Decreased Expression of Liver X Receptor- α in Macrophages Infected with *Chlamydia pneumoniae* in Human Atherosclerotic Arteries in situ

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Key Words

Histopathology • Atherosclerosis • Arteries • Macrophages • Foam cells • *Chlamydia pneumoniae*

Abstract

In in vitro experiments, *Chlamydia pneumoniae* has been shown to infect macrophages and to accelerate foam cell formation. It has been hypothesized that the *C. pneumoniae* infection affects foam cell formation by suppressing the expression of liver X receptors (LXR), but whether such an event occurs in human atherosclerosis is not known. In this study we examined carotid artery segments, obtained by endarterectomy, in which the presence of *C. pneumoniae* was confirmed by both polymerase chain reaction and immunohistochemistry. The expression of LXR- α in macrophages infected with *C. pneumoniae* and macrophages that were not infected was compared using a quantitative immunohistochemical analysis. The analysis revealed a 2.2-fold reduction in the expression of LXR- α in *C. pneumoniae*-infected cells around the lipid cores in atherosclerotic plaques. In the cytoplasm of laser-capture microdissected cells that were im-

munopositive for *C. pneumoniae*, electron microscopy demonstrated the presence of structures with the appearance of elementary, reticulate and aberrant bodies of *C. pneumoniae*. We conclude that LXR- α expression is reduced in *C. pneumoniae*-infected macrophages in human atherosclerotic lesions which supports the hypothesis that *C. pneumoniae* infection might suppress LXR expression in macrophages transforming into foam cells.

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Introduction

Studies over the last decades indicate that immunologically mediated inflammatory responses are crucially involved in the initiation and progression of atherosclerosis and that certain infective agents may play a role in the development of the inflammatory reactions [1]. Among the most extensively studied pathogens are *Chlamydia pneumoniae* (*Chlamydia pneumoniae*), cytomegalovirus and *Helicobacter pylori* [1].

Accumulating evidence indicates that *C. pneumoniae* represents a risk factor for atherosclerosis [1–7]. *C. pneumoniae* is an obligate intracellular Gram-negative pathogen that causes bronchitis, pneumonia and other respiratory tract diseases [8, 9], and it may be transported through the endothelial barrier in the arterial wall via circulating monocytes from the respiratory tract [10–12]. Interaction of *C. pneumoniae*-infected cells with endothelial cells may cause endothelial dysfunction, accompanied by a cascade of pro-inflammatory effects [8, 13, 14]. In the arteries, *C. pneumoniae* has been found to affect various cell types, including resident macrophages, smooth muscle cells and dendritic cells [8, 14–19]. Epidemiological, clinical and experimental studies support a possibility that *C. pneumoniae* infection might aggravate the progression of atherosclerotic lesions [1, 3, 19–29].

As shown in in vitro studies the infection of arterial cells with *C. pneumoniae* results in the induction of the expression of inflammatory cytokines, adhesion molecules and matrix metalloproteinases [8, 29–33]. It has been reported that *C. pneumoniae* infection accelerates macrophage foam cell formation in vitro through the stimulation of low-density lipoprotein (LDL) oxidation and entry of atherogenic LDL to the cytoplasm, indicating that the microorganism represents a causative agent in atherosclerosis [31–33].

Chen et al. [34] and Naiki et al. [35] have hypothesized that *C. pneumoniae* might promote the development of foam cells by triggering pro-inflammatory Toll-like receptor-dependent signaling, but that this process might be inhibited by the activation of liver X receptor (LXR). LXR regulates cholesterol metabolism, controlling reverse cholesterol transport [35, 36–41].

LXR represents the nuclear receptor initially identified in the liver that forms a functional heterodimer with the retinoid X receptor in order to recognize and bind to its hormone response elements [36, 38]. Two isoforms of LXR have been identified and are referred to as LXR- α and LXR- β [36, 38–40]. It is established that LXR- α is expressed in the liver, intestine, adipose tissue and in macrophages, whereas LXR- β is expressed in most tissues [36, 38, 41]. It has been shown that LXR- α null mice display severe abnormalities in lipid and bile acid homeostasis which indicates the role of LXR- α as a sterol sensor and metabolic regulator of cholesterol and lipid homeostasis [36, 38, 42]. An imbalance in reverse cholesterol transport leads to lipid accumulation in peripheral cells, in particular in macrophages residing in the arterial wall, causing foam cell formation [34, 36, 40–44].

