Chlamydial Virulence Determinants in Atherogenesis: The Role of Chlamydial Lipopolysaccharide and Heat Shock Protein 60 in Macrophage-Lipoprotein Interactions

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Data from a spectrum of epidemiologic, pathologic, and animal model studies show that Chlamydia pneumoniae infection is associated with coronary artery disease, but it is not clear how the organism may initiate or promote atherosclerosis. It is postulated that C. pneumoniae triggers key atherogenic events through specific virulence determinants. C. pneumoniae induces mononuclear phagocyte foam cell formation by chlamydial lipopolysaccharide (cLPS) and low-density lipoprotein oxidation by chlamydial hsp60 (chsp60). Thus, different chlamydial components may promote distinct events implicated in the development of atherosclerosis. Data implicating cLPS and chsp60 in the pathogenesis of atherosclerosis are discussed and novel approaches are presented for attempting to elucidate how these putative virulence determinants signal mononuclear phagocytes to modulate lipoprotein influx and modification.

A role for Chlamydia pneumoniae in the pathogenesis of atherosclerosis was suggested first in 1988 by Saikku et al. [1], who demonstrated that patients with coronary artery disease (CAD) were significantly more likely to have serologic evidence for previous C. pneumoniae infection than were matched controls. Subsequent seroepidemiologic studies support the original observation by Saikku et al. [2]; furthermore, the association between C. pneumoniae and atherosclerosis has been strengthened by identification of the organism within atheromas by immunohistochemistry [3] or electron microscopy [4], identification of the C. pneumoniae genome within atherosclerotic lesions by the polymerase chain reaction [3], and isolation of the organism from human atheromatous tissue [5]. In addition, rabbit [6] and apolipoprotein (apo) E knockout murine [7] models have shown that C. pneumoniae can initiate and/or promote the development of atherosclerotic lesions in these animals. These epidemiologic, pathologic, and animal model studies establish a strong association, but not a causal role, for C. pneumoniae in atherosclerosis.

Evidence linking C. pneumoniae infection directly to key atherogenic events has emerged only recently. C. pneumoniae was shown to induce human macrophage foam cell formation and monocyte oxidation of low-density lipoprotein (LDL), implicating this organism as a causative agent in atherosclerosis. It is postulated that C. pneumoniae triggers these atherogenic events through specific virulence determinants. Two such components implicated in the pathogenesis of other chronic inflammatory chlamydial diseases (i.e., trachoma, pelvic inflammatory disease) are chlamydial lipopolysaccharide (cLPS) [8] and chlamydia hsp60 (chsp60) [9]. cLPS and chsp60 both are biologically active in vitro and can induce secretion of inflammatory cytokines or expression of adhesion proteins by endothelial cells, smooth muscle cells, and macrophages [8, 10]. C. pneumoniae, like all chlamydiae, synthesizes abundant amounts of cLPS and chsp60, and both of these components also may be involved in the pathogenesis of atherosclerosis. For example, Linnanmaki et al. [11] demonstrated immune complexes in patients with CAD. More recently, Kol et al. [12] identified the presence of chsp60 within human atheromatous tissue.

We present data that link cLPS and chsp60 to key atherogenic events involving altered mononuclear phagocyte-lipoprotein interactions. Studies aimed at elucidating how these putative virulence determinants signal mononuclear phagocytes to modulate lipoprotein accumulation and oxidation are discussed.

cLPS and Macrophage Foam Cell Formation

Macrophage foam cell formation and C. pneumoniae. Macrophages are central mediators of the inflammatory atheromatous lesion [13]. The hallmarks of early lesions in atherosclerosis are lipid-laden macrophages or foam cells. Macrophages cultured in the presence of native LDL do not accumulate lipids to become foam cells. This is because the native LDL receptor is tightly regulated by intracellular free cholesterol content [14, 15]; receptor-mediated endocytosis of LDL and subsequent ex-
traction of its cholesterol lead to transcriptional down-regulation of LDL receptors.

