BACTERIAL PATHOGENICITY

Search for Chlamydia pneumoniae genes and their expression in atherosclerotic plaques of carotid arteries

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Samples of atherosclerotic tissue from 58 patients undergoing carotid surgery were analysed by tissue culture and PCR for Chlamydia pneumoniae; PCR was performed to detect Omp1, 16S rRNA and HSP-70 genes. To understand the active pathogenic role of C. pneumoniae, a reverse transcriptase-PCR (RT-PCR) assay was applied to detect the specific RNAs expressed either in the replicative form, or in the cryptic form found in chronic infection. The C. pneumoniae omp1 gene, encoding the major outer-membrane protein (MOMP), was detected in 13 of 58 samples. Among these, the result was confirmed in 11 samples after amplification of a further target, the 16S rRNA, and the presence of the HSP-70 gene, encoding heat-shock protein 70, was revealed in only five cases. All the samples were negative for evidence of specific RNAs by RT-PCR. The presence of genomic DNA and absence of specific RNAs in atherosclerotic tissue samples suggests a lack of an active metabolic or persistent infective role for C. pneumoniae. Thus, traces of C. pneumoniae DNA in these samples could be due to a degradative pathway of the host defensive cellular and biochemical mechanisms.

Introduction

The intracellular obligate parasite Chlamydia pneumoniae [1] has been linked to the development of atherosclerotic tissue by studies involving specific antibody detection, electron microscopy, immunohistochemistry, culture and PCR [2–7]. However, clinical trials on patients with coronary artery disease treated with antibiotics active against C. pneumoniae have not confirmed a definitive association between this agent and the risk of myocardial infarction [8–11]. Cardiovascular disease is the most important cause of mortality in the Western world, and although multiple metabolic and genetic factors (high plasma low-density lipoprotein concentration, systemic hypertension, diabetes mellitus, advanced age, male sex) are risk factors for atherosclerosis [12], the possible role of an infectious agent contributing to inflammation at the base of the atheroma has often been postulated [13–16]. In some studies, the relevance of C. pneumoniae in the aetiology of atherosclerosis has been less clear, suggesting that it is more likely to be an ‘innocent’ bystander [17–23]. This study reports the results of a search for three different C. pneumoniae genes, encoding the major outer-membrane protein (omp1), 16S rRNA and HSP-70, and their corresponding RNAs in 58 atherosclerotic tissue samples. The search for specific genes and their transcripts is a strategy that has been adopted previously, mRNA being a promising candidate as a marker of viability in bacteria which might detect the presence of the metabolically active micro-organism in atherosclerotic tissue [24–26]. Indeed the omp1 mRNA may represent the metabolic indicator for an acute and replicative form, while a higher amount of the HSP-70 mRNA could represent the metabolic marker during a chronic infection or in the presence of an abnormal cryptic form of C. pneumoniae [27, 28]. As these mRNAs have a rapid turnover, the study also investigated the presence of ribosomal 16S RNA, which can be considered a marker of permanent metabolic activity and is a relatively more stable RNA [24, 29, 30].

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Materials and methods

Patients and specimens

This study was approved by the Medical Ethics Committee of the Ospedale ‘Le Scotte’ of the University of Siena. A total of 58 patients (males 47, females 11, mean age 67 years, age range 40–88) underwent surgical endarterectomy (at the Institute of Cardiovascular Surgery of University of Siena) for symptomatic severe carotid artery stenosis (>70% narrowing of the lumen) verified by ultrasound examination. Informed consent was obtained from all patients before surgical intervention. Specimens re-

lected from the patients showed histopathological evi-
dence of severe atherosclerosis with fibrosis, calcifi-
cation and ulceration or haemorrhage, or both. Fragments of atheromatous tissue ranging from c. 30 to 45 mg were processed. The tissue was homogenised (BioPulverizer System, BIO 101, Vista, CA, USA) in 1.8–2.0 ml of sterile phosphate-buffered saline and used for C. pneumoniae culture and extraction of DNA and RNA. If not immediately processed, the specimen was frozen at −70°C.

