Transmission of *Chlamydia pneumoniae* infection from blood monocytes to vascular cells in a novel transendothelial migration model

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Abstract

*Chlamydia pneumoniae* uses blood monocytes (PBMC) for systemic dissemination, persists in atherosclerotic lesions, and has been implicated in the pathogenesis of atherosclerosis. During transmigration in a newly developed transendothelial migration model (TEM) *C. pneumoniae*-infected PBMC spread their infection to endothelial cells. Transmigrated PBMC retained their infectivity and transmitted the pathogen to smooth muscle cells in the lower chamber of the TEM. Detection of chlamydial HSP60 mRNA proved pathogen viability and virulence. We conclude that PBMC can spread chlamydial infection to vascular wall cells and we suggest the TEM as a novel tool to analyze host-pathogen interactions in vascular chlamydial infections.

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1. Introduction

In recent years the understanding of the pathomechanisms leading to atherosclerotic disease has changed fundamentally. Besides the known risk factors like hypertension, hyperlipidemia, and diabetes, systemic infection with persistent bacterial (*Chlamydia (Chlamydophila) pneumoniae, Helicobacter pylori*) and viral (HSV, CMV) pathogens is related to proatherosclerotic changes within the vasculature [1]. Seroepidemiological studies described controversial results in the association of seropositivity and atherosclerotic disease progression [2–4]. However, systemic atherosclerotic disease is now considered as a chronic inflammatory process characterized by increased expression of pro-inflammatory and pro-proliferative factors [5].

So far, the obligate intracellular bacterium *C. pneumoniae* is the sole viable pathogen detected in atherosclerotic plaques from coronary artery disease (CAD) patients [6]. Furthermore, blood monocytes (PBMC) have been described as persistently infected with *C. pneumoniae* in up to 26% of CAD patients [7,8]. Using animal models we and others have shown that *C. pneumoniae* uses blood monocytes as a vector for systemic...
dissemination from the lungs to the vasculature [9,10]. Apparently, the chlamydial infection in PBMC is refractory to current antibiotic treatment protocols due to a non-replicative but viable persistent state of the pathogen [11].

In order to investigate interactions between C. pneumoniae infected PBMC and primary vascular endothelial cells and smooth muscle cells we have developed a new transendothelial migration model (TEM) that closely reflects the biological processes in the vascular wall. Briefly, vascular endothelial and smooth muscle cells were grown to confluency in close proximity within a microchamber system, and C. pneumoniae infected PBMC were stimulated to transmigrate through the endothelial cell monolayer towards the vascular smooth muscle cells along a chemotactic gradient of monocyte chemoattractant protein-1 (MCP-1). The aim of this study was to establish a model of vascular chlamydial infection which allows analysis of direct interactions between infected PBMC and the vascular compartment on a molecular and phenotypic level. In its first application we show transmission of chlamydial infection from PBMC to vascular cells as well as sustained transcriptional activity of the transmitted chlamydiae in endothelial and smooth muscle cells within 2–5 days after transmission.

2. Materials and methods

2.1. Culture of C. pneumoniae

The coronary artery isolate C. pneumoniae CV-6 was grown on HEp-2 monolayers as described previously [11]. Briefly, immortalized laryngeal epithelial cells (HEp-2, ATCC CLL 23) were grown in tissue culture plates (Greiner, Frickenhausen/Germany) with growth medium containing Eagle’s minimum essential medium (Sigma, Taufkirchen/Germany), 10% fetal calf serum (Biochrom, Berlin/Germany), L-glutamine (2 mM, Invitrogen, Karlsruhe/Germany), non-essential amino acids (Invitrogen), gentamicin (10 mg/l; Sigma), vancomycin (50 mg/l; Sigma), and amphotericin B (2 mg/l; Sigma). Confluent monolayers were infected with C. pneumoniae and growth medium was replaced by an antibiotic-free medium.

2.2. Culture of endothelial and smooth muscle cells

Primary endothelial and smooth muscle cells were cultured in 1% gelatin-coated culture flasks (Greiner). Human coronary artery endothelial cells (HCAEC) and coronary artery smooth muscle cells (CASMC) were obtained from Clonetics (St. Katharinen/Germany) and maintained in EBM-2 medium and SmGm2 medium (CellSystems, Berlin/Germany).

