Clinical and Experimental Immunology ORIGINAL ARTICLE doi:10.1111/j.1365-2249.2006.03185.x

Chronic *Chlamydia pneumoniae* infection may promote coronary artery disease in humans through enhancing secretion of interleukin-4

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Accepted for publication 13 July 2006
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Summary

Atherosclerosis is an inflammatory response, probably to a range of initiating causes. Chronic infection with *Chlamydia pneumoniae* (C.pn) has been suggested as one cause, but the nature of the association is controversial, in large part due to lack of an identified mechanism to link infection with the atherosclerotic process in man. This study examined 139 consecutive subjects with stable chest pain, with the aim of correlating the serological status of C.pn infection with the pattern of secretion of cytokines from $CD4^+$ T lymphocytes. C.pn seropositive subjects secreted significantly more interleukin (IL)-4 than did those who were C.pn seronegative ($P = 0{\cdot}02$). No significant difference was noted for secreted interferon (IFN)-γ. The amount of secreted IL-4, but not of secreted IFN-γ, correlated positively with the extent of coronary artery disease ($P = 0{\cdot}006$). A similar correlation with secreted IL-4 was not identified with *Helicobacter pylori* infection. These results support the hypothesis that C.pn infection contributes to the inflammatory process responsible for coronary artery atherosclerosis. The method used to detect cytokine secretion involves ligation of CD40L on blood $CD4^+$ T cells, which may have relevance to tissue events.

Keywords: atherosclerosis, *Chlamydia pneumoniae*, *Helicobacter pylori*, IFN-γ, IL-4

Introduction

Atherosclerosis is a major cause of death in the western world. The primary lesion is the atherosclerotic plaque in the intima of particular arteries, and the pathological process is an inflammatory response to injury. Little is known as to what initiates or perpetuates this response. Hallmarks of chronic inflammation are present within both early ('fatty streak' formation) and advanced lesions with plaque rupture, including the presence of T lymphocytes [1]. Both specific antigenic and non-specific stimuli can activate T lymphocytes to drive an inflammatory response through the secretion of cytokines. Recent clinical studies have associated chronic infection, due particularly to *Chlamydia pneumoniae* (C.pn), with atherosclerotic lesions and clinical coronary artery disease [2–4]. What is missing, however, is recognition of a mechanism that could link infection with the progression of disease. Considerable attention has been given to the study of C.pn, but little is known about host factors responding to infection that may promote atherosclerosis. At the same time, however, progress has been made in understanding the pathogenesis of atherosclerosis in terms of the cellular and molecular events that mediate tissue damage, including an interest in the role played by T cell-derived cytokines [5]. The current study was directed at examining the host response to C.pn infection in subjects with coronary artery disease to identify mechanisms that could link infection with disease. The aim of the study was to determine whether the pattern of cytokine secretion differed in C.pn seropositive *versus* seronegative subjects in angiographically defined coronary artery disease and whether the pattern of cytokine secretion varied with the extent of atherosclerosis. A similar analysis with respect to *Helicobacter pylori* infection status is reported as an infection control for specificity of association.

Materials and methods

Subjects studied

Patients studied had stable chest pain, and had been referred for coronary artery angiography to confirm or refute a diagnosis of coronary artery disease (CAD). Patients booked for angiography were approached and invited to participate in

this study. All subjects signed a consent form. The study was approved by the Human Ethics Committees of the University of Newcastle and the Hunter Area Health Department. Blood was taken from the intra-arterial catheter prior to angiography. Based on analysis of the angiogram, subjects were divided into five groups. Group 1 had normal coronary arteries without observed obstruction; group 2 had mild coronary artery atherosclerosis, i.e. areas of obstruction but with no artery obstruction in excess of 50% of lumen size; group 3 had 'one-vessel' disease, i.e. the lumen of one coronary artery was occluded by more than 50%; group 4 had 'two-vessel' disease, i.e. lumen of two coronary arteries were occluded by more than 50%; group 5 had 'three-vessel' disease, i.e. lumen of three coronary arteries were occluded by more than 50%.

Detection of C.pn antibody

Antibody to C.pn was measured by enzyme-linked immunosorbent assay (ELISA) with a preparation of semipurified outer-membrane proteins (Bioclone Australia Pty Ltd, Marrickville, NSW, Australia) using the strictest criteria provided by the manufacturer for a positive result (i.e. IgG and/or IgA antibody with a sample index of $\geq 3{\cdot}0$) [6].

