Enhanced Progression of Early Carotid Atherosclerosis Is Related to Chlamydia pneumoniae (Taiwan Acute Respiratory) Seropositivity

Dirk Sander, MD; Kerstin Winbeck, MD; Jürgen Klingelhöfer, MD; Thorleif Etgen, MD; Bastian Conrad, MD

Background—Chlamydia pneumoniae (Cp) infection has been associated with atherosclerosis and has been proposed as a possible additional cardiovascular risk factor. However, the relationship between Cp seropositivity and the progression of early carotid atherosclerosis is not unequivocally clarified.

Methods and Results—We evaluated the association between serological detection of Cp IgG and/or IgA antibodies and the progression of the intima-media thickness (IMT) of the common carotid artery using duplex ultrasonography in a prospective study with a follow-up of 3 years in 272 consecutive patients with cerebrovascular disease. Cp-seropositive patients showed a significantly enhanced progression of the IMT even after adjustment for other cardiovascular risk factors (0.12 mm/y [95% CI 0.11 to 0.14] versus 0.07 mm/y [0.05 to 0.09]; P < 0.005). Patients with increased C-reactive protein (>0.5 mg/dL) and Cp seropositivity showed the most pronounced IMT progression. Multivariate regression analysis revealed Cp seropositivity to be an independent risk factor for progression of early carotid atherosclerosis. Cox proportional-hazard regression analysis demonstrated a significantly increased rate of cerebrovascular and cardiovascular events in patients with Cp seropositivity, particularly in patients with increased C-reactive protein levels.

Conclusions—Our data support the importance of chronic inflammation and infection for the early stages of atherosclerotic development. (Circulation. 2001;103:1390-1395.)

Key Words: carotid arteries ▪ cardiovascular diseases ▪ atherosclerosis ▪ Chlamydia pneumoniae

Atherosclerosis and related diseases are a major cause of morbidity and mortality worldwide. Differences in the prevalence of conventional risk factors do not account fully for temporal and geographical variations in the prevalence of these diseases.1 Consequently, intense research is focused on seeking other atherogenic risk factors. Epidemiological studies have implicated bacterial infection in the pathogenesis of cardiovascular and cerebrovascular disease. In particular, Chlamydia pneumoniae (Cp) has been suggested to be a causative pathogen. An association between antibodies to Cp and ischemic heart disease (IHD) was first reported in a case-control study from Finland.2 The evidence for Cp as a potential causative agent is strong and is based on findings of numerous seroepidemiological studies,3–6 in vitro animal models,7 and recently, pilot antichlamydial antibiotic intervention trials.8–10 Cp could contribute to pathogenesis by a number of mechanisms11 and even at early stages in the atherogenic process. It could be assumed that the development of atherosclerotic lesions is slow and increases over time.

The use of B-mode ultrasonography offers the opportunity to assess the intima-media thickness (IMT) of the common carotid artery (CCA) as a suitable marker for preclinical atherosclerosis.12 Recently, a large trial identified the IMT as a strong predictor of stroke and myocardial infarction in healthy elderly adults.12 We used this technique to prospectively determine the relationship between Cp seropositivity and progression of early atherosclerotic formation.

Methods

Subjects
From a series of 312 initially evaluated consecutive inpatients >55 years old (mean age 64 years [95% CI 62 to 67 years]; 170 men) admitted to the Department of Neurology, Technical University of Munich, because of a first-ever cerebral ischemia, 290 patients with transient ischemic attacks (TIAs; n=78) or ischemic stroke (n=212) were included in this follow-up study. The study was conducted between 1995 and 1998. Six patients were excluded because Cp testing revealed an acute infection or reinfection, and 8 patients were excluded because a carotid endarterectomy was performed because of high-grade symptomatic carotid stenosis. Follow-up was not possible in 8 patients because these patients decided not to participate further in the study (n=3) or because no further follow-up information could be obtained (n=5). We found no significant differences for age, sex, incidence of Cp seropositivity, and several cardiovascular risk factors between the follow-up group and the
excluded patients. Follow-up was possible for 3 years in 272 of the 290 patients, whereas 18 patients died during the 3-year follow-up period. This study was approved by the local institutional review board. Follow-up information on current health status, medical history, drug use, and former cardiovascular risk factors was obtained by a computerized questionnaire every year on the day of the ultrasound examination at the department of neurology. All fatal and nonfatal events were independently coded by 2 physicians (D.S. or J.K.). Copies of hospital records, autopsy records, and death certificates were available. Cardiovascular events were defined as myocar-
dial infarction and sudden death; cerebrovascular events were defined as recurrent stroke or TIA's. Risk factors determined included smoking status, duration of smoking, arterial hypertension (treatment with antihypertensive medication or documented blood pressure $\geq 140$ mm Hg systolic or $\geq 90$ mm Hg diastolic before admission), diabetes mellitus (treatment with antidiabetic drugs or diagnosis of diabetes during hospital stay), body mass index, prevalent IHD (documented by previous myocardial infarction or angina pectoris, bypass surgery, or $>50\%$ angiographic stenosis of $\geq 1$ major coronary artery), cholesterol, and triglycerides. During follow-up, the medical treatment of the different risk factors was comparable for both subgroups.

