Increased infiltration of \textit{Chlamydophila pneumoniae} in the vessel wall of human veins after perfusion


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\textbf{ABSTRACT}

\textbf{Background} Several studies have suggested an association between \textit{Chlamydophila pneumoniae} (\textit{Cp}) infection and atherosclerosis. A recent study detected \textit{Cp} DNA in the saphenous vein of 12\% of all patients before bypass grafting and in 38\% of failed grafts. We used a system in which human veins were perfused with autologous blood under arterial pressure.

\textbf{Materials and methods} Veins were surplus segments of saphenous veins of coronary artery bypass grafting (CABG) patients. Vein grafts were perfused with the blood of the same patient after CABG procedures. Veins were analysed for \textit{Cp}-specific membrane protein using immunohistochemical and PCR analysis. Veins were analysed before and after perfusion (up to 4 h). The number of \textit{Cp} positive cells was then quantified in the vein layers.

\textbf{Results} \textit{Cp} protein was detected within macrophages only. In non-perfused veins, \textit{Cp} was present in the adventitia in 91\% of all patients, in the circular (64\%) and longitudinal (23\%) layer of the media. No positivity was found in the intima. Perfusion subsequently resulted in a significant increase of \textit{Cp} positive cells within the circular layer of the media that, however, differed strongly between different patients. \textit{Cp} DNA was not detected by PCR in those specimens.

\textbf{Conclusion} \textit{Cp} protein was present in 91\% of veins, but the number of positive cells differed remarkably between patients. Perfusion of veins resulted in increased infiltration of \textit{Cp} into the circular layer. These results may point to a putative discriminating role of \textit{Cp} with respect to graft failure between different patients.

\textbf{Keywords} \textit{Chlamydophila pneumoniae}, coronary artery bypass graft (CABG), saphenous vein.


\section*{Introduction}

\textit{Chlamydophila pneumoniae} (\textit{Cp}), an obligate intracellular living bacterium previously called \textit{Chlamydia pneumoniae}, is a common cause of respiratory tract infection. Population prevalence studies show a widespread geographical distribution of \textit{Cp} infections [1]. A role for \textit{Cp} in the process of atherosclerosis has recently been suggested [2], although contradicting studies have been published in this respect [3]. \textit{Cp} infection of human endothelial cells stimulated transendothelial migration of inflammatory cells \textit{in vitro} [4], and triggered the secretion of inflammatory mediators [5]. Smooth muscle cells responded to \textit{Cp} infection by proliferation [6], while \textit{Cp} infection of monocytes resulted in secretion of cytokines [7]. In addition, human macrophages infected with \textit{Cp} secreted enhanced levels of inflammatory cytokines [8–10]. In line with this, \textit{Cp} was detected by immunohistochemistry in 52\% of diseased arteries while only 5\% of normal arteries were positive [11]. Wong \textit{et al.} found \textit{Cp} DNA in 12\% of saphenous vein specimens before grafting, whereas in 38\% of failed grafts \textit{Cp} DNA was found [12]. \textit{Cp} has also been demonstrated in arteries, i.e. coronary arteries and aortas by electron microscopy [13]. In contrast, in another study using the PCR of post-mortem material no \textit{Cp} was detected in coronary arteries or carotid endarterectomy specimens. The same was true for symptomatic aneurysms of the abdominal aorta [14]. Finally, two studies reported that no \textit{Cp} was detected in atherosclerotic lesions of coronary arteries using immunohistochemistry [15,16]. It is not known whether this is related to technical problems, the specimens that were studied, or geographical differences [3].
Because of the observed association of Cp infection and vein graft failure [17], we undertook this study to determine the presence of Cp at the protein level in the various cell types of the saphenous vein. After initial connection to the arterial circulation, the perfused saphenous vein undergoes rapidly marked morphological changes, which can be mimicked by a pressurized extracorporeal circulation in vitro [18]. Therefore, we extended our study by determining the localization of Cp antigen before and after perfusion of vein segments.

