Endothelial cells are protected against phagocyte-transmitted *Chlamydomphila pneumoniae* infections by laminar shear stress

Gueinzius: Shear stress protects from *C. pneumoniae* infection

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Abstract

The respiratory pathogen *Chlamydomphila pneumoniae* can be detected in atherosclerotic vessels, but the mechanism of dissemination from lung to vasculature remains unknown. Disturbance of vascular shear stress is a risk factor for atherosclerosis. We investigated whether polymorphonuclear neutrophils (PMN) might serve as carriers, transmitting *C. pneumoniae* to endothelial cells and how this is affected by shear stress. PMN were prepared from blood and incubated with *C. pneumoniae*. Real-time PCR and Pathfinder staining showed that after 1 h, 20% of *C. pneumoniae* were ingested and started to form inclusions. When infected PMN were co-incubated with HUVEC for 96 h, 10% of PMN-ingested *C. pneumoniae* were transmitted to HUVEC as shown by PCR and confocal microscopy. Infection of HEp-2 cells with *C. pneumoniae* harvested from HUVEC resulted in *C. pneumoniae* replication and confirmed that the bacteria remained infective. Exposure to laminar shear stress in a rotating cone-and-plate apparatus did not affect the transmission of *C. pneumoniae* from PMN to HUVEC, but led to a 75% reduction of inclusion formation. This can explain the focal distribution of *C. pneumoniae* in the vasculature and links two risk factors of atherosclerosis, i.e. the lack of laminar flow and infection.

Keywords: Atherosclerosis; Endothelial cells; Shear stress; *C. pneumoniae*; Phagocytes

1. Introduction

Atherosclerosis is hallmarked by its focal nature, with atherosclerotic plaques mainly developing at certain artery geometries like bifurcations. Variance of the laminar blood flow (shear stress), like reduced or turbulent flow, is characteristic for these regions, and is supposed to predispose them to early alterations like inflammatory changes [1].

The role of infection with the wide-spread respiratory tract pathogen *Chlamydomphila pneumoniae* in the development of

atherosclerosis has been under controversial discussion for years [2]. Most of the confusion stems from seroepidemiological studies and antibiotic treatment trials. Today, the number of reports showing an association of the anti-*C. pneumoniae* serostatus with coronary artery disease is equal to the number of reports that show the opposite. This can in part be explained by the use of different serological assays that differ in their validity and show large variations in sensitivity, which in consequence results in differently composed study groups [3,4]. Furthermore, the presence of anti-*C. pneumoniae* antibodies does not necessarily indicate a persistently infected state but can also stem from a past infection. This is important to note since many treatment studies are based on serological stratification of study groups. Numerous clinical

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trials have examined the effect of anti-chlamydial antibiotics on the secondary prevention of cardiovascular events, but no benefit was detected [5]. Today it is seriously doubted that antibiotics are capable to eradicate persistent *C. pneumoniae* in atheromas. Firstly, it is questionable whether antibiotics can effectively penetrate the atheroma, and secondly persisting *C. pneumoniae* were shown to be resistant to antibiotic treatment owing to their low metabolic activity [6].

The most convincing results come from animal models in which intranasal infection with *C. pneumoniae* accelerates lesion development and leads to localization of *C. pneumoniae* in the atheroma [7]. These studies are supported by proof of the presence of *C. pneumoniae* in diseased vessels, which was shown by various techniques including culture, implying that the bacteria are indeed still alive [8].

It is still not clear how *C. pneumoniae* disseminate from the lung, the primary target tissue of infection, to the vessel wall. Circulating free *C. pneumoniae* have so far not been detected directly in the blood by PCR, but phagocytes and monocytes were suggested to be possible carriers in several *in vivo* [9,10] and *in vitro* studies [11,12]. Blood monocytes can be PCR-positive for *C. pneumoniae* and can disseminate the infection systemically in mice [10], but *in vitro* infection of monocytes results in growth inhibition [13,14], making it unlikely that monocytes are appropriate to spread *C. pneumoniae* infections to other cell types. A recent publication has shown that PMN effectively take up but do not kill *C. pneumoniae*, which qualifies them as a possible vector system [15].

In the present study we investigated whether PMN can serve as carriers, transmitting *C. pneumoniae* to endothelial cells. Since *C. pneumoniae* are almost exclusively detected in atheromas at the lesion-prone regions of the vessels, we investigated the protective effect of laminar shear stress on the prevention of endothelial cell infection.

