Presence and Severity of Chlamydia pneumoniae and Cytomegalovirus Infection in Coronary Plaques Are Associated With Acute Coronary Syndromes

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SUMMARY

Although an association between Chlamydia pneumoniae (Cpn) or Cytomegalovirus (CMV) infection and coronary atherosclerosis has been reported, such an association is less clear for acute coronary syndromes (ACS). The purpose of this study was to investigate the pathogenic roles of Cpn and CMV infection of coronary plaques in ACS. We divided 38 coronary plaque specimens obtained from 38 patients who underwent directional coronary atherectomy or thrombectomy into an ACS group (n = 21) and a non-ACS group (n = 17). Cpn and CMV in specimens were stained using immunohistochemical techniques and analyzed quantitatively. The detection rate for either Cpn- or CMV-positive cells in ACS patients was slightly higher compared with non-ACS patients. Detection rates for both Cpn- and CMV-positive cells were significantly higher in ACS patients than in non-ACS patients (P = 0.010). Furthermore, the density of Cpn- and CMV-positive cells in plaques was significantly higher in ACS patients than in non-ACS patients (P < 0.003). The results indicate that the presence and severity of Cpn and CMV infection in coronary plaques are greater in patients with ACS compared with non-ACS patients. We conclude that infection with Cpn and CMV in coronary plaques may be involved in the pathogenesis of ACS.  (Int Heart J 2006; 47: 511-519)

Key words: Inflammation, Plaque rupture, Immunohistochemistry, Directional atherectomy, Thrombectomy

INFECTIOUS agents have been implicated as possible causative factors in the development of atherosclerosis.1-4) Many seroepidemiologic,5-9) histopathologic,10-16) and animal17,18) studies have suggested a positive association between...
Chlamydia pneumoniae (Cpn) infection and coronary artery disease (CAD). Cytomegalovirus (CMV) infection is also considered to increase the risk of development of CAD,\(^{19-21}\) including a risk of restenosis after balloon angioplasty,\(^{22,23}\) which may occur through p53 suppression by cytomegalovirus, because the absence of p53 increases vascular neointima formation.\(^{24}\) Although microorganisms such as Cpn and CMV are thought to be associated with chronic coronary artery disease, the role of these pathogens in the initiation of acute coronary syndromes (ACS) is less well defined.

An increase in the inflammatory response in the atherosclerotic plaque is thought to be linked to plaque instability and rupture.\(^{25-27}\) We hypothesized that Cpn and CMV infections in the coronary plaques may play possible roles in the pathogenesis of ACS. If an increase in inflammation within the coronary plaques is induced by infectious agents such as Cpn and CMV, possible roles for a Cpn or CMV infection in the plaques should be confirmed in the setting of ACS. Several seroepidemiologic studies have shown an association between the occurrence of ACS and elevated Cpn IgA titers\(^{28-30}\) as well as elevated CMV IgG titers.\(^ {31} \) However, histopathologic studies of both Cpn and CMV infection in coronary plaque specimens of ACS patients have seldom been performed. A histopathologic autopsy study that demonstrated a large number of Cpn organisms in ruptured plaques suggested possible involvement of Cpn in plaque rupture and development of acute myocardial infarction.\(^ {32}\) This observation prompted us to focus on the number of cells containing Cpn or CMV in coronary plaques. The purpose of this study was to investigate the number of Cpn-containing cells as well as CMV-containing cells in coronary lesion specimens obtained from ACS and non-ACS patients who underwent thrombectomy or directional coronary atherectomy (DCA). Our quantitative comparison of Cpn- or CMV-containing cells between ACS and non-ACS patients represents a step toward understanding the pathogenic role of infectious agents in the occurrence of ACS.

**Methods**

**Patient population:** We studied 38 coronary plaque specimens obtained from 38 patients who underwent DCA or thrombectomy at Toho University Ohashi Medical Center in Tokyo, Japan between June 2002 and November 2002. Specimens and patients were divided into 2 groups on the basis of clinical presentation, electrocardiographic findings, laboratory results, and coronary angiographic findings. The ACS group \((n = 21)\) included patients with acute myocardial infarction (MI, \(n = 9\)) or unstable angina pectoris (UAP, \(n = 12\)). The non-ACS group \((n = 17)\) included patients with stable angina pectoris (SAP, \(n = 15\)) or silent myocardial ischemia \((n = 2)\). Acute MI was defined as the occurrence of typical symp-
symptoms, electrocardiographic evidence (ST elevation of at least 0.1 mV in 2 or more leads), coronary angiographic findings (occlusion of a main coronary artery branch with TIMI grade 0, 1, or 2 flow), and serum creatine kinase (CK) or CK-MB elevation double the upper limit of the normal range. UAP was defined as typical precordial chest pain of Braunwald class IIB or IIIB, angiographic evidence of documented stenosis > 75% based on the American Heart Association classification in 1 or more principal coronary arteries, and no elevation of the serum CK concentrations. SAP was defined as symptoms of typical precordial chest pain and coronary angiographic findings of stenosis > 75% in 1 or more principal coronary arteries. Silent myocardial ischemia was defined as the documentation of myocardial ischemia in the absence of angina with coronary stenosis > 75%.

