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CHRONIC INFECTIONS AND ATHEROSCLEROSIS*

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The inability of traditional risk factors such as hypercholesterolemia, hypertension, and smoking to explain the incidence of atherosclerosis (AT) in about 50% of the cases prompted a search for additional putative risk factors involved in the development of the disease. Infectious agents have long been suspected to initiate/contribute to the process of AT. It has also been suggested that inflammation, either related to infectious agents or independent from infection, may mediate the atherogenic process [1, 2].

Keywords: atherosclerosis, *Chlamydia pneumoniae*, Human cytomegalovirus

In vivo evidence pointing to the involvement of infectious agents in the AT process was first demonstrated in the chicken model. In both normocholesterolemic and hypercholesterolemic chickens, typical AT lesions were caused by infection with an avian herpesvirus, the Marek disease herpes virus (MDHV). Analysis of the arterial tissues further revealed increased levels of total aortic lipids, especially cholesterol, cholesteryl esters and phospholipids, suggesting that the cholesterol uptake of cells in the arterial wall is induced by *in vivo* MDHV infection. Importantly, the atherogenic effect of the virus was shown to be preventable by the use of a herpesvirus vaccine [3–5]. The involvement of infectious agents in the process of human AT was suggested by the detection of the presence of various infectious agents in human AT lesions by seroepidemiological data indicating higher rates of seropositivity to these agents in AT

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patients versus a control population and by animal studies in which AT lesions similar to human AT lesions were induced in animals infected with human pathogens. The most strongly implicated infectious agents are *Chlamydia pneumonia* (*C. pneumoniae*), an obligate intracellular bacterium that causes acute and chronic respiratory diseases, and human cytomegalovirus (HCMV), an ubiquitous herpesvirus, that often infects humans very early in life and establishes latency in the infected host. Other pathogens such as *Helicobacter pylori (H. pylori)* and herpes simplex virus (HSV) have also been suggested as agents contributing to the development of the disease [6–10].

The presence of pathogens in AT lesions

DNA and/or proteins of various pathogens, including *C. pneumoniae*, HCMV and HSV, have been shown to be present in human AT plaques by polymerase chain reaction (PCR) or immunohistochemical techniques. It is noteworthy that, whereas *C. pneumoniae* was not present (or only very rarely) in non-atheromatous tissues, HCMV and HSV DNA were also detected in normal areas of the vessel walls [11–13]. More importantly, *C. pneumoniae* was cultured from the coronary arteries of a patient with coronary AT indicating the presence of live bacteria in the diseased tissues [14]. The cells in the AT lesions that harbor the DNA of these pathogens have not been clearly identified. It is possible that the pathogens infect endothelial and smooth muscle cells of the arterial walls and reside in these cells in a non-replicating form, but these pathogens may well also be present in the monocytes/macrophages and lymphocytes that are the components of the AT lesions. Monocytes are not fully susceptible to HCMV replication, e.g. viral DNA is present in these cells, but viral gene expression occurs only when the monocytes are differentiated into macrophages [15–17]. Differentiation of monocytes may proceed into macrophages at the site of vascular injury, e.g. in the course of coronary angioplastic surgery, leading to the expression of immediate early (IE) or other gene products of HCMV, which then exert their effects on the atherogenic process. Indeed, the expression of IE antigens has been observed in cells of the coronary arteries following angioplastic surgery, suggesting an association with restenosis, an accelerated form of AT, and the reactivation of HCMV-IE antigens in the arterial wall [18]. *C. pneumoniae* DNA has been detected by PCR in the aorta, coronary arteries, carotid artery and arteries of the lower extremities [12], and additionally in the middle cerebral arteries, important sites of cerebral thrombosis [19], indicating that cerebrovascular diseases, including stroke, may also be related to infection of the arterial wall with *C. pneumoniae*. Our results showed that *C. pneumoniae* DNA was present in 5 of 15 AT samples of middle cerebral arteries, but not in control, non-atheromatous tissues (Fig. 1). Transmission electronmicroscopy of

PCR-positive samples revealed the presence of *C. pneumoniae*-like bacterial structures, confirming the PCR results [19].