These regulatory mechanisms have been proposed to maintain a homeostatic level of intracellular cholesterol [16]. However, macrophages infected with *C. pneumoniae* cannot regulate intracellular cholesterol content and accumulate excess levels of exogenous lipids [17]. The precise mechanism of *C. pneumoniae*-induced lipid accumulation and foam cell formation is not known but it involves increased binding and uptake of LDL as assessed by studies using a fluorescent carbocyanine dye (DiI)-labeled LDL (DiI-LDL) [18]. *C. pneumoniae* enhanced cellular uptake and binding of DiI-LDL in a dose-dependent manner with respect to both *C. pneumoniae* and DiI-LDL. Of interest, increased DiI-LDL uptake by infected macrophages can be inhibited by unlabeled native LDL and by other lipoproteins such as high-density lipoprotein (HDL), very low-density lipoprotein, and oxidized LDL in a dose-dependent manner. Furthermore, *C. pneumoniae* increases DiI-LDL uptake and induces foam cell formation in macrophages isolated from native LDL receptor (apoB/E receptor)-deficient mice.

Taken together, these data indicate that *C. pneumoniae* induces macrophage foam cell formation by inducing LDL uptake through mechanisms independent of the classical apoB/E LDL receptor [18]. Furthermore, since native unlabelled LDL inhibits *C. pneumoniae*-induced macrophage DiI-LDL accumulation, it appears that LDL oxidation is not necessary for lipoprotein entry. This view is supported by additional evidence; for example, increased DiI-LDL association occurs even in the presence of exogenous antioxidants such as vitamin E and butylated hydroxytoluene, and oxidation products cannot be detected in the medium throughout the course of the experiment [18]. However, it also is possible that *C. pneumoniae*-infected macrophages minimally modify LDL to facilitate cellular entry of the lipoprotein. If receptor-specific processes are involved in foam cell formation by infected macrophages, then identification of macrophage lipoprotein receptors dysregulated by *C. pneumoniae* will help elucidate the mechanism of enhanced LDL entry.

An intriguing possibility is that *C. pneumoniae* induces foam cell formation not only by promoting lipoprotein binding and entry but also by interfering with the macrophage’s cholesterol efflux machinery. Indeed, chlamydial can disrupt intracellular lipid trafficking by redirecting newly synthesized, membrane-bound lipids to chlamydial inclusions [19–22]. Recent advances in understanding reverse cholesterol transport pathways [23] may permit detailed analysis of macrophage lipid efflux following *C. pneumoniae* infection. For example, impaired cholesterol efflux from macrophage leads to decreased levels of HDL and the presence of foam cells throughout the body in patients afflicted with Tangier disease, a condition linked to defects in the ATP-binding cassette transporter 1 (ABC1) [24–26]. If *C. pneumoniae* dysregulates cholesterol efflux regulatory proteins such as ABC1, then the pathogen could dysregulate cellular cholesterol homeostasis by enhancing lipoprotein entry, down-regulating lipid efflux, or by a combination of both mechanisms (summarized in figure 1).

cLPS in *C. pneumoniae*-induced macrophage foam cell formation. To identify *C. pneumoniae* virulence determinants responsible for *C. pneumoniae*-induced foam cell formation, chlamydial elementary bodies (EBs) were exposed to a variety of treatments before being added to macrophages. Exposure of *C. pneumoniae* EBs to periodate, but not elevated temperatures, inhibited cholesterol ester accumulation and suggested a role for cLPS in foam cell formation [27]. Indeed, purified cLPS

**Figure 1.** Possible involvement of both influx (uptake of low-density lipoprotein [LDL]) and efflux of cholesterol (ABC transporter) in generation of foam cells and pathogenesis of atherosclerosis. One hypothesis is that *C. pneumoniae* up-regulates entry and down-regulates cholesterol efflux (as adapted from [26a]).
Figure 2. Foam cell formation by cLPS- or enterobacterial (e) LPS-treated RAW macrophages. Cell culture methods are described in [27]. Cells were treated with varying concentrations of cLPS (○), Salmonella typhimurium Re595 LPS (●), or Escherichia coli O158:B8 LPS (▼) for 2 h and washed and incubated for 22 h in 10% fetal bovine serum–RPMI 1640 medium with 100 μg/mL low-density lipoprotein. Macrophages were scored for cytoplasmic neutral lipid droplets as described in [27]. * Statistically significant difference vs. similar concentrations of Re LPS and O158:B8 LPS, \( t \) test. = Statistically significant difference vs. 500 ng/mL Re LPS, \( t \) test.

