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Expression of chemokines and adhesion molecules in human coronary artery endothelial cells infected with *Chlamydia* (*Chlamydophila*) *pneumoniae*.

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Running head: *C. pneumoniae* and coronary artery inflammation

Summary

Högdahl M, Söderlund G, Kihlström E. Expression of chemokines and adhesion molecules in human coronary artery endothelial cells infected with *Chlamydia (Chlamydophila) pneumoniae*.

Chlamydia pneumoniae has during recent years been associated with cardiovascular disease and atherosclerosis. Chemokines, leukocyte adhesion proteins and metalloproteinases are significant for chemotaxis and attachment of leukocytes to vessel walls and in stability of atherosclerotic plaques. To determine the ability of *C. pneumoniae* to elicit inflammation in a relevant target host cell, we infected Human Coronary Artery Endothelial Cells (HCAEC) with a clinical isolate of *C. pneumoniae*. Extracellular release of five chemokines, two adhesion proteins and a metalloproteinase were measured at different time points after infection using a cytometric bead assay and ELISA. Secretion of IL-8, MCP-1, MIG, IP-10 and ICAM-1 were significantly increased 48 h after *C. pneumoniae* infection of HCAEC in comparison with uninfected controls. Release of RANTES occurred already 6 h after infection. *C. pneumoniae* did not elicit release of E-selectin and of MMP-1. We conclude that *C. pneumoniae* induces expression of proinflammatory components in HCAEC, that would promote migration of leukocytes towards endothelial cells. This lends support for *C. pneumoniae* to initiate and propagate vascular inflammation in ways that contribute to coronary artery disease.

Key words: Coronary artery inflammation, *C. pneumoniae*, chemokines.

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Introduction

Chemokines (Chemotactic cytokines) are a superfamily of small proteins that bind to G protein-coupled receptors on target cells. They were originally discovered as mediators of directional migration of immune cells to sites of inflammation and injury (1). It is now evident that the function of chemokines extends well beyond the role in leukocyte chemotaxis. They participate in organ development, angiogenesis, leukocyte trafficking and homing, tumorigenesis and metastasis, as well as in immune responses to microbial infection (1).

Chlamydiae are obligate intracellular microbes with a specific developmental cycle inside the host cell. The family *Chlamydiaceae* includes three human pathogenic species, *Chlamydia trachomatis*, *Chlamydia (Chlamydophila) pneumoniae* and *psittaci*. *C. pneumoniae*, primarily a respiratory tract pathogen has in recent years been suggested to be involved in the development of cardiovascular disease and atherosclerosis (2, 3). The organism can infect and multiply within cells usually found in an atheroma, including coronary artery endothelial cells, macrophages and aortic artery smooth muscle cells (4). *C. pneumoniae* is considered to be transported by monocytes from the respiratory tract to the artery vessel wall (5, 6). After successful entry into host cells the bacteria can remain in a dormant, persisting, non-replicating phase and not eliminated by antimicrobial agents (3, 7). Infected cells upregulate expression of adhesion molecules (3, 8) and infection of human endothelial cells stimulates transendothelial migration of leukocytes (9). *C. pneumoniae* infection of endothelial cells also triggers secretion of inflammatory cytokines, the procoagulant tissue factor and plasminogen activator inhibitor-1 (10 - 13). This suggests that *C. pneumoniae* facilitates recruitment of inflammatory cells and modulates procoagulant activity (14).

The expression of leukocyte adhesion molecules on endothelial cells, such as intracellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) is

upregulated during atherosclerotic development (15). The mechanisms that transform a stable atherosclerotic plaque to an unstable involve production of various enzymes that degrade the fibrous cap of the plaque. These include collagenases such as matrix metalloproteinases (MMPs) 1, 8 and 13 (16, 17). The expression of MMP-9 in coronary atherosclerotic plaques is associated with presence of *C. pneumoniae* and MMPs 1 and 3 are overexpressed in smooth muscle cells infected with *C. pneumoniae* (18, 19). Furthermore, *C. pneumoniae* promotes oxidation of low density lipoprotein (LDL) and oxidized LDL increases expression of MMPs in human coronary artery endothelial cells (20, 21). These findings suggest that *C. pneumoniae* upregulates MMPs directly by stimulating cells or indirectly by promoting formation of oxidized LDL.

