Chlamydia, Inflammation, and Atherogenesis

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Atherosclerotic lesions are initiated and progress largely as a result of a chronic, fibroproliferative, inflammatory response. This review discusses how Chlamydia pneumoniae could conceivably contribute to this chronic inflammatory response and reports on recent in vivo and in vitro studies. In vivo studies in mice demonstrate that C. pneumoniae infection is disseminated to the artery wall following infection in the lung by alveolar macrophages. Recent in vitro studies show that infected U937 cells can directly transfer infection to endothelial cells and can indirectly increase the susceptibility of endothelial cells to C. pneumoniae infection. Loading of RAW 264.7 cells with modified forms of low-density lipoprotein increases the resistance of the cells to C. pneumoniae infection and also increases the susceptibility to the combined toxic effects of modified lipids and C. pneumoniae infection.

The initiation and progression of atherosclerotic lesions involves an unusual fibroproliferative inflammatory response. Studies with hypercholesterolemic animal models and analyses of diseased human arteries clearly demonstrate that the initiation and progression of atherosclerosis is largely a result of a dysregulated, fibroproliferative inflammatory response [1]. However, the inflammatory response that is characteristic of atherosclerotic lesions is an unusual type of inflammation. It is initiated and continuously propagated by lipid deposition coupled with other known risk factors and involves macrophages and lymphocytes but not neutrophils. Furthermore, the resulting encapsulation with connective tissue (fibrous cap formation) is the product of smooth muscle cells instead of fibroblasts. The dysregulation of the response is also indicative of the unusual nature of atherosclerosis in that the inflammation is progressive and terminates with complete regression of the plaque or a person's death rather than resolution of the inflammation. Nevertheless, many of the molecular mediators of the inflammation in the artery wall are consistent with those known to play a role in a classical inflammatory response, namely integrins, cytokines, chemokines, growth factors, and connective tissue proteins. However, it is still not clear what induces cellular activation and production of these inflammatory mediators. One possibility is the direct and indirect effects of infection with Chlamydia pneumoniae.

We provide an overview of the molecular and cellular biology of the atherogenic process with emphasis on how C. pneumoniae may contribute to this unusual fibroproliferative inflammatory response. We also discuss some of our recent in vivo and in vitro data, which help explain how C. pneumoniae may contribute to the atherogenic process.

Materials and Methods

C. pneumoniae strain AR-39, a respiratory isolate, was propagated in HL cells and purified by hypaque gradient centrifugation. Purified organisms were resuspended in chlamydial transport medium, sucrose-phosphate-glutamic acid, and stored at −75°C in small aliquots until use. Infectivity titers were assayed in HL cells. A 24-h monolayer of HL cells grown on a 12-mm-diameter coverslip in a 24-well culture plate was inoculated with 0.2 mL of the harvested cells. Inoculated plates were centrifuged for 1 h at 800 g at 25°C. After centrifugation, the inoculum was removed and the culture vial fed with 1 mL of MEM containing 0.6 μg/mL cycloheximide. After 3 days at 35°C, coverslips were removed and fixed with methanol, air dried, and stained with a Chlamydia genus-specific monoclonal antibody conjugated with fluorescein isothiocyanate. Inclusions were counted in 30 random fields at ×400 magnification. The infectivity titers (inclusion-forming units [ifu] per milliliter) were adjusted for the dilution factor and inoculum size and used for comparison. Duplicate or triplicate coverslips were counted in each experiment.

Dissemination studies. Following intranasal or intraperitoneal inoculation of C57BL/6 mice with C. pneumoniae, AR-39 (3 × 10^7 ifu per mouse), the organism was detected by direct plating, isolation, and polymerase chain reaction (PCR) in alveolar and peritoneal macrophages and in peripheral blood mononuclear cells. Alveolar or peritoneal macrophages from infected mice were transferred to the peritoneal cavity of uninfected mice and C. pneumoniae DNA was detected by PCR in lung, thymus, spleen, and abdominal lymph nodes of the uninfected mice as previously described [2].