Detection of *H. pylori* antibody

H. pylori was assessed using anti-*H. pylori* antibody used on acid glycine extract as antigen in an ELISA assay [7].

Measurement of cytokine secretion

Cytokines secreted from blood T lymphocytes were measured following the method described previously [8]. In brief, 150 μl of heparinized whole blood was added to an equal volume of AIM-V serum-free medium (Life Technology, Melbourne, VIC, Australia) in microtitre wells coated with a capture monoclonal anti-human interleukin (IL)-4 antibody. The plate was incubated at 37°C for 24 h. Bound IL-4 was measured by ELISA and interferon (IFN)-γ was measured in culture supernatants by ELISA using monoclonal antibodies [8]. The limit of sensitivity for IL-4 and IFN-γ was 9·4 pg/ml and 4·4 pg/ml, respectively. In this assay, IL-4 and IFN-γ secretion is suppressed completely by anti-$CD4^+$ T cell antibody, by anti-CD40L antibody and by removal of platelets (data not given). Enhanced stimulation by C.pn antigen was tested by adding C.pn elemental bodies (EB) to wells at an optimal concentration of 10 μg/ml [9]. C.pn antigen (strain A03, an isolate from a human with heart disease) was a gift from Professor Peter Timms (Queensland University of Technology).

Measurement of high sensitivity C reactive protein (hsCRP)

Serum hsCRP levels were measured by Beckman Coulter Image (Fullerton, CA, USA) using high sensitivity CRP kits (Beckman Coulter, Fullerton, CA, USA). The lowest limit of detection was 0·2 mg/l.

Statistical methods

CAD was grouped into three categories: no/mild disease, moderate disease (one- or two-vessel disease) or severe disease (three-vessel disease) for the purpose of analysis. Chlamydia and *H. pylori* status was either positive or negative depending on the presence or absence of antibody. The distributions of CRP as well as stimulated and unstimulated IL-4 and IFN-γ were all highly right-skewed. Spearman's rank correlation was used to assess correlation among the unstimulated and stimulated IL-4, IFN-γ and the CRP variables. The non-parametric Kruskal–Wallis test was used to compare IL-4, IFN-γ and CRP values across the three CAD groups, and the rank sum test used for pairwise comparisons when the Kruskal–Wallis test was significant. The rank sum test was used to compare values between the two Chlamydia groups and the two *H. pylori* groups. Analyses were conducted in STATA version 8·0 and a 5% level of significance was used.

Results

Subject groups

One hundred and thirty-nine subjects booked for angiography were approached and all consented to be part of the study.

Group 1 had visually normal coronary arteries [10 subjects: four males (45–60 years) and six females (32–64 years)]; group 2 had mild coronary artery atherosclerosis [40 subjects, 23 male (46–76 years) and 17 female (44–79 years)]; group 3 had one-vessel disease [27 subjects, 14 male (49–67 years) and 13 female (47–77 years)]; group 4 had two-vessel disease [24 subjects, 16 male (54–74 years) and eight female (45–76 years)]; group 5 had three-vessel disease [38 subjects, 29 male (49–81 years) and 9 female (45–77 years)].

Coronary artery disease, infection status and cytokine secretion patterns

As shown in Table 1, unstimulated and stimulated IL-4 increased with increasing severity of coronary artery disease, and this was statistically significant for unstimulated IL-4 ($P = 0{\cdot}006$) but was just short of statistical significance for stimulated IL-4 ($P = 0{\cdot}055$). An apparent increase in IL-4 secretion in those with no/mild disease when stimulated may reflect a small subpopulation of antigen-responsive T cells, not seen in other groups because of the high IL-4 background. Those with severe (three-vessel) CAD had higher unstimulated IL-4 than those with no/mild disease or moderate (one- or two-vessel) disease ($P = 0{\cdot}001$ and

Table 1. Interleukin (IL)-4 concentration in culture supernatants by coronary artery disease (CAD), *Chlamydia* status and *Helicobacter pylori* status.

	IL-4 (pg/ml) (no added C.pn antigen)			IL-4 (pg/ml) (C.pn antigen 10 μg/ml)	
	n	Median (95% CI)	*P**	Median (95% CI)	*P**
CAD					
None/mild	50	37·4 (12·8, 81·6)		51·0 (8·7, 136·9)	
Moderate	51	62·1 (14·8, 115·6)		67·3 (51·8, 125·5)	
Severe	38	140·7 (70·3, 214·7)	0·006	137·5 (67·7, 243·7)	0·055
Chlamydia status					
Seropositive	42	108·8 (62·4, 183·5)		138·1 (69·0, 212·4)	
Seronegative	97	46·0 (20·0, 90·0)	0·020	60·14 (35·3, 113·9)	0·047
H. pylori status					
Seropositive	38	74·0 (24·7, 137·8)		67·0 (17·1, 148·7)	
Seronegative	101	76·2 (35·0, 104·9)	0·559	106·8 (53·7, 132·0)	0·711

Results are expressed as medians and 95% confidence intervals. *The non-parametric Kruskal–Wallis test was used to compare IL-4-value across the CAD groups; *Z*-value from rank sum test was used for *Chlamydia* groups and *H. pylori* groups.

