MICROBIAL PATHOGENICITY

***Chlamydia pneumoniae* in coronary and iliac arteries of Japanese patients with atherosclerotic cardiovascular diseases**

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Recent studies suggest the association of atherosclerotic cardiovascular diseases with *Chlamydia pneumoniae* infection in western populations. It is of great interest whether such an association exists in Asians with their distinct genetic background. Symptomatic patients with coronary heart disease (29) or arteriosclerosis obliterans (10) who underwent directional endo-atherectomy were studied. Atherectomy specimens of coronary and iliac arteries were examined for *C. pneumoniae* by culture, nested PCR and immunohistochemical stain (IHC) with one *Chlamydia* genus-specific, two *C. pneumoniae* species-specific, and two *C. trachomatis* species-specific monoclonal antibodies. Among the 29 patients with coronary artery disease, *C. pneumoniae* was detected in the coronary arteries of 13 by IHC, 16 by PCR and 20 by IHC or PCR, or both. *C. pneumoniae* was also found in the iliac arteries of four patients by IHC, three by PCR and five by IHC or PCR, or both, of the 10 patients with arteriosclerosis obliterans. Attempts to isolate *C. pneumoniae* by culture were unsuccessful. The re-stenotic rate after atherectomy was higher in the *C. pneumoniae*-positive group than in the negative group, but not significantly so. These findings support the high incidence of *C. pneumoniae* in atherosclerotic lesions of symptomatic patients with coronary heart disease and arteriosclerosis obliterans in Asians.

Introduction

Chlamydia pneumoniae is the third species of the genus *Chlamydia* and is known as a leading cause of human acute respiratory tract infections worldwide [1]. *C. pneumoniae* frequently causes sporadic or epidemic community-acquired respiratory infections, such as pharyngitis, sinusitis, bronchitis and pneumonia. Recent sero-epidemiological evidence suggests an association between chronic *C. pneumoniae* infection and several diseases, such as asthma [2, 3], sarcoidosis [4] and atherosclerosis [5–12].

The association of *C. pneumoniae* with atherosclerosis in sero-epidemiological studies is based on the demonstration of elevated IgG or IgA antibody titres to *C. pneumoniae*, or both, in patients with coronary

heart disease, asymptomatic carotid atherosclerosis and ischaemic cerebrovascular disease. All of these studies have shown the adjusted Odds ratio for the association of *C. pneumoniae* with such atherosclerotic categories to be *c.* 2.0 [5–12]. Furthermore, several laboratories have succeeded in detecting *C. pneumoniae* in coronary and aortic atheroma samples, but not in normal arteries, by immunohistochemical staining (IHC), polymerase chain reaction (PCR) and electron microscopy [13–18]. However, there is controversy about the presence of *C. pneumoniae* in atherosclerotic lesions [19]. Whether *C. pneumoniae* has a direct causative role in the development of atherosclerosis also remains to be elucidated. Hitherto, all sero-epidemiological studies as well as studies demonstrating *C. pneumoniae* in atheroma have been made in western countries. Further data are required from other populations, such as from Asians, to consolidate this observation. The aim of the present study was to detect *C. pneumoniae* in atherectomy specimens from symptomatic patients with coronary artery disease and arteriosclerosis obliterans in a Japanese population.

Materials and methods

Subjects and tissues examined

Thirty-nine Japanese patients (aged 45–78 years; 27 males and 12 females) with ischaemic heart disease or arteriosclerosis obliterans treated consecutively by a first atherectomy from September 1993 to March 1994 at Saiseikai Shimonoseki General Hospital, Shimonoseki, Japan were enrolled in this study. The characteristics of these patients are shown in Table 1. All patients had stenotic lesions of the coronary or iliac arteries of >90%. Atherosclerotic plaques resected by directional endo-atherectomy were tested for the presence of *C. pneumoniae* by IHC with monoclonal antibody, PCR and culture. Serum samples were obtained from 25 of the 39 patients for chlamydial serology at the time of atherectomy and stored at -80°C until tested. Patients were monitored for the occurrence of re-stenosis until August 1997.