2. Materials and methods

2.1. Propagation of *C. pneumoniae*

C. pneumoniae strain TW-183, a generous gift from Prof. Jens Kuipers (Division of Rheumatology, Medical School Hannover, Germany), was propagated in HEp-2 cells (ATCC CCL-23) as described [16]. Confluent layers of HEp-2 cells were infected with *C. pneumoniae* in Eagle's MEM (PAA, Coelbe, Germany) supplemented with cycloheximide (1 mg/l, Sigma, Deisenhofen, Germany), 5% heat-inactivated FCS (Biochrom, Berlin, Germany), 1% HEPES (PAA), 1% glucose (Sigma) and 1% non-essential amino acids (PAA). For infection, cells were spun at $1700 \times g$ at 37°C for 1 h. After incubation at 37°C and 5% CO_2 , the infected monolayers were mechanically disrupted. Cell fragments were separated from the supernatant by a brief centrifugation step at $1700 \times g$. The supernatant, containing chlamydial elementary bodies (EBs), was removed and stored at -80°C . To

determine the yield of the EB, DNA was extracted using the DNeasy® Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol and a *C. pneumoniae*-specific real-time PCR was performed as described previously [16]. Quantification of *C. pneumoniae* genome equivalents (GE) in the samples was performed using standard chlamydial DNA, consisting of 1.2 million base pairs per GE, weighing 1.3 fg. The detection limit of the PCR is one chlamydial GE per PCR run, i.e. 100 *C. pneumoniae*/ml. The PCR was confirmed by measuring *C. pneumoniae* IFU in infected HEp-2 cells.

2.2. Preparation of PMN

PMN were isolated from human blood obtained from healthy volunteers. Acute infections were ruled out by differential blood cell count (Pentra60, ABX Technologies, Montpellier, France). Buffy coat leukocytes were separated from the erythrocytes by dextran sedimentation (0.72% Dextran T500, Sigma). After pelleting erythrocytes, the supernatant was centrifuged for 7 min at $650 \times g$. The obtained pellet was re-suspended in $1 \times$ HEPES (PAA), centrifuged twice for 7 min at $650 \times g$ and layered on a discontinuous Percoll gradient (Amersham Pharmacia Biotech, Uppsala, Sweden) consisting of four layers with densities of 1.093 g/ml (97%), 1.088 g/ml (96%), 1.072 g/ml (95%) and 1.059 g/ml (94%). After centrifugation for 15 min at $325 \times g$, the interface between the 94 and the 95% layer was collected and washed in $1 \times$ HEPES. The pellet was re-suspended in RPMI 1640 (Cambrex, Verviers, Belgium) with 10% autologous serum. All steps were performed at room temperature. The purity of PMN achieved by this method was 99% as determined by flow cytometry with a FACSCalibur (BD Biosciences, San Jose, CA, USA).

2.3. Preparation of PBMC

Peripheral blood mononuclear cells (PBMC) of blood from healthy volunteers were prepared using CPT™ Cell Preparation Tubes (Becton Dickinson, Franklin Lakes, USA) according to the manufacturer's instructions. Acute infections were ruled out by differential blood cell count (Pentra60).

2.4. Infection of PMN or PBMC with *C. pneumoniae*

Freshly isolated PMN (1×10^6 /ml) or PBMC (1×10^6 monocytes/ml) were co-incubated with *C. pneumoniae* at a multiplicity of infection (MOI) of 20 (i.e. 2×10^7 EB/ml) in 50 ml tubes (Falcon, Greiner) at 37°C and 5% CO_2 . After 1 h, PMN or PBMC were washed once with medium, further incubated for 4 or 48 h and then PMN or PBMC were washed again thrice. Quantification of the bacterial load in PMN or PBMC was performed after DNA extraction from the third wash fraction or PMN or PBMC by *C. pneumoniae*-specific real-time PCR as described above.

For some experiments PMN were lysed by adding 1 ml of water for 1 min. The PMN remains were spun down by centrifugation at $1100 \times g$ for 10 min. The concentration of bacteria in the supernatant was quantified by real-time PCR and used for further infection of HEp-2 cells as described above.

2.5. IL-8 ELISA

IL-8 was measured in the cell-free supernatants of PMN by in-house sandwich ELISA using antibody pairs against IL-8 (Endogen, Perbio Sciences, Bonn, Germany). Recombinant IL-8 standard was purchased from PeproTech, Tebu, Frankfurt, Germany. Binding of biotinylated antibodies was quantified using streptavidin-peroxidase (Biosource, Camarillo, CA, USA) and the substrate TMB (3,3',5,5'-tetramethylbenzidine, Sigma).

2.6. Endothelial cell cultures

Human umbilical vein endothelial cells (HUVECs) obtained from CellSystems (Remagen, Germany) were cultured in endothelial cell growth medium (EGM, Bio Whittaker Europe/Cambrex, Verviers, Belgium) supplemented with 10% heat-inactivated FCS (PAA), human epidermal growth factor (0.01 $\mu\text{g/ml}$, Bio Whittaker), hydrocortisone (1 $\mu\text{g/ml}$, Bio Whittaker), 1% gentamicin (Bio Whittaker), 1% amphotericin B (Bio Whittaker) and bovine brain extract (12 $\mu\text{g/ml}$, Bio Whittaker). Cells were cultivated in 250 ml tissue culture flasks (Greiner) at 37°C in the presence of 5% CO_2 .

