the ompA sequences obtained from the National Center for Biotechnology Information (NCBI) database (which can be accessed online at http://www.ncbi.nlm.nih.gov). Three of the 5 nPCR-positive cases had symptomatic cerebrovascular disease (cerebral infarct), whereas only 2 of the 10 nPCR-negative cases had symptomatic cerebrovascular disease. The direct cause of death was related to infectious respiratory diseases in 9 of the 15 cases; there was uniform distribution among nPCR-positive and -negative individuals (Table).

TEM of intimal plaques showed structures resembling *C pneumoniae* elementary bodies in 4 of the 5 nPCR-positive atherosclerotic arterial samples. These structures had a pear-shaped appearance with a dense core (Figures 2A and 2B) and were ~0.3 μm in diameter, ie, similar to the size of the elementary bodies detected in control infected tissue culture cells (Figure 2C). None of the 5 nPCR-negative atherosclerotic samples examined by TEM exhibited *C pneumoniae*-like structures.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age, y</th>
<th>C pneumoniae PCR</th>
<th>Organ Manifestations of AT</th>
<th>Diseases Other Than AT</th>
<th>Cause of Death</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>95</td>
<td>+</td>
<td>CI, IHD, severe in aorta</td>
<td>Bilateral chronic pyelonephritis</td>
<td>Pneumonia</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>85</td>
<td>+</td>
<td>CI, severe in aorta, renovascular hypertension</td>
<td>Duodenal ulcer</td>
<td>Pneumonia</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>83</td>
<td>+</td>
<td>CI, IHD, severe in aorta</td>
<td>Cirrhosis of liver</td>
<td>Cardiac failure</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>76</td>
<td>+</td>
<td>IHD, severe in aorta and cerebral arteries</td>
<td>Essential hypertension</td>
<td>Sepsis</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>78</td>
<td>+</td>
<td>Mild in aorta and cerebral vessels</td>
<td>Chronic lymphoid leukemia</td>
<td>Purulent bronchiolitis</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>73</td>
<td>-</td>
<td>IHD, severe in aorta, gangrene of legs</td>
<td>Bilateral chronic pyelonephritis</td>
<td>Pneumonia</td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>84</td>
<td>-</td>
<td>IHD, mild in aorta and cerebral arteries</td>
<td>Essential hypertension</td>
<td>Pneumonia</td>
</tr>
<tr>
<td>8</td>
<td>M</td>
<td>73</td>
<td>-</td>
<td>CI, IHD, moderate in aorta</td>
<td>Essential hypertension</td>
<td>Pneumonia</td>
</tr>
<tr>
<td>9</td>
<td>F</td>
<td>92</td>
<td>-</td>
<td>CI, IHD, atherosclerotic aneurysm in aorta</td>
<td>Bilateral chronic pyelonephritis</td>
<td>AMI</td>
</tr>
<tr>
<td>10</td>
<td>F</td>
<td>85</td>
<td>-</td>
<td>IHD, severe in aorta and moderate in cerebral vessels, mesenteric superior artery</td>
<td>Essential hypertension</td>
<td>Small bowel infarction</td>
</tr>
</tbody>
</table>

11 F 90 – IHD, severe in aorta and cerebral vessels Perforated gastric ulcer Peritonitis
12 M 70 – IHD, severe in aorta and cerebral vessels Diabetes, essential hypertension Pneumonia
13 M 66 – Moderate in aorta and cerebral vessels Diabetes Bilateral acute pyelonephritis
14 M 71 – IHD, severe in aorta and cerebral vessels Disseminated lung cancer Pneumonia
15 M 59 – IHD, severe in aorta and cerebral vessels, gangrene of leg Hypopharynx cancer Pneumonia

AT indicates atherosclerosis; M, male; F, female; CI, cerebral infarct; IHD, ischemic heart disease (critical stenoses in coronaries with or without microscopic foci of myocardial fibrosis and/or with or without myocardial scar); and AMI, acute myocardial infarction.

**Figure 1.** nPCR amplification of *C pneumoniae* ompA gene. Lanes are as follows: 1 to 5, nPCR-positive atherosclerotic samples (206-bp fragments); 6, nPCR-negative sample; 7, negative control; and 8, positive control. M indicates molecular size marker (100-bp DNA ladder, Sigma).
Discussion

*C. pneumoniae* has several features that may lead to the chronic infection of vessel walls and ultimately to atherosclerosis with local thrombosis: the bacterium replicates in vitro in endothelial and smooth muscle cells and macrophages, induces the expression of adhesion molecules, elevates the levels of platelet adhesion and procoagulant activity in endothelial cells, and induces the production of cytokines such as tumor necrosis factor-α, interleukin-1β, and interleukin-6 in monocytes.13–16