Co-culture studies. HMEC-1, a transformed human arterial endothelial cell line, human umbilical vein endothelial cells (HUVEC), and human monocyte-like cells (U937) derived from a histiocytic lymphoma (ATCC CRL1593) were used in these studies.
Endothelial cells were cultured in endothelial cell growth medium (Clonetics, Walkersville, MD) supplemented with human recombinant epidermal growth factor, hydrocortisone, 2% fetal calf serum (FCS), gentamicin, amphotericin, and bovine brain extract. U937 cells were maintained in RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with 10% heat-inactivated FCS and 100 µg/mL each of streptomycin and vancomycin. HL cells were maintained in Eagle MEM (Gibco Laboratories) supplemented with 10% heat-inactivated FCS and 100 µg/mL each of streptomycin and vancomycin.

Endothelial and HL cells were trypsinized from stock cultures and plated at 2 x 10^4 cells/well to achieve a cell monolayer in 24 h in 24-well culture plates. A 12-mm-diameter glass coverslip was placed in each well for ease of staining for microscopic observation and for counting inclusions for assay titers. For inoculations, the culture fluid was removed and the cell monolayer washed three times with Hank’s balanced salt solution; 0.2 mL of inoculum was added per well, and the plate was centrifuged for 1 h at 700–800 g at 25°C. Due to the differences in cell lines, the optimal infectious doses were predetermined and used at an MOI of 1 for HL cells, 10 MOI for HMEC-1 cells, and 100 MOI for HUVEC cells. After centrifugation, the inocula were removed and 1 mL of U937 cells (infected and noninfected) suspended in RPMI 1640 culture medium supplemented with 2% FCS was added to the inoculum at an MOI of 1 for HL cells, 10 MOI for HMEC-1 cells, and 100 MOI for HUVEC cells. After centrifugation, the inocula were removed and 1 mL of U937 cells (infected and noninfected) suspended in RPMI 1640 culture medium supplemented with 2% FCS was added to cell monolayers of either noninfected or preinfected endothelial or HL cells at specified ratios. The plates were incubated in a CO2 incubator at 35°C for 3 days. The U937 cells were then removed by washing before assaying the endothelial and HL cells for infectivity. We measured both the first passage titer indicative of the inclusion counts in the primary endothelial culture and the second passage titration of the infectious units contained in the harvested endothelial cells.

**Foam cell studies.** RAW 264.7 cells (transformed mouse macrophages) were cultured in Dulbecco’s MEM (low glucose) containing 10% fetal bovine serum. They were incubated for 48 h with media containing 30 µg/mL protein of native, oxidized, acetylated, or aggregated human low-density lipopolysaccharide (LDL). Cells were infected with 10^6 ifu/mL of *C. pneumoniae*. Infected cells were quantified after 3 days of culture by using the fluorescence-labeled antibody directed against chlamydial lipopolysaccharide as described above. Numbers of infected cells were compared with non-lipid–loaded RAW cells (100%).

Statistical analyses were done by Student’s t test.

**Results and Discussion**

**Initiation of Lesions**

**Lipid deposition.** In the vast majority of cases, atherosclerotic lesions develop in response to elevated plasma lipoproteins. Our studies with normcholesterolemic mice and rabbits chronically infected with *C. pneumoniae* have shown that *Chlamydia* infection does not cause an elevation of plasma lipids, and in the absence of hyperlipidemia, does not initiate atherosclerotic lesions (Campbell LA, et al., elsewhere this supplement). In hypercholesterolemic animal models, however, the first measurable event in the atherogenic process is a specific trapping and retention of lipoproteins at lesion-prone sites [3, 4]. This presumably occurs as a result of the expression of a particular complement of proteins (e.g., proteoglycans), which have a high affinity for binding lipoproteins [5]. The trapping leads to a localized increase in the concentration of lipoprotein particles and an increased retention time that in turn facilitates both the cellular and spontaneous oxidation of the trapped particles [6].