Human coronary aortic endothelial cells (HCAECs) obtained from (Clonetics, Walkersville, USA) were cultured in endothelial cell growth medium (MV 2, PromoCell, Heidelberg, Germany) supplemented with 10% heat-inactivated FCS (PromoCell), human epidermal growth factor (5 ng/ml, PromoCell), hydrocortisone (0.2 $\mu\text{g/ml}$, PromoCell), vascular endothelial growth factor (0.5 ng/ml, PromoCell), basic fibroblast factor (10 ng/ml, PromoCell), R3 IGF-1 (20 ng/ml, PromoCell), ascorbic acid (1 $\mu\text{g/ml}$, PromoCell) and 1% gentamicin (Bio Whittaker). Cells were cultivated in 250 ml tissue culture flasks (Greiner) coated with 0.1% gelatine (Sigma) at 37°C in the presence of 5% CO_2 .

2.7. Transmission of *C. pneumoniae* from PMN to HUVEC

PMN were infected as described above for 1 h. After one washing step, PMN were further cultured for 4 h and washed thrice. Then, the infected PMN or the PMN supernatant, were transferred to HUVEC, which were seeded at 2.5×10^5 cells/well in 12-well plates (Greiner) 24 h before, at ratios of PMN to HUVEC of 0.1:1 to 10:1. After 96 h of co-incubation, the supernatant was removed and HUVEC were washed five times. DNA was prepared from wash fraction five and from harvested HUVEC and *C. pneumoniae*-specific real-time PCR was performed as described above.

2.8. Pathfinder staining

To visualize chlamydial inclusions, infected PMN were plated on plastic cover slips (Bibby Sterilin Ltd. Stone, Staffordshire, England) for 20 h. 2.5×10^5 HUVEC were seeded one day before and then co-incubated with *C. pneumoniae*-infected PMN for 96 h. After the incubation time the cells were fixed with methanol and stained with Pathfinder[®] solution (Biorad, Munich, Germany) consisting of fluorescein-conjugated monoclonal anti-chlamydial lipopolysaccharide antibodies. Images of infected cells were taken by laser scanning confocal microscopy using a Zeiss LSM 510 Meta. The cells were visualized by their autofluorescence at 543 nm, *C. pneumoniae* inclusions were detected by excitation at 488 nm.

2.9. Application of shear stress

HUVEC were plated in 60 mm $\times$ 15 mm tissue culture dishes (BD Labware, Franklin Lakes, NJ, USA) 24 h prior to infection at 5×10^5 cells/well in EGM (Bio Whittaker). After addition of *C. pneumoniae*-infected PMN, HUVEC were exposed to laminar shear stress in a cone-and-plate apparatus as previously described [17]. A constant shear stress of 5 or 15 dyn/cm² at 37°C and 5% CO_2 for 12 h or 96 h was used to simulate reduced or physiological levels of shear stress. If shear stress was applied for 12 h only, cells were washed twice and cultured further for 84 h. After 96 h, HUVEC were washed five times, harvested, and *C. pneumoniae* were quantified after DNA extraction from the fifth wash fraction and the HUVEC harvest by *C. pneumoniae*-specific real-time PCR as described above. HUVEC incubated with *C. pneumoniae*-infected PMN without shear stress exposure served as controls.

Some experiments were performed with *C. pneumoniae*-infected PBMC instead of PMN. Therefore, *C. pneumoniae*-infected PBMC were incubated with HUVEC at a ratio of 1:1 (HUVEC to monocytes) and were exposed to a constant laminar shear stress of 15 dyn/cm² at 37°C and 5% CO_2 for 96 h.

Some experiments were performed with HCAEC instead of HUVEC. Therefore, HCAEC were plated in 60 mm $\times$ 15 mm tissue culture dishes (BD Labware) 24 h prior to infection at 5×10^5 cells/well in MV 2 (PromoCell). After addition of *C. pneumoniae*-infected PMN, HCAEC were exposed to a constant laminar shear stress of 15 dyn/cm² at 37°C and 5% CO_2 for 96 h.

2.10. Statistics

Statistical analyses were performed using Graph Pad Prism Software, Version 4.0 (Graph Pad Software, San Diego, CA, USA). To compare two groups, a two-tailed unpaired Student's *t*-test was used. All data are given as means $\pm$ S.E.M. $p \leq 0.05$ was considered significant.