*C. pneumoniae* has been detected in atherosclerotic tissues at different sites of the vascular system, including the carotid and coronary arteries, the aortic valves, the aorta, and arteries in the lower extremities. A summary of the results from 23 studies indicated that *C. pneumoniae* was detected in 52% (257 of 497) of diseased arteries and in 5% of control vessels by immunohistochemistry or PCR.3 A recent study on the prevalence of *C. pneumoniae* antigens at multiple locations in the arterial system within the same individual demonstrated a high prevalence of immunoreactivity in the abdominal aorta, iliac arteries, and coronary arteries and a low prevalence in cerebral anterior and posterior arteries but no immunoreactivity in samples of middle cerebral arteries.9 We detected the presence of *C. pneumoniae* DNA in 33% of the diseased middle cerebral arterial samples by nPCR. A review of the autopsy records revealed cerebral infarct in 3 of the 5 nPCR-positive cases, in contrast with only 2 of the 10 nPCR-negative cases. However, the small number of cases did not permit a statistical comparison. A further limitation of the present study is that the detectability of *C. pneumoniae* DNA in the cerebrovascular vessels might be related to age. Because of the younger age of the control individuals and the small numbers of nPCR-positive and -negative cases, statistical comparison is not helpful, and this possibility cannot be excluded. The present study has demonstrated the presence of *C. pneumoniae* DNA in some atherosclerotic samples from middle cerebral arteries, but further studies appear desirable to test for the presence of this organism in these vessels in relation to age and the occurrence of symptomatic cerebrovascular diseases. Because there was no difference in the incidence of respiratory diseases as the direct cause of death among the nPCR-positive and -negative cases, it is improbable that *C. pneumoniae* nPCR positivity was secondary to the infectious diseases, which are rather considered to be terminal-stage diseases that developed a few days before death.

A commercial kit for the molecular detection of *C. pneumoniae* is not available, but the considerations applied in our work suggest that our PCR assay is appropriate.10–12

The detection of pear-shaped structures in *C. pneumoniae* nPCR-positive atherosclerotic samples by TEM suggests that not only was bacterial DNA present but that the complete pathogen was also present in the intimal plaque. A striking aggregation of dense particles indicates an intracellular accumulation of these structures. The additional presence of this microorganism extracellularly suggests either spontaneous autolysis of the cells followed by bacterial flow into the extracellular matrix or the accumulation of these pathogens outside the cell during a certain stage of their life cycle. *C. pneumoniae* organisms were earlier found by electron microscopy in cells and interstitially in atherosclerotic lesions of the aorta or carotid or coronary arteries but not in the adjacent nonatherosclerotic tissue.17

The middle cerebral arteries are important sites of cerebral thrombosis. The presence of *C. pneumoniae* in the atheromatous plaques of the middle cerebral artery does not necessarily mean that the organism is a causative agent of the disease; it rather raises the possibility of a role for this bacterium in the pathogenic process. Such a role would point to the value of antibiotic treatment as a means of attenuating cerebrovascular diseases relating to *C. pneumoniae*. Tests on a larger number of cases and age- and sex-matched controls appear warranted, extending to the *C. pneumoniae* serostatus and the presence of *C. pneumoniae* DNA and antigens in vessels other than middle cerebral arteries in the same subjects.

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4. Burian K, Kis Z, Virok D, Endresz V, Prohaszka Z, Duba J, Berencsi K, Horvath L, Romics L, Fust G, et al. Independent and joint effects of antibodies to human heat-shock protein 60 and *Chlamydia pneumoniae* DNA in the cerebrovascular vessels might be related to age. Because of the younger age of the control individuals and the small numbers of nPCR-positive and -negative cases, statistical comparison is not helpful, and this possibility cannot be excluded. The present study has demonstrated the presence of *C. pneumoniae* DNA in some atherosclerotic samples from middle cerebral arteries, but further studies appear desirable to test for the presence of this organism in these vessels in relation to age and the occurrence of symptomatic cerebrovascular diseases. Because there was no difference in the incidence of respiratory diseases as the direct cause of death among the nPCR-positive and -negative cases, it is improbable that *C. pneumoniae* nPCR positivity was secondary to the infectious diseases, which are rather considered to be terminal-stage diseases that developed a few days before death.

