Serological Association Between Chlamydia pneumoniae Infection and Age-Related Macular Degeneration

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Background: Age-related macular degeneration (ARMD) is a leading cause of blindness in the United States, but the mechanisms that initiate and promote the disease remain ill defined. There are several risk factors that ARMD shares with atherosclerosis, and these diseases may have similar pathogenic mechanisms that involve inflammation. Chlamydia pneumoniae, a prokaryotic pathogen that causes chronic inflammation is now emerging as a risk factor in the development of cardiovascular diseases. It is therefore plausible that this microorganism also contributes to the pathogenesis of ARMD.

Methods: To examine if C pneumoniae infection is associated with ARMD, serum samples from 25 consecutive patients with ARMD and from 18 without the disease were collected and assayed for the presence of the antibodies to C pneumoniae elementary bodies, Chlamydia trachomatis heat shock protein 60 (cHsp60), C trachomatis heat shock protein 10 (cHsp10), Escherichia coli GroEL, and E coli GroES.

Results: A serological association was found between ARMD and anti–C pneumoniae antibodies (P=.047) but not between ARMD and the anti–C trachomatis or anti–E coli heat shock protein antibodies. The association remained statistically significant after adjusting for age and smoking, both established risk factors for ARMD.

Conclusions: These data indicate that C pneumoniae infection may be associated with ARMD. Further studies on larger cohorts of individuals are necessary to determine if this pathogen plays a role in the pathogenesis of ARMD.

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with healthy control subjects. Second, the organism can be detected within atherosclerotic lesions by immunohistochemistry, polymerase chain reaction, and electron microscopy; furthermore, the pathogen has been isolated from atherosclerotic lesions and propagated in vitro. Importantly, the organism has been detected in atheromatous tissue (but not in normal arterial tissue), isolated from a multitude of sites, including coronary arteries, carotid endarterectomy specimens, and abdominal aortic aneurysms. Third, in vitro studies indicate that the organism has the capacity to modulate cellular lipoprotein metabolism, induce inflammatory cytokine cascades, and alter cell-cell interactions to contribute to atherogenesis. Finally, studies using animal models show that C. pneumoniae can promote lesion initiation and promotion, and antibiotic treatment of infected animals can prevent the development of atherosclerotic lesions.

Since ARMD involves inflammation, with features similar to atherogenesis, the current study examined if ARMD is associated with C. pneumoniae infection.

STUDY DESIGN AND PATIENT POPULATION

A case-controlled trial was conducted at the Veterans Affairs (VA) Hospital Eye Clinic in Palo Alto, Calif. All patients older than 55 years visiting the VA Hospital eye clinic between January 1, 2001, and June 1, 2001, were eligible for the study. Patients were enrolled consecutively to either the case group (ARMD patients) or the control group (non-ARMD patients). The case group was composed of 25 patients with clinical evidence of ARMD as determined on funduscopy by a staff retina specialist. The control group consisted of 18 patients without clinical evidence of ARMD on funduscopy. Written informed consent for collection and use of blood for research was obtained from each patient in the study. The study protocol was approved by the VA Hospital Institutional Review Board (Palo Alto).

DEMOGRAPHIC AND POTENTIAL ARMD RISK FACTORS

We gathered interview and medical record–review data on several ARMD and cardiovascular risk factors to assess potential confounding influences. Assessed factors were age and sex, as well as a history of smoking, diabetes, hypertension, hyperlipidemia, and coronary artery disease. Tobacco use was defined as current use or past smoking history of more than 5 pack-years. Diabetes was defined as a fasting blood glucose level greater than 126 mg/dL (7.0 mmol/L) on 2 separate occasions, a glycosylated hemoglobin level greater than 7.5%, or use of anti diabetic therapy. Hypertension was defined as a history of systolic blood pressure higher than 160 mm Hg, diastolic blood pressure higher than 90 mm Hg, or use of antihypertensive therapy. Hyperlipidemia was defined as a history of total cholesterol greater than 200 mg/dL (5.17 mmol/L), a low-density lipoprotein level greater than 130 mg/dL (1.3 g/L), or use of lipid-lowering therapy. A history of coronary artery disease was noted if the patient had a history of stable angina, unstable angina, myocardial infarction, coronary angioplasty, or coronary artery bypass grafting.