**Coronary specimens:** Eighty-seven specimens (2 or 3 specimens from each patient) from the culprit lesions were obtained by DCA or thrombectomy. Conventional DCA was performed with an 8Fr Atherocath GTO device (Guidant, Santa Clara, CA, USA) or an 8Fr guidewire catheter-compatible FLEXI-CUT directional atherectomy device (Guidant) in patients with stable angina pectoris and silent myocardial ischemia. In patients with acute myocardial infarction and unstable angina, an occlusive balloon type distal protection device (PercuSurge GuardWire, Medtronic Inc, Santa Clara, CA, USA) and a Rescue thrombectomy catheter (SciMed/Boston Scientific Co., Maple Grove, MN, USA) were used to obtain the coronary specimens. The quality and size of each specimen were assessed, and lesions that were too small (less than 1 × 1 mm) or inappropriately stained were excluded (n = 8). There were no significant differences in the quality and size of specimens between the ACS and non-ACS groups. After counting Cpn-positive or CMV-positive cells and measuring the area in all of the specimens, the maximum number of infected cells (/mm²) per patient was used for quantitative comparisons (n = 38).

**Immunohistochemical analysis:** All specimens were embedded immediately in optimal cutting temperature compound and snap-frozen in liquid nitrogen for storage at −80°C. Frozen specimens were cut into 4-µm thick sections with a cryostat microtome. The frozen sections were affixed to lysine-coated slides. Some specimens were stained with hematoxylin and eosin. In other specimens, immunohistochemical staining was carried out as described below.

Frozen sections adjacent to their hematoxylin and eosin-stained counterparts were fixed in acetone and a Cpn-specific monoclonal primary antibody (RR-402; Dakopatts, Copenhagen, Denmark) against the major outer membrane protein of Cpn. Sections were stained using the avidin-biotin complex immunoperoxidase method with a Dako-LSAB kit (Dakopatts) and then were counterstained with Mayer's hematoxylin. Adjacent sections with no primary antibody...
exposure were used as negative controls. Other adjacent sections were stained using a CMV-specific primary antibody (CH2-DDG9, Dakopatts) and a macrophage-specific monoclonal primary antibody (CD-68, Dakopatts) using a similar procedure.

A cell was considered to stain positively when it contained immunoreactivity evident by light microscopy. To compare the number of Cpn-positive or CMV-positive cells between ACS and non-ACS groups, we digitally stored microscopic images of sections in a computer, using a Polaroid PDMC (II)/OL camera (Olympus, Tokyo). The entire tissue section area was measured using Scion Image software (Scion, Friderick, MD, USA). Cpn-positive and CMV-positive cells were counted in the entire tissue section and reported as the mean number of positive cells per square millimeter.

**Statistical analysis:** For continuous variables, data are presented as the mean ± SD for normally distributed variables and as medians for non-normally distributed variables. Proportions are used to present categorical variables. The unpaired Student’s t test and the Mann-Whitney U test were used for comparison of normally and non-normally distributed variables, respectively, between 2 groups, with the chi-square test being used to compare proportions of categorical variables. All tests were 2-tailed, and a value of P less than 0.05 was considered to indicate statistical significance. SPSS II software (SPSS Japan Inc., Tokyo) was used for these analyses.