$P = 0{\cdot}02$, respectively). Stimulated IL-4 level was also higher in those with severe CAD than in those with no/mild disease ($P = 0{\cdot}02$). IFN-γ did not vary with severity of CAD (Table 2).

IL-4 was higher for participants who were Chlamydia-positive relative to those who were Chlamydia-negative (Table 1). This reached statistical significance for both unstimulated and stimulated groups. The similar level fold increase in IL-4 secretion in seropositive subjects in the absence or presence of added antigen may reflect high backgrounds due to CD40/CD40L ligation, or a degree of endogenous stimulation in C.pn-positive subjects. IFN-γ did not vary significantly with Chlamydia status (Table 2).

IL-4 levels were similar for participants who were seropositive or seronegative for *H. pylori* (Table 1). Unstimulated and stimulated IFN-γ levels were significantly lower for *H. pylori*-positive patients, compared to those who were *H. pylori*-negative (Table 2).

Correlation of CRP with severity of CAD and infection status

CRP did not vary with CAD level or Chlamydia status or *H. pylori* status ($P = 0{\cdot}085$, $P = 0{\cdot}346$ and $P = 0{\cdot}183$, respectively).

The proportion of Chlamydia-positive or *H. pylori*-positive patients did not vary with severity of CAD (data not shown: $\chi^2_2 = 1{\cdot}07$, $P = 0{\cdot}584$; $\chi^2_2 = 3{\cdot}46$, $P = 0{\cdot}177$, respectively). Those who were Chlamydia-positive were more likely to be *H. pylori*-positive than those who were Chlamydia-negative (38% *versus* 23%), although this was not statistically significant at the 5% level ($\chi^2_2 = 3{\cdot}51$, $P = 0{\cdot}061$).

Discussion

The objective of this study was to test the hypothesis that C.pn infection promotes atherosclerosis by initiating a host

Table 2. Interferon (IFN)-γ concentration in culture supernatants by coronary artery disease (CAD), *Chlamydia* status and *Helicobacter pylori* status.

	IFN-γ (pg/ml) (no added C.pn antigen)			IFN-γ (pg/ml) (C.pn antigen 10 μg/ml)	
	n	Median (95% CI)	*P**	Median (95% CI)	*P**
CAD					
None/mild	50	2·3 (0, 10·0)		20·8 (1·5, 40·3)	
Moderate	51	0 (0, 5·4)		15·5 (4·5, 46·5)	
Severe	38	0·5 (0, 5·8)	0·598	15·3 (3·4, 25·4)	0·913
Chlamydia status					
Seropositive	42	5·2 (0, 8·4)		22·5 (9·0, 51·7)	
Seronegative	97	0 (0, 2·4)	0·098	14·4 (3·1, 24·7)	0·102
H. pylori status					
Seropositive	38	0 (0, 0)		2·4 (0, 12·7)	
Seronegative	101	3·0 (0, 6·0)	0·005	24·9 (15·0, 39·8)	0·004

Results are expressed as medians and 95% confidence intervals. *The non-parametric Kruskal–Wallis test was used to compare IFN-γ-value across the CAD groups; *Z*-value from rank sum test was used for *Chlamydia* groups and *H. pylori* groups.

inflammatory response, detectable through the quantification of cytokines secreted from circulating T lymphocytes. The results support this hypothesis, as seropositive subjects had a significantly increased level of secretion of IL-4. While sIFN-γ was higher for the Chlamydia-positive than for the Chlamydia-negative group, this did not reach statistical significance at the 5% level ($P = 0{\cdot}098$), possibly because the sample size was not large. A secondary observation noted that the level of IL-4 secreted from circulating T cells varied with the extent of atherosclerosis, as observed in the coronary angiogram. However, while overall the level of IL-4 increased with increasing degree of disease, this did not show a linear trend or dose–response relationship.

The patients studied were referred for angiography to investigate stable chest pain considered likely to be due to coronary artery disease. Subjects were classified as C.pn antibody-positive or -negative, based on the results of a commercial ELISA assay that detects antibody to C.pn outer membrane proteins. There is no 'gold standard' antibody assay for chronic C.pn infection, as all are imperfect with respect to sensitivity and specificity. The assay we have used has 85% concordance with the microimmunofluorescence assay, which has been used in other studies of C.pn and coronary atherosclerosis. This latter assay has a higher sensitivity but lower specificity compared to the ELISA assay used in the current study [6]. Rigorous diagnostic cut-off levels were used in our study, making it possible that the seronegative group as defined may include some chronically infected individuals. Blocking studies, adding anti-CD4 and anti-CD40L antibody to the assay, inhibited IL-4 and IFN-γ secretion. This means that high secretion levels reflect ligation of $CD40^+$ blood cells, presumably platelets, by CD40L-bearing $CD4^+$ T cells [10,11]. Failure to detect an increase in IL-4 in stimulated cultures probably reflects the high level of *in-vivo* activation through CD40L–CD40 interaction.

