**Chlamydia pneumoniae** Infection Increases Adherence of Mouse Macrophages to Mouse Endothelial Cells In Vitro and to Aortas Ex Vivo

Naohisa Takaoka,† Lee Ann Campbell, Amy Lee, Michael E. Rosenfeld, and Cho-Chou Kuo

*Department of Pathobiology and Epidemiology and Department of Pathology, University of Washington, Seattle, Washington*

Received 14 September 2007/Returned for modification 23 October 2007/Accepted 26 November 2007

Interactions between monocytes/macrophages and endothelial cells play an important role in the pathogenesis of atherosclerosis, and the adherence of monocytes to the arterial endothelium is one of the early events in atherogenesis. In the present study, peritoneal macrophages harvested from green fluorescent protein (GFP) transgenic mice were used to analyze how *Chlamydia pneumoniae* infection affects the adherence of GFP-macrophages to mouse endothelial cells in vitro and to the aorta from normolipidemic and hyperlipidemic mice ex vivo. In vitro studies showed that *C. pneumoniae*-infected GFP-macrophages adhered better than uninfected macrophages to endothelial cells and GFP-macrophages adhered better to infected than uninfected endothelial cells. The ex vivo studies showed that *C. pneumoniae*-infected macrophages adhered better than uninfected macrophages to aortas from both normolipidemic and hyperlipidemic C57BL/6J mice and apolipoprotein E (ApoE)-deficient mice. In contrast, adherence of *C. pneumoniae*-infected macrophages to the aortas of intercellular adhesion molecule 1 (ICAM-1) knockout mice was not enhanced, suggesting that ICAM-1 is crucial for activation of the adherence of *C. pneumoniae*-infected macrophages to the endothelium. In conclusion, the present study defined a homing mechanism by which *C. pneumoniae* promotes the adherence of mononuclear phagocytes to the endothelium at the site of atherosclerotic lesion formation to promote the progression of atherosclerosis.

*Chlamydia pneumoniae* is an obligate intracellular gram-negative bacterium and is primarily a respiratory pathogen. Seroepidemiological studies have shown an association of *C. pneumoniae* antibody and atherosclerosis (28). The association of *C. pneumoniae* and atherosclerosis has been strengthened by detection (17) and isolation (26) of the organism from atherosclerotic lesions. Studies in animal models of atherosclerosis showed that intranasal inoculation of hyperlipidemic mice accelerates the progression of atherosclerosis (7, 21). In addition, animal experiments indicate that *C. pneumoniae* may be disseminated from the lungs to atherosclerotic lesions in the artery via circulating monocytes (5, 22).

Atherosclerosis is a disease of chronic inflammation and a major cause of coronary heart disease and stroke. Early events in lesion development include endothelial activation, which can be triggered by risk factors such as hypercholesterolemia. This results in leukocyte recruitment to the endothelium and migration into the subendothelium (18). The interaction of monocytes/macrophages with the endothelium is promoted by the expression of receptors for adhesion molecules on monocytes/macrophages, which mediate adherence to the corresponding adhesion molecules on endothelial cells, such as intercellular adhesion molecule 1 (ICAM-1), vascular adhesion molecule 1 (VCAM-1), E-selectin, and P-selectin (6, 8, 18).

Leukocyte recruitment and activation of the expression of proinflammatory cytokines characterize the early process of atherosclerosis (18). Infection of human monocytes or macrophages with *C. pneumoniae* has been shown to enhance the adhesion of monocytes/macrophages to human endothelial cells (2, 10, 12, 20). Furthermore, infection of endothelial cells with *C. pneumoniae* has been shown to up-regulate the expression of E-selectin, ICAM-1, and VCAM-1 (11) and stimulate rolling, adhesion, and transmigration of human neutrophils or monocytes (14, 19, 23).

In this study, peritoneal macrophages from green fluorescent protein (GFP) transgenic mice on a C57BL/6J background were used as a tool to analyze how *C. pneumoniae* infection affects the adherence of GFP-macrophages to mouse endothelial cells in vitro and how hyperlipidemia affects the adherence of *C. pneumoniae*-infected macrophages to the mouse aorta ex vivo. In addition, the role of ICAM-1 in the adherence of *C. pneumoniae*-infected macrophages was studied with ICAM-1 knockout mice.