3. Results

3.1. Infection of phagocytes with *C. pneumoniae*

To investigate whether PMN can serve as carriers transmitting *C. pneumoniae* to endothelial cells, PMN were prepared from human whole blood and infected with *C. pneumoniae* *in vitro* for 1, 4 or 48 h. To quantify the number of *C. pneumoniae* taken up by PMN, a *C. pneumoniae*-specific real-time PCR was performed. To avoid falsification of the results by free EB, the PMN were washed thrice before preparation of DNA. After 1 h, PMN had ingested about 20% of the avail-

able *C. pneumoniae* and an increase of the bacterial load from 1 to 48 h post infection as well as an increase in PMN life span was observed (Fig. 1a), indicating that the bacteria were alive and able to replicate. In line with the publication by van Zandbergen et al. [15], we measured IL-8, which was reported to increase the life span of infected PMN [18], in the supernatant of infected and non-infected PMN after 48 h. The infection of PMN resulted in a 10-fold increase of IL-8 release compared to non-infected controls (0.22 ± 0.10 ng/ml versus 1.97 ± 0.31 ng/ml; $n=6$, $p \leq 0.001$). Pathfinder staining of the infected PMN revealed that almost all PMN contained *C. pneumoniae* inclusions after 20 h of infection (Fig. 1b). How-

Fig. 1. Infection of PMN with *C. pneumoniae*. PMN were infected with *C. pneumoniae* at a MOI of 20 and (a) 1 h and 48 h post infection DNA was prepared and the *C. pneumoniae* load of infected PMN was quantified by real-time PCR. Data are means $\pm$ S.E.M. of $n=7$ different donors performed on three different days. (b) 20 h post infection, PMN were stained with Pathfinder solution and analyzed by confocal microscopy. Inclusions are labeled in green. (c) 4 h post infection, the PMN were lysed and the supernatant was used to infect HEp-2 cells in different titers. After 96 h, DNA was prepared from infected HEp-2 cells and real-time PCR was performed. Data are means $\pm$ S.E.M. of $n=8$ replicates.

ever, since previous papers had reported a strong bactericidal activity of PMN against *C. trachomatis* [19,20], we have further confirmed that the *C. pneumoniae* in PMN-inclusions were still infective. Therefore, the PMN were lysed after 4 h of infection by adding water and spun down by centrifugation. Different volumes of the supernatant were then used to infect HEp-2 cells. As shown in Fig. 1c, the infectivity of the supernatant increased by titrating, confirming that the PMN indeed contained infective *C. pneumoniae*.

3.2. Infected phagocytes transmit *C. pneumoniae* to endothelial cells

To determine whether infected PMN could transmit bacteria to endothelial cells, we co-cultured *C. pneumoniae*-infected PMN with HUVEC. PMN were infected for 4 h with *C. pneumoniae*, washed thrice and transferred to HUVEC in ratios of 0.1:1 and 1:1. After 96 h of co-incubation, stained *C. pneumoniae* inclusions were detectable in HUVEC by confocal microscopy (Fig. 2a). To determine the num-

Fig. 2. Transmission of *C. pneumoniae* from PMN to HUVEC. HUVEC were co-incubated in duplicates with *C. pneumoniae*-infected PMN at a ratio of (a) 1:1 or (b) 0.1:1 and 1:1. (a) 96 h post infection, HUVEC were stained with Pathfinder solution and analyzed by confocal microscopy. Inclusions are labeled in green. (b) DNA was prepared from PMN infected with *C. pneumoniae* for 4 h and from HUVEC after 96 h of co-incubation with infected PMN and real-time PCR was performed. White bars represent the amount of *C. pneumoniae* ingested by PMN before transfer to HUVEC at the ratios 0.1:1 and 1:1. The HUVEC harvest is given in black bars. Data are means ± S.E.M. of $n=3$ for different PMN donors and $n=6$ for HUVEC harvest.

Fig. 3. Reinfection of HEp-2 cells by *C. pneumoniae* harvested from HUVEC. HUVEC were harvested 96 h after co-incubation with *C. pneumoniae*-infected PMN. The HUVEC harvest was diluted 5-fold and used for infection of HEp-2 cells. DNA was prepared from the HUVEC harvest, the dilution and the HEp-2 harvest 96 h post infection and analyzed by real-time PCR. Data are means ± S.E.M. of $n=3$.

ber of *C. pneumoniae* in HUVEC, DNA extraction and *C. pneumoniae*-specific real-time PCR was performed. To exclude falsification of the results by remaining PMN or free *C. pneumoniae*, the HUVEC were washed five times and DNA was prepared from the fifth wash fraction and as well analyzed by *C. pneumoniae*-specific real-time PCR. The fifth wash fraction was negative in all cases (data not shown). The transmission of *C. pneumoniae* to HUVEC correlated with the number of PMN used for co-incubation (Fig. 2b). Control experiments confirmed that the PMN supernatant was not infective (data not shown). In both cases (ratio 0.1:1 or 1:1) about 10% of the *C. pneumoniae* that were contained in the PMN were transmitted to the HUVEC. Incubation of PMN with HUVEC in ratios between 0.1:1 and 10:1 showed that the transmission of *C. pneumoniae* from PMN to HUVEC can be further increased by using more infected PMN (data not shown).

