INTERNATIONAL JOURNAL OF IMMUNOPATHOLOGY AND PHARMACOLOGY Vol. 17, no. 3, 301-306 (2004)

DETECTION OF *CHLAMYDIA PNEUMONIAE* IN ATHEROSCLEROTIC CORONARY ARTERIES

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Received April 13, 2004 - Accepted August 28, 2004

***Chlamydia pneumoniae* has recently been associated with the development of coronary heart diseases by sero-epidemiological studies and by direct detection of the organism in atherosclerotic tissues. The aim of our study was to employ a semi-nested PCR approach to investigate the presence of *C. pneumoniae* in both normal and atherosclerotic coronary arteries of humans obtained at autopsy. Moreover, we have evaluated the role of infection with *C. pneumoniae* in relation to the extent of coronary atherosclerosis. One hundred and eighty coronary artery specimens were collected at autopsy from 60 consecutive subjects (three arterial segments from each subject). Atherosclerosis in each arterial segment was graded histologically by the Stary classification. Thirty normal coronary arteries were also taken at autopsy as control. PCR results evidenced the presence of *C. pneumoniae* DNA in atherosclerotic coronary arteries in 19 (31.7%) of 60 subjects examined, while none of the 30 subjects with non-atherosclerotic tissues was positive (p=0.001). Moreover, of the 180 atherosclerotic specimens examined, *C. pneumoniae* DNA was detected in 3.4% (2/59) of mild atherosclerotic lesions, and in 14.0% (17/121) of advanced atherosclerotic lesions (p=0.05). Our results demonstrate that the presence of *C. pneumoniae* DNA may be associated with the severity of coronary atherosclerosis.**

Chlamydia pneumoniae is an obligate intracellular pathogen which causes respiratory infections world-wide (1, 2). In addition to its role in respiratory disease, there is growing evidence that *C. pneumoniae* may be involved in the pathogenesis of atherosclerosis. It has been suggested that, after colonization of the respiratory tract, *C. pneumoniae* may diffuse through the bloodstream and, by leukocyte infiltration, may migrate into normal vessel walls or into vessels presenting pre-existing atheromatous lesions (3-5). The role of *C. pneumoniae* in the development and/or in the progression of atherosclerosis remains uncertain, despite the above evidence, the sero-epidemiological studies which suggest a direct association between *C. pneumoniae* infection and coronary heart disease (CHD), and that *C. pneumoniae* has been demonstrated in atherosclerotic lesions by electron microscopy, immunohistochemistry, and polymerase chain reaction (PCR), (6-10).

Additionally, *C. pneumoniae* has been reported in plaques obtained during atherectomy (11), in carotid endarterectomy samples (12-13), as well as in the walls of abdominal aortic aneurysms (14). Furthermore, *C. pneumoniae* has been detected in coronary artery fatty streaks and atherosclerotic lesions obtained at autopsy, even though some studies have failed to detect the organism in these tissues (8-9, 15).

Key words: Chlamydia pneumoniae, atherosclerosis, polymerase chain reaction, coronary arteries

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0394-6320 (2004)

The aim of our study was to investigate the presence of *C. pneumoniae* DNA in normal and atherosclerotic coronary arteries, obtained at autopsy, and to evaluate the role of the infection with *C. pneumoniae* in relation to the extent of coronary atherosclerosis.

MATERIALS AND METHODS

Study design

Between June 2001 and December 2002, 180 coronary artery specimens from 60 consecutive subjects (three specimens from each subject) were collected at autopsy and examined. The subjects were aged from 44 to 90 years and included 45 males (mean age 64 ± 12 years) and 15 females (mean age 68 ± 15 years). Of the 60 subjects considered, 32 (53.3%) died from causes related to CHD, while 28 (46.7%) died from non-CHD related causes.

Experiments were carried out on three left anterior descending coronary artery segments that were collected for each subject. Arterial segments were removed at different distances to their origin: about 0.5 cm (sample I); about 2.5-3 cm (artery intermediate tract, sample II); and about 5-6 cm (terminal opening vessels, sample III). Thirty normal coronary arteries were taken from 30 age and sex-matched subjects who died from non-CHD related causes, as a control. All samples were divided in two aliquots, one half was frozen at –70°C for PCR analysis, while the other half was directly processed for histological examination.

Histological examination

Each specimen was fixed in 10% buffered formalin, embedded in paraffin and stained with haematoxylin and eosin. The severity of atherosclerosis was graded according to the Stary classification (16). In this classification, lesions range from Grade 1 (initial lesion) to Grade 6 (plaque rupture, fissure, or hemorrhage). Subsequently, sections were divided in two groups: mild atherosclerotic lesions (Grades 1 to 3), and advanced atherosclerotic lesions (Grades 4 to 6).

