Induction of Interleukin-18 in Atherosclerotic Patients: A Role for *Chlamydia pneumoniae*

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Key Words

Atheroma · Cytokine · Inflammation · mRNA · Bacteria

Abstract

Objectives: The present work explored gene expression and spontaneous induction of the inflammatory cytokine interleukin-18 (IL-18) in atherosclerotic patients. In addition, the effect of the chlamydial antigen heat shock protein 60 (HSP60) and lipopolysaccharide (LPS) on the induction of this mediator was examined. **Subjects and Methods:** Detection of IL-18 mRNA and protein level were assessed by in situ hybridization and immunohistochemistry, respectively, in 15 patients with coronary artery disease undergoing angiograms and 15 matching controls. **Results:** These experiments showed significantly high levels of spontaneously expressed IL-18 mRNA and high protein levels in patients compared to healthy controls ($p < 0.0005$). Cells stimulated with chlamydial HSP60 (CHSP60) and LPS showed a significantly high expression of IL-18 at the mRNA level ($p < 0.0005$ for CHSP60 and $p < 0.005$ for LPS) and an increased production of IL-18 at protein level ($p < 0.0005$ for CHSP60 and $p < 0.005$ for LPS). **Conclusion:** This study demonstrated de novo synthesis of the inflammatory cytokine IL-18 in atherosclerosis and, furthermore, that chlamydial antigens might play a role in the immunopathological events in this disease by generating more inflammatory mediators such as IL-18.

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Introduction

Atherosclerosis is a pathological process that takes place in the major arteries and is the underlying cause of heart attacks, stroke and peripheral artery disease [1]. It is no longer considered a disorder of lipid accumulation, but a disease process characterized by the dynamic interaction between endothelial dysfunction, subendothelial inflammation and the 'wound healing response' of the vascular smooth muscle cells [2]. The principal cell types of the artery wall, the endothelial cells, the smooth muscle cells and the monocytes/macrophages, are major factors in the events involved in initiation and evolution of the atherosclerotic plaque [3]. Other important factors are platelets and lymphocytes, which have a modulating influence on smooth muscle cells, endothelial cells and macrophage behavior. The earliest detectable lesions, called fatty streaks, contain macrophage foam cells that are derived from recruited monocytes. More advanced atherosclerotic lesions, called fibrofatty plaques, are the result of continued monocyte recruitment and smooth muscle cell migration and proliferation. Variable numbers of CD4⁺ T cells are found in atherosclerotic lesions with cytokines secreted by T helper 1 (Th1)- or Th2-type cells. Th1-type cytokines, as for example interferon- γ (IFN- γ), have a tendency to produce the proinflammatory responses responsible for killing intracellular parasites and for perpetuating autoimmune responses. The

Th2-type cytokines such as interleukins-4, 5 and 13 are associated with the promotion of IgE and eosinophilic responses in atopy [4]. Both Th1- and Th2-type cells can have a profound influence on macrophage gene expression within atherosclerotic plaques [5].

Interleukin-18 (IL-18) is a key cytokine that mediates several inflammatory disorders. For example, several autoimmune diseases are thought to be mediated in part by IL-18. In addition, IL-18 appears to be involved in ischemia, including acute renal failure in human beings. Animal studies also support the concept that IL-18 is a key factor in models of lupus erythematosus, atherosclerosis, graft-versus-host disease, and hepatitis [6]. Recent studies additionally showed that plasma IL-18 is increased in postinfarction patients and is associated with coronary atherosclerosis [7].

A number of risk factors for atherogenesis, including infectious agents, have been shown to exert their influence via inflammatory mechanisms. There is growing evidence that *Chlamydia pneumoniae* may be involved in the pathogenesis of atherosclerosis, as several studies have demonstrated the presence of the organism in atherosclerotic lesions [8]. *C. pneumoniae* are Gram-negative, obligate intracellular bacteria that depend on their host cell for growth and prolonged survival [9]. *C. pneumoniae* is 1 of 3 chlamydial species that cause human disease [10]. It is a common human pathogen transmitted by aerosol droplets and can lead to upper respiratory tract infections, including pharyngitis, bronchitis, sinusitis, community-acquired pneumonia, and otitis media [11]. *C. pneumoniae* can initiate and propagate inflammation in ways that could contribute to atherosclerosis. Infected leukocytes may serve to disseminate an infection from the lung to other susceptible tissues including arteries [12, 13]. *C. pneumoniae* also may influence atheroma biology by modulating macrophage-lipoprotein interactions. Infected macrophages ingest excess low-density lipoprotein to become cholesteryl ester-laden foam cells, the hallmark of early lesions in atherosclerosis [14, 15]. In addition, *C. pneumoniae* induces monocytes to oxidize lipoproteins, converting them to highly atherogenic forms [16]. It is suggested that *C. pneumoniae* triggers key atherogenic events through their specific virulence determinants, the chlamydial lipopolysaccharide (LPS) and chlamydial heat shock protein 60 (CHSP60). The *C. pneumoniae*-induced foam cell formation is mediated chiefly by LPS, whereas lipoprotein oxidation occurs mainly by CHSP60 [17].