2.3. Isolation of human PBMC

PBMC obtained from buffy coat blood from healthy adult volunteers were diluted 1:6 in pyrogen-free saline over Histopaque 1077 (Sigma) and enriched to >94% purity by Ficoll (Sigma) and Percoll (Amersham, Freiburg/Germany) gradients. Fresh PBMC were washed twice in saline and suspended in RPMI1640 medium supplemented with 10 mg/l gentamicin and 10 mM L-glutamine, non-essential amino acids and seeded in culture flasks (Greiner). Non-adherent cells were removed by washing procedures after 2 h incubation at 37 °C and 5% CO₂ with RPMI1640 medium. Harvested PBMC were adjusted to 1 × 10⁶ cells/ml and infected with C. pneumoniae at a concentration of 4 IFU/cell for 24 h. PBMC from all donors were demonstrated to be free from chlamydial DNA by screening aliquots in a real-time PCR (LightCycler®, Roche Molecular Biochemicals, Mannheim/Germany) of the 16S rRNA gene region.

2.4. Transendothelial migration model

For transmigration experiments 5 × 10⁴ HCAEC were seeded onto 1% gelatin-coated 6.5 mm Transwell Clear™ inserts (Corning Costar, Bodenheim/Germany) and CASMC (5 × 10⁴) were cultured in the lower chambers of the Transwells on sterile glass coverslips (Fig. 1). After the confluency of the endothelial cell monolayers had been assessed by measuring their permeability to FITC-labeled bovine serum albumin [12], medium in the lower chambers was changed against EBM-2 medium, containing MCP-1 (Sigma; 5 ng/ml). Preconditioned monocytes at a density of 1 × 10⁶/ml were added to the upper chamber and the rate of transmigration was determined by counting cells that adhered to the underside of the polycarbonate membrane and to the CASMC monolayer after 4, 24, 38, and 48 h. The average number of transmigrated cells from a total of 10 high-power fields (HPFs; 1000× magnification) was projected to the area of the chamber (n = 3). Transmission of replicative C. pneumoniae infection from PBMC to HCAEC and CASMC was evaluated after 3 and 5 days by anti-chlamydial fluorescence staining as described below. Chlamydial HSP60 and 16S rRNA mRNA expression was analyzed on HCAEC and CASMC monolayers after repeated washing steps to take off remaining PBMC.

2.5. Fluorescence microscopy

Chlamydial infection of PBMC, HCAEC and CASMC was determined by fluorescence staining with FITC-labeled anti-Chlamydia-LPS monoclonal antibody (DAKO, Hamburg/Germany). To ascertain intracellular chlamydial infection, CASMC were double-stained with
rhodamine–phalloidine for actin-filaments (Tebu, Frankfurt/Germany) and chlamydial LPS mAB (kindly provided by H. Brade, Research Center Borstel, Germany) visualized with a FITC-labeled goat anti-mouse IgG antibody (Sigma). A two-color fluorescence assay (Live/dead Kit, MolecularProbes, Eugene/OR) was used to determine the viability of PBMC, HCAEC and CASMC over 120 h as instructed by the manufacturer. Briefly, Transwell inserts and sterile glass coverslips on the bottom of the microchamber were incubated with both fluorescent dyes in darkness for 45 min. Dye solution was removed, cells were fixed with 1% paraformaldehyde for 10 min and stored at −4°C. Cells with intact membranes are stained green with the highly membrane-permeant dye Syto 9®, whereas cells with damaged membranes are stained red due to an increased penetration of propidium iodide.

2.6. Real-time PCR

*C. pneumoniae* mRNA from HCAEC and CASMC was isolated using the NucleoSpin RNA II Kit (Macherey-Nagel, Düren/Germany). Extracted RNA was reverse transcribed into cDNA with random primers and reverse transcriptase according to the manufacturer’s protocol (Roche First-Strand PCR Kit, Mannheim/Germany). The expression of the chlamydial HSP60 and 16S rRNA gene was analyzed using the LightCycler® Detection System (Roche Molecular Biochemicals; i.e. 16S rRNA: forward [TCG CCT GGG AAT AAG AGA GA]; reverse [AAT GCT GAC TTG GGG TTG AG]; HSP60: forward [TTG GAT ATC AGG TTT GTT TCC TAA G]; reverse [AGA CTT CCT TCC AGT TTT ACA ACA A]). PCR was run using the following protocol: 95°C for 10 min, 40 cycles of 95°C for 10 s, 60°C for 5 s, 72°C for 10 s. In a dissociation protocol single peaks were confirmed in each of the amplified sequences to exclude non-specific amplification. Each sample was additionally amplified without prior RT as a control for the absence of DNA in the RNA preparation. PCR products were separated on a 2% agarose gel for visualisation.