Laboratory Examinations

Nonfasting blood samples were drawn from each subject within 6 hours after hospitalization. Serum was separated by centrifugation within 6 hours and stored at $-20^\circ\text{C}$ until analysis. Cp (TWAR) titers were measured by microimmunofluorescence using Maxiscreeen Chlamydia MIF slides (IO International Ltd) and fluorescenc conjugated Cp species-specific antihuman immunoglobulins initially and after 3 years of follow-up. Only an even pattern of elementary body fluorescence was regarded as positive. In every batch of slides tested, 2 control serum preparations known to be positive for this organism and 2 negative control subjects were each applied to 2 slides. IgA titers $\geq 1:16$ and IgG titers $\geq 1:64$ were taken as positive according to previous studies using microimmuno flourescence techniques to analyze the relationship between carotid atherosclerosis and Cp.\textsuperscript{13,14} Acute infection or reinfection just before testing was presumed to be indicated by titers of IgG $\geq 1:512$ and IgM $\geq 1:8$.

C-reactive protein (CRP) concentration was measured initially within 6 hours after hospitalization and every year on the day of the ultrasound examination. CRP was determined in serum specimens with a Dimension RxL clinical chemistry analyzer with CRP Flex reagent cartridges. The assay range was 0.05 to 12 mg/dL. A concentration $\geq 0.5$ mg/dL was defined as pathologically increased according to the reference values of our laboratory.

Ultrasound Imaging

The initial duplex ultrasonography and the follow-up investigations (every year) were performed by the same investigat-ors using a 7.5-MHz linear array transducer. Both internal carotid arteries were categorized as normal, plaques (1% to 29% reduction), moderate (30% to 70% reduction), and severe stenosis ($>70\%$ reduction) according to the ECST criteria.\textsuperscript{15} The measurements of CCA IMT were done according to the ARIC study protocol.\textsuperscript{16} When an optimal longitudinal image was obtained, it was stored on a videotape. This procedure was repeated 3 times for each side.\textsuperscript{17,18} The longitudinal B-scans frames were digitized and analyzed with a computerized image analysis system by an investigator blinded to the Cp results. IMT measurements were performed 8 to 18 mm proximal to the tip of the flow divider. In this 1-cm segment, 11 measurements of the IMT of the far wall were automatically attempted at 1-mm increments with the image analysis system, and the IMT of the segment was estimated as the mean of these 11 measurements.\textsuperscript{17,18} To enhance the reproducibility of carotid measures, standardized interrogation angles were used according to the recommendations described previously.\textsuperscript{16} From the average of 3 images per artery, a mean lumen diameter and a mean IMT (1/2 [left plus right]) were taken as measures of current lumen diameter and wall thickness of the CCA. In every patient, the follow-up measurements were performed at the same location as in the initial measurement. The Spearman correlations between all the IMT measurements at baseline and all the measurements performed 3 years later were 0.88 (Cp-positive) and 0.84 (Cp-negative), indicating a good reproducibility of the IMT measurements during follow-up. The intraindividual reproducibility between the 3 baseline IMT measurements was high ($r=0.95$). The progression of early carotid atherosclerosis was defined as the difference between the last and first IMT measurements and was normalized as the change of IMT per year.

