Distribution of *Chlamydia pneumoniae* Infection in the Atherosclerotic Carotid Artery

Katsuhiro Yamashita, MD; Kazunobu Ouchi, MD; Mutsunori Shirai, MD; Toshikazu Gondo, MD; Teruko Nakazawa, MD; Haruhide Ito, MD

**Background and Purpose**—*Chlamydia pneumoniae* infection has recently become noteworthy in relation to atherosclerosis. We investigated by immunohistochemistry the distribution of *C pneumoniae* infection in the atherosclerotic carotid artery.

**Methods**—Twenty carotid atherosclerotic lesions that were resected during carotid endarterectomy were investigated. Parallel sections were stained immunohistochemically with monoclonal antibodies for a *C pneumoniae*–specific antigen, macrophages, and smooth muscle cells.

**Results**—Immunoreactivity for the *C pneumoniae*–specific antigen was observed in 11 of 20 specimens (55%), and intense immunoreactivity was observed in 7 of 20 (35%). *C pneumoniae* infection was observed in endothelial cells, macrophages and in smooth muscle cells that had migrated into the atheromatous plaque, as well as in smooth muscle cells and small arteries in the media underlying the atheromatous plaques. *C pneumoniae* infection was most prominently observed in smooth muscle cells. The severity of the infection as demonstrated by immunohistochemistry was not significantly related to general risk factors for atherosclerosis.

**Conclusions**—*C pneumoniae* widely infects endothelial cells, macrophages, and smooth muscle cells in the atherosclerotic carotid artery. The results of the present study can help us to understand how *C pneumoniae* infection contributes to the progression of carotid atherosclerosis. (*Stroke. 1998;29:773-778.*)

**Key Words:** atherosclerosis ▪ carotid arteries ▪ *Chlamydia pneumoniae* ▪ infection

*Chlamydia pneumoniae*, a gram-negative bacteria, frequently causes community-acquired respiratory infections, such as pharyngitis and pneumonia. There are epidemics of *C pneumoniae* infection every 5 to 7 years, with 50% to 70% prevalence of seropositivity in adults, and most adults are infected 2 to 3 times in their lifetime.\(^1,2\)

Recently, *C pneumoniae* has been linked to an atherosclerotic disease. Since Saikku et al\(^3\) first reported that chronic *C pneumoniae* infection is a risk factor for coronary heart disease, a number of investigations have shown a positive relationship between *C pneumoniae* infection and atherosclerosis.\(^4-14\) Shor et al\(^9\) detected by electron microscopy bodies of *C pneumoniae* in lipid-rich areas of fibrous plaques and in intimal smooth muscle cells of the coronary arteries. The organism has also been detected in atherosclerotic lesions by immunohistochemistry, the polymerase chain reaction method, and cell culture.\(^10-15\) Moreover, the presence of *C pneumoniae* in carotid atherosclerotic lesions and its possible contribution to ischemic cerebrovascular diseases have been reported.\(^16-18\) However, the detailed distribution of *C pneumoniae* infection in the carotid arterial wall and atheromatous plaque remains to be elucidated. The distribution of *C pneumoniae* infection may indicate how it contributes to the progression of atherosclerotic lesions.

The aim of the present study was to clarify the distribution of *C pneumoniae* infection in carotid arterial walls and atheromatous plaques, which were resected during carotid endarterectomy (CEA) by immunohistochemistry, with use of monoclonal antibodies for a *C pneumoniae*–specific antigen, macrophages, and smooth muscle cells. In addition, we investigated the relationship between the severity of the infection, as demonstrated by immunohistochemistry, and general risk factors for atherosclerosis.

**Materials and Methods**

Twenty specimens of carotid atheromatous plaque were obtained during CEAs performed in 19 patients with symptomatic severe (>70%) carotid artery stenosis. The severity of carotid artery stenosis in these patients was verified by conventional carotid angiography. The symptoms of patients were transient ischemic attacks in 8 of 19 and minor stroke in 11 of 19 patients. The specimens were embedded in paraffin after fixation with 10% buffered formaldehyde and cut serially to expose coronal planes of the carotid artery and atheromatous plaque. Eight serial sections 6 μm thick were prepared from each specimen for the following: hematoxylin-eosin staining, elastica–van Gieson staining to visualize...
elastic fibers, immunohistochemical staining, and terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) staining to detect apoptotic cells.