**MATERIALS AND METHODS**

*Cells and animals.* The buffers used were Hanks’ balanced salt solution (HBSS; NaCl, 8 g; glucose, 1 g; KCl, 0.4 g; KH2PO4, 60 mg; Na2HPO4, 48 mg; MgSO4·7H2O, 0.2 g; CaCl2, 0.11 g [per liter, pH 7.2]), a glucose-potassium-phosphate (GKBP) solution (HBSS containing no Ca2+ or Mg2+), and chlamydia transport medium SPG (0.2 M sucrose, 0.8 mM KH2PO4, 6.7 mM Na2HPO4, 5 mM L-glutamic acid; pH 7.4). The culture media used were (i) RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum and 100 μg/ml each streptomycin and vancomycin and (ii) Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 5% fetal calf serum and 100 μg/ml each streptomycin and vancomycin. Cell lines used were a continuous human epithelial cell line (HL cells) obtained from
Adherence of *C. pneumoniae*-infected macrophages to endothelial cells in vitro. *C. pneumoniae* infection of macrophages was shown to stimulate adherence of macrophages to uninfected endothelial cells. Figure 1A shows adherent green macrophages under a fluorescence microscope. The increase in the number of adherent green macrophages in the infected group (infected at an MOI of 0.1) over the uninfected group can be readily discerned (Fig. 1A). In addition, in the infected group, clusters of two to four macrophages were observed. Quantification of adherent macrophages showed significant increases of 2.0- to 3.8-fold in the number of macrophages that adhered to endothelial cells when macrophages were infected at MOIs of 0.1, 0.5, 1.0, and 10 (Fig. 1B; done in triplicate).

### Adherence of macrophages to *C. pneumoniae*-infected endothelial cells in vitro

*C. pneumoniae* infection of endothelial cells was shown to increase the adherence of uninfected macrophages from female mice to endothelial cells. Endothelial cells were infected at MOIs of 0.1, 0.5, 1.0, and 10 (Table 1). Significant adherence increases of 2.0- and 1.8-fold were observed at MOIs of 1 and 10, respectively (*P < 0.01*). A similar magnitude of enhancement was observed when GFP-macrophages from male mice were used. The increases were 1.7- and 2.3-fold at MOIs of 1 and 10, respectively (*P < 0.01* and *P < 0.05*).

### Adherence of *C. pneumoniae*-infected macrophages to the aortas of normo- and hyperlipidemic mice ex vivo

*C. pneumoniae* infection of macrophages resulted in significant increases in the adherence of macrophages to the aortas of normolipidemic C57BL/6J mice fed a chow diet (1.4-fold, Fig. 2A), hyperlipidemic ApoE-deficient mice fed a chow diet (1.3-fold, Fig. 2A), and C57BL/6J mice fed an atherogenic diet (1.6-fold, Fig. 2B). The adherence of uninfected macrophages to the aortas of hyperlipidemic, ApoE-deficient mice in comparison to those of normolipidemic C57BL/6J mice was also shown to increase slightly, but the increase was not statistically significant (Fig. 2A). The plasma cholesterol levels were 54 ± 7 mg/dl in C57BL/6J mice fed a chow diet, 310 ± 41 mg/dl in ApoE-deficient mice fed a chow diet, and 112 ± 22 mg/dl in C57BL/6J mice fed an atherogenic diet.

### Adherence of *C. pneumoniae*-infected macrophages to the aortas of ICAM-1 knockout mice ex vivo

To study the role of adhesion molecules in *C. pneumoniae*-associated atherosclerosis, the adherence of *C. pneumoniae*-infected macrophages to the aortas of ICAM-1 knockout mice fed an atherogenic diet was evaluated. The plasma cholesterol levels of ICAM-1 knockout mice were 159 ± 12 mg/dl. Infected macrophages adhered no better than uninfected macrophages to the aortas of ICAM-1 knockout mice (Fig. 2B). This was in contrast to the 1.6-fold increase in adherence (*P < 0.05*) observed with *C. pneumoniae*-infected macrophages in comparison to uninfected macrophages in hyperlipidemic C57BL/6J mice (Fig. 2B). These findings indicate that ICAM-1 may play a role in *C. pneumoniae*-accelerated atherosclerosis. In contrast, no decrease in the adherence of uninfected macrophages was observed in ICAM-1-deficient mice in comparison to C57BL/6J mice. This finding may suggest a compensatory effect from other adhesion molecules which were activated in ICAM-1 knockout mice by hyperlipidemia induced by the atherogenic...
Hyperlipidemia has been shown to activate the expression of endothelial adhesion molecules VCAM-1 and ICAM-1 (8).