The inclusions which developed in HUVEC after co-incubation with *C. pneumoniae*-infected PMN appeared to be small and no significant replication of *C. pneumoniae* was observed after 96 h (18 ± 10^4 *C. pneumoniae*/ml after 24 h versus 33 ± 10^4 *C. pneumoniae*/ml after 96 h, $n=6$, $p>0.05$). To determine whether the *C. pneumoniae* in these inclusions were still infective, *C. pneumoniae* were harvested from HUVEC, diluted 5-fold and used for infection of HEp-2 cells. As indicated in Fig. 3, *C. pneumoniae* were still able to infect and replicate in HEp-2 cells, leading to a 100-fold increase of the bacterial load after 96 h. Therefore, we conclude that the small chlamydial inclusions in HUVEC might represent morphological aberrant bodies, containing persistent rather than replicative *C. pneumoniae*.

3.3. Shear stress inhibits the PMN-mediated transmission of *C. pneumoniae* to endothelial cells

To investigate the effect of laminar shear stress on the transmission of *C. pneumoniae*-infection from PMN to HUVEC, HUVEC were co-incubated with *C. pneumoniae*-

Fig. 4. Laminar shear stress inhibits the PMN-mediated transmission of *C. pneumoniae* to endothelial cells. *C. pneumoniae*-infected PMN were transferred to HUVEC or HCAEC at a ratio of 1:1 and co-incubated for 96 h with 15 dyn/cm² shear stress in a shear stress apparatus or left without shear stress. After 96 h, DNA was prepared from the HUVEC or HCAEC harvest and analyzed by real-time PCR. Data are means ± S.E.M. from five independent experiments; $n = 6$ for HUVEC without shear stress, $n = 5$ for HUVEC with shear stress, $n = 5$ for HCAEC ± shear stress. Asterisks (**) indicate significance versus HUVEC or HCAEC without shear stress.

infected PMN for 96 h and exposed to physiological levels (15 dyn/cm²) of laminar shear stress of during the whole period of co-incubation. The amount of *C. pneumoniae* in the HUVEC harvest was compared to that in HUVEC which had not received shear stress. As demonstrated in Fig. 4, shear stress exposure led to a significantly reduced bacterial transmission from PMN to HUVEC. It was excluded by measuring host cell DNA that this result was due to varying numbers of HUVEC cells in the different wells. If the shear stress force was reduced from 15 to 5 dyn/cm² no significant inhibition of transmission of bacteria could be observed anymore.

Since monocytes are also discussed as potential candidates to disseminate *C. pneumoniae* to other organs the identical experiment was performed with *C. pneumoniae*-infected PBMC, instead of PMN. After 1 h, PBMC had ingested about 50% of the available *C. pneumoniae*, but transferred only 0.01% of the *C. pneumoniae* that were contained to the HUVEC during co-culture. Shear stress exposure, like in the case of PMN, led to a 4-fold reduction of bacterial transmission (data not shown).

In vivo, *C. pneumoniae* are exclusively detected in aortic endothelial cells. Therefore, we have reproduced the shear stress experiments using HCAEC instead of HUVEC. Co-incubation of HCAEC with *C. pneumoniae*-infected PMN led to an even stronger transmission of *C. pneumoniae* to HCAEC than to HUVEC, which was, like for HUVEC, significantly inhibited by exposure to laminar shear stress (Fig. 4).

Next, we asked whether the transmission of *C. pneumoniae* from PMN to HUVEC was inhibited in general under shear stress or whether the establishment of *C. pneumoniae* inclusions was inhibited by shear stress. For this purpose, HUVEC were co-incubated with *C. pneumoniae*-infected PMN with or without shear stress exposure for 12 h. After 12 h, shear stress was withdrawn and all cells were cultured further for 84 h to allow the completion of a whole infection cycle. After 96 h, no significant treatment-dependent difference between the amount of *C. pneumoniae* in the

HUVEC could be observed (4×10^4 *C. pneumoniae*/ml without shear stress versus 3×10^4 *C. pneumoniae*/ml with 12 h shear stress, $n = 5$, $p > 0.05$), indicating that shear stress exposure did not inhibit the transmission of *C. pneumoniae* from PMN to HUVEC and thus must interfere with the formation of *C. pneumoniae* inclusions. This conclusion is further supported by our finding that shear stress exposure of HUVEC that were first incubated with infected PMN for 12 h without shear stress, is still able to significantly reduce inclusion formation (data not shown).