**Immunohistochemical Staining for C. pneumoniae Infection and Identification of Cell Types With Infection**

Three parallel sections of each specimen were immunohistochemically stained with the following monoclonal antibodies: CF-2, a Chlamydia genus-specific monoclonal antibody that recognizes Chlamydia lipopolysaccharide (Washington Research Foundation) diluted 1:1000; AY-6, a C. pneumoniae-specific monoclonal antibody that recognizes a 53-kDa outer membrane protein (Hitachi Chemical), diluted 1:1000; and Virostat 1641, a C. trachomatis-specific monoclonal antibody that recognizes a major outer membrane protein (Virostat), diluted 1:100. Another two parallel sections from each specimen were also immunohistochemically stained to identify the cell types infected by C. pneumoniae, with two monoclonal antibodies: anti-human α-smooth muscle actin (Dako), diluted 1:50, and anti-human macrophage KP-1 (Dako), diluted 1:10. The immunoreactivity was detected by immunoperoxidase staining through use of the streptavidin-biotin-peroxidase method (LSAB kit; Dako) and was visualized with diaminobenzidine tetrahydrochloride as the chromogen. Control slides with HeP-2 cell monolayers infected with C. pneumoniae or C. trachomatis were processed in parallel with the tissue sections. Finally, sections were counterstained with hematoxylin. Immunoreactivity for C. pneumoniae infection was assessed in the whole area of each section according to the following four grades: 3+, intense immunoreactivity in numerous cells; 2+, positive immunoreactivity in a moderate number of cells (10 to 100 cells); 1+, weak immunoreactivity in a few cells; and 0, no immunoreactivity.

**In Situ End Labeling of Fragmented DNA (TUNEL Staining)**

TUNEL staining was performed with use of an in situ apoptosis detection kit (ApoTag Plus; Oncor Inc) to detect cells with DNA fragmentation. Briefly, deparaffinized sections were incubated with 20 μg/mL proteinase K for 15 minutes at room temperature to digest the proteins in the specimens, washed with water, and treated with 2% vol/vol hydrogen peroxide for 5 minutes to extinguish the endogenous peroxidase activity. Immediately afterward, the sections were washed with distilled water and immersed in equilibration buffer, then in terminal deoxynucleotidyltransferase enzyme solution for 1 hour at 37°C, followed by stop/wash buffer. Subsequently, 35 μL of digoxigenin-peroxidase solution was applied to each slide, and peroxidase was detected by staining with 0.025% wt/vol diaminobenzidine. Sections were finally counterstained with hematoxylin. Negative controls were treated with distilled water instead of the terminal deoxynucleotidyltransferase enzyme.

**Risk Factors**

The general risk factors for atherosclerosis, smoking, hypertension, hypercholesterolemia, diabetes mellitus, and complications of ischemic heart diseases were investigated for each patient. The severity of each risk factor was graded as follows: current smoker (>20 cigarettes/day), former smoker, and never smoked; with hypertension and without hypertension; severe hypercholesterolemia of >250 mg/dL, moderate hypercholesterolemia of 220 to 250 mg/dL, and no hypercholesterolemia; severe diabetes mellitus with systemic complications such as retinal and renal diseases, moderate diabetes mellitus without systemic complications, and no diabetes mellitus.

**Statistical Analysis**

The relationship between immunoreactivity for C. pneumoniae infection in the atherosclerotic carotid artery and general risk factors for atherosclerosis was investigated using multiple regression analysis, and a value of P<0.05 was considered to indicate statistical significance.

**Results**

The intensity of CF-2 (a Chlamydia genus-specific monoclonal antibody) immunoreactivity was the same as that of AY-6 (a C. pneumoniae-specific monoclonal antibody) in all specimens. Eleven of 20 specimens (55%) were positive for CF-2 and AY-6 immunoreactivity, and intense AY-6 immunoreactivity (grade 3) was observed in 7 of 20 specimens (35%) (Table 1).