Additionally, when the proximal end of the venous graft was anastomosed to the ascending aorta, small pieces of the aortic lesions were removed by punch biopsy from 12 patients (aged 57–65 years) who were undergoing coronary artery by-pass surgery (CABS) from January 1994 to September 1994 and were examined by PCR and IHC. Although macroscopically less advanced atherosclerotic lesions were chosen for anastomoses, four of the aortic punched-out samples were classified as having intimal thickening and eight as having mild atheromatous plaques. Coronary artery samples of 10 paediatric patients who died of asphyxia, drowning or bacterial meningitis (age range 1 day–2 years) and two biopsy samples of the aorta from patients with aortic regurgitation (aged 19 and 22 years) were used as non-atherosclerotic controls.

Atherectomy specimens were immediately placed into *Chlamydia* transport medium SPG [20] and frozen at -80°C for isolation and PCR or into buffered formalin 10% for IHC.

IHC

After paraffin-embedding, all samples were sliced into 5- μm sections and stained by immuno-peroxidase by the avidin-biotin-peroxidase method with the DAKO LSAB kit (DAKO, Glostrup, Denmark). Slides of each specimen were stained with the following five monoclonal antibodies (MAbs): CF-2, a genus-specific MAb to chlamydial lipopolysaccharide (Washington Research Foundation, Seattle, USA) at 1 in 1000 dilution; two *C. pneumoniae*-specific MAbs, RR-402 (Washington Research Foundation) and AY-6 (53 kD outer-membrane protein-specific, Hitachi Chemical, Tokyo, Japan), both at a 1 in 1000 dilution; and two *C. trachomatis*-specific MAbs, KK-12 (Washington Research Foundation) and 1651 (MOMP specific, Virostat, ME, USA), both at a 1 in 100 dilution. Control slides with HEp-2 cell monolayers infected with *C.*

pneumoniae or *C. trachomatis* were run in parallel with each staining batch.

PCR

Two resected lesions were homogenised with a new tissue homogeniser for each patient and centrifuged at 15 000 rpm for 30 min. Pellets were resuspended in 100 μl of PCR buffer containing 50 mM KCl, 10 mM Tris-Cl, pH 8.3, NP40 0.5%, Tween 20 0.5% with proteinase K 1 mg/ml and incubated at 55°C for 1 h. After the reaction mixture was heated at 95°C for 10 min, DNA was extracted twice with equal volumes of phenol-chloroform-isoamyl alcohol and precipitated with isopropanol by standard methods and dissolved in 100 μl of H_2O [21]. The nested PCR test to detect the *C. pneumoniae*-specific 474-bp *Pst*I fragment reported by Campbell *et al.* [22] was used in this study. The first PCR was done with the HL-1 and HR-1 primer set and the second PCR by the newly designed primer set, ON-1, 5'-TTGAGCATATTCGTGAGG-3'; ON-2, 5'-GTACAGTTTCTCCGTTAG-3'. The sizes of the products of each PCR were 437 bp and 190 bp. The PCR amplification mixture contained 0.5 μM primers, 100 μM dNTPs, 5 mM MgCl_2 and 1 U Taq DNA polymerase (Takara, Kyoto, Japan) for the first and the second PCR. Twenty μl of sample were used for the first PCR and 2 μl of the first PCR product for the second PCR. The samples were denatured initially at 94°C for 5 min and the mixtures were subjected to 30 thermal cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min with a 5-min additional extension for the last cycle. PCR products were evaluated by DNA size and restriction pattern with *Acc*I digestion. This PCR technique allows the detection of 5.0×10^{-3} ifu for *C. pneumoniae* strain TW-183.

Isolation by culture

The resected atherosclerotic plaques were homogenised in SPG with a tissue homogeniser and inoculated on to a HEp-2 cell monolayer in 96-well microtitration plates by centrifugation at 1700 *g* for 1 h followed by cycloheximide (1 $\mu\text{g}/\text{ml}$) treatment as described previously [23]. After incubation for 72 h at 35°C , plates were fixed and stained by the indirect fluorescent antibody technique with *C. pneumoniae*-specific MAb RR-402. At least three cell passages were done for each specimen.