CLASSIFICATION OF ARMD

A staff retina specialist classified ARMD into nonneovascular or neovascular stages of disease. Nonneovascular ARMD was defined as macular drusen or the presence of geographic atrophy without choroidal neovascularization or scarring. Neovascular ARMD was defined as the appearance of a CNVM or scar on funduscopy and angiography.

SPECIMEN COLLECTION

Ten milliliters of blood was collected by venipuncture, and the serum was separated and stored frozen at −70°C. Samples were encoded, and laboratory personnel were masked to clinical information on the patients. Samples were shipped in dry ice to the University of Wisconsin (Madison) for serological analysis as described in the following subsections.

ANTIGENS

A panel of antigens was tested. Chlamydia trachomatis heat shock proteins (cHsps) 10 and 60 and Escherichia coli and GroEL were obtained from Stressgen Biotechnologies Corp (Victoria, British Columbia). Chlamydia trachomatis Hsp10 and Hsp60 were purified as previously described. The C. pneumoniae whole organisms (isolate TW187) were grown in HeLa cells, and the infectious stage of the organism known as elementary bodies (EBs) was harvested as described elsewhere and stored at −80°C until used.

ENZYME-LINKED IMMUNOSORBENT ASSAYS

The enzyme-linked immunosorbent assay (ELISA) method used was a modification of that previously reported. Briefly, Immulon 2 plates (Dynex Technologies, St Paul, Minn) were coated with 0.5 µg of each antigen in phosphate-buffered saline for 48 hours at 4°C. After this period, plates were washed 3 times with buffer containing phosphate-buffered saline and 0.1% Tween 20, using a Labsystems Wellwash 4 Mk 2 plate washer (Labsystems Inc, Helsinki, Finland), then blocked for 90 minutes at 37°C with phosphate-buffered saline, 3% ovalbumin (grade 2), and 0.1% Tween 20. Plates were then washed 3 times and incubated for 1 hour at 37°C with a 1:250 dilution of patient serum samples in phosphate-buffered saline, 0.1% ovalbumin (grade 5), and 0.05% Tween 20. Following this step, plates were washed 3 times, followed by incubation with alkaline phosphatase–conjugated goat antihuman IgG (Jackson Immunoresearch Laboratories, West Grove, Pa) for 30 minutes at 37°C. Finally, plates were washed 3 times, followed by a rinse with Tris-buffered saline. The substrate P-nitrophenylphosphate (SigmaFAST tablets; Sigma Chemical Co, St Louis, Mo) was added and incubated for 30 minutes at 37°C. Absorbance was read as optical density (OD) at 405 nm on a Perkin Elmer HTS 7000 Bio Assay Reader (Perkin Elmer Systems, San Francisco, Calif). For each serum sample, the OD value of a phosphate-buffered saline–coated well that had no antigen (antigen-blank) was subtracted from the values for all of the test wells for that antigen. Triplicate-blank test OD values for each antigen were averaged and reported for each patient. Laboratory personnel performing the ELISA test were masked to clinical information on the patients.

STATISTICAL METHODS

The ELISA results of seroreactivity to each antigen were reported as OD from the assay measurements. Seroreactivity to each antigen between case and control groups was evaluated by 2-tailed t tests. Correlation between seroreactivity and age was assessed by a linear regression analysis. Multivariate re-
gession was used to adjust for those variables independently associated with ARMD. Sigmapstat 2.0 (SPSS Inc, Chicago, Ill) software was used to calculate $P$ values from 2-tailed $t$ tests and regression models. Sigmaplot 7.0 (SPSS) and Prism (GraphPad Software Inc, San Diego, Calif) software were used to graph data.

### RESULTS

#### DEMOGRAPHICS

Several ARMD and cardiovascular risk factors were evaluated. Univariate analysis confirmed that established risk factors for ARMD occurred more frequently in patients as compared with controls (Table 1; age: $P < .001$; smoking: $P = .049$). Frequency of hypertension, diabetes, coronary artery disease, and hyperlipidemia was similar between the 2 groups.