**Materials and methods**

**Vein graft tissue**

Veins were surplus segments of harvested saphenous veins of patients who underwent coronary artery bypass grafting (CABG). Vessel specimens were collected in the operating room under sterile conditions for histopathological examination. Part of the vein graft segments were perfused with autologous blood after CABG procedures in an experimental set-up (Fig. 1) [18]. Vein graft segments were analysed before perfusion (n = 22) and after different perfusion time points, i.e. 1 h (n = 14), 2 h (n = 14) and 4 h (n = 12), respectively.

Our study was approved by the Ethics Committee of the VU Medical Centre and the OLVG, Amsterdam.

**Immunohistochemistry**

Paraffin embedded vein sections (4 μm thick) were mounted on microscope slides and deparaffinized for 10 min in xylene at room temperature and dehydrated through descending concentrations of ethanol. Endogenous peroxidase activity was blocked by incubation in 0.3% (v/v) H2O2 in methanol for 30 min. Tissue sections were subjected to antigen retrieval by boiling in 10 mM sodium citrate buffer, pH 6, for 10 min in a microwave oven. After that tissue sections were incubated for 30 min with a chlamydia block-buffer. Antibody and normal serum were diluted in phosphate buffered saline (PBS) containing 1% (w/v) bovine serum albumin (BSA). Tissue sections were preincubated for 10 min with normal rabbit serum, followed by incubation for 1 h with 5 g mL⁻¹ monoclonal antibody directed against Cp specific membrane protein [clone RR-402, Washington Research Foundation (WRF)] in PBS/BSA 1% overnight at 4°C.

After washing in PBS, sections were incubated for 30 min with a biotin-conjugated secondary antibody (rabbit antimouse biotin 1 : 500 dilution). After washing in PBS, slides were incubated with streptavidin-biotin complex (sABC; 1 : 1000 dilutions) for 1 h and were visualized with 3,3′-diaminobenzidine (DAB; 0.1 mg mL⁻¹, 0.02% H2O2). Slides were counterstained with haematoxylin and mounted with Depex.

Serial sections of veins were also stained to detect macrophages. These sections were boiled in sodium citrate buffer (10 mM, pH 6.0) for 10 min, and subsequently stained with mouse monoclonal antibody CD68 (1 : 400 dilution) (Kp1; Santa Cruz Biotechnology, CA, USA). Slides were then stained via a streptavidin-biotin complex and visualized with DAB as described above.

To confirm the specificity of the immunostaining, parallel sections were incubated with 1% PBS/BSA alone without adding RR-402 or CD68. Next, the sections were incubated with biotin-conjugated rabbit antimouse biotin, and subsequently with streptavidin-biotin complex and were visualized with DAB. The controls were all negative (not shown).

**Immunoscoring**

Immunoscoring was performed by two independent investigators (KK, HWMN). Cp and macrophages were scored for anatomical localization and amount of Cp positive cells. On microscopic examination, the amount of Cp positive cells were separately analysed in all four layers namely, intima, longitudinal muscular layer, circular muscular layer and adventitia. In each patient, three sections of each vein were analysed. An average score of the amount of Cp positive cells/vein was calculated by multiplying the scores of all three sections and then dividing it by three.

Notably, only those sections in which the total circumference of the whole of the vein was visible were analysed.
**Tissue processing and DNA extraction**

DNA extraction from veins was performed according to the standard method with optimized procedures. DNA concentration and purity were estimated spectrophotometrically at optical density (OD) 260/280 using an Eppendorf Biophotometer (Hamburg, Germany).

**Chlamydia pneumoniae detection by PCR**

*Cp* was detected by PCR targeting the 16S-rRNA gene, using the sense primer CP16S1 (5’-AAT AAT GAC TTC GGT TGT TAT TTA G-3’ (annealing temperature 55 °C), antisense primer CP16S4 5’-CTC AAC CGA AAG GTC CG-3’ (annealing temperature 55 °C) and probe CHP 16S 5’-GTA GTG TAA TTA GGC ATC TAA TA-3’.

