Matrix metalloproteinase-9 expression is associated with the presence of *Chlamydia pneumoniae* in human coronary atherosclerotic plaques

G Arno, J C Kaski, D A Smith, J P Akiyu, S E Hughes, C Baboonian

**Objective:** To investigate the association between *Chlamydia pneumoniae* and matrix metalloproteinase-9 (MMP-9) in atherosclerotic plaques.

**Design:** 31 coronary atherosclerotic plaque specimens were studied by immunohistochemistry, polymerase chain reaction (PCR), and reverse transcription PCR for the presence of *C pneumoniae* antigen and genomic DNA, and of MMP-9 protein and transcripts.

**Results:** Immunohistochemical analysis identified a strong association between the presence of *C pneumoniae* antigen and production of MMP-9 in coronary atherosclerotic plaques (p = 0.001). Furthermore, analysis of the intralosomal amount of *C pneumoniae* and MMP-9 indicated an increased number of cells positive for MMP-9 in arterial sections that had increased *C pneumoniae* positivity (p < 0.05).

**Conclusions:** This study provides evidence of an association between expression of MMP-9 and the intravascular presence of *C pneumoniae* and may suggest a potential pathological mechanism whereby *C pneumoniae* may contribute to the progression of coronary atherosclerosis.
15 minutes, washed briefly with phosphate buffered saline, and incubated with the primary antibodies. The primary antibodies, prepared in phosphate buffered saline containing four drops/ml biotin (Avidin/Biotin blocking kit, Vector Labs), were mouse anti-
C pneumoniae major outer membrane protein (clone RR402, Dako), mouse anti-MMP-9 (clone 56–2A4, Oncogene Biosciences), or mouse IgG3 (Sigma) used as a negative control. After an overnight incubation at 4°C, the sections were washed and incubated with biotin conjugated rabbit anti-mouse immunoglobulins (Dako) followed by streptavidin/fluorescein isothiocyanate (Dako).

Sections were then labelled with antibodies to the macrophage marker CD68 (Dako), α smooth muscle cell actin (Sigma), or endothelial cell CD31 (Dako) for one hour at room temperature, followed by incubation with biotin conjugated rabbit anti-mouse immunoglobulins (Dako) and streptavidin/7-amino-4-methylcoumarin-3-acetate (Jackson Labs). Immunostaining with the anti-C pneumoniae, anti-MMP-9, and anti-CD68 and the presence of foam cells were assessed by using the following grades: 1+, 2+, 3+, and 4+, corresponding to immunoreactivity in 1–10, 11–20, 20–30, and over 30 cells per ×400 microscope field, and 0 for negative sections. To determine the stage of atherosclerosis, sections were analysed by haematoxylin and eosin staining and the plaques were classified according to the recommendations by Stary et al.25

Table 1  Summary of immunohistochemical analysis, PCR, RT-PCR, and plaque grade data

<table>
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<tr>
<th>Patient</th>
<th>Age (years)/sex</th>
<th>Macrophage grade</th>
<th>Foam cell grade</th>
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<th>MMP-9 RT-PCR</th>
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</table>

Immunohistochemistry data shown as grades 0 to 4+.
*Classified according to the recommendations by Stary et al.25
C pneumoniae, Chlamydia pneumoniae; F, female; M, male; MMP-9, matrix metalloproteinase-9; NT, not tested; PCR, polymerase chain reaction; RT-PCR, reverse transcription polymerase chain reaction.

Positive control slides of C pneumoniae infected Hep-2 cells (Dako) were also analysed.

Detection of C pneumoniae DNA by polymerase chain reaction

C pneumoniae genomic DNA was detected by nested polymerase chain reaction (PCR). Briefly, 5 mm long artery sections were digested for three hours at 55°C in 5% sodium dodecyl sulfate and 400 μl/m proteinase K in tris-EDTA buffer pH 8.0 followed by phenol chloroform DNA extraction and ethanol precipitation. After DNA quantification, 500 μg of DNA was subjected to nested PCR with oligonucleotide primers specific for the major outer membrane protein of C pneumoniae (first round: sense, 5′-TACAAAGGCTTGCTTGGAGG-3′; antisense, 5′-GGAGTCCAAAATGTITAGG-3′. Second round: sense, 5′-TTAATAGTGTAGTACCAATA-3′; antisense, 5′-ATCTCACGGCAGTATAGTT-3′) according to procedures described elsewhere.25 The resulting 207 base pair amplicons were analysed by agarose gel electrophoresis. Amplification of the housekeeping gene myoglobin served as a control confirming the integrity of the extracted DNA.25

Detection of MMP-9 transcript by reverse transcription PCR

MMP-9 transcription was analysed by reverse transcription (RT) -PCR as described.25 Briefly, total RNA was isolated from 5 mm long artery sections with ultrapure TRIzol (Gibco) and reverse transcribed into cDNA with the Superscript II preamplification kit (Gibco). The cDNA was subjected to PCR with oligonucleotide primers specific for human MMP-9 (sense, 5′-CAGTTCACCCCTCTAGAGC-3′; antisense, 5′-GCCACTTGTCGGCAGGATT-3′).25 To confirm the integrity of the extracted RNA, transcription of the housekeeping gene
MMP-9 and C pneumoniae

RESULTS

Immunohistochemical analysis of human coronary artery specimens

Analysis of human coronary atherosclerotic lesions by immunohistochemical staining showed an association between intraluminal presence of C pneumoniae and increased expression of MMP-9. Immunoreactive chlamydial antigen was detected in 22 of 31 specimens (71%) and immunoreactive MMP-9 was detected in 27 of 31 specimens (86%) (table 1). The positive chlamydial staining was re-examined and confirmed in 18 available specimens with the C pneumoniae antibody and a second monoclonal antibody to a distinct chlamydial antigen (fig 1). Incubation of specimens with negative control mouse antibodies resulted in no staining. The four samples that were negative for MMP-9 were also negative for C pneumoniae (table 1). These data support that detection of MMP-9 in coronary atherosclerotic plaques is associated with the intraluminal presence of C pneumoniae (p = 0.001). Furthermore, analysis of the intraluminal amount of C pneumoniae positivity and MMP-9 production by the grading system outlined above indicated that the number of cells positive for MMP-9 was apparently increased in arterial sections that had a higher level of C pneumoniae positivity. Analysis of these data showed that there was a significant association between the extent of chlamydial infection and the amount of MMP-9 production (p < 0.05).