Fig. 1. Nested PCR (nPCR) amplification of *C. pneumoniae* ompA gene
Lane 1, nPCR-positive atherosclerotic middle cerebral artery sample (207 bp fragment) amplified as described [19, 72]; lane 2, nPCR-negative control cerebral artery sample; lane 3, nPCR-positive control prepared from *C. pneumoniae* infected McCoy cells; lane 4, nPCR-negative control prepared from uninfected McCoy cells; lane 5, molecular size marker (100 bp ladder; Sigma, St. Louis, MO)

Association demonstrated by seroepidemiological studies

Saikku et al. [20] revealed an association between serum antibodies to *C. pneumoniae* and myocardial infarction and other forms of coronary artery disease. In the past 12 years, research groups in various countries have reported similar findings concerning coronary artery disease, myocardial infarction and AT disease in other areas of the vascular system, including the aorta, the carotis and arteries in the brain and lower extremities [21]. A few reports have also been published which did not confirm a serological association between AT and *C. pneumoniae,* probably because of the old age of the patient and control subjects with high background level of *C. pneumoniae* antibodies, or an ongoing *C. pneumoniae* epidemy in the study population [21]. In Hungary, elevated levels of *C. pneumoniae* antibody have been reported in AT patients [22]. In our studies significantly higher levels of IgG antibodies specific to *C. pneumoniae* ($p=0.021$), but not to HCMV ($p=1.00$), have been documented in patients with coronary artery disease verified by coronarography versus control patients. The difference between cases and controls for *C. pneumoniae* antibodies remained significant after adjustment of the data for age and sex, with a relative risk factor (odds ratio) of 2.2 [23]. The association of the cell-mediated immune (CMI) response to *C.*

pneumoniae and AT disease has been poorly studied, probably because of the technical difficulties posed by the determination of *C. pneumoniae*-specific CMI, and especially cytotoxic T lymphocytes.

Elevated levels of HCMV-specific antibodies in AT patients have been observed in several studies [6, 24], suggesting that, as a result of a periodic reactivation of the latent virus, or frequent re-infections, high HCMV antibody levels might be associated with AT. However, most of the cases in which an association was demonstrated between the disease and HCMV antibodies were defined as coronary restenosis after atherectomy or the development of lesions in transplanted hearts, and the association between primary coronary artery AT and CMV antibodies is less convincing [8, 23, 25]. In some studies levels of antibodies to *H. pylori* were elevated in patients with myocardial infarction, coronary stenosis or stroke, but failure to make appropriate adjustment for potential confounders, or uncertainty arising in consequence of the small numbers of cases and controls, mean that the epidemiological evidence provided by these studies for or against a solid association can not be regarded as convincing [6, 9, 25].

Animal models

Since AT is a multifactorial disease with a long progression period, the Koch postulates are difficult to fulfil. Nevertheless, several publications have documented inflammatory lesions similar to early AT lesions in humans in the arterial walls of mice or rabbits infected with murine CMV (MCMV), a biologically and structurally related virus to HCMV, or *C. pneumoniae,* respectively. For example, MCMV induced such lesions in normocholesterolemic BALB/c mice and in mice fed with a cholesterol diet. The lesions were significantly larger and increased in number if the mice were mildly immunosuppressed by gamma-irradiation at the time of infection, indicating that prolonged infection is beneficial for the development of the disease [26]. The serum low density lipoprotein (LDL) level was also significantly higher in mice infected with MCMV, as compared with that in mice that were not infected, suggesting that the lipid metabolism in the MCMV-infected host is also impaired, which might also be a component in the development of the disease [26]. Likewise, we (Fig. 2) and others have shown that repeated *C. pneumoniae* infections induce inflammatory lesions and early AT plaques in mice and rabbits [27, 28]. Both *C. pneumoniae* and MCMV infection accelerate the progression of AT in apolipoprotein-E (apo-E)-deficient mice, which develop the disease spontaneously [29, 30]. In the rat transplantation model, AT development was faster in animals receiving aortic allografts if the animals were infected with rat CMV [31]. The degree of neointima thickening was increased in rat