In in vitro experiments, *C. pneumoniae*-induced foam cell formation was found to be blocked by pharmacologic LXR agonist treatment [34, 35, 37]. This observation supports a possibility that *C. pneumoniae* infection affects foam cell formation by suppressing the expression of LXR [34, 35, 37]. Whether *C. pneumoniae* infection of macrophage foam cells in human atherosclerotic lesions relates to the altered expression of LXR is unknown. The present study was carried out in order to examine a possible relationship between *C. pneumoniae* infection and the expression of LXR- α by macrophages in human atherosclerotic lesions in situ.

Materials and Methods

Tissue Specimens

Archival tissue specimens, some characteristics of which were reported previously [15, 16, 44], were used in the present study and represented carotid artery tissues obtained by endarterectomy from 39 patients whose ages ranged from 52 to 75 years (24 males and 15 females) [15]. The study was carried out in accordance with the principles outlined in the Helsinki Declaration and informed consent was obtained from each patient [15]. Each tissue specimen was divided into three parts. One tissue part was embedded in OCT compound, frozen in liquid nitrogen and stored at -70°C until cryostat sectioning. A second tissue part from each specimen was fixed in 10% buffered formalin, processed and embedded in paraffin wax. The remaining tissue parts were used for electron microscopic analysis as detailed below. Histologically, analyzed specimens contained various types of atherosclerotic lesions, including types V and VI, according to Stary et al. [45]; in some specimens, areas of undiseased intima were adjacent to atherosclerotic lesions.

The presence or absence of *C. pneumoniae* in these tissue specimens was established by a combination of immunohistochemistry and PCR, that was carried out according to Campbell et al. [22] as reported previously [15]. In that study [15], in the 60 arterial specimens analyzed, *C. pneumoniae* was identified by both immunohistochemistry and PCR in 17 specimens (28%) (group I); in 2 specimens (3%), *C. pneumoniae* was identified by only immunohistochemistry (group II); in 19 specimens (32%), *C. pneumoniae* was detected by only PCR (group III); in 22 specimens (37%), no *C. pneumoniae* was detected by either technique (group IV). The negative controls were free of *C. pneumoniae* [15]. It was found that the presence of *C. pneumoniae* in the arterial specimens did not relate to the age or sex of the patient, the length of clinical history or any clinical data available [15].

Of the 39 archival tissue specimens selected for the present analysis, 17 contained *C. pneumoniae* as shown by both immunohistochemistry and PCR (PCR⁺/IH⁺ specimens; group I of our earlier study [15]); in the remaining 22 specimens selected for the present analysis, no *C. pneumoniae* was detected by either technique (PCR⁻/IH⁻ specimens; group IV of our earlier study [15]).

Antibodies and Immunohistochemical Procedures

Anti-*C. pneumoniae* monoclonal antibody (DakoCytomation; clone RR402) was used to detect *C. pneumoniae* in the sec-

tions as detailed previously [15]. Avidin-biotin complex (ABC) method was utilized for single immunostaining and negative controls were carried out according to the recommendations of Dowell et al. [46] as previously described [15].