In view of the depicted potential role for IL-18 in inflammation in general and the suggested role in athero-

sclerosis in addition to the possibility that infection with *C. pneumoniae* may be an underlying contributing cause for the development of atherosclerosis, the present work studied the spontaneous induction of IL-18 at the gene and protein levels and, furthermore, examined the effect of chlamydial antigens on the expression of this cytokine.

Subjects and Methods

Study Participants

Fifteen patients who presented to the Bahrain Defense Force (BDF) Hospital with coronary artery disease undergoing angiograms (age >35 years) were included in the study after the purpose of the study and its implications were fully explained to them. Control subjects comprised healthy individuals (n = 15). Characteristics and clinical data of patients and controls are included in table 1. All subjects (patients and controls) were asked to complete and sign a consent form indicating their acceptance to participate in the study. For patients, 15 ml of venous blood samples were collected in EDTA tubes after an angiogram was done and pathological changes were confirmed, and were stored at room temperature pending lymphocyte isolation. Similarly, 15 ml of venous blood was collected from each control.

Lymphocyte Isolation

For lymphocyte isolation, peripheral blood cells were isolated by carefully overlaying the diluted blood on Ficoll and centrifugation at 3,200 rpm for 20 min at room temperature, followed by discarding the plasma layer and collecting the buffy coat layer. Peripheral blood lymphocytes were then washed 3 times with 1 × [phosphate-buffered saline pH 7.2 (PBS)].

Cell Cultures

Lymphocytes were maintained in culture medium RPMI 1640 (ICN, Biomedicals) supplemented with 5% fetal bovine serum, 1 M Hepes buffer, 50 µmol mercaptoethanol, 2 ml of 200 mM L-glutamine, and 5 IU/5 µg penicillin streptomycin (ICN, Biomedicals). The cells were transferred to adhesion slides (BioRad Lab, Munich, Germany) and were stimulated by 5 pg chlamydial LPS and *Escherichia coli* or 5 ng CHSP60 (gift from Dr. M. Majeed, Division of Medical Microbiology, Linköping University, Sweden). This concentration was recommended by the provider and after further testing by using different concentrations was found to be the optimum. The cells were then incubated overnight at 37°C in 5% CO₂. Some negative control cells were left without stimulation. The positive control cells were stimulated by 5 µg of phytohemagglutinin (PHA; Sigma, Germany).

Detection of Cytokine mRNA Expression by *in situ* Hybridization

In situ hybridization was performed as previously described [18]. Briefly, 200-µl aliquots of suspensions containing 4 × 10⁵ mononuclear cells were plated into round-bottomed microtiter plates (Nunc) in triplicate. Ten-microliter aliquots of 5 pg chlamydial LPS or 5 ng CHSP60 or *E. coli* LPS or PHA were added to appropriate wells. After culture for 24 h, the cells were washed,

Table 1. Characteristics of patients and controls

Characteristics	Patients (n = 15)	Controls (n = 15)
Age, years	54.3	49.9
Gender		
Female	6 (35.71%)	6 (35.71%)
Male	9 (64.29%)	9 (64.29%)
Hypertension, %	84.4	0
Diabetes, %	59.4	0
Hyperlipidemia, %	65.9	0
Glucose (3.4–6.1), mmol/l	7.6	4.6
Triglycerides (0.11–2.15), mmol/l	1.8	0.9
Cholesterol (3.88–6.47), mmol/l	5.4	3.8
LDL cholesterol (<3.4), mmol/l	3.8	1.8
HDL cholesterol (female = 0.91–1.168; male = 0.91–2.07), mmol/l	1.17	1.0
WBC (4.4–11 × 10 ³), /μl	8.72	6.98
Platelet count (150–450 × 10 ⁶), /μl	250.8	230.9