CMV-infected rats after carotid balloon injury as compared with that in uninfected animals [32]. Thus, animal models comprise a difficult but very useful approach to the establishment of a strong relationship between infectious agents and the AT process. The results suggest that *C. pneumoniae* and CMV initiate an inflammatory process in the arteries, which may lead to the development of AT and these pathogens can also enhance an ongoing AT process.

Fig. 2. Aortic changes following repeated *C. pneumoniae* inoculations of mice
Panel A: section of the aorta from an uninfected mouse (H&E, ×500), note the smooth lining of the aorta; Panel B: section of the aorta from a mouse 2 weeks after 4 *C. pneumoniae* inoculations (4 weeks apart) (H&E, ×500), note the thickening of the intima and subendothelial inflammatory lesion (arrows)

Possible mechanisms of initiation/acceleration of the AT process by infectious agents

Mechanisms by which certain pathogens might be involved in the development of the AT process are based on A) the presence of these pathogens in the AT lesions leading to direct effects, or B) the hypotheses that indirect, systemic effects of the pathogens residing outside the vascular system are involved in the development of the disease, and the presence of the pathogens is not necessary in the vessel wall. Obviously, direct and indirect effects may also act in concert in the development of the disease.

A) Assuming the presence of the pathogens in the vessel walls, such direct effects include an increased mitotic activity and the proliferation of semipermissive and

permissive non-human and human cells, such as human vascular smooth muscle cells, following HCMV infection [33, 34]. One of the molecular mechanisms by which HCMV may lead to an increased cellular proliferation is the physical binding of the IE2 product of the virus to the tumor suppressor protein p53, thereby inhibiting its function [18, 35, 36] and *C. pneumoniae* infection [37] of cultured coronary artery smooth muscle and other cells inhibits apoptosis, leading to an accumulation of the infected cells, which may initiate vessel wall lesions or may increase the size of AT lesions.

Direct effects might be involved in the lipid accumulation by infected cells. A number of herpesviruses, including HSV and CMV, have been shown to decrease intracellular cholesteryl ester hydrolysis, or to enhance the incorporation of oxidized LDL [26, 38–40]. *C. pneumoniae* promotes the accumulation of cholesteryl esters in monocytes/macrophages when infected cells are incubated in the presence of LDL, resulting in the development of foam cells, early components of AT lesions [41].

The presence of pathogens, including CMV and HSV, in the vessel walls might induce an increased expression of adhesion molecules such as selectins, intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) that are essential for leukocyte-endothelial cell interactions [32, 42–44]. *C. pneumoniae* has been found to replicate in human macrophages, endothelial cells and arterial smooth muscle cells [45] and to cause the upregulation of these adhesion molecules [46]. The stimulated expression of adhesion molecules enhances the adhesion of monocytes and lymphocytes to the vessel walls. These monocytes and lymphocytes then produce inflammatory cytokines that contribute to the development of the disease. Further, the infected endothelial cells change their normally anticoagulant functions into procoagulant functions with an elevated level of thrombin production and a decreased rate of thrombomodulin production. Importantly, altered endothelial cells produce molecules that are involved in the oxidation of LDL, further increasing the proatherosclerotic functions of these cells [47, 48].

Dendritic cells probably deserve special attention as regards the development of AT lesions since AT plaques are rich in dendritic cells [49] and since HCMV infects monocyte/dendritic cell precursors present in the bone marrow and the virus genome persists in these cells [17, 50] with possible reactivation on vascular injury. No data are available concerning the interaction between *C. pneumoniae* and dendritic cells. However, *C. trachomatis* and *C. psittaci*, species closely related to *C. pneumoniae*, induce the secretion of high levels of tumor necrosis factor-α (TNF-α) and the maturation of dendritic cells [51]. If similar effects are exerted on dendritic cells by *C. pneumoniae,* a further mechanism(s) could be suggested for the contribution of *C.*

pneumoniae to a disturbance within the plaque milieu, which might lead to thrombosis or other complications of AT.