### Table I. Patient Characteristics

<table>
<thead>
<tr>
<th></th>
<th>Total n=38</th>
<th>ACS n=21</th>
<th>non-ACS n=17</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong></td>
<td>65.6 ± 2.7</td>
<td>67.8 ± 2.3</td>
<td>63.7 ± 2.4</td>
<td>0.47</td>
</tr>
<tr>
<td><strong>Male</strong></td>
<td>34 (89.5)</td>
<td>19 (90.5)</td>
<td>15 (88.2)</td>
<td>0.76</td>
</tr>
<tr>
<td><strong>BMI, kg/m²</strong></td>
<td>24.5 ± 0.7</td>
<td>23.8 ± 0.8</td>
<td>25.3 ± 1.3</td>
<td>0.32</td>
</tr>
<tr>
<td><strong>Ejection fraction, %</strong></td>
<td>58.1 ± 2.6</td>
<td>55.0 ± 3.2</td>
<td>64.1 ± 3.7</td>
<td>0.05</td>
</tr>
<tr>
<td><strong>Smoker</strong></td>
<td>28 (73.7)</td>
<td>15 (71.4)</td>
<td>13 (76.5)</td>
<td>0.98</td>
</tr>
<tr>
<td><strong>Hyperlipidemia</strong></td>
<td>21 (55.3)</td>
<td>9 (42.9)</td>
<td>12 (70.6)</td>
<td>0.17</td>
</tr>
<tr>
<td><strong>Hypertension</strong></td>
<td>15 (39.5)</td>
<td>8 (38.1)</td>
<td>7 (41.2)</td>
<td>0.89</td>
</tr>
<tr>
<td><strong>Diabetes mellitus</strong></td>
<td>14 (36.8)</td>
<td>6 (28.6)</td>
<td>8 (47.0)</td>
<td>0.25</td>
</tr>
<tr>
<td><strong>HbA1c, %</strong></td>
<td>5.5 ± 0.3</td>
<td>5.3 ± 0.2</td>
<td>5.9 ± 0.6</td>
<td>0.30</td>
</tr>
<tr>
<td><strong>Lesion (LAD/LCx/RCA)</strong></td>
<td>26/5/7</td>
<td>14/2/5</td>
<td>12/2/3</td>
<td>0.60</td>
</tr>
<tr>
<td><strong>Thrombectomy</strong></td>
<td>22 (57.9)</td>
<td>20 (95.2)</td>
<td>2 (11.8)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td><strong>DCA</strong></td>
<td>16 (42.1)</td>
<td>1 (4.8)</td>
<td>15 (88.2)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Data are presented as the mean ± SD or as number (%) of patients. ACS indicates acute coronary syndromes; BMI, body mass index; HbA1c, glycosylated hemoglobin; LAD, left anterior descending artery; LCx, left circumflex artery; RCA, right coronary artery; and DCA, directional coronary atherectomy.
RESULTS

The characteristics of the 21 ACS and 17 non-ACS patients are summarized in Table I. There were no significant differences with respect to distribution of age, gender, or conventional risk factors for coronary atherosclerosis between the ACS and non-ACS groups.

As shown in Table II, the rate of detection of Cpn-positive cells in the ACS group was slightly higher than in the non-ACS group, although the difference was

| Table II. Immunohistochemistry of Cpn and CMV: Patient and Plaque Analysis in ACS and Non-ACS |
|-----------------------------------------------|-----------------------------------------------|
| Overall | ACS | non-ACS | P |
| n = 38 | n = 21 | n = 17 |  |
| Cpn-positive patient | 26 (68.4) | 17 (81) | 9 (52.9) | 0.13 |
| CMV-positive patient | 23 (60.5) | 16 (76.2) | 7 (41.2) | 0.25 |
| Both Cpn and CMV-positive patient | 21 (55.3) | 16 (76.2) | 5 (29.4) | 0.010 |
| Section area, mm² | 2.7 ± 2.0 | 2.4 ± 1.9 | 3.3 ± 2.1 | 0.24 |
| Cpn-positive cells/mm² | 3.7 | 7.2 | 0.7 | 0.0012 |
| CMV-positive cells/mm² | 2.9 | 6.6 | 1.1 | 0.0026 |

Data are presented as number (%) of patients, mean ± SD in section area, and median in cell number per millimeter square. ACS indicates acute coronary syndromes; Cpn, chlamydia pneumoniae; and CMV, cytomegalovirus.