Cells with AY-6 immunoreactivity were observed both in the intima near the atheromatous plaque and in the media, which was partially removed during the CEA procedure. The border between the intima and the media was clearly demonstrated by elastica–van Gieson staining. AY-6 immunoreactivity was observed in endothelial cells of the intima near the atheromatous plaque (Fig 1). Within the atheromatous plaque, macrophages and smooth muscle cells that had migrated from the media into the plaque showed AY-6 immunoreactivity (Figs 2 and 3); cells with AY-6 immunoreactivity were identified by immunohistochemical staining of adjacent sections for KP-1 and α-smooth muscle actin. Macrophages with C. pneumoniae infection were predominantly located in the subendothelial region. Numerous cells with AY-6 immunoreactivity observed in the media were smooth muscle cells underlying the atheromatous plaques as well as those of small arteries that supply the carotid arterial wall (Figs 4 and 5). These smooth muscle cells were also identified by immunohistochemistry for α-smooth muscle actin. The cells with C. pneumoniae infection formed clusters that were distributed in a patchy pattern in the atheromatous plaque and the media.

The immunoreactivity for Virostat 1641 (a C. trachomatis-specific monoclonal antibody) was not observed in the atheromatous plaque or in the media of any of the specimens. Only a few scattered TUNEL-positive cells were seen in the subendothelial region of the atheromatous plaques in two specimens with intense AY-6 immunoreactivity (data not shown).

The risk factors for atherosclerosis of each patient are shown in Table 1. The positivity of each risk factor for all 19 patients was 31.6% (6 of 19) for smoking, 73.7% (14 of 19) for hypertension, 31.6% (6 of 19) for diabetes, and 10.5% (2 of 19) for hypercholesterolemia. Ischemic heart disease was present in 3 of 19 patients (15.8%). None of these risk factors correlated significantly with AY-6 immunoreactivity, that is, with the severity of C. pneumoniae infection.

**Discussion**

In the present study, C. pneumoniae infection of the carotid arterial wall and the atheromatous plaque was observed in 55% of the patients. Although this frequency of infection is very similar to those reported in other studies,9-11,13,14 C. pneumoniae infection was not observed in all our patients. It is more likely that the results of the histopathologic examination underestimate rather than overestimate the frequency of C. pneumoniae infection, because only a very small region with a 6-μm thickness of each atherosclerotic lesion was examined.

We demonstrated that C. pneumoniae infected both the intima near an atheromatous plaque and the media of the
carotid artery. Infection was localized to endothelial cells in the intima and to macrophages and smooth muscle cells in the atheromatous plaque. This finding is similar to those reported for in vivo studies of an aorta and a coronary artery and in vitro studies, although this is the first report of the infection in endothelial cells in vivo. Macrophage seem to play an important role in the transportation of *C pneumoniae* bodies and progression of atherosclerosis. It is thought that *C pneumoniae* is taken up by macrophages in the pharynx or the lung after a chronic infection and transported via the blood to the subendothelial region through the injured endothelium of the artery. According to the hypothesis of Ross, macrophages in the intima produce some cytokines and growth factors (such as platelet-derived growth factor) and elicit migration of smooth muscle cells from the media to the intima, as well as an inflammatory response that subsequently leads to the progression of atherosclerosis. *Chlamydial* organisms survive and multiply in macrophages, and such a chronic infection of *C pneumoniae* in macrophages is believed to enhance the proliferative and inflammatory processes of atherosclerosis by inducing some cytokines and lipoproteins.

Infection by *C pneumoniae* was observed not only in smooth muscle cells that had migrated into the atheromatous plaque but also in smooth muscle cells in the media underlying the atheromatous plaques. The susceptibility of smooth muscle cells to *C pneumoniae* infection has been demonstrated in previous in vitro studies. The outcome of widespread infection of *C pneumoniae* in smooth muscle cells is unclear compared with that in macrophages. Growth factors such as platelet-derived growth factor in atherosclerotic lesions are also produced and released by smooth muscle cells, and this function of smooth muscle cells may be enhanced by chronic *C pneumoniae* infection.

Infection by *C pneumoniae* of the wall of small arteries in the media is interesting. It is generally believed that the *C pneumoniae* infection was observed not only in smooth muscle cells that had migrated into the atheromatous plaque but also in smooth muscle cells in the media underlying the atheromatous plaques. The susceptibility of smooth muscle cells to *C pneumoniae* infection has been demonstrated in previous in vitro studies. The outcome of widespread infection of *C pneumoniae* in smooth muscle cells is unclear compared with that in macrophages. Growth factors such as platelet-derived growth factor in atherosclerotic lesions are also produced and released by smooth muscle cells, and this function of smooth muscle cells may be enhanced by chronic *C pneumoniae* infection.