In addition, for specificity issues, we also determined the presence of *C. trachomatis* DNA by plasmid sense primer 5’AGA GTA CAT CGG TCA ACG A-3’, antisense primer 5’-TCA CAG CGG TTG TCT GAA GCA-3’, probe CHP 16S 5’-GTA GTG TAA TTA GGC ATC TAA TA-3’, and chromosomal targeting (*omp1* gene) by nested PCR as described previously, using the NLO and NRO primers for initial amplification followed by one microlitre of the generated PCR product for nested amplification (increasing sensitivity of the PCR), using the primers sero1A and sero2A generating a PCR product of 1 KB which was checked for its length using agarose gel electrophoresis.

For monitoring PCR sensitivity, serial dilutions from 10 to 0.01 IFU were used as controls in each PCR.

**Statistical analysis**

Data analysis was performed with SPSS for windows version 11.5. The data were normally distributed and the student’s T-test was used to calculate the significance of the differences. A P-value (two sided) of less than 0.05 was considered to be significant.

**Results**

**Detection on Chlamydia pneumoniae by immunohistochemistry**

For immunohistochemical validation of the RR-402 antibody, HeLa cells were cultured with *Cp* (10–0.01 IFU) and subsequently embedded in paraffin and stained with the RR-402 antibody. As can be seen in Fig. 2(a), numerous cells stained positive for *Cp*.

We subsequently analysed the RR-402 antibody in the human vein segments. In non-perfused veins, *Cp* was already detected in the adventitia in 91% of all patients (Figs 2b,d, 3a). Notably *Cp* was detected only within macrophages (Fig. 2e), but not all macrophages were *Cp* positive. In these non-perfused veins, the circular layer of the media was positive for *Cp* in 64% of all patients (Fig. 3b), whereas in the longitudinal layer of the media *Cp* was detected in 23% of all patients (Fig. 3c). No *Cp* was detected in the intima (not shown). Remarkably, the amount of *Cp* positive cells differed strongly between different patients in the circular layer, but especially in the adventitia (Fig. 3).

Subsequent perfusion under an arterial hydrostatic pressure of 60 mmHg caused a considerable dilatation of the vein, and during perfusion complete loss of the luminal endothelial cell layer, in agreement with our previous observations [18]. Interestingly, this perfusion resulted in an increase in the number of vein segments positive for *Cp* in the circular layer, from 64 to 91% (see Figs 2c, 4a), while in the adventitia an increase from 91% to 100% was found. The number of vein segments positive for *Cp* in the longitudinal layer did not change after perfusion.

Perfusion also induced a significant increase in the amount of *Cp* positive cells in the circular layer of the media when compared with non-perfused veins (Fig. 4b), that was not found in the adventitia, longitudinal layer of the media or the intima. This increase was found after 1 h of perfusion, without a further significant increase in the amount of *Cp* positive cells at longer time points of perfusion.

**Detection on Chlamydia pneumoniae by PCR**

Remarkably, *Cp* PCR data of the veins were negative while positive controls, both pure *Cp* DNA and DNA derived from embedded *Cp* cultured material (see above), were positive with expected sensitivities. In addition, also the *Ct* plasmid and *omp1* PCRs, performed for specificity, were negative.