Sets of parallel sections were immunostained with anti-*C. pneumoniae* antibody and anti-LXR- α antibody, specific for the LXR- α isoform (Santa Cruz Biotechnology; sc-34386). In order to determine an appropriate dilution of anti-LXR- α antibody for further investigation, tissue sections were initially immunostained with LXR- α antibody in different dilutions which showed that when sections were stained with the antibody in 1:10 or 1:20 dilutions, LXR- α immunopositivity was seen in all cells around the lipid cores in atherosclerotic plaques but when a 1:100 dilution of the antibody was applied, it was possible to visually distinguish cells that intensely expressed LXR- α and cells that displayed a low intensity of LXR- α expression (see Results section). Therefore, for all further analyses performed in the present study, immunohistochemical procedures were carried out using LXR- α antibody in a 1:100 dilution. Appropriate negative controls were carried out in all series of experiments. Double immunostaining utilized a combination of anti-*C. pneumoniae* antibody and anti-LXR- α in order to examine a possible association of *C. pneumoniae* location with LXR- α expression. In brief, after visualization of LXR- α , sections were washed with 0.1 M glycine-hydrochloric acid buffer, pH 2.2, and then incubated with anti-*C. pneumoniae* antibody. After rinsing in Tris-phosphate-buffered saline, the sections were incubated with biotinylated secondary antibody and then with alkaline phosphatase-conjugated streptavidin (Dako) or with avidin-biotin complex (Dako). A combination of the peroxidase-anti-peroxidase and alkaline phosphatase-anti-alkaline phosphatase (APAAP) techniques was also used. Controls were as for single immunostaining. Double immunostaining procedures were carried out as described in earlier publications [47, 48]. Double immunohistochemistry was also performed to visualize combinations of *C. pneumoniae* antigen and CD68 (anti-CD68, M0876; 1:50 dilution; Dako) and *C. pneumoniae* antigen and CD14 (anti-CD14, M0825; 1:20 dilution; Dako).

Computerized Quantitative Analysis of Relative LXR- α Expression

A computerized quantitative analysis of relative LXR- α expression was carried out at $\times 400$ magnification using the Image-Pro Plus image analysis program (Media Cybernetics). Five serial sections from each tissue sample were used for the analysis. The expression of LXR- α in each section was measured in pixels per 7 standard microscopic fields (0.04 mm² each) around the lipid cores in atherosclerotic plaques and the results were presented as means from all sections of the sample. Statistical comparison of LXR- α expression between the areas that displayed *C. pneumoniae* immunopositivity in consecutive tissue sections and the adjacent areas which were free from *C. pneumoniae* immunopositivity was performed by t test using Prism® 5 (GraphPad Software).

Computerized Quantitative Analysis of C. pneumoniae Immunopositivity

In order to compare *C. pneumoniae* immunopositivity levels in infected cells that intensely expressed LXR- α (LXR- α ^{high} cells) with *C. pneumoniae* immunopositivity levels in infected cells that were characterized by low-intensity LXR- α expression (LXR- α ^{low}

cells), a computerized quantitative analysis was carried out in double immunostained sections (anti-*C. pneumoniae*/anti-LXR- α) using the Image-Pro Plus image analysis program (Media Cybernetics). *C. pneumoniae* immunopositivity levels in individual cells were measured in standard microscopic fields (2,500 μ m² each) and the results were presented as means from all parameters analyzed in each sample. Statistical analysis was performed by t test using Prism 5 (GraphPad Software).

Electron Microscopy

Small pieces of tissue were fixed in 1% glutaraldehyde (Sigma) in phosphate-buffered saline (PBS), pH 7.4. Electron microscopy was performed according to the procedures detailed in our earlier publications [49, 50]. The ultrastructural features typical for different cell types were used in order to identify the nature of cells as used previously [49–51].

Electron Microscopic Immunohistochemistry

For electron microscopic immunohistochemistry, tissue pieces taken from some specimens were fixed in 0.2% glutaraldehyde and 3% paraformaldehyde in PBS (pH 7.4), and embedded in LR White resin according to the protocol described by Keita et al. [52]. Postembedding immunohistochemistry on ultrathin sections was carried out using anti-*C. pneumoniae* antibody according to the technique of Keita et al. [52] with gold-labeled secondary antibody prepared according to Simmons et al. [53]. Colloidal gold particles were prepared by the reduction of a solution of HAuCl₄ by a mixture of sodium citrate and tannic acid [53]. The contrast of ultrathin sections was increased by treatment with uranyl acetate.