4. Discussion

Atherosclerosis is widely regarded as an inflammatory disease, but the precise initiators and sustainers of inflammation are still unknown. A role of the respiratory tract pathogen *C. pneumoniae* as risk factor for atherogenesis has been discussed for two decades now. It is tempting to speculate that *C. pneumoniae*, which infect the vessel wall, lead to the activation of otherwise resting endothelial and smooth muscle cells, unbalancing vascular homeostasis and contributing to sustained inflammation. However, the route of dissemination of *C. pneumoniae* from the lung to the vasculature is unclear. Here we demonstrate that PMN can serve as carriers for *C. pneumoniae*, transmitting the bacteria to endothelial cells, leading to intracellular inclusions, which contain viable *C. pneumoniae*. Control experiments with gradient-purified *C. pneumoniae* had been performed to exclude that remaining HEp-2 components led to falsification of the results. Furthermore, this development of inclusions is diminished by exposure to laminar shear stress, which can explain the focal distribution of *C. pneumoniae* in the vasculature, since atherosclerotic lesions develop mainly at sites of disturbed laminar shear stress.

The association of *C. pneumoniae* infections with atherosclerosis is mainly supported by animal models, which provide causal evidence [7] and by direct detection of the pathogen in the lesions [8]. These studies demonstrate the presence of infective *C. pneumoniae* in the vessel wall, although they cannot explain whether and at what stage they might contribute to the pathogenesis of atherosclerosis. Several *in vitro* studies have reported the profound pro-inflammatory effects of *C. pneumoniae* infections on atherosclerosis-relevant cells like monocytes, endothelial and smooth muscle cells [21–25]. Furthermore, key events in atherogenesis have been associated with the presence of *C. pneumoniae*, like the oxidation of low density lipoprotein by *C. pneumoniae*-infected endothelial cells [26], the enhancement of the inflammatory action of oxidized low density lipoprotein [27] or the stimulation of reactive oxygen species in platelets by *C. pneumoniae* [28]. *In vivo*, *C. pneumoniae* were detected in endothelial cells [29], smooth muscle cells [30] and in T-lymphocytes located in the atherosclerotic plaque [31]. The *C. pneumoniae* burden in carotid arteries was shown to be associated with upregulated levels of plaque

IL-6 and serum C-reactive protein [32]. Taken together, these results implicate that the presence of *C. pneumoniae* in vascular cells or in immune cells, which have invaded the inflamed tissue, contribute to sustained stimulation of inflammation.

To date it is unclear, how *C. pneumoniae*, which infect primarily the epithelial cells of the upper respiratory tract, reach the large arteries. PMN are immune cells with the most potent phagocytic and anti-bacterial activity and are recruited rapidly and in large numbers to the site of infection. Indeed, as demonstrated in the present study, *in vitro* co-culture of PMN with *C. pneumoniae* results in the uptake of bacteria, which can then be transmitted to endothelial cells by simple co-incubation. In contrast, PBMC, which are also able to potentially ingest *C. pneumoniae*, are much less effective in transmitting *C. pneumoniae* to the endothelial cells. It is surprising that phagocytosis of *C. pneumoniae* does not result in killing of the bacteria by PMN, but rather in multiplication of the pathogen and in a prolonged half-life of the PMN, making them suitable shuttles. These *in vitro* results are further supported by an *in vivo* study in which intranasal infection of mice with *C. pneumoniae* resulted in the recruitment of PMN into the lungs and in subsequent *C. pneumoniae* infection of PMN. In that model, *C. pneumoniae* did not only replicate in PMN, but PMN also enhanced the replication of *C. pneumoniae* in bronchial epithelial cells [33]. It is not known whether PMN, after entering a tissue, can re-enter the blood circulation. Due to their normally short half-life of a few hours, this seems rather improbable. However, *in vitro* infection with *C. pneumoniae* extends their half-life up to two days and leads to autocrine IL-8 production, which was first reported by van Zandbergen et al. [15] and confirmed here. It might be further speculated that *C. pneumoniae* induce a redirection of PMN towards the vasculature. So far, no study has investigated whether circulating PMN in humans might carry *C. pneumoniae*. Most monocyte studies were done with blood monocytes from healthy volunteers or patients with coronary artery disease. In case of PMN, it seems reasonable to investigate blood PMN from patients with an acute respiratory tract infection, since at this time point the infiltration of PMN and the chance for *C. pneumoniae* uptake and distribution should be maximal.

The fact that the PMN- and PBMC-mediated infection of endothelial cells can be efficiently prevented by exposure to laminar shear stress is a reasonable explanation for the focal distribution of *C. pneumoniae* in the vasculature and represents another important piece to the puzzle. It is suggested that *C. pneumoniae*-infected PMN or PBMC can be targeted to the vessel wall, but that infection is normally prevented by the laminar blood flow. Together with results from *in vitro* and *in vivo* studies this argues for a causal role of *C. pneumoniae* in atherogenesis, but this conclusion still remains speculative.