There is no evidence to date that infection with *C. pneumoniae* contributes to the initial trapping and retention of lipoproteins in the artery wall. However, recent data suggest that infection with *C. pneumoniae* can stimulate cellular oxidation of LDL [7]. Secretion of the enzymes myeloperoxidase and 15-lipoxygenase by resident arterial macrophages can contribute to the oxidation of LDL [8, 9]. On the basis of results of previous studies with *Chlamydia trachomatis* and neutrophils [10], it is conceivable that infection of resident arterial macrophages with *C. pneumoniae* also induces expression of both myeloperoxidase and 15-lipoxygenase and in this way facilitates the oxidation of trapped lipoproteins (Kalayoglu MV, et al., elsewhere this supplement). In addition, infection of macrophages with *Chlamydia psittaci* stimulates a respiratory burst [11]. Thus, assuming there is a similar response to infection with *C. pneumoniae*, both the resulting superoxide and hydrogen peroxide could also partially contribute to the oxidation of the trapped lipoproteins [12].

**Leukocyte adherence and diapedesis: the initial phase of the inflammatory response.** The trapping of LDL is temporarily associated with an increase in the number of leukocytes that adhere to the endothelial surface [13]. The adherence and subsequent diapedesis of leukocytes is likely supported by the expression on the endothelium of leukocyte-specific adhesion molecules, such as vascular cell adhesion molecule-1 [14, 15], expression of the integrin counter receptors for these adhesion molecules by leukocytes, and by expression of leukocyte-specific chemoattractants, such as monocyte chemotactic protein (MCP)-1 by all of the different cell types resident within the

**Table 1.** Detection of *C. pneumoniae* DNA by polymerase chain reaction in tissues of C57BL/6J mice 3 days after intraperitoneal transfer of macrophages from infected mice.

<table>
<thead>
<tr>
<th>Tissues of recipient mice</th>
<th>Intranasal to alveolar macrophages</th>
<th>Intraperitoneal to peritoneal macrophages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung</td>
<td>1/4</td>
<td>3/4</td>
</tr>
<tr>
<td>Thymus</td>
<td>0/4</td>
<td>3/4</td>
</tr>
<tr>
<td>Spleen</td>
<td>3/4</td>
<td>4/4</td>
</tr>
<tr>
<td>Abdominal lymph nodesa</td>
<td>4/4</td>
<td>3/4</td>
</tr>
</tbody>
</table>

**NOTE:** Alveolar macrophages from 10 intranasally infected donor mice were collected 3 days after inoculation. Peritoneal macrophages from 10 intraperitoneally infected donor mice were collected 7 days after inoculation. Fractions represent no. positive over no. tested. Table adapted from [2].

a Abdominal lymph nodes include superior mesenteric, renal, and paraaortic nodes.
In addition to oxidation, the trapped LDL may also undergo modifications that include aggregation, complexation with matrix proteins and immunoglobulins, and glycosylation [28]. The macrophages, and to a lesser extent the endothelial cells and smooth muscle cells within the artery wall, express a variety of receptors that recognize the different types of modified lipoproteins. These receptors are now collectively known as scavenger receptors and include the classical types I and II scavenger receptors (type A), the SRB1 and CD-36 family of receptors (type B) [29, 30], and a recently described receptor (LOX-1), which is expressed by both endothelial cells and macrophages [31, 32]. Expression of the type A scavenger receptors is increased during the differentiation of monocytes into macrophages and is facilitated by the presence of the hematopoietic factor, macrophage-colony stimulating factor (MCSF) [33]. The type B scavenger receptors are induced by lipid accumulation and in particular by oxidized LDL [34, 35]. Of interest, C. pneumoniae infection stimulates LDL uptake by cultured macrophages [36] but it is not known whether infection causes differentiation and an up-regulation of expression of scavenger receptors that would account for the stimulatory effect of C. pneumoniae on foam cell formation.

Paradoxically, we recently observed that accumulation of modified lipoproteins by RAW mouse macrophages inhibits the subsequent infection of the cells by C. pneumoniae (figure 2). This suggests that once macrophages are transformed into foam cells, they become poor hosts for perpetuating and disseminating C. pneumoniae infection. It is unclear to what extent foam cells as opposed to non-lipid–loaded macrophages support infection within atherosclerotic lesions.

Lesion Progression

**Foam cell activation and proliferation.** Many of the foam cells within the lesions become activated to express a large number of factors that likely play important roles in stimulating the progression of atherosclerotic lesions. These include interleukin-1, MCP-1, MCSF, tumor necrosis factor (TNF–α), and leukin-1, MCP-1, MCSF, tumor necrosis factor (TNF–α), and interferon-γ.