Identification of C pneumoniae genomic DNA and MMP-9 transcripts in human coronary artery specimens

Nested PCR analysis identified the presence of C pneumoniae genomic DNA in four of 31 specimens (13%). Although this detection rate is lower than that of the immunohistochemical analysis, all four samples that were positive by nested PCR were also positive by C pneumoniae immunostaining. RT-PCR analysis of total cellular RNA showed the presence of MMP-9 transcripts in 16 of 28 specimens (57%). Comparison of the RT-PCR and immunohistochemical detection of MMP-9 showed a significant linear trend (p = 0.024). All four specimens positive for C pneumoniae by nested PCR showed MMP-9 production by both RT-PCR and immunohistochemistry.

Figure 1  Immunohistochemical staining of 5 μm sections of a coronary endarterectomy specimen with monoclonal antibodies to two distinct antigens of Chlamydia pneumoniae. Sequential sections stained with (A) fluorescein isothiocyanate (FITC) labelled C pneumoniae major outer membrane protein antibody (clone RR402) and (C) on IgG3 isotype control antibody. Sequential sections of the same specimen stained with (B) FITC labelled C pneumoniae heat shock protein-60 antibody (clone A57-B9) and (D) an IgG1 isotype control antibody. Arrows indicate positively stained cells. L, artery lumen. (Original magnification ×400).
DISCUSSION

This study provides evidence of an association between increased MMP-9 production and the presence of *C. pneumoniae* in human coronary atherosclerosis and may provide evidence for a potential mechanism by which the bacteria may accelerate or exacerbate coronary atherosclerotic disease.

*C. pneumoniae* has been associated with atherosclerosis and coronary artery disease, although the mechanism by which the bacterium may affect disease initiation or progression is poorly understood. Recent evidence shows that *C. pneumoniae* can infect, survive in, and stimulate macrophages, smooth muscle cells, and endothelial cells, resulting in foam cell formation and MMP-9 production by macrophages in vitro. MMP-9 is an important contributing factor to atherosclerotic disease and is upregulated in atherosclerotic plaques, particularly during remodelling, in unstable plaques and in macrophage, foam cell, and smooth muscle cell rich areas. It is suggested that MMP-9 is associated with exacerbation of atherosclerosis and advancement of disease by breaking down matrix components and by inducing instability and rupture of the fibrous cap.

The data described here suggest that there is a strong association between the presence of *C. pneumoniae* and MMP-9 production. A significant association was found between the extent of intralesional *C. pneumoniae* and the amount of MMP-9 production. When serial sections of tissue were examined, *C. pneumoniae* and MMP-9 were co-localised to the same regions of the plaque. There was no evidence in these specimens to suggest that *C. pneumoniae* is associated with increased macrophage infiltration or foam cell formation. As MMP-9 is expressed by macrophages as well as smooth muscle cells it was not possible to ascribe the increase in MMP-9 expression to macrophage infiltration alone. The use of immunohistochemical as well as molecular techniques allowed confirmation of the association between infection with *C. pneumoniae* and upregulation of MMP-9 transcription.

Although a proportionally lower number of samples was found positive by *C. pneumoniae* PCR, this is a finding well documented by others and may reflect rapid degradation of chlamydial genomic DNA and persistence of chlamydial membrane proteins within the plaque, a phenomenon that has been shown to occur with the use of in situ DNA nick end...
The results of the work described here are in agreement with previously published findings. *C. pneumoniae* has been shown to increase the capacity of macrophages to produce MMP-9 by stimulating the expression of this enzyme in human monocye derived macrophages.22 In mouse macrophages, *C. pneumoniae* and its 60 kDa heat shock protein stimulate tumour necrosis factor α and MMP-9 production.23 *C. pneumoniae* has been shown to activate the nuclear factor κB pathway in vascular smooth muscle cells and endothelial cells.24 In a study by Song and colleagues,25 in four atherosclerotic aortic tissue specimens immunoreactivity for MMP-9 and cyclooxygenase-2 co-localised with immunoreactivity for *C. pneumoniae*. Choi and colleagues34 recently observed expression of MMP-9 co-localised with *C. pneumoniae* staining in diseased aorta and carotid artery specimens.

In contrast however, Petersen and colleagues35 did not observe any association between a positive DNA test for *C. pneumoniae* and activity of MMP-2 and MMP-9 in aortic aneurysms.35 However, the authors used separate blocks of tissue for detection of *C. pneumoniae* DNA and MMP activity. The advantage of the present study is that the MMP-9 and *C. pneumoniae* could be detected in sequential 5 μm sections by immunohistochemistry.

In conclusion, the data presented here provide evidence of an association between increased MMP-9 production and intraleisonal presence of *C. pneumoniae* in coronary atherosclerosis and may provide increased evidence for a potential mechanism by which the bacterium may accelerate or exacerbate atherosclerotic disease.

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