Chlamydia culture

Flat-bottomed tubes containing a confluent monolayer of cycloheximide-treated HEP-2 cells (CCL-23; Ameri-
can Type Culture Collection, Rockville, MD, USA) were inoculated with 200–μl of homogenised sample as described elsewhere [31]. Twelve blind passages were performed 72 h after infection in triplicate; a 200-μl portion of resuspended infected cells was stored at −20°C for DNA extraction. At 72 h and 5 days after each blind passage, the infected monolayer was fixed in cold acetone and stained with genus- and species-

specific monoclonal antibodies (MAbs) for C. pneu-
moniae (Argene Biosoft, Varilhes, France) for ex-

amination by immunofluorescence. C. pneumoniae (CWL-
029 ATCC VR 1310) was propagated in HEP-2 cells; 200 μl of the infected suspension, containing 2 × 10^5 inclusion-forming units (ifu)/ml, were included in each culture assay as a positive control. The same amount of infected cells was added to a suspension (2 ml) of homogenised atherosclerotic tissue negative for C. pneumoniae and used as a simulated positive control for DNA and RNA extraction in the molecular assays with a final titre of 2 × 10^5 ifu/ml.

DNA extraction and PCR

Portions (400–600 μl) of the homogenised tissue sample were subjected to DNA extraction following the procedure described in the QIAamp Tissue Kit (Qiagen, Hilden, Germany). The DNA extracted was tested in PCR for a 268-bp fragment of the human β-
globin gene [32] to verify the suitability of the DNA specimen for amplification. Bacterial DNA was de-
tected with sets of primers specific for different regions of the C. pneumoniae genome: primers CpnA/Cpn3

specific for a 408-bp fragment of the omp1 gene [33];

primers CpnA/CpnB specific for a 463-bp fragment of the 16S rRNA gene [34]; and the primers Hsp70-1 (5′-

AAGTCGTAAGCTCCTACTC-3′, nt 112–132; MWG-Biotech GmbH, Ebersberg, Germany) and Hsp70-2 (5′-CTTCTACAAAGTCTCCTCA-3′, nt 858–878; MWG-Biotech) specific for a 766-bp fragment of the HSP-70 gene. The PCRs were performed as follows: denaturation at 94°C for 1 min, annealing at 52°C, 54°C and 56°C for 50 s with the primer pairs Cpn1/Cpn3, CpnA/CpnB, Hsp70-1/Hsp70-2 respecti-

vely, and primer extension at 72°C for 1 min. Five μl of the Cpn1/Cpn3, CpnA/CpnB, Hsp70-1/Hsp70-2 PCR products were subjected to nested amplification (nPCR) (30 cycles at 94°C for 45 s, at 58°C for 30 s and at 72°C for 40 s) with inner primers Cpn1/Cpn2 [24], Cpn/CpnD (CpnC: 5′-CAAGACGCGCCACCTTACAG-3′, nt 1041–1060; CpnD: 5′-GGTCAAAC CAACTCCCAATGA-3′, nt 1352–1372 MWG-Biotech), Hsp70-3/Hsp70-4 (Hsp70-3: 5′-CGATGTGGCGCTTCAAAGTG-3′, nt 312–331; Hsp70-4: 5′-CTAGACCGTCGACAG-3′, nt 661–680; MWG-Biotech) specific for fragments of 339, 331 and 359 bp of the

omp1, 16S rRNA and HSP-70 genes, respectively. All the PCR protocols were executed, including the dUTP-

uracil-N-glycosylase contamination prevention system (Promega). PCR was performed according to the recommended guidelines [35], including three negative controls and one positive control in each assay, starting from the nucleic acid extraction step. Amplification products were analysed by electrophoresis through an agarose 2% gel by standard methods [36]. This procedure was applied to each sample in duplicate. All the pairs of specific primers used for the detection of C. pneumoniae DNA had been tested previously on respiratory samples shown to be positive by Cpn1/Cpn3 PCR. The molecular diagnostic results were always confirmed.