Statistical Analysis

All values are given as mean and 95% CI. Independent t tests were used to test differences between the 2 groups. Adjustment for multiple comparisons was done by the Bonferroni method. The variation in IMT between subgroups according to age, pack-years of smoking, CRP, prevalent IHD, cholesterol, triglycerides, and systolic and diastolic blood pressure was tested with an ANCOVA using SYSTAT (Systat Inc). Because the CRP was highly skewed (Kolmogorov-Smirnov test), the CRP levels were natural-log transformed before further analysis. Linear multivariate regression analysis was performed by forward selection followed by backward elimination of covariates, resulting in an equation in which only covariates that significantly increase the predictability of the dependent variable are included. All covariates included in the final model were tested for interactions with each other and were corrected for collinearity if necessary. Age, pack-years of smoking, diabetes, cholesterol, triglycerides, systolic and diastolic blood pressure values, CRP, IHD, and Cp seropositivity were selected as independent variables; the IMT as the dependent variable. The IMT data were checked for normality (Kolmogorov-Smirnov test; not significant) and were entered into the model as continuous values. The outcome events studied were fatal plus nonfatal cardiovascular (myocardial infarction, sudden death) and cerebrovascular (recurrent TIA or stroke) morbidity events. Survival curves were estimated by the Kaplan-Meier product-limit method. Hazard ratios were calculated with the Cox proportional-hazard regression model. A calculated difference of $P<0.05$ was considered to be statistically significant.

Results

Overall, 125 patients (46%) were Cp-seropositive (IgG $\geq 1:64$ and/or IgA $\geq 1:16$). Cp IgG seropositivity was observed in 123 patients (45%) and Cp IgA seropositivity in 112 (41%). No significant differences between the Cp-seropositive patients and the Cp-seronegative group were found for several cardiovascular risk factors (Table 1). In contrast, Cp-positive patients had a larger initial CRP and initial IMT and an increased incidence of ischemic heart disease (Table 1). In both groups, no significant CRP changes were observed during the follow-up CRP measurements. Even after 3 years of follow-up, the Cp-positive patients showed significantly increased CRP values compared with the Cp-negative group (0.69 [0.66 to 0.72] versus 0.58 [0.54 to 0.62]; $P<0.01$). The Cp-positive patients showed a significantly larger progression of IMT (Table 1). This association remains nearly unchanged after adjustment for the other risk factors used (Table 1). There were distinct differences, however, in the extent of IMT progression depending on the CRP level (Figure 1). Patients with increased CRP ($\geq 0.5$ mg/dL) and Cp seropositivity showed the most pronounced IMT progression (Figure 1). Comparing the different groups by ANCOVA and post hoc testing (Tukey-Kramer) revealed significant differences for IMT progression between seropositive patients with normal CRP (n=75) and seronegative patients with normal CRP (n=102, $q=8.7$, $P<0.001$) as well as seropositive patients with elevated CRP (n=50) and
seronegative patients with elevated CRP (n=45, q=6.4, P<0.001). In contrast, no significant differences for IMT progression were found between seropositive patients with normal CRP and seronegative patients with elevated CRP (Figure 1).

To evaluate the influence of the different risk factors on IMT progression, a stepwise multivariate regression analysis was performed (Table 2). In addition to Cp seropositivity (IgA and IgG), CRP, systolic blood pressure, age, diabetes, and pack-years of smoking were also significantly correlated with the IMT progression (Table 3). The association between IgA seropositivity and IMT was better than for IgG seropositivity (Table 2). All other risk factors tested did not significantly increase the predictability of the regression. The predicted model accounted jointly for 44% of the variation in IMT progression. No significant changes of the regression analysis were observed when IgG and IgA seropositivity are entered into the regression model as separate variables.

During follow-up, 36 (12.5%) of the 290 patients developed fatal (n=11) and nonfatal cardiovascular (myocardial infarction [n=7]; sudden death [n=1]) and cerebrovascular (recurrent TIA [n=8] or stroke [n=20]) events. Kaplan-Meier survival analysis (Figure 2) revealed a significantly higher rate of events in patients with Cp seropositivity even after adjustment for CRP and the other significant risk factors. Cox proportional-hazard regression analysis demonstrated a significantly increased rate of cardiovascular and cerebrovascular events in patients with Cp seropositivity (Table 3), particularly in patients with increased CRP levels. This association was weakened but
remained significant after correction for age, pack-years of smoking, systolic blood pressure, diabetes, and prevalent IHD (Table 3).

**Discussion**

In this prospective study, we observed a significant relationship between progression of CCA IMT and the incidence of future cardiovascular events and Cp seropositivity. The findings remain constant after control for age, sex, and several other conventional cardiovascular risk factors and imply that Cp infection is associated with the progression of early stages of carotid atherosclerosis in patients >55 years old with prevalent cerebrovascular disease. Strategies to take account of potential confounding variables are essential in studies of Cp antibodies. We therefore used multivariate regression analysis techniques to control for possible confounding variables. The multivariate model, however, includes Cp seropositivity as a strong and independent predictor of IMT progression.