PCR detection of C. pneumoniae DNA

Genomic *C. pneumoniae* DNA was detected in all samples by a semi-nested PCR methodology using total DNA preparations obtained from arterial tissues as templates, as has been previously described (4). DNA was extracted as described by Sambrook et al. (17). Briefly, frozen arterial tissues were finely minced and homogenized in DNA extraction buffer containing 10 mM Tris-HCl (pH 8.0), 100 mM EDTA, and 0.5% SDS. Proteinase K was added to a final concentration of 100 μg/ml and samples were incubated at 56°C overnight, followed by a 10 minute treatment at 98°C. After phenol-chloroform extraction and ethanol sodium acetate precipitation, the DNA was finally suspended in distilled water.

Semi-nested PCR was then performed by use of species-specific HL-1/HR-1 primer pair for the first round of 40 amplifications. The seminested primer pair HM-1/HR-1, which yields a 229-bp product, was then used in the subsequent 40 amplification cycles (18). Positive PCR results were confirmed by a nested-PCR assay (19) using the species-specific Cpn A/Cpn B primer pairs for the first round of 30 amplifications. The nested primer Cpn 1/Cpn 2, which yields a 268-bp product, was then used in the following 30 amplification cycles.

Samples that scored negative were further analysed by PCR, either spiked with *C. pneumoniae* DNA or assayed for the presence of the β-globin DNA gene (to assess the presence of human cellular DNA in the PCR mixtures). Each sample was tested in duplicate. For a specimen to be recorded as positive for the presence of *C. pneumoniae* DNA, at least one of two duplicate samples needed to be PCR positive. In order to minimize the risk of contamination, sample handling and DNA extractions, PCR amplifications and electrophoresis were carried out in separate rooms equipped with UV hoods.

Statistical analysis

Results were subjected to χ^2 analysis (significance at $p=0.05$), with Yates correction, to compare frequency distribution. When the minimum estimated expected value was less than 5, the Fisher exact test was used.

RESULTS

Histological examination

Each coronary artery specimen was subjected to histological examination and the severity of the atherosclerotic lesions (Stary classification) was determined, as described in the Materials and Methods section. Of the 180 atherosclerotic coronary segments examined 59 (32.8%) were classified as mild atherosclerotic lesions and 121 (67.2%) as advanced atherosclerotic lesions. Of the 59 mild atherosclerotic lesions, 37 were obtained from

Tab. I. *Severity of atherosclerosis in 180 coronary arteries, according to the Stary classification.*

LESIONS[a]	Subjects		Total (n= 180)
	CHD-related deaths	Non-CHD-related deaths	
Mild	37 (62.7%)	22 (37.3%)	59 (32.8%)
Advanced	59 (48.8%)	62 (51.2%)	121(67.2%)

[a]*Mild, atherosclerotic lesions classified Stary grades 1 to 3; Advanced, atherosclerotic lesions classified Stary grades 4 to 6.*

Tab. II. *Prevalence of C. pneumoniae DNA in subjects with atherosclerotic coronary arteries[a].*

	Subjects[b]		
	CHD-related deaths (n=32)	Non-CHD-related deaths (n=28)	Total (n=60)
PCR$^+$	12 (37.5%)	7 (25%)	19 (31.7%)

[a]*C. pneumoniae DNA was not detected in any of the 30 subjects with normal coronary arteries (p=0.001).*

[b]*Subjects dead of causes related to CHD versus subjects dead of non-CHD related causes, p=not significant.*

PCR$^+$, number of subjects with atherosclerotic coronary arteries scoring PCR-positive for C. pneumoniae DNA.

subjects who died from causes related to CHD and 22 from subjects who died from non-CHD related causes. As for the 121 advanced atherosclerotic lesions, 59 were from subjects who died from CHD, and 62 from subjects who died from non-CHD related causes (Tab. I).

Detection of C. pneumoniae DNA by PCR

Normal and atherosclerotic coronary arteries were examined for the presence of *C. pneumoniae* DNA by a semi-nested PCR approach, as described in the Materials and Methods section. The results obtained are shown in Table II and Figure 1. *C. pneumoniae* DNA was found in 19 (31.7%) of the 60 subjects with atherosclerotic coronary arteries, while none of the 30 subjects with normal coronary arteries were found to be positive (p=0.001) (Tab. II). Of these 19 subjects, *C. pneumoniae* DNA was found in 12 of 32 (37.5%) subjects who died from causes related to CHD, and in 7 of the 28 (25.0%)

Tab. III. *Presence of C. pneumoniae DNA in atherosclerotic coronary segments according to the severity of the lesions.*

	MILD LESIONS[a] Segments[b]				ADVANCED LESIONS[a] Segments[b]			
	I	II	III	**Total**	I	II	III	**Total**
CHD-related deaths								
PCR$^+$	1/10	0/13	0/14	**1/37**	4/22	2/19	5/18	**11/59**
Non-CHD related								
PCR$^+$	1/7	0/6	0/9	**1/22**	1/21	2/22	3/19	**6/62**
Total	**2/17**	**0/19**	**0/23**	**2/59**	**5/43**	**4/41**	**8/37**	**17/121**

[a]Mild lesions, Stary grades 1 to 3 according to the Stary classification; Advanced lesions, Stary grades 4 to 6.
[b]Arterial segments were taken at different distances: about 0.5 cm from its origin (sample I); about 2.5-3 cm (artery intermediate tract, sample II); and about 5-6 cm (terminal opening vessels, sample III).
PCR$^+$, number of PCR-positive samples for C.pneumoniae DNA/total number of segments analysed.