Prolonged laminar shear stress has been shown to generate an anti-oxidant, anti-proliferative, anti-apoptotic and pro-differentiative endothelial cell type via the regulation of gene transcription [34]. In "lesion-prone regions", these

beneficial effects of laminar shear stress are reduced. As demonstrated, shear stress exposure does not prevent the transmission of *C. pneumoniae* to endothelial cells but does inhibit the establishment of inclusions. Therefore, it is less likely that the regulation of endothelial surface molecules, like adhesion proteins, by shear stress is responsible for the effect. It is known that, shortly after infection, *C. pneumoniae* starts to influence host cell metabolism, especially redirects its energy sources and induces rearrangements of the cytoskeleton [35,36]. Whether effects like these are prevented by shear stress remains to be investigated. The hypothesis that deviations from the laminar blood flow render the vasculature more susceptible to *C. pneumoniae* infection also supports the possibility that vascular infection occurs as an early event in atherogenesis. Otherwise the focal distribution of *C. pneumoniae* can only be explained by secondary bystander effects, preferentially infecting already diseased tissue. This seems less likely since *C. pneumoniae*, as intracellularly replicating energy parasites, need healthy tissue for efficient multiplication.

In conclusion, this study links for the first time two putative risk factors for the initiation of atherogenesis, i.e. lack of laminar shear stress and infection of endothelial cells with *C. pneumoniae*, and explains both a possible route of infection and the selective localization in atherosclerotic lesions.

Acknowledgements

We thank Dr. Sonja von Aulock for the critical reading of the manuscript and Teodora Baris for her help with the endothelial cell cultures. We thank PD Dr. Jan Rupp and Prof. Matthias Maas for prompt support with HCAEC.

Funding: This study was supported by the "Eliteprogramm für Postdoktorandinnen und Postdoktoranden der Landesstiftung Baden-Württemberg gGmbH". CH was supported by a M. von Wrangell fellowship for habilitation.

References

- [1] Cunningham KS, Gotlieb AI. The role of shear stress in the pathogenesis of atherosclerosis. *Lab Invest* 2005;85:9–23.
- [2] Ieven MM, Hoymans VY. Involvement of *Chlamydia pneumoniae* in atherosclerosis: more evidence for lack of evidence. *J Clin Microbiol* 2005;43:19–24.
- [3] Hermann C, Graf K, Groh A, Straube E, Hartung T. Comparison of eleven commercial tests for *Chlamydia pneumoniae*-specific immunoglobulin G in asymptomatic healthy individuals. *J Clin Microbiol* 2002;40:1603–9.
- [4] Peeling RW, Wang SP, Grayston JT, et al. *Chlamydia pneumoniae* serology: interlaboratory variation in microimmunofluorescence assay results. *J Infect Dis* 2000;181:S426–9.
- [5] Kuppuswamy VC, Gupta S. Antibiotic therapy for coronary heart disease: the myth and the reality. *Drugs Today (Barc)* 2005;41:677–85.
- [6] Higgins JP. *Chlamydia pneumoniae* and coronary artery disease: the antibiotic trials. *Mayo Clin Proc* 2003;78:321–32.
- [7] de Kruif MD, van Gorp EC, Keller TT, Ossewaarde JM, ten Cate H. *Chlamydia pneumoniae* infections in mouse models: relevance for atherosclerosis research. *Cardiovasc Res* 2005;65:317–27.