The ability of *C. pneumoniae* to induce inflammation in human coronary artery endothelial cells has, to the best of our knowledge, not previously been investigated. To systematically investigate expression of chemokines, adhesion molecules and MMP-1 in this relevant target host cell, we infected human coronary artery endothelial cells with *C. pneumoniae* and analysed expression of these molecules.

Materials and Methods

Host cells, chlamydia isolate and infectious procedures.

This was as described by Schöier et al. 2006 (22). Briefly, *C. pneumoniae*, isolate T45 (obtained from the respiratory tract in a patient during a *C. pneumoniae* outbreak in northern Sweden) was propagated in HEp-2 cells, titrated for inclusion forming units (IFU) and stored at -70 ° C. Titration for IFU was performed both in HEp-2 cells and in Human Coronary Artery Endothelial Cells (HCAEC) (Clonetics, Walkersville, MA, USA) and showed that development of complete chlamydial inclusions was about 10 times less efficient in HCAEC than in HEp-2 cells. Two morphological, immunoreactive forms of *C. pneumoniae* were

observed associated with HCAEC; round to oval intracellular inclusions and spots/aggregates [22]. Multiplicities of infection (MOI) 1 and 10, as titrated in HEp-2 cells, of *C. pneumoniae* were added to mycoplasma-free HCAEC prior to centrifugation at 3000g for 1h. After incubation for 2 h at 37 ° C, the medium was discarded and fresh growth medium without cycloheximide was added. The cells were then incubated for 6, 24 or 48 h at 37 ° C in cycloheximide –free growth medium and supernatants from 3 separate experiments were collected for analysis. Supernatants from uninfected cells, incubated and treated as infected cultures were used as controls.

Flow cytometry and ELISA.

Quantification of chemokines was performed using the BD™ Cytometric Bead Assay, Human Chemokine Kit I with antibodies specific for interleukin 8 (IL-8), regulated on activation, normal T-cell expressed and secreted (RANTES), monokine induced by interferon- γ (MIG), monocyte chemoattractant protein -1 (MCP-1) and interferon- γ inducible protein 10 (IP-10) (BD Biosciences, San Diego, CA, USA). Supernatants from the tissue culture wells were added to antibody-coated beads, and subsequently analysed by flow cytometry using a FACSCalibur™ flow cytometer (BD Biosciences). Data analysis was performed by BD Biosciences Cell Quest soft ware.

E-selectin, intercellular adhesion molecule 1 (ICAM-1) and pro-matrix metalloproteinase 1 (pro-MMP-1) from the cell culture supernatants were determined by ELISA with reagents from R&D System (Minneapolis, MN, USA).

In most cases duplicate analyses were used in flow cytometry and ELISA. However, duplicates from the same experiments showed only small variations. When protein concentrations were outside the standard curve samples were diluted.

Statistics.

Data were expressed as mean and standard deviations. Statistical analysis was carried out using ANOVA (Fisher).

Results

The ability of *C. pneumoniae* to induce a chemokine (IL-8, MCP-1, RANTES, IP-10 and MIG) response in HCAEC was determined in supernatants from infected cells. The release of all 5 chemokines significantly increased from cells infected with MOI 1 after 48 h incubation compared with uninfected cells (Fig. 1). IL-8, RANTES and IP-10 were also released to a higher extent after 48 h at MOI 10 compared with uninfected cells. Levels of MCP-1, RANTES and MIG were lower at MOI 10 than at MOI 1 after 48h with incubation with *C. pneumoniae*. For RANTES, a significant increase in secretion occurred already after 6 h at MOI 10. At 24 h, the increase in release of RANTES and of the other 4 chemokines from infected cells did not reach statistical significance.

C. pneumoniae stimulated secretion of ICAM-1 at MOI 1 and MOI 10 after 48 h incubation compared with uninfected cells (Fig. 2). No upregulation of E-selectin expression above background levels was observed (data not shown).

Pro-MMP-1 secretion increased with incubation time but there was no differences between *C. pneumoniae*-infected and uninfected cells.