RNA extraction and RT-PCR

Portions (800–1100 μl) of the homogenised tissue samples which were C. pneumoniae DNA-positive were subjected to guanidinium isothiocyanate-phenol-

chloroform RNA extraction as described elsewhere [37]. The average yields of total RNA obtained ranged from 18 to 30 μg. After RNA extraction and deoxy-
ribonuclease 1 RNAase-free (Sigma) digestion, the extract was suspended in 100 μl of DEPC-treated distilled sterile water. To establish the quality of the nucleic acid extracted and purified after DNA diges-
tion, 10 μl of the RNA sample were examined by RT-

PCR assay to detect the human ubiquitous glyceralde-
hyde-3-phosphate-dehydrogenase (GAPDH) mRNA

[38, 39]. Then 10 μl of the samples which were positive for the GAPDH mRNA were examined by RT-PCR with the antisense primers Cpn3, CpnB and Hsp70-2 (0.25 mmol/L). The CDNA was synthesised in a 25-μl reaction volume containing KCl 50mmol/L, Tris-HCl 10mmol/L (pH 8.3), MgCl2 5mmol/L, deoxy-
ribonucleoside triphosphate mixture 1mmol/L, RNAase inhibitor (Promega, Madison, USA) 20 U and Moloney murine leukemia virus reverse transcriptase (Promega) 40 U. The mixture was incubated at 37°C for 30 min and then denatured for 3 min at 94°C and cooled quickly in ice. At the same time, multiple negative controls (RNA extracted from uninfected HEp-2 cells and reagent control) were included, from the extraction step onwards. This procedure was applied to each sample in duplicate. All the pairs of specific primers used in the search for C. pneumoniae RNA had been tested by RT-PCR on the positive respiratory samples previously tested by Cpn1/Cpn3 PCR. The specific RNAs were always detected. The sensitivity of the C. pneumoniae RT-nested PCR (RT-nPCR) assay was then evaluated with 200 μl of the simulated positive control sample containing $2 \times 10^5$ ifu/ml. This sample was then serially diluted and subjected to the same RNA extraction procedure and DNAase treatment as the clinical samples.

Results

No chlamydia antigens were detected by immunofluorescence in the HEp-2 cells infected with homogenised tissue samples. To confirm this result, the same samples were analysed for the presence of C. pneumoniae DNA after each blind passage on cell culture. nPCR for the omp1 gene was negative. This nPCR was sufficiently sensitive to detect DNA corresponding to $4 \times 10^{-1}$ ifu of C. pneumoniae in a simulated positive control as well as in a suspension of infected cells. A sample corresponding to 400 ng of DNA extracted from atheromas was tested by PCR. However, in these samples containing more DNA, amplification of the human β-globin gene was unsuccessful, probably due to the presence of non-specific inhibitors [35]. Duplicate PCRs for the omp1, 16S rRNA and HSP-70 genes were negative in all 58 samples. The subsequent nested amplifications showed the presence of a 339-bp fragment of the omp1 gene in only 13 samples, two of which gave a positive results only in a single assay. The nested 16S rDNA PCR with CpnC/CpnD primers confirmed the positive results in 11 of 13 samples; in one of them the result was not confirmed in duplicate samples. The HSP-70 nPCR was positive in both duplicates for only 5 of the 13 samples (Fig. 1). The results are summarised in Table 1. The RT-nPCRs amplified fragments up to the dilution corresponding to $4 \times 10^{-1}$ ifu in the simulated positive sample, showing a sensitivity level similar to that obtained for DNA detection by nPCR (Fig. 2). The clinical samples that were positive in at least one of the PCRs performed on the different DNA targets were examined for the corresponding RNAs. To avoid a false positive signal due to the incidental presence of bacterial DNA during the total RNA extraction, the samples were digested with DNAase. All samples were positive for ubiquitous GAPDH mRNA, but none was positive for the detection of specific C. pneumoniae mRNA or rRNA (Table 1).

Discussion

Atherosclerosis develops as a response of the vessel wall to injury [12–16]. Fabricant et al. [40] showed that chickens experimentally infected with an avian

![Fig. 1. Gel electrophoresis of three representative samples subjected to nested PCR. Lanes 1–3, products of omp1, HSP-70 and 16S rRNA gene PCRs performed on sample no. 1; 4–6, products of omp1, HSP-70 and 16S rRNA gene PCRs performed on sample no. 2; 7–9, products of omp1, HSP-70 and 16S rRNA gene PCRs performed on sample no. 3; 10, negative controls; 11, blank controls, M, mol. wt standard (Φ × 174 digested with HaeIII).](image)

Table 1. Detection of C. pneumoniae genes and their RNA in atherosclerotic specimens by nested PCR and RT-nested PCR