B) Certain experimental data suggest that systemic effects of viral/bacterial infections contribute to the development of the disease, and the actual presence of the pathogen in the vascular wall might not be essential. Such indirect effects include elevated levels of circulating cytokines, acute phase reactants, humoral and/or CMI responses to a pathogen that interacts with cellular components of the vessel wall through molecular mimicry, and increased levels of human heat shock proteins (HSPs) and antibodies to HSPs. Thus, *C. pneumoniae* replicates in *in vitro* cultures of human endothelial cells and activates the production of monocyte chemotactic protein-1 (MCP-1) and IL-8. Relating to elevated levels of chemotactic proteins, a significantly increased transendothelial neutrophil and monocyte migration was observed in *C. pneumoniae*-infected endothelial cells [52, 53]. Interestingly, although *C. trachomatis* strains A and E replicated well in these endothelial cells, the upregulation of MCP-1 and IL-8 was unique to *C. pneumoniae*, and *C. trachomatis* strain L2 caused only a slight increase in transendothelial neutrophil migration. These results suggest that the role of *C. trachomatis* in the development of AT is less significant than that of *C. pneumoniae* [52]. Further, the adherent fraction of human peripheral blood mononuclear cells (PBMCs) cultured *in vitro* for at least 3 days, consisting of dendritic cells and monocytes/macrophages, replicated *C. pneumoniae* and secreted increased levels of TNF-α, IL-1b, IL-6 and interferon-α (IFN-α) [54]. The ability of *C. pneumoniae* to survive inside mononuclear cells suggests the importance of these cells *in vivo*. For example, *C. pneumoniae* could escape from host defense mechanisms and be transported from the infection site to remote target tissues, e.g. the vascular wall. The increased production of cytokines by these cells suggests a role of monocytes/macrophages in the development of the disease. However, it seems that the fate of *C. pneumoniae* is strongly dependent on the host cells, since infected monocytes carry *C. pneumoniae* inclusions at various developmental stages, whereas the development of the infective elementary bodies, the infectious forms of *C. pneumoniae*, is inhibited. On the other hand, macrophages support the full replication of *C. pneumoniae* [55]. Consistent with this observation, a highly differentiated human monocytic cell line, MonoMac, is susceptible to the full replication of *C. pneumoniae* and long-term infection can be established in these cells. Thus, monocytes/macrophages may contribute not only to the dissemination of *C. pneumoniae*, but also to host defense and immunoreactive mechanisms and to the maintenance of long-lasting local inflammation in the vessel walls [56]. However, a recent study which addressed the question of how the bacteria are transported from the

lungs to the cardiac vessels established that the cell population within the PBMCs that harbors *C. pneumoniae* DNA is predominantly the circulating CD3+ T cell population, and to a lesser extent the adherent population consisting of monocytes/macrophages and dendritic cells. The significance of these results remains to be determined, but they support the hypothesis that *C. pneumoniae* is transported from the lungs via the cellular route [57]. Concerning the humoral or CMI responses induced by antigenic peptides of a pathogen, cross-reactions with epitopes of the host cells causing immune injury of the host have been postulated. Similar mechanisms have been suggested for the development of a number of autoimmune diseases, such as diabetes mellitus and Guillain-Barré syndrome [58, 59]. Interestingly, antigenic mimicry between chlamydia peptides and cardiomyocytes is implicated in the development of cardiomyopathy [60].