counted and applied onto restricted areas of electronically charged glass slides (ProbeOn slides; Fisher Scientific, Pittsburgh, Pa., USA). Synthetic oligonucleotide probes (Scandinavian Gene Synthesis, Köping, Sweden) were labeled using ³⁵S-deoxyadenosine-5'-α-(thio)-triphosphate with terminal deoxynucleotidyl transferase (Amersham). To increase the sensitivity of the method, a mixture of four different probes was employed for each cytokine. The oligonucleotide sequences were obtained from GenBank using MacVector software. Cells were hybridized with 10⁶ cpm of labeled probe per 100 μl of hybridization mixture. After emulsion autoradiography, development and fixation, the coded slides were examined by dark-field microscopy for positive cells containing more than 15 grains per cell in a star-like distribution. The intracellular distribution of the grains was always checked by light microscopy and expressed as numbers per 10⁴ mononuclear cells. In many positive cells, the grains were so heavily accumulated within and around the cells that it was not possible to count every single grain. In cells adjudged negative, the numbers of grains were mostly 0–2 per cell, with the grains scattered randomly over the cells and not distributed in a star-like fashion. There were therefore no difficulties in differentiating between positive and negative cells. Variation between duplicates was <10%. A control probe used in parallel with the cytokine probe on cells from each rat revealed no positive cells.

Intracellular Detection of IL-18 by Immunohistochemistry

Cell fixation was performed in methanol for 5 min. Slides were incubated with avidin-biotin blocking kit (Vector Laboratories, Burlingame, Calif., USA) to block endogenous biotin or biotin-binding proteins. Cells were permeabilized with 0.1% saponin dissolved in PBS to allow the intracellular access of the cytokine-specific antibody. A total of 30 μl IL-18-specific monoclonal antibody (mouse anti-human IL-18; R&D Systems, Oxon, UK) diluted in PBS-saponin to a final concentration of 5 μl/ml was added and allowed to incubate overnight at 4°C followed by several washes in PBS. Nonspecific staining by the second-step biotinyl-

ated goat antibody caused by Fc interactions was prevented by a subsequent incubation with 1% normal goat serum (Dakopatts, Glostrup, Denmark) dissolved in PBS-saponin for 30 min at room temperature. The biotin-conjugated secondary antibodies were then added. The cells were incubated with an avidin-biotin horseradish peroxidase complex (Vectastain, Vector Laboratories) for 30 min in the dark at room temperature. A color reaction was developed by 3-diaminobenzidine tetrahydrochloride (DAB; Vector Laboratories) and stopped after 2–10 min by washes in PBS. The cells were counterstained with hematoxylin and the slides were left to dry before mounting in DPX (Fluka, Switzerland). The immunocytochemically stained cells were examined in a Leica RXM microscope (Leica, Wetzlar, Germany) equipped with a 3CDD color camera (Sony, Tokyo, Japan). Counting of cytokine-producing cells was performed manually using 100× objective. The frequency of cytokine-expressing cells was assessed by examination of at least 10,000 cells.

Statistical Analysis

Student's unpaired t test was used to measure statistical significance between the two groups. In all tests *p* < 0.05 was taken as the level of significance.

Results

The results of this study showed significantly high levels of spontaneously expressed IL-18 at mRNA level in patients compared to healthy controls (*p* < 0.0005) (fig. 1a, 2a, b). While cells stimulated with CHSP60 showed higher significant differences (*p* < 0.0005) between patients and healthy controls, lower significance for the cells which were stimulated with chlamydial LPS (*p* < 0.005) was depicted (fig. 1a). PHA was used as a control, but there were no significant differences between IL-18 mRNA levels induced in the cells after stimulation with PHA between patients and healthy controls. However, mRNA for IL-18 detected after PHA stimulation was much higher in both patient and healthy control groups when compared to nonstimulated cells (fig. 1a). Another important control was exposure of the cells from patients and controls to LPS from *E. coli* and the data did not show significant statistical differences between the patients and healthy control subjects (fig. 1a).