Figure 3. NF-α secretion by cLPS- or enterobacterial (e) LPS-treated monocytes. A. Human monocytes freshly isolated as described in [36] were treated with varying concentrations of cLPS (○) or Escherichia coli O158:B8 LPS (●), cultured in RPMI in absence of serum, and assayed for tumor necrosis factor (TNF)-α concentration in supernatant after 24 h by commercial kit. * Statistically significant difference vs. similar concentrations of cLPS, \( t \) test. TNF-α secretion was assessed directly from whole blood ex vivo [29] treated with varying concentrations of cLPS (○), Salmonella typhimurium Re595 LPS (●), or E. coli O158:B8 LPS (▲) as described. Data in B are representative of 2 similar experiments.

was sufficient to induce cholesterol ester accumulation and foam cell formation, and the LPS antagonist lipid X inhibited C. pneumoniae– and cLPS–induced lipid uptake [27]. Additional studies showed that cLPS was sufficient to induce specific Dil-LDL uptake by macrophages in a manner similar to C. pneumoniae [18]. Taken together, these data suggest that cLPS is a major component of C. pneumoniae responsible for macrophage foam cell formation.

cLPS is a major structural component of all chlamydial species [8]. It contains a pentaacetyl-1,4'-diphosphoryl lipid A moiety instead of the classical hexaacetyl-1,4'-diphosphoryl lipid A of enterobacteria [8], which predicts that it should have low biologic activity. This prediction is based on examination of the structure-to-function relationship of “toxic” LPS, where a change in the structure of hexaacetyl-1,4'-diphosphoryl lipid A in any direction leads to lower activity [28]. Thus, a monophosphoryl lipid A is only 1% as toxic as diphosphoryl lipid A, and heptaacetyl, pentaacetyl, and tetraacetyl lipid A as also are
much less active than the model structure. Indeed, compared with enterobacterial LPS (eLPS), cLPS is a weak inducer of tumor necrosis factor (TNF)-α secretion from whole blood ex vivo [29]. Preliminary experiments suggest that cLPS also is weaker than eLPS in inducing macrophage foam cell formation (figure 2). Ingalls et al. [29] postulated that the relatively weak potency attributed to cLPS accounts for chronic inflammation characteristic of persistent chlamydial infections. This view certainly could be extended to include chronic *C. pneumoniae* infection in the pathogenesis of atherosclerosis.

Understanding why cLPS and eLPS exhibit differences in biologic activity may help elucidate how cLPS activates macrophages to accumulate lipoproteins. Although cLPS is less potent than eLPS in eliciting TNF-α production from whole blood (or monocytes), it is relatively more effective than eLPS in inducing TNF-α secretion from monocytes cultured in serum-free conditions (figure 3). This observation suggests that cLPS interacts differently with known serum LPS-binding proteins compared with eLPS, perhaps due to its unique pentaacyl-1,4-diphosphoryl lipid A moiety.

**chsp60 and Mononuclear Phagocyte LDL Oxidation**

Mononuclear phagocyte LDL oxidation and *C. pneumoniae*. A key atherogenic event in the pathogenesis of atherosclerosis is widely considered to be the oxidation of lipoproteins, particularly LDL [30]. Native LDL modified by oxidation acquires highly atherogenic properties to injure atheroma cell types, promote smooth muscle cell proliferation and foam cell formation, and serve as chemotactic factors for inflammatory leukocytes [31]. Furthermore, all atheroma cell types can oxidize LDL to render it more atherogenic [30, 31]. A variety of clinical and animal studies indicate that oxidation of LDL can occur in vivo [32–34], and some evidence exists to suggest a cardioprotective effect of antioxidants in humans [35]. Therefore, oxidation of LDL, a biologically plausible mechanism of LDL modification, may explain why a high blood level of native LDL is the primary risk factor for atherosclerosis.

If *C. pneumoniae* promotes oxidation of LDL, then a role for the pathogen in atherogenesis would be strengthened. To test this hypothesis, human monocytes were infected with increasing concentrations of *C. pneumoniae* and cultured in the presence of LDL. *C. pneumoniae* was found to induce monocyte oxidation of LDL in a dose-dependent manner by three different methods used to assess oxidation [36]. LDL oxidation was enhanced with longer incubation periods and with increasing concentrations of LDL, whereas it was inhibited by the antioxidant α-tocopherol (vitamin E). Of interest, superoxide production was not necessary to induce LDL oxidation by infected monocytes. Taken together, these data indicate that *C. pneumoniae* is capable of activating mononuclear phagocytes to promote the oxidation of LDL, thereby suggesting another pathogenic mechanism for this organism in atherogenesis [36].