Tab. IV. *Prevalence of C. pneumoniae DNA in atherosclerotic coronary lesions[a].*

	Lesions[a]			p
	Mild (n=59)	**Advanced (n=121)**	**Total (n=180)**	
PCR$^+$	2 (3.4%)	17 (14.0%)	19 (10.5%)	0.05

[a]Mild lesions, Stary grades 1 to 3, according to the Stary classification; Advanced lesions, Stary grades 4 to 6.
PCR$^+$, PCR-positive samples for C. pneumoniae DNA.
p, statistical significance.

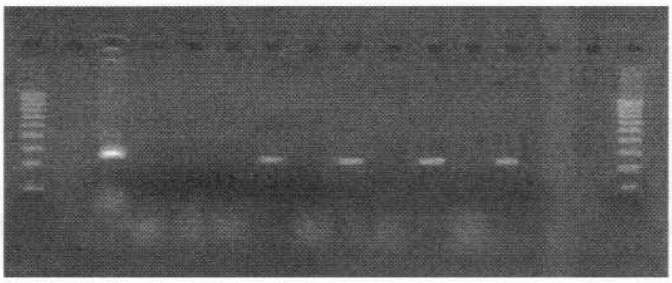

Fig. 1. *Nested PCR on atherosclerotic coronary arteries. Representative agarose gel showing the detection of a specific 268-bp fragment. Lane 5, 7, 9 and 11, samples that scored positive for the presence of C. pneumoniae DNA; lanes 3, 4, 6, 8 and 10, samples that scored negative; lane 1, positive C. pneumoniae DNA control (see Materials and Methods for details); lane 2, negative control.*
M: DNA molecular weight marker (100-bp ladder).

subjects who died from non-CHD related causes (p=not significant).

Table III shows the presence of *C. pneumoniae* DNA in the different segments of atherosclerotic coronary artery specimens classified according to the severity of the lesions. Of the 59 segments classified as mild atherosclerotic lesions, *C. pneumoniae* DNA was detected in only 2 segments (3.4%), while 17 (14%) of the 121 segments classified as advanced atherosclerotic lesions were found to be positive. This difference in chlamydial DNA detection between mild and advanced lesions was statistically significant (p=0.05) (Tab. IV).

No significant correlation was observed between the presence of C. pneumoniae DNA and the distance of the atherosclerotic coronary segments from their origin.

DISCUSSION

To our knowledge, this is the first study in Italy evaluating the presence of *C. pneumoniae* DNA in normal and atherosclerotic coronary arteries obtained at autopsy and the role of *C. pneumoniae* infection in relation to the extent of coronary atherosclerosis.

Major results of this study strongly suggest that *C. pneumoniae* can be associated with atherosclerosis since it was detected in atherosclerotic tissues and was not found in normal coronary arteries (p=0.001), and that the presence of the microorganism appears to be correlated with severity of coronary atherosclerosis (p=0.05).

The overall prevalence of *C. pneumoniae* DNA detected in our study was 31.7% (19 out of the 60 subjects with atherosclerotic coronary arteries).

Differences in PCR-detection of *C. pneumoniae* DNA in atherosclerotic coronary tissues have been reported. In these studies, detection rates varies considerably, ranging from 0% to 79% (9, 15, 20). These wide differences may possibly reflect a poor standardization of PCR methodology between the different laboratories. However, differences in prevalence of *C. pneumoniae* DNA might also be due to a focal localization of the organism in vascular tissues. In our study, a patchy distribution of *C. pneumoniae* in the atherosclerotic lesions was observed, with no segment of the left anterior descending coronary artery having significantly more *C. pneumoniae* present. As previously observed in other studies our results suggest that *C. pneumoniae* is localized within these lesions (9, 13), and highlight the importance of testing multiple segments along coronary arteries of the same subject. We would, therefore, recommend searching for *C. pneumoniae* DNA in whole coronary artery as a method to improve the change of detecting this organism.

As for the association between *C. pneumoniae* and the extent of coronary atherosclerosis, several studies have demonstrated a significant association between *C. pneumoniae* and the severity of coronary atherosclerosis, while others have failed to demonstrate such an association (10, 20-21). Additionally, our study established a statistically significant correlation between advanced atherosclerotic coronary arteries and the presence of *C. pneumoniae* DNA.

In conclusion, even if further studies are needed to assess the precise role of *C. pneumoniae* in the genesis of atherosclerosis, the results reported in this study further support the observation that this organism may play a role either in the formation, and/or in the progression, of atherosclerosis. With this in mind, it is possible to hypothesize that *C. pneumoniae* might interfere with the normal cellular physiology, triggering chronic tissue inflammation and leading to damage of vessel endothelia, as a possible mechanism.

ACKNOWLEDGEMENTS

This work was supported by Faculty 60% funds granted to R.S. - project "Studio sul meccanismo di apoptosi indotto da *Chlamydia pneumoniae*".

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