Laser-Capture Microdissection and Electron Microscopy of C. pneumoniae-Immunopositive Cells

For laser-capture microdissection, tissue segments fixed in a solution containing 4% paraformaldehyde and 0.05% glutaraldehyde in PBS were cut into thin slices (about 1.5 mm in thickness). Immunohistochemical reaction using anti-*C. pneumoniae* antibody was carried out in the floating tissue slices by using APAAP and ABC techniques. After the immunohistochemical procedures were completed, the tissue slices were washed in PBS and re-fixed in 0.5% glutaraldehyde in PBS. After being washed in PBS, the tissue slices were oriented and embedded in OCT compound in liquid nitrogen. After being cut on a cryostat, sections were placed onto slides covered with polyethylene-naphthalate membranes (PALM Microlaser Technologies; 1,440–1,000). A PALM Laser-MicroBeam System (PALM Microlaser Technologies), which enabled the contact-free isolation of single cells, was used for microdissection. Cells that showed immunopositive staining with the anti-*C. pneumoniae* antibody and were located around the lipid cores were microdissected and catapulted into lids of 0.5-ml reaction tubes by using the laser pressure catapulting technique of the instrument. Preparation of microdissected cells for electron microscopy was carried out according to a modified technique described by Grant and Jerome [54], as used previously [55]. All of these lids containing the microdissected cells were fixed in 1% glutaraldehyde in PBS, postfixed in 1% OsO₄ and embedded in Araldite. Serial ultrathin sections of the embedded lids were then cut and placed on formvar-coated grids. The ultrathin sections were stained with uranyl acetate and lead citrate and were examined with the aid of an electron microscope.

Fig. 1. Foci of the arterial wall with signs of early atherosclerotic alterations that are immunopositive for the presence of *C. pneumoniae*. **a** Location of *C. pneumoniae* in the intima along the luminal surface of the arterial wall. **b** Detail of **a**. **c** Focal aggregates of cells immunopositive for the presence of *C. pneumoniae* located in the superficial layer of the intima. **d** Detail of **c**. ABC immunoperoxidase technique; visualization of *C. pneumoniae* with antibody RR402 (Dako) and DAB (brown reaction product); Counterstaining with Mayer's hematoxylin. Scale bars = 250 μ m. **e** Double immunostaining showing colocalization of *C. pneumoniae* immunopositivity and CD68 antigen. *C. pneumoniae* was visualized using ABC immunoperoxidase reaction and DAB (brown reaction product), while CD68 antigen was visualized using Fast red substrate kit (rose reaction product). Counterstaining with Mayer's hematoxylin. Scale bar = 20 μ m.

Results

Association of C. pneumoniae with Monocytes/Macrophages in Arterial Tissue Specimens

In all 17 IH⁺/PCR⁺ tissue specimens, *C. pneumoniae* immunopositive cells were found to be located in groups or cell aggregates that were irregularly distributed throughout the arterial intima. In some sites of the arterial specimens, *C. pneumoniae* immunopositive cells were found to be present only in the superficial layer of the intima along the luminal surface of the vessels (fig. 1a–d). As this was shown by double immunostaining that utilized a combination of anti-*C. pneumoniae* antibody and anti-CD68, *C. pneumoniae* immunopositivity was associated with macrophage type cells (CD68⁺) that displayed the appearance of foam cells (fig. 1e). In some ar-

terial segments, round shaped cells were observed in close proximity to the luminal surface and double immunostaining, utilizing a combination of anti-*C. pneumoniae* antibody and anti-CD14, demonstrated that these cells represented monocytes, some of which contained *C. pneumoniae* immunopositive granules in their cytoplasm (fig. 2a). The presence of co-localized CD14 antigen and *C. pneumoniae* immunopositivity was seen in these areas in cells located in the subendothelial space of the intima (fig. 2a). Electron microscopic analysis of the corresponding areas of the arterial wall demonstrated close apposition of monocytes to endothelial cells, under which in the subendothelial space, foam cells with inclusions containing structures that resembled *C. pneumoniae* microorganisms at different stages of their development were seen (fig. 2b, c).

Fig. 2. Attachment of monocytes to the surface of the arterial wall that displays the presence of *C. pneumoniae*. **a** Double immunostaining showing a close apposition of a monocyte (CD14⁺) to the surface of the arterial wall, the subendothelial layer of which shows immunopositivity for *C. pneumoniae*. Note the co-localized CD14 and *C. pneumoniae* immunopositivity in the subendothelial space (shown by small arrows). *C. pneumoniae* was visualized using ABC immunoperoxidase reaction and DAB (brown reaction product), while CD14 antigen was visualized using Fast red substrate kit (rose reaction product). Counterstaining with Mayer's hematoxylin. Scale bar = 10 μ m. **b** Attachment of monocyte to the endothelial monolayer, the subendothelial space of which contains foam cells with 'lipid droplets' and inclusions containing structures resembling *C. pneumoniae* microorganism at different stages of development. Note the cell processes formed by the monocyte in the direction to the endothelium. **c** Detail of **b**. Electron microscopy. Scale bar = 3 μ m.