<table>
<thead>
<tr>
<th>Number of patients</th>
<th>omp1</th>
<th>16S</th>
<th>HSP-70</th>
<th>omp1</th>
<th>16S</th>
<th>HSP-70</th>
</tr>
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<tbody>
<tr>
<td>(total = 58)</td>
<td>DNA</td>
<td>rDNA</td>
<td>DNA</td>
<td>mRNA</td>
<td>RNA</td>
<td>mRNA</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>6</td>
<td>+</td>
<td>+</td>
<td>–</td>
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<tr>
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<td>–</td>
<td>ND</td>
<td>ND</td>
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* positive; –, negative; ND, not determined.  
Two samples were not confirmed in duplicate.  
One sample was not confirmed in duplicate.
ABSENCE OF C. PNEUMONIAE RNA INATHEROMAS

protein family, is increased [27, 28]. Such a condition may exist in persistent, chronic infection or in atheromatous tissue, where a complex interaction between different cytokines and cell types occurs [44, 45]. The detection of specific mRNAs to demonstrate organism viability [24–26] in those samples with detectable C. pneumoniae DNA would help to clarify these observations. The present study was unable to detect specific RNA in any of the 13 samples positive for omp1, HSP-70, or 16S rRNA genes. One explanation as to why mRNA was not detected, even in the presence of the corresponding DNA, is that some mRNAs may be subject to rapid turnover. However, the absence of a more stable RNA, such as the 16S rRNA, confirms the absence of whole bacteria in biological samples.

The inflammatory process which occurs during the formation of atheroma could relate to other immune-pathogenetic mechanisms such as molecular mimicry between a persistent chlamydial antigen and host peptides [46], or to the induction of metabolic mechanisms, which do not require the viability of the bacterium [47, 48]. The results of the present study suggest that the C. pneumoniae DNA present in atherosclerotic tissue at the time of specimen collection does not correlate with an active replicative form nor a cryptic or silent form, but it could indicate the incidental presence of the micro-organism in the immune system cells. The presence of C. pneumoniae DNA may reflect the migration of immune cells containing non-viable C. pneumoniae into the inflamed vascular tissue.

References

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herpes virus developed vascular lesions similar to those of human atherosclerosis. Subsequently other investigators have recognised infectious agents (herpes virus, cytomegalovirus, Helicobacter pylori and C. pneumoniae associated with atherosclerosis and cerebrovascular disease [13, 41, 42]. Detection rates of C. pneumoniae in atherosclerosis have varied widely depending on the method used. No C. pneumoniae DNA in atherosclerotic lesions has been found in other studies [18–23]. As other studies have produced contradictory results, the possible role of C. pneumoniae in asymptomatic and chronic infection [27] is controversial. The present study analysed DNA extracted from atheromatous tissue samples by the PCR technique specific for three different targets: omp1, 16S rRNA and HSP-70 genes of C. pneumoniae. C. pneumoniae DNA was present in very few copies as shown by the fact that positive results were obtained only when nPCR was performed. Moreover, the same results were not always confirmed in duplicate for all the samples. The use of such a highly sensitive PCR raises the possibility of false-positive reaction. The use of multiple negative controls from the nucleic acid extraction phase onwards and working in accordance with the recommended guidelines would seem to exclude low levels of incidental contamination.

Under stress conditions, such as exposure to penicillin or interferon-γ, chlamydiae seem to enter a viable but non-culturable state leading to a persistent abnormal cryptic infection [27, 43]. This would explain the failure to culture C. pneumoniae in the present study. Transcription and translation are not affected, but the expression of MOMP is reduced and one of the other Omp proteins is inhibited [27]. On the other hand, the production of stress proteins, such as the heat-shock protein family, is increased [27, 28]. Such a condition may exist in persistent, chronic infection or in atheromatous tissue, where a complex interaction between different cytokines and cell types occurs [44, 45]. The detection of specific mRNAs to demonstrate organism viability [24–26] in those samples with detectable C. pneumoniae DNA would help to clarify these observations. The present study was unable to detect specific RNA in any of the 13 samples positive for omp1, HSP-70, or 16S rRNA genes. One explanation as to why mRNA was not detected, even in the presence of the corresponding DNA, is that some mRNAs may be subject to rapid turnover. However, the absence of a more stable RNA, such as the 16S rRNA, confirms the absence of whole bacteria in biological samples.