Serology

Titres of IgG, IgA and IgM antibody to *C. pneumoniae* were measured in serum by a micro-immunofluorescence test [24]. Formalinised whole elementary bodies of *C. pneumoniae* strain TW-183 were used as an antigen.

Data collection and analysis

All data were verified by a retrospective review of the patient records. Statistical comparisons were performed by the χ^2 test with Yates' correction for categorical variables. Fisher's exact test was used when the minimum estimated value was < 5 . Continuous variables were analysed with the Mann-Whitney test. A p value < 0.05 was considered significant.

Results

Demographic data for the atherosclerotic patients undergoing a first atherectomy are listed in Table 1. The only apparent difference in clinical characteristics between patients with coronary artery disease and those with arteriosclerosis obliterans was the greater rate of habitual smoking in the latter, although this and other differences were not statistically significant. *C. pneumoniae* was detected in 55% (16 of 29) by PCR and 46% (13 of 28) by IHC of atherectomy specimens from coronary arteries of patients with ischaemic heart disease. Of the samples from patients with arteriosclerosis obliterans of iliac arteries, *C. pneumoniae* was demonstrated in 30% (3 of 10) by PCR and 40% (4 of 10) by IHC. Overall, *C. pneumoniae* was detected in 69% (20 of 29) of atherosclerotic coronary arteries and in 50% (5 of 10) of atherosclerotic iliac arteries by either PCR or IHC, or both (Table 2). All attempts to isolate *C. pneumoniae* by culture were unsuccessful. Of the 12 punch samples of aortic lesions obtained during

CABS, one (8%) was positive for *C. pneumoniae* by PCR and IHC. This *C. pneumoniae*-positive sample was classified as a mild atherosclerotic plaque. None of the 10 control samples from paediatric patients or of the two aortic biopsy samples was positive by IHC or PCR or both. Representative results of IHC are shown in Fig. 1.

In IHC no discrepant results among the three MABs (CF-2, RR-402, and AY-6) were observed. All samples were negative in IHC with two *C. trachomatis* species-specific MABs.

Serum IgG, IgA and IgM antibodies against *C. pneumoniae* were positive in 25 (100%), 7 (28%) and none (0%), respectively, of the 25 patients who were studied serologically. The mean and range of IgG serum titres of *C. pneumoniae* were 280 and 32–4096, respectively, and 10 patients (40%) had IgG titres ≥ 512 . The range of IgA serum titres in seven positive patients was 8–64. The rate of positivity for antibodies to *C. pneumoniae* did not differ significantly between patients positive or negative for *C. pneumoniae* in their coronary or iliac arteries.

The clinical characteristics and distribution of risk factors for atherosclerosis according to positivity for *C. pneumoniae* in atherectomy specimens are listed in Table 3. The rate of re-stenosis after atherectomy was higher in the *C. pneumoniae*-positive group (13 of 25, 52%) than in the negative group (4 of 14, 29%), although this difference was not statistically significant

Table 1. Characteristics of 39 patients undergoing atherectomy in the present study

Characteristic	Coronary heart disease	Arteriosclerosis obliterans	p
Number of patients	29	10	
Gender (male: female)	19:10	8:2	
Age: mean (range)	65 (47–78)	64 (45–73)	
Indication for atherectomy (n)	Unstable angina pectoris (14) Myocardial infarction (15)	Arteriosclerosis obliterans (10)	
Stenotic lesion (n)	Left coronary artery (22) Right coronary artery (7)	Iliac artery (10)	
Cardiovascular risk factor (percent of patients)			
Hypertension	62	70	NS
Hyperlipidaemia	38	20	NS
Diabetes mellitus	31	20	NS
Habitual smoking	55	80	NS
Family history of CAD	68	60	NS

CAD, coronary artery disease; NS, not significant.