**Table 1.** Summary of virulence determinants in mononuclear phagocyte-lipoprotein interactions.

<table>
<thead>
<tr>
<th>Effect on mononuclear phagocyte-lipoprotein interactions</th>
<th>Putative mechanisms of dysregulation</th>
<th>Host cell signaling receptor and associated carrier proteins</th>
<th>Chlamydial lipopolysaccharide (LPS)</th>
<th>Chlamydial hsp60</th>
</tr>
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<tbody>
<tr>
<td>Major <em>C. pneumoniae</em> component that induces mononuclear phagocyte LDL uptake and subsequent foam cell formation</td>
<td>1. Increased expression of promiscuous lipoprotein receptor</td>
<td>Unknown. Pathway may be different than enterobacterial LPS signaling</td>
<td>Major <em>C. pneumoniae</em> component that induces mononuclear phagocyte oxidation of LDL</td>
<td>2. Does not involve the classical native LDL (apoB/E) receptor</td>
</tr>
<tr>
<td>Largely unknown. <em>C. pneumoniae</em>-induced LDL oxidation is superoxide independent</td>
<td>2. May involve myeloperoxidase, lipooxygenase, or NADPH oxidase systems</td>
<td>2. May involve disruption of cholesterol efflux mechanisms (i.e., dysregulation of ABC1 transporter)</td>
<td>3. May involve disruption of cholesterol efflux mechanisms (i.e., dysregulation of ABC1 transporter)</td>
<td>3. Does not involve the classical native LDL (apoB/E) receptor</td>
</tr>
<tr>
<td>1. Increased expression of promiscuous lipoprotein receptor</td>
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**NOTE.** LDL, low-density lipoprotein; apo, apolipoprotein.

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**chsp60 in C. pneumoniae–induced monocyte LDL oxidation.** Exposure of *C. pneumoniae* to elevated temperatures, but not UV light, greatly reduced the capacity of the organisms to induce monocyte LDL oxidation [36]. This observation suggested that cLPS did not play a major role in *C. pneumoniae*–induced monocyte LDL oxidation. Instead, it was hypothesized that an inflammatory chlamydial protein could activate monocytes to oxidize LDL. The 60-kDa chsp60 is one such inflammatory protein that induces mononuclear phagocyte LDL oxidation.

**Table 2.** Effect of truncated versions of chlamydial hsp60 in tumor necrosis factor (TNF)-α induction by using human peripheral blood ex vivo assay.

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Induction of TNF-α</th>
<th>Portion of Molecules</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native chsp60</td>
<td>++ +</td>
<td>αα 1-544</td>
</tr>
<tr>
<td>F1</td>
<td>+ + +</td>
<td>αα 1-544</td>
</tr>
<tr>
<td>F2</td>
<td>+ + +</td>
<td>αα 101-544</td>
</tr>
<tr>
<td>F3</td>
<td>+ +</td>
<td>αα 201-544</td>
</tr>
<tr>
<td>F4</td>
<td>–</td>
<td>αα 301-544</td>
</tr>
<tr>
<td>F5</td>
<td>–</td>
<td>αα 401-544</td>
</tr>
<tr>
<td>(−) Control</td>
<td>– to +</td>
<td>–</td>
</tr>
<tr>
<td>(+) Control</td>
<td>+ + +</td>
<td>+ + +</td>
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</tbody>
</table>

**NOTE.** Human heparinized whole blood was plated in 96-well plates (160 μL) and 40 μL of appropriate dilutions of hsp60 or truncated versions were added (700–175 nM). Supernatant fluids were collected 24 h later and used in commercial ELISA format to measure TNF-α induction.

a Negative controls were either buffer alone or heat-treated versions of proteins and peptides.

b Positive controls were appropriate concentrations of *Escherichia coli* lipopolysaccharide (0.5–2.0 μg/mL).
Figure 4. LPS and hsp60 both act to stimulate proinflammatory signal transduction pathways. LPS pathway involves several steps that include fluid-phase binding (LBP), linking to host cell co-receptor (CD14), and activation of signaling receptor (Tlr 2 and/or 4). Similar steps may be involved in induction of proinflammatory and atherogenic steps mediated by hsp60, but details have not been identified. Traf-6, tumor necrosis factor receptor-associated factor 6; IRAK, immune response-activated kinase; NIK, NF Kappa B-inducing kinase; IKK, I Kappa kinase (as adapted from [41]).