To ensure that the mRNA expression resulted in actual protein production generated during the immunopathogenetic events of the disease, the level of IL-18 protein was measured by immunohistochemistry. As found in the measurement of mRNA for IL-18, a significantly higher level of spontaneously expressed IL-18 in patients compared to healthy controls was demonstrated (*p* < 0.0005) (fig. 1b, 2c, d). Also, cells stimulated with CHSP60 showed higher significant differences (*p* < 0.0005) and

Fig. 1. a Numbers of mRNA-expressing cells for IL-18 among mononuclear cells from atherosclerotic patients challenged with chlamydial and *E. coli* LPS and HSP60. Means $\pm$ SD are shown. **b** Numbers of immunopositive cells for IL-18 among mononuclear cells from atherosclerotic patients challenged with chlamydial and *E. coli* LPS and HSP60. Means $\pm$ SD are shown. ** $p < 0.005$, *** $p < 0.0005$.

lower significance for the cells which were stimulated with LPS ($p < 0.005$) (fig. 1b). Again, PHA was used as a control and showed no significant differences in IL-18 levels between the patient and control groups, but increased levels when compared to nonstimulated cells (fig. 1b). Furthermore, LPS from *E. coli* stimulation did not reveal significant protein production of IL-18 (fig. 1b).

Discussion

The present work explored the spontaneous induction of a potential inflammatory cytokine in atherosclerotic patients at gene and protein levels. In addition, the effects of the chlamydial antigen HSP60 and LPS on the induction of this mediator were examined. This inflammatory mediator was measured because atherosclerosis is considered as an unusual form of chronic inflammation occurring within the artery wall [19].

Evidence that inflammation is a risk factor for cardiovascular diseases is based on studies that show conditions caused by inflammatory states are at increased risk of cardiovascular disease. This has led to the search for inflammatory markers which could be of predictive value in terms of outcome, enabling early clinical intervention and thus, an important mediator as the inflammatory cytokine IL-18 was selected for this study. IL-18 is a costimulatory factor for production of IFN- γ [20], an important mediator of protective immunity to chlamydial infection that has been suggested to play a critical role in inhibiting chlamydial growth [21]. The data showed spontaneous upregulation of IL-18. This spontaneous production of IL-18 was not increased upon in vitro stimulation with specific chlamydial components. This is most probably due to in vivo prestimulation as demonstrated by the spontaneous induction since these cells were already triggered in vivo and thus could not be additionally activated ex vivo even when different doses of the antigen were used.

Fig. 2. **a** Cells spontaneously expressing mRNA for the cytokine IL-18 produced by human mononuclear cell culture on slide. **b** Sense control. **c** Intercellular immunohistochemistry staining an IL-18 produced by human mononuclear cell culture on slide. **d** Nonstimulated cells (negative control). The photographs were taken using bright-field microscopy. $\times 200$.

The in vivo upregulation of the inflammatory cytokine IL-18 is understandable in view of the role of inflammation in mediating the atherosclerotic process [19]. IL-18 was recently shown to enhance atherosclerosis in apolipoprotein E(-/-) mice through release of IFN- γ , which was demonstrated to be a potent enhancer of atherogenesis [22]. Other results suggested a major role for IL-18 in atherosclerotic plaque destabilization leading to acute ischemic syndromes [23].

On the other hand, this cytokine may have a meandering role in controlling the infection by inducing the cytokine IFN- γ , which is necessary for the immunity to chlamydia. Thus, as a result of a host parasite interaction process immunopathology develops, which is atherosclerosis in this case, but the eventual response may indirectly contribute to the resolution of the infection.

Data presented here provide evidence for the role of IL-18 in atherosclerosis as produced by mononuclear cells. Considering such a role for IL-18 and the recent

findings indicating the potential role of infectious agents in the pathogenesis and progression of atherosclerosis and the fact that among different microorganisms suspected, *C. pneumoniae* had been implicated as the most plausible pathogen to have a link in lesion development of atherosclerosis [24], we addressed the induction of the selected cytokine (spontaneously and in response to the chlamydial antigen HSP60 and LPS). The data illustrated the role of the chlamydial antigens HSP60 and LPS in directing the immune response to inflammation with a Th1 type of response.

The significant induction of IL-18 in patients with atherosclerosis could be compared to healthy individuals; this suggests that cells from atherosclerotic patients may specifically recognize these antigens suggesting the presence of chlamydia in patients with atherosclerosis as previously depicted [8]. This was confirmed by the absence of a significant difference between patients and healthy subjects when using LPS from *E. coli* and also using PHA as a positive control. Hence, another confirmation for the role of chlamydia in the pathogenesis of atherosclerosis is provided since chlamydial antigens stimulated essential inflammatory cytokines.

Conclusion

This work demonstrates the production of the inflammatory cytokine IL-18 spontaneously and after challenges with chlamydial antigens. The recognition of the chlamydial antigens by mononuclear cells from patients with atherosclerosis and the ensuing production of IL-18 suggest a role for chlamydia in the immunopathogenetic events occurring in atherosclerosis.

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