Peculiarities of Association between LXR- α Expression and C. pneumoniae Immunopositivity in Macrophage Foam Cells

In atherosclerotic plaques, *C. pneumoniae* immunopositivity was most frequently observed in cells located around the 'lipid cores'. These areas were composed of CD68⁺ cells which, as this was shown by electron microscopy, were presented by macrophage origin cells with various amounts of 'lipid droplets' in their cytoplasm. Typically, the areas which were 'rich' in *C. pneumoniae* immunopositivity were adjacent to areas which displayed low levels of *C. pneumoniae* immunopositivity or even were completely free of *C. pneumoniae* immunopositivity (fig. 3a). Analysis of parallel consecutive sections stained with anti-LXR- α antibody indicated that the areas that were rich in *C. pneumo-*

niae immunopositivity displayed low levels of LXR- α expression and, in contrast, the areas that were 'poor' in *C. pneumoniae* immunopositivity were characterized by notable levels of LXR- α expression (fig. 3a, b). Although this relationship between *C. pneumoniae* immunopositivity levels and LXR- α expression was obvious in some consecutive sections (fig. 3a, b), such a relationship was questionable in other consecutive sections. Therefore, we further undertook a computerized quantitative analysis of LXR- α expression levels in plaque areas that corresponded to the areas that, in consecutive sections, were identified as poor or rich in *C. pneumoniae* immunopositivity. The quantitative analysis of *C. pneumoniae* immunopositivity around the lipid cores revealed that LXR- α expression in areas that were rich in *C. pneumoniae* immunopositive foam cells was approximately

Fig. 3. *C. pneumoniae* immunopositivity (a) and LXR- α expression (b, c) in atherosclerotic plaques. Consecutive sections immunostained with anti-*C. pneumoniae* antibody RR402 (a) and anti-LXR- α antibody (b). Note that areas containing *C. pneumoniae* immunopositivity display notably lower levels of expression of LXR- α . b The marked areas show different levels of LXR- α expression. ABC immunoperoxidase technique; *C. pneumoniae* was visualized using antibody RR402 and DAB (brown reaction product), while LXR- α was visualized using anti-LXR- α antibody and DAB (brown reaction product); counterstaining with Mayer's hematoxylin. Scale bars = 250 μ m. c Relative expression of LXR- α in atherosclerotic lesions, evaluated as a number of pixels per standard field (0.04 mm²) using computerized quantitative analysis (see Materials and Methods). C.p.⁺; P = *C. pneumoniae* PCR⁺/IH⁺ specimens; C.p.⁺; R = *C. pneumoniae* rich areas in *C. pneumoniae* PCR⁺/IH⁺ specimens; C.p.⁺ = *C. pneumoniae* PCR⁺/IH⁺ specimens; C.p.⁻ = *C. pneumoniae* PCR⁻/IH⁻ specimens (see Results section).

2.2 times lower than LXR- α expression in areas corresponding to the areas with poor *C. pneumoniae* immunopositivity (186.8 ± 24.8 and 85.2 ± 16.7 , respectively; $p = 0.0018$; fig. 3c).