Discussion

During the last two decades there has been an increasing amount of intriguing reports for a role of *C. pneumoniae* in the development and progression of atherosclerosis and coronary artery disease. This is based on clinical studies linking the presence of either antibodies against *C. pneumoniae* to the disease process or presence of the bacterium in atherosclerotic

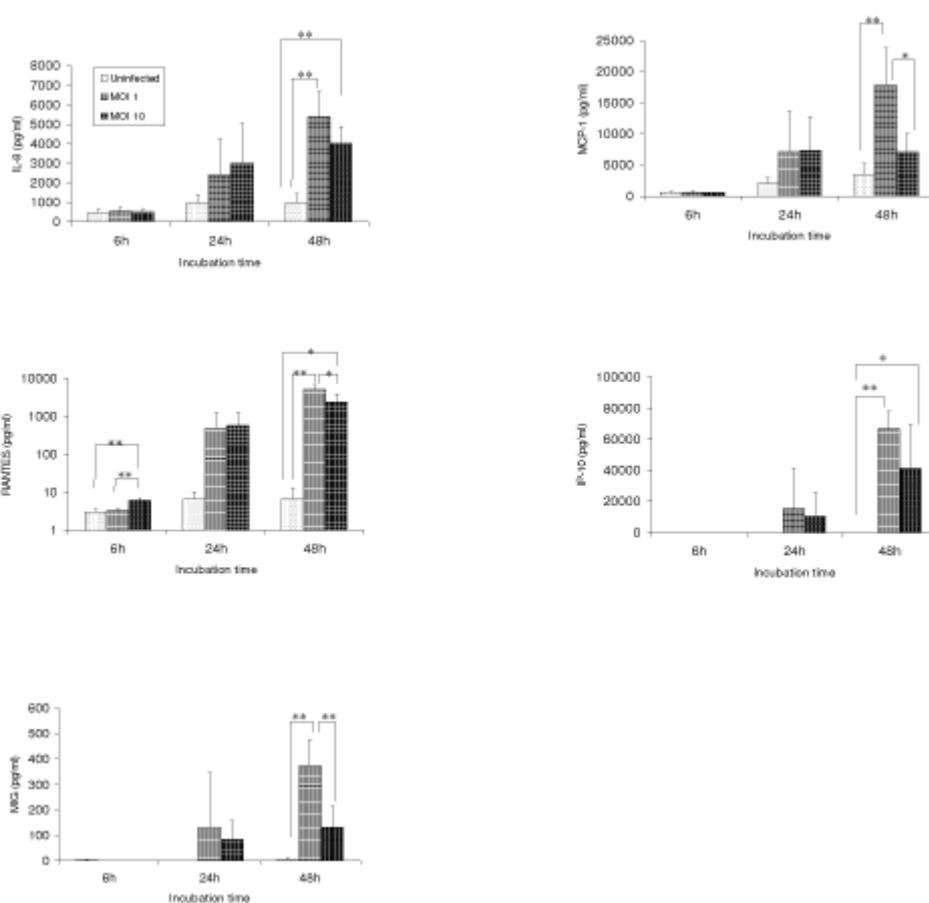


Fig. 1. Secretion of IL-8, MCP-1, RANTES, IP-10 and MIG, from HCAE cells after infection with *C. pneumoniae* T45. Chemokine concentrations from cell culture supernatants were determined by flow cytometry at indicated time points and inocula. Each value represents the mean + SD from 3 separate experiments. * $p < 0.05$ and ** $p < 0.01$.

lesions (3). Several in vitro investigations have shown that *C. pneumoniae* is capable of infecting vascular endothelial cells, smooth muscle cells, and macrophages and initiate inflammatory activation of these cells, a process that results in increased expression of adhesion molecules, procoagulant tissue factor, plasminogen activator inhibitor-1, inflammatory cytokines, and chemokines (14, 23). Despite initial promising results, long-term antibiotic treatment, at least with macrolides or quinolones of patients with documented coronary artery disease has little or no beneficial effect on secondary prevention of