The second observation was that there was a relationship between the extent of coronary artery atherosclerosis and the amount of T cell-secreted cytokine. It was noted that the 'spontaneous' secretion of IL-4 (but not IFN-γ) was linked positively to the extent of coronary artery disease as defined by angiography, with a fourfold increase in median levels in subjects with three-vessel disease compared with those with normal arteries or one-vessel disease. The similar ratios of secreted IL-4 in seropositive *versus* seronegative subjects for all grading groups, including those with angiographically normal vessels, is consistent with a C.pn effect irrespective of the atheroma 'load'. Analysis of T cell receptor (TCR) β-chain variable (Vβ) gene segments of circulating activated T cells in patients with stable angina showed a polyclonal expansion of antigen-driven $TCRV\beta^+$ T cells with greater antigenic relevance to C.pn antigens, heat shock protein (HSP)60, autologous coronary plaque proteins and oxidized low density lipoprotein (LDL), compared to patients with unstable angina [12]. It is therefore possible that the high IL-4 levels may relate to chronic *in-vivo* polyclonal T cell activation mediated by a number of plaque antigens, including C.pn and HSP60 homologues, amplified by CD40–CD40L interaction.

How do these observations support the argument that C.pn augments atherosclerosis? The association between the level of IL-4 secretion and the extent of coronary atherosclerosis irrespective of the C.pn status suggests that plaque inflammation is driven, at least in part, by Th2 lymphocytes. CD40 is expressed on intimal endothelial cells, foam cells, macrophages and smooth muscle [13] and on platelets [10,11]. The extent of CD40 and CD40L expression correlates with the severity of disease and the number of circulating activated $CD4^+$ T cells [13,14]. Therefore, ligation of CD40 on these cells by CD40L on Th2 committed $CD4^+$ cells could activate IL-4 secretion [15–17]. Several recent studies have shown that T cells reactive to C.pn and HSP60 homologues are present within atherosclerotic plaques [12,18–22], capable of secreting both IFN-γ and IL-4/IL-5 (i.e. a Th0 profile) [23]. Demonstration of infection of antigen-presenting cells infected with C.pn within plaque suggests that a signal from these cells drives on the IL-4 response [24]. A number of mechanisms whereby IL-4 can promote atherosclerosis have been described, including mouse models [25–27] and identification of a number of pro-atherogenic mechanisms [28–33]. The association between C.pn infection and a Th2 response differs from the dominant Th1 response described with other Chlamydia species, such as *C. trachomatis* [34].

C.pn is one of several chronic infections claimed to promote coronary atherosclerosis – indeed it is unclear whether C.pn makes a specific contribution or contributes to the total 'infection load' [35]. While there was a trend towards a co-association of infection with C.pn and *H. pylori*, the latter was not associated with an increase in IL-4 secretion, consistent with recent studies [36]. There was also no difference in IL-4 secretion in subjects with positive serology for *P. gingivalis*, an organism linked to periodontitis and atheroma (P. Ford, University of Queensland, unpublished results). Co-existence of infection has not been noted before – it may reflect a common environmental exposure, and could account for the purported linkage between atherosclerosis and *H. pylori*. The reason for a negative association between seropositivity for *H. pylori* and both 'spontaneous' and C.pn stimulated secretion of INF-γ is unclear, but underlines the complexity and interaction of factors modulating atheroma growth.

Demonstration of cytokine abnormalities linked to both C.pn infection and the extent of coronary atherosclerosis is important in the broader discussion on pathogenesis and treatment of coronary artery disease, as two recently reported antibiotic intervention trials (PROVE IT and ACES studies) which together include about 16 000 subjects, failed to demonstrate benefit [37,38]. The use of intermittent single drug therapy, as used in these studies, is inappropriate

for the eradication of a chronic intracellular bacterial infection; the lack of change in antibody titre in the PROVE IT [38] trial reflects this ineffective therapy. The cytokine results in the current study support the conclusion of Kaski in his review of these therapy intervention studies, that despite the results from therapeutic trials, 'chronic infections are likely to be important – as triggers of inflammation and immune responses' [39]. The role of C.pn in promoting coronary artery disease may be most relevant to the growth of atheroma, rather than in facilitating plaque rupture with its attendant link to acute clinical events.

Acknowledgements

We wish to thank the Cardiologists of the John Hunter Hospital, B. Bastian, G. Bellamy, T. Singham, G. Warner, J. Silberberg and S. Thambar, for their cooperation in giving access to data and blood samples. The study was funded in part by the National Heart Foundation.

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