**DISCUSSION**

This study demonstrates that *C. pneumoniae* infection of macrophages promotes the adherence of macrophages to endothelial cells in vitro and ex vivo in the mouse system. The experimental findings from this study support our hypothesis that *C. pneumoniae* disseminates from the lungs to atherosclerotic plaques. The data suggest that *C. pneumoniae* infection enhances the adherence of macrophages to endothelial cells, which may contribute to the progression of atherosclerosis.

**TABLE 1.** *C. pneumoniae* infection of endothelial cells increases adherence of uninfected GFP-macrophages

<table>
<thead>
<tr>
<th>MOI</th>
<th>No. of adherent macrophages*</th>
<th>Fold increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>375 ± 58</td>
<td>1.0</td>
</tr>
<tr>
<td>0.1</td>
<td>440 ± 33</td>
<td>1.2</td>
</tr>
<tr>
<td>0.5</td>
<td>522 ± 173</td>
<td>1.4</td>
</tr>
<tr>
<td>1.0</td>
<td>763 ± 72^b</td>
<td>2.0</td>
</tr>
<tr>
<td>10</td>
<td>682 ± 87^b</td>
<td>1.8</td>
</tr>
</tbody>
</table>

*a Average cell count per 20 fields at a magnification of ×200; n = 3.

*b P < 0.01.
In this study, adherence of macrophages to endothelial cells was also increased in C. pneumoniae-infected endothelial cells in comparison to uninfected endothelial cells. Similar findings were observed by other investigators (14, 19, 23). These reports showed that infection of human endothelial cells with C. pneumoniae stimulates rolling, adhesion, and transmigration of human neutrophils or monocytes (14, 19, 23).

How C. pneumoniae infection promotes the adherence of GFP-macrophages was not analyzed in this study. Previously, Kalayoglu et al. showed that C. pneumoniae infection of human monocytes activates the expression of the integrin β2 adhesion molecules (10). Subsequently, May et al. (20) reported that C. pneumoniae infection of human monocytes up-regulates very late antigen 4, lymphocyte function-associated antigen 1 (LFA-1), and macrophage antigen 1 (Mac-1) or urokinase receptor and increases the adhesion of human monocytes to human umbilical vein endothelial cells. It has been shown that C. pneumoniae infection induces gamma interferon (27) and that exposure of monocytes to gamma interferon enhances their adhesiveness to endothelial cells and activates LFA-1 (CD11a/CD18), Mac-1 (CD11b/CD18), CD14, and t-selectin on monocytes (30). Therefore, it is logical to conclude that C. pneumoniae may use this pathway to promote macrophage adherence.

The ex vivo studies were consistent with the in vitro studies described in this report and reports by other investigators (2, 10, 12). The significant findings from the present study were twofold. (i) C. pneumoniae infection of macrophages increased adherence to the aortas of normolipidemic and hyperlipidemic mice (Fig. 2A and B), and (ii) C. pneumoniae infection failed to enhance the adherence of GFP-macrophages to the aortas of ICAM-1 knockout hyperlipidemic mice (Fig. 2B). The first finding is consistent with our previous in vivo studies showing that C. pneumoniae accelerates hyperlipidemia-induced atherosclerosis (3, 21, 22). The second finding is consistent with studies demonstrating that C. pneumoniae infection may activate LFA-1 and/or Mac-1, the ligand of ICAM-1 (29). Therefore, the results of the ex vivo study with ICAM-1 knockout mice suggests that ICAM-1 is critical for C. pneumoniae-infected mononuclear phagocytes to infiltrate atherosclerotic lesions. This results in promoting a cascade of events, such as C. pneumoniae-induced production of proinflammatory cytokines and other proatherogenic factors by infiltrating macrophages induced by C. pneumoniae, which contribute to the progression of atherosclerosis.

In conclusion, by using GFP-macrophages, this study demonstrates that C. pneumoniae infection of macrophages enhances the adherence of macrophages to endothelial cells in vitro and the aortas of normolipidemic and hyperlipidemic mice ex vivo and that ICAM-1 is critical for the adherence of C. pneumoniae-infected macrophages to the aorta.

ACKNOWLEDGMENTS

This study was supported by National Institutes of Health grants HL-56036 and AI-43060. We thank Mark Berry and Angela Lam for technical support.

REFERENCES