### Table 2. Enhancement of susceptibility of human arterial endothelial cells (HMEC-1) to infection with C. pneumoniae (AR-39) by coculturing with human monocytes (U937): determination of an optimal ratio of U937 to HMEC-1 cells.

<table>
<thead>
<tr>
<th>Ratio</th>
<th>0</th>
<th>0.25</th>
<th>0.5</th>
<th>2.5</th>
<th>5</th>
<th>10</th>
<th>25</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>1.1</td>
<td>3.8</td>
<td>3.3</td>
<td>2.2</td>
<td>1.9</td>
<td>1.6</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>1.1</td>
<td>2.5</td>
<td>5.4</td>
<td>2.8</td>
<td>2.1</td>
<td>0.9</td>
</tr>
<tr>
<td>Average</td>
<td>1</td>
<td>1.1</td>
<td>3.2</td>
<td>4.4</td>
<td>2.5</td>
<td>2.0</td>
<td>1.3</td>
</tr>
</tbody>
</table>

* Relative to cultures in absence of U937 cells (ratio of U937/HMEC = 0).

Table 3. Transmission of infection to human arterial endothelial (HMEC-1) and HL cells by coculturing with C. pneumoniae (AR-39)–infected human monocytes (U937 cells).

<table>
<thead>
<tr>
<th>Duration (days)</th>
<th>Infectivity titers (ifu)</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HMEC</td>
<td>HL</td>
</tr>
<tr>
<td>0</td>
<td>89</td>
<td>3567</td>
</tr>
<tr>
<td>1</td>
<td>25</td>
<td>15</td>
</tr>
<tr>
<td>2</td>
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<td>60</td>
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<tr>
<td>3</td>
<td>4305</td>
<td>1107</td>
</tr>
<tr>
<td>4</td>
<td>492</td>
<td>233</td>
</tr>
</tbody>
</table>

NOTE. U937 cells were infected at MOI of 1 and identical nos. of U937 cells were added to wells containing HMEC and HL cells. U937 cells were removed by washing before harvest of HMEC and HL cells. Second passage titration of infectious units contained in harvested cells was measured in HL cells 3 days after coculturing.

a Immediately after 1 h adsorption.

b Due to direct infection by organisms attached to but not phagocytized by U937 cells.
platelet-derived growth factor (PDGF), transforming growth factor (TGF)-β, fibroblast growth factor (FGF), tissue factor, and a variety of stress proteins [1, 37]. As noted, we do not yet know what induces this activation in vivo. However, in vitro studies again suggest that intracellular oxidative stress may be involved. It can contribute to both signal transduction cascades and the activation of transcription factors [38]. Infection with *C. pneumoniae* also likely contributes to expression and/or activation of these factors [39]. Surprisingly, a significant number of macrophages within atherosclerotic lesions are also activated to proliferate [40]. Again, local expression of MCSF may be responsible for the macrophage proliferation, and in vitro studies have shown that oxidized LDL can induce DNA synthesis in macrophages [41]. It is not known whether infection with *C. pneumoniae* also induces macrophages to proliferate.

T cells in the lesions also become activated [42]. Furthermore, macrophages can present antigens derived from oxidized lipoproteins or oxidized cellular proteins to T cells isolated from human plaques and stimulate T cell activation and proliferation [43]. Whether T cells obtained from atherosclerotic lesions become activated in response to macrophage presentation of *C. pneumoniae*-derived antigens has not been determined.

Smooth muscle cell migration, proliferation, and connective tissue synthesis. One hallmark of the transition of the fatty streak into a more advanced lesion is the formation of the fibrous cap [44]. The formation of the fibrous cap is likely a protective smooth muscle cell adaptive response to changes in the patterns of blood flow and/or an attempt to reduce the influx of lipoproteins and inflammatory cells. Formation of the fibrous cap is dependent on a localized increase in the smooth muscle cell and connective tissue content of the lesions and involves the migration of smooth muscle cells from the media into the intima coupled with a phenotypic switch from a contractile cell to a cell type that produces extracellular matrix and produces large amounts of connective tissue [45, 46]. Evidence suggests that macrophage-derived factors may play a role in the induction of this phenotypic switch [47]. In particular, the growth factors PDGF, FGF, and TGF-β are expressed by activated macrophages in the lesions [1] and may contribute to stimulating the smooth muscle cell migration, proliferation, and connective tissue synthesis. The expression of these factors may also be in response to oxidative stress within the macrophages [38] or to activation by *C. pneumoniae*.