Table 2. Results of detection of *C. pneumoniae* from atherectomy specimens of atherosclerotic lesions

Sample tested	Number of positive results/number tested (%)			
	PCR	IHC	PCR or IHC or both	Culture
Atherectomy specimens of				
coronary heart disease (n = 29)	16/29 (55)	13/28 (46)	20/29 (69)	0/18 (0)
arteriosclerosis obliterans (n = 10)	3/10 (30)	4/10 (40)	5/10 (50)	0/6
Punch biopsies of aortic lesions in patients for CABS	1/12 (8)	1/12 (8)	1/12 (8)	NT
Control* (n = 12)	0/2 (0)	0/12 (0)	0/12 (0)	NT

IHC, immunohistochemistry; CABS, coronary artery bypass surgery; NT, not tested.

*Control, autopsy samples of coronary arteries from 10 paediatric patients and two biopsy specimens of aorta from patients aged 19 and 22 years with aortic regurgitation as control without atherosclerosis.

Fig. 1. A representative feature of immunohistochemical staining for *C. pneumoniae* in atherosclerotic lesions resected by atherectomy. **a**, positive staining (dark stain) with *C. pneumoniae*-specific MAb AY-6, localised in the deep layer of the atheroma ($\times 180$). **b**, no staining of the same tissue as **a** with *C. trachomatis*-specific MAb KK-12 ($\times 180$). **c**, negative staining with AY-6 in the coronary artery sample from a 6-month-old patient who died from sudden infant death syndrome (negative control, $\times 180$).

($p = 0.10$). Clinical factors such as age, sex, habitual smoking, hyperlipidaemia, hypertension, a family history of coronary artery disease and serum IgG or

IgA antibody titres against *C. pneumoniae* did not affect significantly the incidence of tissue positivity for *C. pneumoniae* in atherectomy specimens. A

Table 3. Demographic data and distribution of risk factors for atherosclerosis of patients with or without *C. pneumoniae* in atherectomy specimens

Parameter	<i>C. pneumoniae</i> positive group (n = 25): number (%)	<i>C. pneumoniae</i> negative group (n = 14): number (%)	p value
Mean age (SD)	63.9 (6.6)	65.4 (10.9)	NS
Male/female	20/5	7/7	NS
IgG antibody to <i>C. pneumoniae</i> geometric mean titre (SD)	1:294 (1:184)	1:256 (1:128)	NS
Diagnosis			
Unstable angina	12 (48)	7 (50)	NS
Post-myocardial infarction	8 (32)	2 (14)	NS
Arteriosclerosis obliterans	5 (20)	5 (36)	NS
Re-stenotic lesion	13 (52)	4 (29)	NS
Habitual smoking	17 (68)	7 (50)	NS
Hypertension	17 (68)	8 (57)	NS
Hyperlipidaemia	10 (40)	3 (21)	NS
Diabetes mellitus	10 (40)	1 (7)	<0.05
Family history of CAD	17 (68)	8 (57)	NS

NS, not significant; CAD, coronary artery disease.

statistically significant positive association was noted only between the rate of diabetes mellitus and a positive tissue test for *C. pneumoniae*.

Discussion

There is controversy about the presence of *C. pneumoniae* in atheroma of coronary arteries [19]. The results of this study support its presence in atherosclerotic lesions of coronary and iliac arteries in symptomatic patients. The rate of tissue positivity for *C. pneumoniae* in this study, i.e., 69% in atherosclerotic coronary arteries and 50% in atherosclerotic large arteries in Japanese patients, is comparable to the 51–79% found in previous studies of western populations [13–18]. This is the first report of positive results of atherosclerotic tissue tests for *C. pneumoniae* in Asians, whose seropositive rate by age is similar to that in western populations [25]. Furthermore, the positivity rate by PCR and IHC for *C. pneumoniae* of aortic atherosclerotic lesions from patients undergoing CABS was lower (8%) than that of atherectomy specimens of symptomatic patients in the present study ($p = 0.01$). In addition, the degree of atherosclerosis in the lesions was less.

The control tissue specimens examined were not age-matched to the case specimens in this study, although none was *C. pneumoniae*-positive. In a previous study with age- and sex-matched coronary artery segments from normal young adults, most of the atheromatous plaques were positive for *C. pneumoniae*, but no normal control coronary artery was positive [26]. These findings provide evidence for a possible involvement of *C. pneumoniae* in the pathogenesis of atherosclerosis in the coronary and iliac arteries.