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References

14. Kaukoranta-Tolvanen SS, Ronni T, Leinonen M, Saikku P, Laitinen K. Association of Chlamydia pneumoniae infection and coronary disease risk. A smaller roll of the PCR-positive specimens. Note also that the control samples demonstrated presence of Chlamydia pneumoniae DNA, whereas none of the control samples did. Moreover, 3 of the 5 polymerase chain reaction (PCR)-positive cases (60%) had symptomatic disease, whereas only 2 of the 10 PCR-negative cases (20%) were symptomatic. Electron microscopy, furthermore, demonstrated presence of Chlamydia pneumoniae in 4 of 5 of the PCR-positive specimens.
organism simply reflects age; it would have been ideal to have had age-matched controls without atherosclerosis. Still, the organism appears to have been found more often in the atherosclerotic cerebral vessels.

Discovery of Chlamydia pneumoniae DNA by PCR in tissue from the MCA is not surprising. Several studies have found evidence of Chlamydia pneumoniae in arterial tissue taken from sites throughout the body, including the coronary arteries, aorta and femoral arteries. Closer to the brain, Chlamydia pneumoniae has been identified using PCR and immunohistochemical techniques in carotid atherosclerotic tissue taken from endarterectomy specimens. The organism, notoriously difficult to culture, has also been cultured from the carotid artery. A 1997 review of studies of Chlamydia pneumoniae in atherosclerotic tissue found that 257 of 495 samples of atheromatous tissue (52%) were positive for Chlamydia pneumoniae, while only 6 of 118 nonatheromatous specimens (5%) were positive. A more recent autopsy study sampled 33 arterial sites throughout the body of each of 24 elderly individuals and found Chlamydia pneumoniae immunostaining in at least one artery in all subjects. Almost all arteries were affected in at least some individuals, although the prevalence was greatest in the abdominal aorta, iliac arteries, and coronary arteries. Importantly, the prevalence was highest at sites of greatest luminal stenosis, and in sites typically affected clinically. The organism was found in only 2% of all large cerebral vessels in that study, however, compared with 33% of coronary vessels, and not in any of the MCA specimens. The present study is thus the first to find Chlamydia pneumoniae in MCA tissue. It is of interest that the organism was found so much more commonly in the MCA tissue in the study by Virok et al, but this may simply reflect the different patient population, which in their sample included 15 patients with moderate or severe MCA stenosis.

Of course, the presence of Chlamydia pneumoniae in arterial tissue cannot itself establish this, or any other organism, as a causative agent in atherosclerosis. Lipid-rich atherosclerotic tissue may simply be an attractive resting ground for the organisms, or they may be brought in by circulating macrophages entering the developing plaque. Chlamydia pneumoniae, according to this critique, is simply an “innocent bystander,” and not involved directly in the pathogenic process. A small but growing body of experimental and other animal evidence is available to support the pathogenic role of Chlamydia pneumoniae in atherosclerosis, however. The presence of chlamydial lipopolysaccharide, for example, facilitates conversion of macrophages to foam cells and increases oxidative metabolism of LDL, potentially damaging endothelium. Chlamydia pneumoniae has also been shown in vitro to induce human peripheral blood monocytes to secrete proinflammatory cytokines known to participate in the atherosclerotic process. In experimental models, rabbits inoculated with Chlamydia pneumoniae develop atherosclerotic lesions while controls do not, and azithromycin, a macrolide antibiotic active against chlamydiae, retards this process.

There is also evidence to suggest that some of the adverse effects of infections on atherosclerosis could be caused indirectly by immunological mechanisms, without the persistence of organism itself in the vessel wall—what has been called a “hit and run” effect. In rats undergoing arterial balloon injury and infected with rat cytomegalovirus, for example, arterial wall thickening progresses even after infection is completed and cytomegalovirus DNA is no longer detectable in the vessel.

Currently, several large-scale clinical trials are ongoing among coronary disease patients to assess whether antibiotic therapy can prevent recurrent events. Atherosclerosis, however, has several logically distinct, if continuous, phases, including atherogenesis, progression, and plaque rupture, the most common precipitant of an acute event. Different mechanisms may be operative in each of these phases. A trial directed at reducing clinical events, therefore, even if negative, cannot prove that infectious agents are not involved in the earlier processes of atherogenesis or progression.

No clinical trials in stroke have yet been initiated. It is important to remember, however, that while the term “brain attack” has heuristic value, a stroke is not simply a “heart attack” of the brain. Again, even if negative, trials in heart disease cannot definitively answer the question of the association of Chlamydia pneumoniae and stroke. The etiologies of stroke are more heterogeneous than those of coronary artery disease, and it is possible that there are differential effects on the two. Recent data from the National Health and Nutrition Examination Surveys, for instance, suggest an association of periodontal disease with stroke but not heart disease.

The study by Virok et al, then, is important in being the first to focus on the presence of this organism in the brain’s vessels and thus in its recognition that research in stroke should begin with an investigation of the brain’s arteries, and not simply those elsewhere in the body. Further studies of the prevalence of this organism in brain arteries and its clinical consequences are needed.

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