3. Results and discussion

*Chlamydia pneumoniae* efficiently infects cells of the atherosclerotic plaques (macrophages, endothelial cells, smooth muscle cells) and implements its characteristic intracellular cycle of replication [13]. Activation of NF-κB in *C. pneumoniae* infected endothelial cells and smooth muscle cells mediates the secretion of inflamma-
tory and pro-coagulant factors (tissue factor, plasminogen activator inhibitor-1) in vitro, all of which are known to promote atherosclerosis [14,15]. Furthermore, *C. pneumoniae* induces the differentiation from PBMC to macrophages and the formation of lipid-loaded foam cells, which represent a major cellular component of atherosclerotic lesions [16,17]. Thus, it is justified to investigate the pathogenetic role of vascular chlamydial infection in more detail, taking into account the functional relevance of blood monocytes as a vector and a host cell for chlamydiae. We therefore established a microchamber model of vascular chlamydial infection, which reflects the architecture of the arterial wall in the conjunction of primary human endothelial and smooth muscle cells and infected PBMC. This system reliably permitted tracking of migration-related interactions between *C. pneumoniae* infected blood monocytes and vascular cells on the molecular and the phenotypical level.

### 3.1. Transendothelial migration of *C. pneumoniae* infected PBMC

Transmigration of *C. pneumoniae* infected PBMC through endothelial monolayers occurred in a time dependent manner. Fig. 2 shows the percentage of *C. pneumoniae* infected and non-infected PBMC that transmigrated through the HCAEC monolayer on MCP-1 attraction. Transmigration of infected PBMC to the lower chamber was comparatively delayed as *C. pneumoniae* infected PBMC initially remained more adherent to endothelial cells in the upper chamber. Facilitated adhesion of chlamydia infected monocytes to endothelial cells had previously been reported by Kaul et al. [18] in coinubcation experiments. Transmigration of infected PBMC was completed after 48 h. Interestingly, transmission of chlamydial infection was not restricted to endothelial cells during the prolonged transmigration process. PBMC were clearly able to transmigrate through the endothelial monolayer and remain infective for vascular smooth muscle cells.

#### 3.2. Transmission of replicative infection from *C. pneumoniae* infected PBMC to endothelial and smooth muscle cells during transendothelial migration

Fluorescence microscopy of chlamydial LPS showed replicative *C. pneumoniae* infection of 10.1 ± 3.3% HCAEC within 3 days after the beginning of the transmigration process (Fig. 3(a)). Transmigrated *C. pneumoniae* infected PBMC adhered to CASMC on the bottom of the lower chamber after 2 days (Fig. 3(b)), resulting in replicative infection in 8.5 ± 2.4% of cells after 5 days. Fluorescent double-staining for *C. pneumoniae* and actin filaments proved intracellular infection of the CASMC (Fig. 3(c)) discerning it from persistent infection in PBMC. Co-incubation with the supernatants of *C. pneumoniae* infected PBMC did not result in chlamydial infection of CASMC. Infected PBMC, endothelial and smooth muscle cells remained viable for 5 days as shown with Live/dead staining (Fig. 3(d)). Chlamydial HSP60 mRNA expression in HCAEC and CASMC, as a marker of pathogen viability and virulence [19], was retained after transmission from PBMC during the observation period of the TEM.

#### 3.3. Transcriptional activity of *C. pneumoniae* within the TEM

*Chlamydia pneumoniae* specific mRNA expression was analyzed in HCAEC (day 2 and day 3) and CASMC (day 4 and day 5) after removal of the transmigrated PBMC. Sustained expression of HSP60 mRNA proved transcriptional activity of *C. pneumoniae* in HCAEC and CASMC within 5 days after beginning of PBMC transmigration in the TEM (Fig. 4). Amplification of the chlamydial 16S rRNA gene served as a control for *C. pneumoniae* detection. Chlamydial 16S rRNA and HSP60 mRNA was not detected in the supernatants of infected PBMC after 3 and 5 days (data not shown). This system to study the kinetics of vascular chlamydial infection also permits to analyze the host cell response in a controlled environment that reflects the in vivo process in migration of leukocytes across endothelial cells. Uriarte et al. [20] could recently show that pre-treatment of *C. pneumoniae* infected endothelial cells with fluoroquinolones significantly reduced neutrophil and monocyte migration due to inhibition of IL-8 and MCP-1 production. As therapeutic prevention of cardiovascular diseases depends on the reduction of known risk factors, the eradication of *C. pneumoniae* in chronic

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**Fig. 2.** Migration kinetics of *C. pneumoniae* infected and non-infected PBMC through an endothelial cell monolayer in the TEM assay. PBMC were initially infected with *C. pneumoniae* (Cp) at a rate of 82.5 ± 4.9%. In comparison to non-infected PBMC transmigration of infected PBMC was delayed due to an enhanced adhesion to the endothelial cell monolayer on the Transwell membrane (representative experiment; n = 3).
vascular infection would be a central target for medical intervention if a causative role could be established.

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