#### ANTIBODY TITERS

We used ELISA to measure seroreactivity to C pneumoniae EBs, C trachomatis antigens (cHsp10, cHsp60), and E coli antigens (GroES, GroEL) (Table 2). Significantly increased antibodies to C pneumoniae EBs were present in patients with ARMD compared with patients without ARMD ($P = .047$; Table 2; Figure 1). In contrast, antibody titers to 2 C trachomatis antigens (cHsp10 and cHsp60) and 2 E coli antigens (GroES and GroEL) were similar between patients with and without ARMD (Table 2). When anti–C pneumoniae antibodies were examined by quintiles, 7 (28%) of 25 patients with and 2 (11%) of 18 patients without ARMD had levels measured in the highest fifth quintile (Figure 2). Patients in the highest quintiles were similar in age ($P = .57$) and history of smoking ($P = .25$) as compared with patients in the lower quintiles. In addition, these patients were equally likely to have neovascular ARMD as compared with patients with ARMD in the lower quintiles ($P = .30$).

Additional analyses were performed to determine if age and smoking may have confounded the association between anti–C pneumoniae antibodies and ARMD. Linear regression showed no correlation between age as a function of anti–C pneumoniae antibodies for all patients ($r^2 = 0.002; P = .80$), patients with ARMD ($r^2 = 0.083; P = .16$), or patients without ARMD ($r^2 < 0.001; P = .95$). To determine if smoking was a confounder, antibody levels of both groups were analyzed by subgroups of smokers and nonsmokers. Nonsmokers with ARMD were more likely to have higher anti–C pneumoniae antibodies compared with nonsmokers without ARMD ($P = .03$). In addition, multivariate analysis adjusting for age and smoking showed that anti–C pneumoniae antibodies remained significantly associated with ARMD ($P = .049$). Importantly, there was no statistically significant difference between levels of chlamydial or E coli Hsp antibodies and presence of ARMD after adjusting for age and smoking (not shown).

#### COMMENT

Data presented in this case-control study suggest that patients with ARMD were more likely to have higher levels of anti–C pneumoniae antibodies compared with patients without ARMD. The association between C pneumoniae antibody levels and ARMD remained significant after adjusting for age and smoking. Antibodies to 2 C trachomatis and 2 E coli antigens did not show correlation with ARMD, suggesting that the association was specific to C pneumoniae elementary bodies. Of note, the highest levels of C pneumoniae antibodies (fifth quintile) were observed in 28% of patients with vs 11% of patients without, although there were only 8 to 9 patients in individual quintile groups. Those patients with antibody levels in the highest quintiles were similar in age, stage of ARMD, and prevalence of atherosclerosis risk factors compared with patients with lower antibody levels.

Several limitations exist in this study. First, the small sample size and case-control design of the study did not

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**Table 1. Demographics and Potential Risk Factors in Patients With and Without ARMD**

<table>
<thead>
<tr>
<th>Demographics</th>
<th>All Subjects (N = 43)</th>
<th>Subjects Without ARMD (n = 18)</th>
<th>Subjects With ARMD (n = 25)</th>
<th>$P$ Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ± SD age, y</td>
<td>74.6 ± 8.5</td>
<td>68.7 ± 7.7</td>
<td>78.8 ± 6.2</td>
<td>&lt; .001</td>
</tr>
<tr>
<td>Male sex, %</td>
<td>98</td>
<td>100</td>
<td>96</td>
<td>.40</td>
</tr>
<tr>
<td>Potential risk factors, %</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypertension</td>
<td>81</td>
<td>72</td>
<td>88</td>
<td>.20</td>
</tr>
<tr>
<td>Diabetes</td>
<td>37</td>
<td>44</td>
<td>32</td>
<td>.42</td>
</tr>
<tr>
<td>Coronary artery disease</td>
<td>42</td>
<td>33</td>
<td>48</td>
<td>.35</td>
</tr>
<tr>
<td>Smoking</td>
<td>51</td>
<td>33</td>
<td>64</td>
<td>.049</td>
</tr>
<tr>
<td>Hyperlipidemia</td>
<td>70</td>
<td>66</td>
<td>72</td>
<td>.72</td>
</tr>
</tbody>
</table>

Abbreviation: ARMD, age-related macular degeneration.