We also compared the levels of LXR- α expression between PCR⁺/IH⁺ and PCR⁻/IH⁻ tissue specimens randomly measuring the levels of LXR- α expression in areas around the lipid cores (no parallel sections stained with anti-*C. pneumoniae* were used for this analysis). The computerized quantitative analysis failed to demonstrate statistical difference between LXR- α expression in PCR⁺/IH⁺ and PCR⁻/IH⁻ specimens (225.8 ± 26.1 vs. 263.3 ± 25.5 ; $p = 0.3171$; fig. 3c). However, a comparison of the levels of LXR- α expression in *C. pneumoniae* poor areas

in PCR⁺/IH⁺ specimens with LXR- α expression levels in PCR⁻/IH⁻ specimens did reveal statistical difference (186.8 ± 24.8 vs. 263.3 ± 25.5 ; $p = 0.0417$). Likewise, a comparison between the levels of LXR- α expression in *C. pneumoniae* rich areas in PCR⁺/IH⁺ specimens and LXR- α expression levels in PCR⁻/IH⁻ specimens demonstrated significantly higher LXR- α expression in *C. pneumoniae* rich areas in PCR⁺/IH⁺ than that in areas in PCR⁻/IH⁻ specimens (85.2 ± 16.7 vs. 263.3 ± 25.5 ; $p < 0.0001$). No statistical difference was shown between the levels of LXR- α expression in *C. pneumoniae* poor areas in PCR⁺/IH⁺ specimens and the levels of LXR- α expression in areas randomly taken around the lipid cores in PCR⁺/IH⁺ specimens (186.8 ± 24.8 vs. 225.8 ± 26.1 , accordingly;

Fig. 4. Comparative analysis of *C. pneumoniae* immunopositivity in LXR- α^{high} and LXR- α^{low} cells performed by computerized quantitative analysis in double immunostained sections (anti-*C. pneumoniae*/anti-LXR- α) in *C. pneumoniae* PCR⁺/IH⁺ specimens (see Materials and Methods as well as Results sections). **a** Double immunohistochemistry. *C. pneumoniae* was visualized using ABC immunoperoxidase reaction and DAB (brown reaction product), while LXR- α was visualized using Fast red substrate kit (rose reaction product). The arrows show cells that intensely express LXR- α . **b, c** Details of **a**. Scale bar = 20 μm .

Fig. 5. Ultrastructural identification of *C. pneumoniae* inclusions in cells located around the lipid cores in atherosclerotic plaques. **a** *C. pneumoniae* immunopositivity (shown by arrows) in inclusions revealed by electron microscopic immunohistochemistry utilizing immunogold technique. **b, c** *C. pneumoniae* inclusions identified in the cytoplasm of laser-capture microdissected *C. pneumoniae* immunopositive cells. **d, e** Details of **c**. In **c**, note the fusion of inclusions containing *C. pneumoniae* organisms at different stages of development. Electron microscopy. Scale bars = 0.5 μm .

$p = 0.2865$). LXR- α expression in areas randomly taken around the lipid cores in PCR⁺/IH⁺ specimens was found to be 2.7 times higher than LXR- α expression in *C. pneumoniae* rich areas in PCR⁺/IH⁺ specimens (225.8 ± 26.1 vs. 85.2 ± 16.7 ; accordingly; $p < 0.0001$).

Single immunostaining, utilizing anti-LXR- α antibody in a 1:100 dilution (see Materials and Methods section), demonstrated a marked variation in the intensity of LXR- α expression in cells located around the lipid cores: while some cells intensely expressed LXR- α in their nuclei, in others, LXR- α immunopositivity was low. Similarly, double immunostaining of PCR⁺/IH⁺ specimens utilizing an anti-LXR- α /anti-*C. pneumoniae* antibody combination showed that the intensity of the expression of LXR- α in *C. pneumoniae* immunopositive cells varied markedly (fig. 4a). Whereas the expression of LXR- α in some *C. pneumoniae* immunopositive cells was strong in the nuclei (fig. 4a, b), other *C. pneumoniae* immunopositive cells displayed low levels of LXR- α expression or LXR- α expression was practically undetectable visually (fig. 4a, c). A semi-quantitative evaluation of the double immunostained sections suggested that cells that intensely expressed LXR- α contained markedly low numbers of *C. pneumoniae* immunopositive inclusions in their cytoplasm (fig. 4a–c). Further comparison of *C. pneumoniae* immunopositivity levels between LXR- α^{high} and LXR- α^{low} cells, performed by computerized quantitative analysis, confirmed that in LXR- α^{high} cells the level of *C. pneumoniae* immunopositivity was 3.3 times lower than that in LXR- $\alpha^{\text{(low)}}$ cells (17.2 ± 2.9 vs. 56.1 ± 7.5 , accordingly; $p < 0.0001$; fig. 4d).