Cell necrosis and apoptosis. Another hallmark of the transition to an advanced atherosclerotic lesion is the formation of a lipid-rich necrotic core [44]. One key cellular event that may be associated with the formation of the necrotic core is the necrotic or apoptotic death of cells at the base and margins of the plaque. There is substantial evidence that many macrophages and smooth muscle cells located adjacent to a developing core contain fragmented DNA [48]. Many of these cells also contain large amounts of lipid oxidation products. The fragmentation of the DNA may be a response to the accumulation of excess oxidized lipids resulting in a terminal oxidative stress. DNA fragmentation may also be the result of the activation of the programmed cell death/apoptosis pathway in response to the localized expression of factors such as TNF-α and Fas ligand or increased formation of the sphingolipid ceramide [38]. *C. pneumoniae* infection may also contribute to cell death within atherosclerotic plaques, but to date there is no evidence that dying cells within the lesions are also infected with *C. pneumoniae*. Furthermore, previous studies showed that...
Cells were infected with *C. pneumoniae* proteins for 48 h before infection with 10⁸ ifu of arterial wall cells from the lungs. *C. pneumoniae* atherogenic process and disseminate with regard to macrophages that play a variety of roles in the arterial wall.

### Conclusions

Oxidized LDL accumulation of oxidized LDL followed by infection with *C. pneumoniae*. Effects of preincubation with native and oxidized low-density lipoprotein (LDL) and infection with *C. pneumoniae on RAW 264.7 cell protein and cholesterol concentrations.

<table>
<thead>
<tr>
<th>LDL concentration (µg/mL)</th>
<th>Protein cellular concentration (µg/mL)</th>
<th>Cholesterol (µg/µg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>200</td>
<td>505</td>
</tr>
<tr>
<td>Native LDL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>236</td>
<td>699</td>
</tr>
<tr>
<td>20</td>
<td>248</td>
<td>984</td>
</tr>
<tr>
<td>30</td>
<td>252</td>
<td>992</td>
</tr>
<tr>
<td>Oxidized LDL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>261</td>
<td>812</td>
</tr>
<tr>
<td>20</td>
<td>321</td>
<td>1389</td>
</tr>
<tr>
<td>30</td>
<td>118</td>
<td>1712</td>
</tr>
</tbody>
</table>

NOTE. Raw cells were preincubated with designated concentration of lipoproteins for 48 h before infection with 10⁷ ifu of *C. pneumoniae* strain AR-39. Cells were infected with *C. pneumoniae* for 3 days before measurement of cell protein and cholesterol content. Data are mean of triplicate wells.

*C. pneumoniae* infection induces resistance to apoptotic stimuli in HeLa 229 cells [49]. However, preliminary data from our in vitro studies with RAW mouse macrophages have shown that accumulation of oxidized LDL followed by infection with *C. pneumoniae* kills more cells than either oxidized LDL or *C. pneumoniae* infection alone (table 4).

Another functional hallmark of the transition to a more advanced lesion is a loss of normal vascular tone [50]. This is likely due to a reduced availability of nitric oxide, possibly associated with an increased formation of reactive oxygen intermediates by macrophages [51, 52]. Increased production of superoxide for example will complex the available nitric oxide to form the highly toxic peroxynitrite [53]. Thus, stimulation of the respiratory burst in macrophages in response to *C. pneumoniae* could both inhibit nitric oxide-mediated vasodilation and contribute to cell death and formation of the necrotic core.

### Notes

**Table 4.** Effects of preincubation with native and oxidized low-density lipoprotein (LDL) and infection with *C. pneumoniae* on RAW 264.7 cell protein and cholesterol concentrations.

**References**