In contrast to our findings, 2 recent cross-sectional studies in asymptomatic healthy individuals from an urban population observed no significant relationship between Cp seropositivity and IMT. Several factors can account for these different results: In both studies, healthy subjects without known cardiovascular disease and of younger age were investigated. In our patients, a more advanced atherosclerosis could be assumed. Moreover, we studied the progression rate of the IMT over ≥3 years, whereas both of the other investigations were cross-sectional. Our findings indicate that the association between CP seropositivity and early atherosclerosis may be more pronounced in high-risk patients for cardiovascular disease, more enhanced atherosclerosis, and probably older patients with longer infection times. Accordingly, 2 cross-sectional investigations observed a relationship between Cp IgG seropositivity and asymptomatic carotid atherosclerosis in individuals with more pronounced atherosclerotic lesions.

Studies like the present one, based on selected populations, obviously have natural shortcomings and restrictions. All patients suffered from cerebrovascular disease and were >55 years old. The results may therefore not be generalizable to other age groups or healthy subjects. Conversely, the strengths of this study are the homogeneous population, its prospective design, and careful follow-up. Various techniques are available to detect CP antibodies. In this study, we used the most widely taken microimmunofluorescence assay to determine Cp seropositivity, which has been used in

<table>
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<th>TABLE 2. Determinants of CCA IMT</th>
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<td><strong>Coefficient [95% CI]</strong></td>
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<td>Cp IgG seropositivity</td>
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<td>Cp IgA seropositivity</td>
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<td>Log CRP</td>
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<td>Systolic blood pressure</td>
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Values in brackets indicate 95% CI. The coefficient gives estimates for how much the dependent variable (IMT) will change if the respective variable is increased by 1 and the other variables were held constant.
immunological response in patients with advanced disease. Concern persists that a finding of Cp antigen or DNA or an association due to cross-reactions with antigens. Chlamydial lipopolysaccharide, which can produce spurious interpretation by an expert microscopist but avoids criticism of tests on the basis of chlamydial immune complexes or techniques, Cp antigen was detected in smooth muscle cells, in coronary artery.23 In addition to these studies, our findings point to an important role of chronic Cp infection even for the progression of early carotid atherosclerosis. In our investigation, we considered high IgG antibody levels to be markers of previous infections. Assessment of the chronicity of an infection is a complicated issue, however, and it is not clear whether increased levels of IgG antibodies reflect the duration of the infection, reactivation of a latent infection, reinfection, or some unknown immunological features of the host. The persistent presence of elevated IgA titers and specific immune complexes has been shown to reflect chronic Cp infection more exactly than detection of IgG antibodies.24 Using a multivariate approach, we observed a stronger association between IgA seropositivity and IMT progression compared with IgG. Similarly, Markus et al observed a significant relationship between Cp seropositivity and carotid artery stenosis (>50%) only for IgA. These findings may underscore that detection of IgA is a better parameter for chronic infection.

A further important finding of our study is that the IMT progression and risks associated with Cp seropositivity were strongly modified by CRP, a serological marker of chronic inflammation. We observed the most enhanced progression of IMT in patients with both Cp seropositivity and increased CRP levels. There is increasing evidence that one of the primary mechanisms in atherogenesis may be inflammation.25 CRP has been found to predict the risk of future myocardial infarction and stroke.26 It has been suggested that Cp is related to the pathogenesis of atherosclerosis by causing chronic systemic inflammation.11,21 Recently, the detection of Cp-reactive T lymphocytes in carotid atherosclerotic plaques suggests that cell-mediated immunity to Cp plays a role in the atherosclerotic process and that this process may involve autoimmunity.11 In addition, it could be demonstrated that treatment with roxithromycin seems to be effective in reducing the bacterial burden of Cp within carotid atherosclerotic plaques.10 These findings point to a possible chronic inflammation induced by Cp as a cause of the enhanced rate of IMT progression.

B-mode ultrasonography provides the opportunity to relate risk factors to atherosclerosis in patients with early lesions. Ultrasonographically determined increased IMT of the CCA was previously validated as a marker of atherosclerosis12 and was identified as a strong predictor of stroke and myocardial infarction in healthy adults >65 years old.12 Thus, it is possible that the link between Cp seropositivity and increased cardiovascular risk was the enhanced development of atherosclerotic lesions in the carotid and probably coronary beds. In conclusion, our data support the importance of chronic inflammation and infection for the early stages of atherosclerotic development.

References


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