Distribution and Ultrastructural Identification of C. pneumoniae in Monocytes/Macrophages in the Arterial Wall

In the arterial wall, *C. pneumoniae* immunopositivity was presented mostly by granular staining located in the cytoplasm of cells. In the superficial layer of the intima, association of *C. pneumoniae* immunopositivity with CD14 was evident as shown by anti-*C. pneumoniae*/anti-CD14 double immunostaining. However, CD14⁺ cells were identified predominantly in the superficial layers of the intima, whereas CD14 immunopositivity levels were relatively low in atherosclerotic plaques around the lipid cores. Around the necrotic cores, *C. pneumoniae* immunopositivity was observed in cells that consistently displayed the presence of CD68 antigen.

In order to confirm that these *C. pneumoniae* immunopositive cells indeed represented cells infected with *C. pneumoniae*, we performed electron microscopic immu-

nocytochemical analysis. Electron microscopic immunocytochemistry demonstrated the presence of *C. pneumoniae* antigen within vacuoles of some cells, although the fine ultrastructure was not readily visible in these ultrathin sections (fig. 5a). The fine ultrastructure of *C. pneumoniae* immunopositive cells was examined in cells which were microdissected from areas around the lipid cores, using the laser-capture microdissection technique. Electron microscopic analysis showed that the microdissected cells contained structures, the appearances of which were typical of inclusions, elementary bodies and reticulate bodies of *C. pneumoniae* (fig. 5b–e), described in publications of other investigators [56–58]. In some *C. pneumoniae*-infected cells, the cytoplasm contained a large number of *C. pneumoniae* inclusions (fig. 5c). In such cells, the surrounding membranes of neighbouring *C. pneumoniae* inclusions were often apposed to each other (fig. 5c). The microdissected cells did not contain a basement membrane, ‘dense’ bodies along the plasma-membrane or visible microfilaments (fig. 5b–e), indicating their macrophage nature [50, 51, 59].

Discussion

The present study demonstrated a 2.2-fold reduction in the expression of LXR- α in *C. pneumoniae*-infected macrophage foam cells located around the lipid cores in atherosclerotic plaques in human carotid arteries. This is in accordance with in vitro findings and the hypothesis that *C. pneumoniae* infection affects foam cell formation by suppressing the expression of LXR [34, 35].

The examination of cells that intensely expressed LXR- α revealed markedly lower amounts of *C. pneumoniae* immunopositive inclusions in their cytoplasm, compared with that in macrophage foam cells that did not display LXR- α expression (in tissue sections immunostained with anti-LXR- α antibody in a 1:100 dilution). This observation provides further support to the possibility that *C. pneumoniae* might suppress LXR- α expression in macrophages. An alternative explanation might be that macrophages which differently express LXR- α in atherosclerotic lesions might represent different subpopulations of macrophages characterized by an unequal susceptibility to *C. pneumoniae* infection.

Accumulating knowledge indicates that in an organ or a tissue, the population of macrophages might consist of subpopulations with different gene expression patterns and different potentials for promoting pathological events [60–64]. A number of recent studies suggest that