- [8] Campbell LA, Kuo CC. *Chlamydia pneumoniae*—an infectious risk factor for atherosclerosis? *Nat Rev Microbiol* 2004;2:23–32.
- [9] Gieffers J, van Zandbergen G, Rupp J, et al. Phagocytes transmit *Chlamydia pneumoniae* from the lungs to the vasculature. *Eur Respir J* 2004;23:506–10.
- [10] Moazed TC, Kuo CC, Grayston JT, Campbell LA. Evidence of systemic dissemination of *Chlamydia pneumoniae* via macrophages in the mouse. *J Infect Dis* 1998;177:1322–5.
- [11] Azenabor AA, Job G, Adedokun OO. *Chlamydia pneumoniae*-infected macrophages exhibit enhanced plasma membrane fluidity and show increased adherence to endothelial cells. *Mol Cell Biochem* 2005;269:69–84.
- [12] Rupp J, Koch M, van Zandbergen G, et al. Transmission of *Chlamydia pneumoniae* infection from blood monocytes to vascular cells in a novel transendothelial migration model. *FEMS Microbiol Lett* 2005;242:203–8.
- [13] Gaydos CA, Summersgill JT, Sahney NN, Ramirez JA, Quinn TC. Replication of *Chlamydia pneumoniae* in vitro in human macrophages, endothelial cells, and aortic artery smooth muscle cells. *Infect Immun* 1996;64:1614–20.
- [14] Wolf K, Fischer E, Hackstadt T. Degradation of *Chlamydia pneumoniae* by peripheral blood monocytic cells. *Infect Immun* 2005;73:4560–70.
- [15] van Zandbergen G, Gieffers J, Kothe H, et al. *Chlamydia pneumoniae* multiply in neutrophil granulocytes and delay their spontaneous apoptosis. *J Immunol* 2004;172:1768–76.
- [16] Mueller M, Postius S, Thimm JG, et al. Toll-like receptors 2 and 4 do not contribute to clearance of *Chlamydia pneumoniae* in mice, but are necessary for the release of monokines. *Immunobiology* 2004;209:599–608.
- [17] Dimmeler S, Fleming I, Fisslthaler B, et al. Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature* 1999;399:601–5.
- [18] Kettritz R, Gaido ML, Haller H, et al. Interleukin-8 delays spontaneous and tumor necrosis factor- α -mediated apoptosis of human neutrophils. *Kidney Int* 1998;53:84–91.
- [19] Yong EC, Chi EY, Chen WJ, Kuo CC. Degradation of *Chlamydia trachomatis* in human polymorphonuclear leukocytes: an ultrastructural study of peroxidase-positive phagolysosomes. *Infect Immun* 1986;53:427–31.
- [20] Yong EC, Klebanoff SJ, Kuo CC. Toxic effect of human polymorphonuclear leukocytes on *Chlamydia trachomatis*. *Infect Immun* 1982;37:422–6.
- [21] Prochnau D, Rodel J, Hartmann M, Straube E, Figulla HR. Growth factor production in human endothelial cells after *Chlamydia pneumoniae* infection. *Int J Med Microbiol* 2004;294:53–7.
- [22] Kim MP, Gaydos CA, Wood BJ, et al. *Chlamydia pneumoniae* enhances cytokine-stimulated human monocyte matrix metalloproteinases through a prostaglandin E2-dependent mechanism. *Infect Immun* 2005;73:632–4.
- [23] Rupp J, Hellwig-Burgel T, Wobbe V, et al. *Chlamydia pneumoniae* infection promotes a proliferative phenotype in the vasculature through Egr-1 activation in vitro and in vivo. *Proc Natl Acad Sci USA* 2005;102:3447–52.
- [24] Shi Y, Tokunaga O. *Chlamydia pneumoniae* (*C. pneumoniae*) infection upregulates atherosclerosis-related gene expression in human umbilical vein endothelial cells (HUVECs). *Atherosclerosis* 2004;177:245–53.
- [25] Yang X, Coriolan D, Schultz K, Golenbock DT, Beasley D. Toll-like receptor 2 mediates persistent chemokine release by *Chlamydia pneumoniae*-infected vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* 2005;25:2308–14.
- [26] Ditttrich R, Dragonas C, Mueller A, et al. Endothelial *Chlamydia pneumoniae* infection promotes oxidation of LDL. *Biochem Biophys Res Commun* 2004;319:501–5.
- [27] Nazzari D, Cantero AV, Therville N, et al. *Chlamydia pneumoniae* alters mildly oxidized low-density lipoprotein-induced cell death in human endothelial cells, leading to necrosis rather than apoptosis. *J Infect Dis* 2006;193:136–45.
- [28] Kalvegren H, Bylin H, Leanderson P, et al. *Chlamydia pneumoniae* induces nitric oxide synthase and lipoxygenase-dependent production of reactive oxygen species in platelets. Effects on oxidation of low density lipoproteins. *Thromb Haemost* 2005;94:327–35.
- [29] Alakarppa H, Surcel HM, Laitinen K, et al. Detection of *Chlamydia pneumoniae* by colorimetric in situ hybridization. *Apmis* 1999;107:451–4.
- [30] Gaydos CA. Growth in vascular cells and cytokine production by *Chlamydia pneumoniae*. *J Infect Dis* 2000;181(Suppl. 3):S473–8.
- [31] Mosorin M, Surcel HM, Laurila A, et al. Detection of *Chlamydia pneumoniae*-reactive T lymphocytes in human atherosclerotic plaques of carotid artery. *Arterioscler Thromb Vasc Biol* 2000;20:1061–7.
- [32] Johnston SC, Zhang H, Messina LM, Lawton MT, Dean D. *Chlamydia pneumoniae* burden in carotid arteries is associated with upregulation of plaque interleukin-6 and elevated C-reactive protein in serum. *Arterioscler Thromb Vasc Biol* 2005;25:2648–53.
- [33] Rodriguez N, Fend F, Jennen L, et al. Polymorphonuclear neutrophils improve replication of *Chlamydia pneumoniae* in vivo upon MyD88-dependent attraction. *J Immunol* 2005;174:4836–44.
- [34] Wasserman SM, Topper JN. Adaptation of the endothelium to fluid flow: in vitro analyses of gene expression and in vivo implications. *Vasc Med* 2004;9:35–45.
- [35] Dautry-Varsat A, Subtil A, Hackstadt T. Recent insights into the mechanisms of *Chlamydia* entry. *Cell Microbiol* 2005;7:1714–22.
- [36] Balana ME, Niedergang F, Subtil A, et al. ARF6 GTPase controls bacterial invasion by actin remodelling. *J Cell Sci* 2005;118:2201–10.