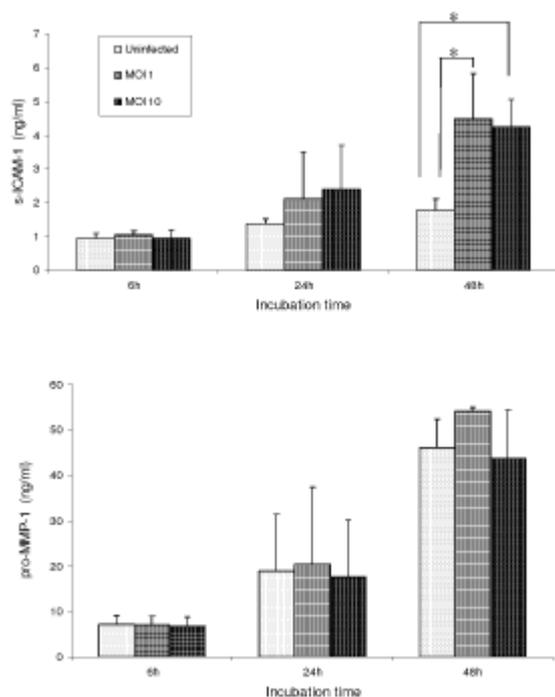


Fig. 2. Secretion of ICAM-1 and pro-MMP-1 from HCAE cells after infection with *C. pneumoniae* T45. Protein concentrations from cell culture supernatants were determined by ELISA at indicated time points and inocula. Each value represents the mean + SD from 3 separate experiments. * $p < 0.05$.

cardiovascular events (24, 25). However, these seemingly contradictory findings do not preclude an etiopathogenic role of *C. pneumoniae* in atherosclerosis.

We systematically investigated the release of inflammatory mediators induced by *C. pneumoniae* from human coronary artery endothelial cells, a relevant target cell. Previous studies have used other cells such as human aortic artery and umbilical vein endothelial cells (8, 10, 12, 13, 26-28). Using a flow cytometry immunoassay the 5 chemokines, IL-8, MCP-1, MIG, IP-10 and RANTES were measured simultaneously, thus avoiding interassay variations. RANTES was released after 6 h, but a significant increase of the other 4 chemokines occurred only after the longest incubation time, 48 h. This is in contrast to other bacteria that usually elicit a more rapid and transient proinflammatory cytokine response after infection of different cell types (29, 30). Other investigators have noticed a faster induction of IL-8 and MCP-1 after infection with *C. pneumoniae*, however in these cases other host cells than

HCAECs were used (12, 13). Also, different *C. pneumoniae* isolates vary in ability to induce a chemokine response at least in umbilical vein endothelial cells (13). On the other hand the IL-8 response from a human lymphoid cell line was suppressed after persistent infection with *C. pneumoniae* (31).

Chemokines investigated in this work attract neutrophils, monocytes and T-lymphocytes which are the leukocytes found in the early atheroma. In accordance with current opinion of atheroma development these leukocytes accumulate in the subendothelial space, where the monocytes are converted to macrophages that ingest modified, oxidized LDL, subsequently leading to the formation of foam cells, a principal component of the atherosclerotic plaque (32, 33). Oxidized LDL behaves as a potent proinflammatory agent and stimulates the synthesis of various cytokines from endothelial and smooth muscle cells. These attract monocytes and T-lymphocytes to the activated vessel wall (34).

In this study *C. pneumoniae* infection of HCAEC induced release of ICAM-1 but not of E-selectin. Surface expression of both of these adhesion molecules have previously been reported in *C. pneumoniae* infected human aortic and umbilical vein endothelial cell (8, 26, 27). We measured extracellular release and not surface expression of E-selectin and the first time point of measurement was after 6 h when expression of E-selectin starts to decline (28). These methodological variations may explain lack of E-selectin detection from HCAEC in our study. Oxidized LDL can stimulate the expression of leukocyte adhesion molecules such as E-selectin, ICAM-1 and VCAM-1 in endothelial cells (35). The finding that endothelial *C. pneumoniae* infection promotes oxidation of LDL suggests an indirect way for *C. pneumoniae* to elicit upregulation of the cytokine and adhesion molecule responses (20). We found no upregulation of MMP-1 in *C. pneumoniae* infected HCAEC. However, other investigators have reported increased expression of MMP-1 and other metalloproteinases by e.g. smooth

muscle cells after *C. pneumoniae* infection (18). Thus, chlamydia infection may modify stability of atheromatous plaques.

In conclusion, important chemoattractants promoting migration of leukocytes to the endothelium are expressed during, *C. pneumoniae* infection of HCAEC. These results support findings that *C. pneumoniae* has the capacity to initiate or propagate inflammation in ways that contribute to coronary artery disease.

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