the population of macrophages in atherosclerotic lesions is heterogeneous [60–64]. It has been shown that monocytes can differentiate in vitro into specific macrophage subpopulations in alternative cytokine environments, in particular, in response to granulocyte-macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF) [62–64]. Waldo et al. [64] have shown that human monocytes can be differentiated in vitro into two divergent macrophage phenotypes in response either to GM-CSF or M-CSF and that these macrophage phenotypes have potentially alternative functions in the promotion of atherosclerosis. Both phenotypes are capable of accumulating lipids and forming foam cells in vitro but they possess alternative inflammatory characteristics and encompass divergent regulation of cholesterol homeostasis [64]. The macrophage phenotype formed in response to GM-CSF has been found to express 5 times more LXR- α than the M-CSF-dependant phenotype [64]. Analyzing human coronary arteries, Waldo et al. [64] identified CD68⁺/CD14⁺ and CD68⁺/CD14⁻ macrophage subpopulations and suggested that their alternative functions develop in response to GM-CSF and M-CSF, respectively. In coronary arteries, the CD68⁺/CD14⁺ macrophage subpopulation predominates in atherosclerotic lesions, whereas the CD68⁺/CD14⁻ macrophage subpopulation predominates in intimal areas devoid of disease [64]. In tissue specimens of atherosclerotic carotid arteries analyzed in the present study, both CD14 and CD68 antigens were consistently observed in the superficial layer of the intima but the areas around the lipid cores were presented by foam cells, the vast majority of which displayed the presence of CD68 antigen only. LXR- α expression varied markedly in macrophages in this plaque zone, with LXR- α^{high} and LXR- α^{low} subpopulations being identified. This might imply that the population of macrophages in atherosclerotic lesions is more heterogeneous than it is currently thought. CD68⁺/CD14⁻ macrophages might be presented by different subpopulations. One cannot exclude that CD68⁺/CD14⁻/LXR- α^{high} cells might be less susceptible to *C. pneumoniae* infection than CD68⁺/CD14⁻/LXR- α^{low} cells.

C. pneumoniae is able to grow in vitro within a wide range of human cell types, including monocytes/macrophages, dendritic cells, endothelial cells and smooth muscle cells [2, 14, 17, 56, 65]. The presence of *C. pneumoniae* in these cell types was detected in human arterial wall as well [7, 9, 15, 18, 25, 65, 66]. However, the course of infection might be different in different cell types in atherosclerotic lesions. A recent study [65] re-

vealed that the infectious cycle of *C. pneumoniae* behaves differently between monocytic and endothelial cells. It has been suggested that, while the intracellular pathogen remains in a persistent form within monocytes, it can differentiate and proliferate within endothelial cells, indicating that endothelial cells are a preferred environment for *C. pneumoniae* [65]. Bellmann-Weiler et al. [65] showed that nutritive factors such as iron have subtle effects on *C. pneumoniae* biology in endothelial but not monocytic cells and that *C. pneumoniae* responds differently to immune stimulation by IFN- γ , modulation of iron availability and restriction of tryptophan in endothelial versus monocytic cells. Bellmann-Weiler et al. [65] suggested that monocytes may not be a good environment for the multiplication of *C. pneumoniae* but rather act as a shuttle for *C. pneumoniae* in the circulation to reach endothelial cells.

It is believed that *C. pneumoniae*-infected monocytes circulating in the blood penetrate the arterial wall and eventually cause widespread distribution of the microorganism in atherosclerotic lesions [10–12, 67]. This possibility is supported by experimental studies [28, 68, 69]. To our knowledge, the present study is the first report that visualizes the ‘attachment’ of monocytes to the luminal surface of arteries, the subendothelial space of which contains *C. pneumoniae*. In atherosclerotic plaques, *C. pneumoniae* was found to be distributed irregularly throughout the intima which might indicate that different zones in atherosclerotic lesions are characterized by the local microenvironmental conditions that differently affect the development potential of *C. pneumoniae*. In agreement with a number of earlier reports [25, 31, 40], the present study showed that *C. pneumoniae* is frequent in plaque zones consisting of macrophage origin cells. The ultrastructural observations provided morphological evidence that *C. pneumoniae* might not only survive but also develop in macrophages in human atherosclerotic lesions. In accordance with this, in vitro experiments showed that *C. pneumoniae* infection of monocytes induces their differentiation into macrophages [8, 31–33]. The mechanisms by which *C. pneumoniae* might affect macrophage function have been examined [8, 33, 36, 70–73] and it has been also shown that infection of macrophages with *C. pneumoniae* in vitro accelerates foam cell formation [24, 30–33, 73–76].

Uptake of modified lipids, primarily modified LDL, such as oxidized LDL, by means of scavenger receptors and by receptor-independent mechanisms is critical in the formation of foam cells from macrophages [43, 59, 63–66, 77–79]. LXRs antagonize this process by promot-

ing cholesterol efflux via the upregulation of the ABC family transporters, resulting in enhanced reverse cholesterol transport [36, 43, 73, 79, 80]. The present study indicates that in human atherosclerosis, reverse cholesterol transport in macrophages might be affected by *C. pneumoniae* infection.

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