Many data suggest the involvement of HSPs and antibodies to HSPs in the development of AT. For example AT lesions were induced by the immunization of rabbits with human HSP60 [61], which is induced in humans by various forms of stress, including hypertension, oxidized LDL and smoking. Bacterial HSPs display a high homology to human HSPs and may induce humoral and CMI responses that cross-react with host cell HSPs, causing endothelial or smooth muscle cell injuries in the vessel walls that lead to AT. However, no direct data are available to support the hypothesis that pathogens induce AT through molecular mimicry. In fact, our data concerning the interactions of coronary arterial diseases and antibodies to human HSP60 or *C. pneumoniae* suggest that antibodies to human HSP60 or *C. pneumoniae* are independent risk factors, and reveal that the simultaneous presence of both antibodies may result in a highly elevated joint effect promoting development of the disease (odds ratio: 82.0) [23]. Additional infection-related secreted factors, such as TNF-α, which induces a high expression of adhesion molecules in the endothelial cells, and elevated levels of matrix metalloproteinases, which degrade the connective tissue and weaken the fibrous cap on the atheromatous plaques, might also contribute to the development of AT, or to plaque instability and rupture, a usual cause of death from AT [62, 63]. Indeed, our results indicate elevated levels of IFN-γ and TNF-α in lung suspensions of mice inoculated intranasally with *C. pneumoniae* [64], suggesting that *C. pneumoniae* might exert an atherogenic effect through this mechanism. Elevated levels, and localization in the AT lesion, of C-reactive protein, an acute phase reactant and indicator of inflammation, are also suggested to be contributors to, or indicators of AT [65]. Other systemic effects include mechanisms based on the increased expression and release of various growth factors from fibroblasts and smooth muscle cells infected with HCMV. Some of these proteins have not been fully characterized [66], but certain CMV-induced growth factors have been characterized at the mRNA level as basic

fibroblast growth factor, acidic growth factor, platelet-derived growth factor (PDGF) or PDGF receptor [34].

Multiple pathogens in the development of AT

It should perhaps not be considered as a chance observation that a number of pathogens have been shown to be associated with the development of the disease by the seroepidemiological data and detection of the pathogens in the AT lesion. It is quite possible that many pathogens are able to exert an atherogenic effect by local infection of the vascular cells or by a systemic effect that involves the induction of inflammatory cytokines, acute phase reactants or some other mechanisms. Consecutive infections might be related to the progression of the disease, but if various pathogens are present in the arterial wall at the same time, molecular interactions between these infecting pathogens might also contribute to the exacerbation of AT lesions. Indeed, the simultaneous presence of *C. pneumoniae* and *H. pylori* [67] or *C. pneumoniae,* HCMV and HSV-1 [68] has been observed in AT areas of the coronary arteries.

Conclusions

Local and systemic inflammation and chronic infection with common pathogens such as *C. pneumoniae* and HCMV are strongly associated with the development of AT, a highly prevalent disease worldwide. These pathogens might also contribute to the complications of AT, including plaque instability and rupture. These associations are suggested by an increasing number of seroepidemiological data, by demonstration of the presence of DNA or proteins of these pathogens in AT plaques, and by animal models revealing the initiation or exacerbation of existing AT disease when the animals are infected with these pathogens. The effects of *in vitro* infections on the cellular components of AT plaques, vascular endothelial cells, smooth muscle cells and monocytes/macrophages, e.g. the induction of various adhesion molecules, cytokines and growth factors, indicate the mechanisms by which these pathogens function *in vivo*.

However, solid evidence for the causative role of infectious agents is missing and it seems premature to initiate anti-microbial strategies in an attempt to cure or attenuate AT diseases. In fact, a small number of clinical trials designed to test whether macrolides known to be effective against *C. pneumoniae* infections mitigate coronary artery disease have resulted in conflicting results, i.e. a reduction in clinical events in the antibiotic-treated vs the control group [69, 70], or no differences in clinical events

[71]. Additional human and animal studies to identify further pathophysiological associations among infections, inflammation and autoimmunity induced by infectious illnesses, the genetic factors of the host, and the process of development of AT are clearly needed.

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