Several discrepant results of PCR and IHC were observed in the present study. Of 25 *C. pneumoniae*-positive samples, 11 were positive by both PCR and

IHC, eight by PCR only and six by IHC only. The same discrepancy was shown in previous studies [14, 26]. This discrepancy may be caused by the use of different pieces of atherectomy samples for each test, because it has been reported that *C. pneumoniae* is present in localised areas of a lesion [26]. Another possibility is that several specimens may contain PCR inhibitors, despite attempts to remove them by phenol-chloroform DNA extraction.

C. pneumoniae could not be isolated by culture from atherectomy samples, even though fresh atherectomy specimens were frozen and handled by the same methods that have been used successfully to isolate the organism from nasopharyngeal specimens. So far, failure to isolate *C. pneumoniae* from coronary arteries has been reported in two studies. Autopsy samples were used in one [15] and atherectomy samples in the other [19]. All studies, including the present study, failed to isolate a living organism. However, Ramirez *et al.* recently reported the isolation of a strain of *C. pneumoniae* from an atherosclerotic coronary artery of a fresh explanted heart of a patient undergoing heart transplant [27]. γ -Interferon, tumour necrosis factor and nutrition deprivation induce persistent, non-productive chlamydial infection [28]. Furthermore, even though it has been established that in *C. trachomatis* infections, chlamydial antigen or DNA is sometimes detected by non-cultural diagnostic tests that do not depend on chlamydial viability, viable *C. trachomatis* organisms cannot be isolated from children with moderate to severe active trachoma [29]. The present study used one genus-specific and two species-specific MAbs for IHC and obtained exactly the same results. As the three MAbs are directed against different outer-membrane epitopes, the outer membrane of *C. pneumoniae* would seem to be intact. It is possible that most of the *C. pneumoniae* in atherosclerotic lesions may be present in a persistent, non-productive form and only a small portion in a productive, culturable form.

In a South African study, Puolakkainen *et al.* [30] reported that *C. pneumoniae* antigen and DNA were detectable in coronary arterial atherosclerotic lesions more frequently in patients with low IgG titres against *C. pneumoniae* than in those with higher IgG titres. In both the Seattle study [14] and the present Japanese study, *C. pneumoniae* IgG antibody titres did not significantly affect the incidence of a positive tissue result for *C. pneumoniae*. Although the reason for this discrepancy is unknown, further studies are needed to elucidate whether *C. pneumoniae* serology may provide guidelines for the presence of *C. pneumoniae* in atherosclerotic lesions.

Campbell *et al.* [14] suggested a higher *C. pneumoniae*-positive rate in re-stenotic tissues, but Muhlestein *et al.* [17] found a lower re-stenosis rate in *C. pneumoniae*-positive tissues, although the differences were not statistically significant in these studies. These lesions had not been examined for evidence of *C. pneumoniae* before the development of re-stenosis. In contrast, the present study examined native lesions and then followed the patients for subsequent re-stenosis for 3–4 years. A higher, but not significantly higher, re-stenosis rate was found in patients with a *C. pneumoniae*-positive test ($p = 0.10$). Although atherectomy has been replaced by percutaneous transluminal coronary angioplasty with stent implantation, further prospective studies of large numbers of samples will be required to clarify this finding.

An earlier sero-epidemiological study did not find any association between *C. pneumoniae* seropositivity and coronary heart disease in diabetic patients [31]. However, a statistically significant positive association was noted in the present study between the rate of diabetes mellitus and a positive tissue test for *C. pneumoniae*. It is well known that host defences are impaired in patients with diabetes mellitus. Abnormalities in adherence [32], chemotaxis [33], phagocytosis [32] and intracellular oxidative killing [34] contribute to the impaired function. Therefore, host defences against *C. pneumoniae*, a gram-negative intracellular bacterium, might be weak in diabetic patients and frequent or persistent *C. pneumoniae* infections may occur.

In conclusion, these results give further support to the presence of *C. pneumoniae* in atherosclerotic lesions of coronary and large arteries in symptomatic patients with atherosclerotic cardiovascular diseases. Further studies are required to clarify the direct causative role of *C. pneumoniae* in the pathogenesis of atherosclerosis.

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