**Table 2. Antibody Titers to Chlamydia pneumoniae Whole Organisms, Chlamydia trachomatis cHsp10 and cHsp60, and Escherichia coli GroES and GroEL in Patients With and Without ARMD**

<table>
<thead>
<tr>
<th>Mean ± SD antibody titers (OD reading)</th>
<th>All Subjects (N = 43)</th>
<th>Subjects Without ARMD (n = 18)</th>
<th>Subjects With ARMD (n = 25)</th>
<th>$P$ Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-cHsp10</td>
<td>0.069 ± 0.303</td>
<td>0.127 ± 0.451</td>
<td>0.026 ± 0.112</td>
<td>.287</td>
</tr>
<tr>
<td>Anti-GroES</td>
<td>0.131 ± 0.307</td>
<td>0.063 ± 0.314</td>
<td>0.166 ± 0.303</td>
<td>.391</td>
</tr>
<tr>
<td>Anti-cHsp60</td>
<td>0.213 ± 0.338</td>
<td>0.241 ± 0.457</td>
<td>0.193 ± 0.226</td>
<td>.651</td>
</tr>
<tr>
<td>Anti-GroEL</td>
<td>0.338 ± 0.350</td>
<td>0.297 ± 0.349</td>
<td>0.368 ± 0.355</td>
<td>.516</td>
</tr>
<tr>
<td>Anti-Chlamydia pneumoniae</td>
<td>0.374 ± 0.274</td>
<td>0.276 ± 0.159</td>
<td>0.444 ± 0.319</td>
<td>.047</td>
</tr>
</tbody>
</table>

Abbreviations: ARMD, age-related macular degeneration; cHsp10 and cHsp60, chlamydial heat shock proteins 10 and 60; GroES and GroEL, Escherichia coli Hsps; OD, optical density.
permit the association to be conclusive, or allow the performance of a detailed multivariate analysis. In addition, the study was conducted in a VA hospital setting, where the majority of patients were male and had several comorbid conditions; these factors make it difficult to apply the conclusions to the general population. Furthermore, anti–C trachomatis Chsp60 levels were not elevated in patients with ARMD, even though Hsp60 may cross-react between species. Epidemiological studies on a large cohort of men and women would allow detailed subgroup analysis, help confirm or refute the borderline-significant (P = .047) association between ARMD and elevated anti–C pneumoniae antibodies, and determine which, if any, chlamydial antigens may contribute to the pathogenesis of ARMD.

Since the pathogenesis of ARMD may involve inflammatory processes, it is plausible that infectious agents may initiate or propagate the disease by chronic inflammation. The hallmark of chlamydial disease is persistent infection and chronic inflammation. Thus, C pneumoniae may contribute to ARMD either by infecting subretinal tissues or through production of locally acting inflammatory mediators from distant sites of infection, such as the lung or arteries. Inflammatory cells, including macrophages, can be detected in retinal pigment epithelium basement membranes of patients with ARMD, and C pneumoniae may travel within mononuclear phagocytes to tissues expressing inflammatory markers. Indeed, the organism has been shown to increase adherence of infected monocytes to vascular endothelial cells through modulating integrin adhesion molecule interactions. The organism can infect a variety of cell types, including endothelial cells, macrophages, and fibroblasts, and expresses inflammatory moieties such as Chsp60 and lipopolysaccharide to dysregulate cellular lipid metabolism, induce lipoprotein oxidation, initiate inflammatory cytokine cascades and matrix metalloproteinases, and modulate adhesion molecule expression. The pathogenesis of ARMD involves alterations in subretinal lipid metabolism and oxidation, as well as expression of several inflammatory modulators, thus, C pneumoniae may mediate many of these events if present in local tissue. Ongoing studies are aimed at determining if the pathogen can be detected in ARMD tissue and propagated from extracted CNVM.

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