![Figure 1. Case 2. AY-6 (a *C pneumoniae*-specific monoclonal antibody) immunoreactivity in endothelial cells (arrows). Weak immunoreactivity was also observed in form cells in the intima (below the arrows) (original magnification ×100, bar=50 μm).](image)

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**Immunoreactivity for CF-2, AY-6, and Virostat Monoclonal Antibodies in the Atherosclerotic Carotid Artery and Risk Factors for Atherosclerosis in 19 Patients**

<table>
<thead>
<tr>
<th>Case</th>
<th>Age/Sex</th>
<th>CF-2</th>
<th>AY-6</th>
<th>Virostat</th>
<th>Smoking</th>
<th>Hypertension</th>
<th>Diabetes</th>
<th>Hypercholesterolemia</th>
<th>Ischemic Heart Disease</th>
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<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
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<td>3+</td>
<td>3+</td>
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<td>–</td>
<td>–</td>
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</tr>
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<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<td>–</td>
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<tr>
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<td>61/M</td>
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<td>Current</td>
<td>+</td>
<td>–</td>
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<tr>
<td>18</td>
<td>78/F</td>
<td>–</td>
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<td>+</td>
<td>–</td>
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<tr>
<td>19</td>
<td>68/M</td>
<td>2+</td>
<td>3+</td>
<td>–</td>
<td>Never</td>
<td>+</td>
<td>–</td>
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</tr>
</tbody>
</table>

CF-2 indicates *Chlamydia* genus–specific monoclonal antibody; AY-6, *C pneumoniae*–specific monoclonal antibody; and Virostat, *C trachomatis*–specific monoclonal antibody. Under Immunoreactivity, – indicates no staining; 1+, positive staining in a few cells; 2+, positive staining in moderate number of cells; and 3+, intense staining in numerous cells. Under Risk Factors (and Ischemic Heart Disease), + indicates presence of and – indicates absence of risk factor (or disease).
pneumoniae body is transported to the carotid arterial wall and the atheromatous plaque predominantly by macrophages that can pass through injured endothelium. However, another route of infection, in which C pneumoniae invades an atheromatous plaque and carotid arterial wall from small arteries in the media (vasa vasorum), is possible.

Recently, many investigations have shown the involvement of apoptosis of both macrophages and smooth muscle cells in atherosclerosis. Although TUNEL staining can cause overestimation of apoptosis, some cells in the atherosclerotic lesions surely undergo apoptotic cell death, and in addition, bacterial infection is a well known cause of apoptosis. However, only a few TUNEL-positive cells were observed in the atheromatous plaques in the present study, which is consistent with other reports; thus, there was a considerable discrepancy between the number of TUNEL-positive cells and the number of cells infected by C pneumoniae. The relationship between atherosclerosis and C pneumoniae infection probably does not involve the process of apoptosis.

We investigated well-characterized general risk factors for atherosclerosis (hypertension, hypercholesterolemia, diabetes, and cigarette smoking) for each patient, and none of these risk factors was related to the severity of C pneumoniae infection as demonstrated by immunohistochemistry, even though hypertension was observed in 74% of patients. This result is similar to those reported in previous studies of C pneumoniae infection of the coronary artery and does not support a positive relationship between C pneumoniae infection and atherosclerosis. However, some serological investigations of C pneumoniae infection have provided evidence for a significant relationship between infection and athero-
sclerosis based on the correction of general risk factors for atherosclerosis. Therefore, *C pneumoniae* infection can be related to carotid atherosclerosis independently of the general risk factors and will become the next risk factor for atherosclerosis.

In conclusion, we investigated the distribution of *C pneumoniae* infection in atherosclerotic carotid lesions resected during CEA. Infection by *C pneumoniae* was widespread in endothelial cells, macrophages, and smooth muscle cells that had migrated into the atheromatous plaque. In addition, smooth muscle cells and small arteries in the media of the carotid artery were also infected. This investigation has not necessarily established the etiologic role of *C pneumoniae* infection in the development of atherosclerosis, and further studies are required to confirm all of Koch’s postulates, which establish the etiologic role of *C pneumoniae* infection in atherosclerosis. However, clinical evidence that *C pneumoniae* infection contributes to atherosclerosis is accumulating, and an animal model of *C pneumoniae* infection in the atherosclerotic lesion has been developed. The demonstration of the distribution of *C pneumoniae* infection in the present study, together with the animal model of *C pneumoniae* infection, will help to clarify how *C pneumoniae* infection contributes to the progression of carotid atherosclerotic lesions.

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