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 Akinyu, 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 intraluminal 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.

METHODS

Patients and samples

Human coronary endarterectomy specimens were obtained from 24 patients (21 men, mean age 63 (8) years) requiring elective urgent coronary artery bypass surgery. A further seven human coronary artery samples were obtained from the explanted hearts of patients undergoing cardiac transplantation (seven men, mean (SD) age 44 (11) years). Venous samples from restenotic bypass grafts were excluded. Samples were stored at 4°C until being processed on the same day as surgery. The study protocol was approved by the local ethics committee and all participants gave written informed consent.

Histology and immunohistochemistry

Serial 5 μm cryostat sections of atherosclerotic coronary arteries were analysed by immunofluorescence double labelling. Sections were cut, air dried on multisport slides, and frozen until use. For immunohistochemical analysis, sequential sections were double stained for C pneumoniae and CD68, MMP-9 and CD68, negative control and α smooth muscle cell actin, or CD31 and negative control. Sections were initially blocked with rabbit serum (Dako) containing four drops/ml avidin solution (avidin/biotin blocking kit, Vector Labs) for

Abbreviations: MMP, matrix metalloproteinase; PCR, polymerase chain reaction; RT-PCR, reverse transcription polymerase chain reaction.
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/7-amino-4-methylcoumarin-3-acetate (Jackson Labs) 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 recommendation published by Stary and colleagues.24

To validate the findings and rule out the possibility of false positive specimens caused by artefactual staining, 18 of 22 C pneumoniae positive specimens were re-examined for chlamydial antigen with the mouse C pneumoniae monoclonal antibody (RR402) and a further mouse C pneumoniae monoclonal antibody (clone A57-B9, Affinity Bioreagents Inc) specific for the 60 kD heat shock protein of C pneumoniae. 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 sulphate and 400 µl 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’-TTACAAGGCTTG CTGTAGG-3’; antisense, 5’-GCCATCCAAAATGTTAAAGGC. Second round: sense, 5’-TTATATGAGTGTTACAATA-3’; antisense, 5’-ATCTAGGCGTAGTAGTTTAT-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.26 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’-CAGTGCACCCTCCAGAGG-3’; antisense, 5’-GCCACTTGTCGGGCGATAAGG-3’).26 To confirm the integrity of the extracted RNA, transcription of the housekeeping gene

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

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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.
glyceraldehyde-3-phosphate dehydrogenase was analysed as a control (sense, 5’-ACACATCCATGCTACATCC-3’; antisense, 5’-GCTGTTCTGCTTCAAG-3’). The resulting PCR amplicons were analysed by agarose gel electrophoresis.

Statistical analysis
Statistical analysis was carried out with the SPSS version 10 for Windows (SPSS Inc, Chicago, Illinois, USA). Results were analysed as categorical data with the $\chi^2$ test and Fisher’s exact test where necessary. A probability value of $p < 0.05$ was considered significant.

RESULTS

Immunohistochemical analysis of human coronary artery specimens
Analysis of human coronary atherosclerotic lesions by immunohistochemical double 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.

To determine the cellular localisation of C pneumoniae and MMP-9, double immunostaining was carried out with C pneumoniae antibody or anti-MMP-9 antibody with CD68 antibody, and sequential sections were immunostained with a smooth muscle cell actin and anti-CD31 antibodies. Chlamydial antigen co-localised with macrophages (fig 1), smooth muscle cells, and endothelial cells. MMP-9 was observed in macrophages, smooth muscle cells, and endothelial cells (figs 2 and 3). Both MMP-9 and C pneumoniae were often observed within macrophage rich regions in the plaque (fig 2) and in areas of extensive smooth muscle cell infiltration such as the shoulder regions of the plaque. Analysis of serial sections showed that MMP-9 co-localised with C pneumoniae in 13 of 19 specimens (68%). Immunohistochemical analysis of macrophage infiltration and foam cell accumulation by CD68 staining (table 1) showed that there was a significant correlation between the extent of macrophage infiltration and foam cell accumulation ($p < 0.05$). There was, however, no significant association between macrophage infiltration and presence of intraluminal C pneumoniae or MMP-9. There was also no significant association between foam cell accumulation and C pneumoniae infection.

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 RR602) and (C) an IgG1 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 intraluminal *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...
labelling (terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling).31
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 monocyte-derived macrophages.28 In mouse macrophages, C pneumoniae and its 60 kDa heat shock protein stimulate tumour necrosis factor α and MMP-9 production.29 C pneumoniae has been shown to activate the nuclear factor κB pathway in vascular smooth muscle cells and endothelial cells.30 In a study by Song and colleagues31 in four atherosclerotic aortic tissue specimens immunoreactivity for MMP-9 and cyclooxygenase-2 were localised with immunoreactivity for C pneumoniae. Choi and colleagues recently observed expression of MMP-9 co-localised with C pneumoniae staining in diseased aorta and carotid artery specimens.

In contrast however, Petersen and colleagues32 did not observe any association between a positive DNA test for C pneumoniae and activity of MMP-2 and MMP-9 in aortic aneurysms.33 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 intraluminal 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.

ACKNOWLEDGEMENTS

This work was funded by British Heart Foundation grant number CH/ 92013.

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