**Discussion**

In the present study, our aim was to investigate the effect of perfusion of human saphenous veins on the putative positivity for *Cp*. Surprisingly we found, using immunohistochemistry, that non-perfused saphenous veins, used as a bypass graft, were already positive for *Cp* protein in the adventitia in 91% of all patients. Remarkably, the amount of *Cp* positive cells differed strongly between different patients (Fig. 4a). A high prevalence (67%) of *Cp* has also been described in circulating macrophages in the adventitia of coronary arteries [19]. Two seroepidemiological studies have shown that at least 75%–80% of the patients are seropositive for *Cp* in the case of coronary artery stenosis [20,21]. Macrophages seem to play an important role in the transportation of *Cp*. Indeed, we found *Cp* exclusively within macrophages in the veins studied. It is also known that the adventitia frequently contains macrophages, which thus can explain the high number of patients positive for *Cp* [22]. Remarkably, DNA analysis for *Cp* was negative in these veins. This is in contrast with the recent study of Wong et al. who could detect *Cp* DNA in saphenous veins, although in only 12% of their patients [12]. However, it is known from the literature that the amount of *Cp* positive vessels is significantly higher using immunohistochemistry than using DNA analysis [23].
Regarding the differences in the amount of Cp positive veins, we cannot exclude a priori that the prevalence of Cp in veins in England (Wong et al.) [12] is different compared with its prevalence in the Netherlands (our study). Furthermore, the presence of Cp antigen in the absence of Cp DNA suggests that antigens, rather than viable bacteria, persist in atherosclerotic lesions [24]. This suggests that antibiotical therapy in this respect might not be successful.

It has to be taken into account that positive immunohistochemical results can be explained by aspecific cross-reactivity [25]. For example, one might suggest that the Cp antibody cross-reacts with a macrophage epitope as we only detected Cp positivity within macrophages. However, we found that not all macrophages were Cp positive.

We also found a significant increase of Cp in the circular layer of the media subsequent to perfusion (Fig. 4b). This might also point to a role of Cp in graft failure but to establish this follow-up studies are warranted.

It has been shown indeed that monocyte-derived macrophages can serve as a vehicle for dissemination of Cp. It has been hypothesized that those infected monocytes might migrate out of the vasa vasorum into the adventitia of human saphenous vein grafts [26]. Alternatively, it has been suggested that Cp does not selectively enter via the luminal side of the veins, but also enters
into the adventitia of the perfusion vein wall via the vasa vasorum [27]. This latter suggestion is in line with our results showing the numerous \(Cp\) positive cells in the adventitia and the significant increase of \(Cp\) positivity after perfusion in the circular layer of the media, but not in the longitudinal layer. Notwithstanding this, we cannot rule out the possibility that during perfusion influx of infected monocytes via the luminal side of the vein also contributes to the occurrence of \(Cp\) positive monocytes/macrophages in the longitudinal layer of the media. Our data are in favour of the hypothesis that migration of \(Cp\) positive macrophages into the vein wall contributes to local inflammation and finally to the narrowing of veins [28,29].

It has been shown that \(Cp\) can affect immune reactions and in the short term \(Cp\) infected macrophages might secrete inflammatory cytokines [7]. Furthermore, \(Cp\) can activate the innate immune system via Toll-like receptors (TLRs), the sensors of innate immunity [30].

In the long term \(Cp\) infection might also affect the development or progression of atherosclerosis in veins through altering the functional characteristics of a variety of cell types populating

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Figure 3 Percentage of \(Cp\) positive patients. The amount of \(Cp\) positive cells for each patient in (a) adventitia (ADV), (b) circular layer (Circ. L.) and (c) longitudinal layer (Long. L.). No positivity was found in the intima.
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atherosclerotic lesions [31]. In particular, Cp can induce foam cell formation and activate monocytes to oxidize lipoproteins, converting them to highly atherogenic forms [32]. Furthermore, Cp may trigger antibody-mediated cytotoxicity through an immunological cross-reaction between itself and autoantigens [33]. Macrophages can undergo cytolysis, resulting in the release of infectious elementary bodies [34] which are capable of infecting and replicating within all atheroma cell types, including resident macrophages and smooth muscle cells. The latter eventually results in vascular smooth muscle cell proliferation [35].

Pilot clinical trials of preventive antibiotic treatment in patients with coronary disease did not find a beneficial effect of the intervention [36,37]. This observation may be explained by the fact that Cp is present within macrophages, mostly as residual parts of Cp. This is also likely to be the case in the veins we studied, and may explain the absence of Cp DNA.

In conclusion, these observations therefore suggest that anti-inflammatory therapy rather than antibiotic treatment may be more beneficial in vein graft patients, as it has been hypothesized that Cp has pro-inflammatory effects on the affected vessels [36,37].

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