protein implicated in the pathophysiology of chronic chlamydial infections [9]; furthermore, it recently was localized within human atheromatous lesions [12]. When the role of chsp60 in LDL oxidation was investigated, it was found that chsp60 induced monocyte LDL oxidation in a dose-dependent manner [36]. Another chsp (chsp10) did not induce monocyte LDL oxidation. It was concluded that chsp60 is a C. pneumoniae component that induces monocytes to oxidize LDL, thus identifying a second virulence determinant potentially important to the pathogenesis of atherosclerosis (table 1).

chsp60 is a highly immunogenic chlamydial protein that provokes a strong humoral and cellular immune response, and the immunopathology resulting from these responses likely leads to the adverse sequelae of chlamydial infection [9]. In fact, anti-chsp60 antibody isolated from patients with CAD cross-reacts with human hsp60 to mediate endothelial cytotoxicity in the presence of complement [37]. However, chsp60 also may promote inflammation by directly activating host cells, since chsp60 alone is sufficient to induce LDL oxidation [36] and matrix metalloproteinase production [12] by mononuclear phagocytes; cytokine production by mononuclear phagocytes, endothelial cells and smooth muscle cells [10]; and adhesion molecule expression by endothelial cells [10]. Therefore, chsp60 may function in two ways to promote atherosclerotic heart and vessel disease: first by direct antigenic stimulation and second as a signal transducer that results in activation of cells within atheromatous lesions.

How chsp60 interacts with host cells to transduce activating signals remains unknown. There have been no reports of hsp60 receptors, although the presence of specific receptors is suggested by studies with Legionella pneumophila hsp60; L. pneumophila hsp60 mediates invasion into HeLa cells via specific receptor-ligand interactions [38, 39] and induces interleukin-1β mRNA via protein kinase C signaling [40]. An initial approach to identify cellular receptors capable of interacting with hsp60 is to determine which region of the protein is sufficient for cellular signaling. To this end, truncated versions of chsp60 were used to stimulate TNF-α secretion from whole blood ex vivo (table 2). It was found that chsp60 fragments F1 (full-length chsp60), F2 (amino acids [aa] 101–544), and F3 (aa 201–544) induced TNF-α secretion to varying degrees, whereas fragments F4 (aa 301–544) and F5 (aa 401–544) did not induce any appreciable TNF-α production. These data suggest either a conformation-dependent requirement for activity or a role for the aa 201–300 region in directing cellular signal transduction. Further work will be required to distinguish between these possibilities and identify the smallest peptide sufficient to induce cell activation, which may be useful in identifying a chsp60 receptor.

Conclusions and Future Directions

To establish a cause-effect relationship between C. pneumoniae and atherosclerosis, it is first necessary to understand how the pathogen can initiate or promote the disease. Two putative pathogenic mechanisms involve the capacity of C. pneumoniae to dysregulate mononuclear phagocyte-lipoprotein interactions. In the presence of high concentrations of LDL, a known risk factor for development of CAD, C. pneumoniae induces mononuclear phagocyte foam cell formation [17, 18, 27] and LDL oxidation [36]. Importantly, foam cell formation appears to occur independently of LDL oxidation, permitting analysis of specific chlamydial virulence determinants responsible for each
event. Two chlamydial components associated with chronic inflammatory chlamydial infections are involved in these processes: cLPS is the major chlamydial component that induces mononuclear phagocyte foam cell formation [27], whereas chsp60 induces mononuclear phagocyte oxidation of LDL [36] (table 1). Thus, specific chlamydial virulence determinants may promote atherogenesis by dysregulating specific mononuclear phagocyte functions. What remains to be understood is how cLPS and chsp60 signal mononuclear phagocytes to dysregulate cell-lipoprotein interactions.

It is likely that the signaling pathways between cLPS and chsp60 overlap since similar cellular activities are invoked by both components (figure 4). Unique chsp60- and cLPS-specific signaling proteins may be uncovered by conducting experiments in the presence or absence of serum or specific carrier proteins. chsp60-specific cellular receptors also may be identified by determining which region of chsp60 is sufficient to activate the host cell. Ultimately, understanding how cLPS and chsp60 promote atherogenic events such as foam cell formation and LDL oxidation may elucidate how C. pneumoniae contributes to the pathogenesis of atherosclerosis.

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References