Figure. Immunohistochemistry of coronary specimens obtained from an acute coronary syndrome (ACS) patient (A-C) and a non-ACS patient (D-F). Cells with immunoreactivity are shown in brown. All sections were counterstained with Mayer’s hematoxylin. A, D: Sections stained with a Chlamydia pneumoniae (Cpn)-specific monoclonal antibody (RR-402; Dakopatts, Copenhagen, Denmark). B, E: Adjacent sections stained with a Cytomegalovirus (CMV)-specific monoclonal antibody (CH2-DDG9; Dakopatts). C, F: Adjacent sections stained for a macrophage marker (CD-68; Dakopatts). Immunostained cell density was higher in the specimen from the ACS patient than from the non-ACS patient. Original magnification; × 400. Cpn indicates chlamydia pneumoniae; and CMV, cytomegalovirus.
not statistically significant (81.0% versus 52.9% of specimens, $P = 0.13$) and the rate of detection of CMV-positive cells was slightly higher in the ACS group than in the non-ACS group (76.2% versus 41.2%, $P = 0.25$). Importantly, the detection rates for both Cpn- and CMV-positive cells were higher in ACS patients than in non-ACS patients (76.2% versus 29.4%, $P = 0.010$). Furthermore, as shown in Table II, Cpn-positive cells were significantly more numerous in the ACS group than in the non-ACS group (median, 7.2 versus 0.7 cells/mm², $P = 0.0012$). This was also true for CMV-positive cells (median, 6.6 versus 1.1 cells/mm², $P = 0.0026$). In 6 specimens from 6 ACS patients we detected more macrophages in adjacent sections corresponding to a greater number of Cpn- and CMV-positive cells, while fewer macrophages were apparent in sections adjacent to those with fewer Cpn- and CMV-positive cells. Examples of the immunohistochemical staining in an ACS and a non-ACS patient are shown in the Figure.

**DISCUSSION**

The present study demonstrated the frequent detection of both Cpn-positive and CMV-positive cells in ACS patients compared with non-ACS patients. Furthermore, the number of Cpn- and CMV-positive cells in coronary plaques was greater in ACS patients than in non-ACS patients. The findings suggest that a greater burden of Cpn and CMV infection in coronary plaques may contribute to the development of ACS compared with the development of stable chronic coronary artery disease. While Cpn or CMV has been demonstrated within atherosclerotic lesions by various techniques and Cpn or CMV infection is thought to be associated with CAD, debate continues as to whether the organisms are causative pathogens or merely innocent bystanders in the setting of CAD. Whether Cpn or CMV infection of an atheroma can trigger an acute event also is disputed. Higuchi, et al detected Cpn in vessel segments with a ruptured plaque obtained at autopsy using immunohistochemical staining and Macchuavello's method. These 2 methods demonstrated a similar number of Cpn-positive cells, which validated counting Cpn-positive cells by immunohistochemical staining as a reliable method. To date, most immunohistochemical studies have not focused on the number of cells stained with Cpn or CMV antibodies, although there are a few studies that have used semiquantitative methods to assess the presence of Cpn or CMV. The present study represents the first quantitative comparison of Cpn-positive and CMV-positive cells between ACS and non-ACS groups.

Gattone, et al showed that subjects who had been exposed to a greater number of infectious pathogens have particularly unfavorable profiles with an increased risk of CAD and myocardial infarction. Furthermore, Miya, et al...
demonstrated that patients with higher titers of Cpn are at a greater risk for ACS than those with lower titers of Cpn. These findings allowed us to hypothesize that more intense infection of a vulnerable plaque with Cpn and CMV may contribute to plaque rupture causing ACS.

Experimental and clinical studies suggest differences between Cpn or CMV infection in the mechanisms responsible for the development of chronic atherosclerosis and the rupture of vulnerable plaques. In the initial stages of atherosclerosis, Cpn infection can induce monocytes to oxidize lipoproteins, which are highly atherogenic, and induce endothelial dysfunction and expression of various inflammatory markers and adhesion molecules. Cpn heat shock protein 60 enhances the expression of matrix-degrading metalloproteinases (MMPs), and MMPs degrade fibrous cap and can cause plaque rupture. CMV replication also may induce plaque instability because CMV infection can lead to increased polymorphonuclear leukocyte adhesion to endothelium.

Our study has some limitations. It is unclear whether Cpn- or CMV-containing cells are macrophages, endothelial cells, or smooth muscle cells. Double staining for Cpn and macrophages would be helpful for determining whether the cells are macrophages or not, but could not be performed in these series because of a limited quantity of specimens. Localization of Cpn- or CMV-containing cells also is unclear because of the limitations of methods, such as DCA or thrombectomy, for acquiring coronary specimens. The evidence of existence of Cpn- or CMV-positive cells at the shoulder of a ruptured plaque or a ruptured region of fibrous cap might support a stronger association between infections by these pathogens to coronary plaque and ACS.

**Conclusion:** Immunohistology frequently detected infectious agents, such as Cpn and CMV in coronary plaques. Such pathogens were more prevalent in plaques associated with ACS and with high densities of Cpn and CMV within the plaque.

**REFERENCES**


