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Review

Infection and atherosclerosis. An alternative view on an outdated hypothesis

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Abstract:
Already at the beginning of the 20th century, a potential role for microbes in vascular diseases was suggested. However, not until the late '70 of that century, much attention has been paid to this infection hypothesis. Then, predominantly based on the pioneering work of Fabricant et al., evidence for a contributing or even initiating role for microbes in atherosclerosis, as well as other vascular diseases, was accumulating. Also, the seminal paper by Saikku and co-workers, demonstrating serological evidence of an association of C*hlamydia pneumoniae*, an obligate intracellular respiratory gram-negative bacterium, with chronic coronary heart disease and acute myocardial infarction, significantly boosted the research on the infection hypothesis. Since then, numerous papers have been published demonstrating associations between a large variety of pathogens and atherosclerotic disease. Furthermore, many molecular mechanisms have been suggested by which microbes may affect atherogenesis. Nevertheless, in recent large randomised prospective trials, evaluating the efficacy of antibiotic treatment for the secondary prevention of coronary events, no reduction in the rate of cardiovascular events was observed, thereby seriously challenging the validity of the infection hypothesis. Nevertheless, the large body of supporting evidence, which has accumulate over the past decades, should not be ignored and maybe we should look at the hypothesis, and in particular the mechanisms by which microbes affect the disease, from a different angle.

Key words:
infection, atherosclerosis, toll-like receptors, cytomegalovirus, chlamydia

Introduction

The notion that infections play a role in the development of atherosclerosis is surprisingly old. Already at the end of the 19th century, Gilbert and Lion concluded that infections merit an important place in the etiology of human atheromatous arteritis as injection of a pathogen in a rabbit aorta resulted in the formation fatty streak-like lesions [26]. Moreover, at the beginning of the previous century, it has also been suggested that infections may contribute to the development of cardiovascular diseases [22, 57, 58]. Nevertheless, this was not until the seventies that experimental evidence supporting this hypothesis became available when Fabricant and colleagues showed that infection of chickens with an avian herpes virus produces typical atherosclerotic lesions in the main arteries [20]. Since then, several pathogens have been related to the development of cardiovascular diseases, such as *Helicobacter pylori*, the periodontal pathogen *Porphyromonas gingivalis*, hepatitis A virus, influenza virus and various herpesviridae [11, 30, 42, 48, 49]. As a prominent member of the latter, cytomegalovirus has frequently been associated with cardiovascular diseases [9, 66]. Even more often, serological,

histological and experimental evidence has pointed to a role for *Chlamydia pneumoniae* (Cpn), an obligate intracellular Gram-negative bacteria, in the development of atherosclerosis [11]. In the next paragraphs we will mainly focus on these two pathogens and their potential role in cardiovascular diseases. Yet, recently serious doubt has been raised about a the direct role of infections, and Cpn in particular, in cardiovascular diseases primarily due to the negative results of some large antibiotic trials examining the efficacy of antibiotic treatment for the secondary prevention of cardiovascular events [4]. This raised the idea that the infection hypothesis eventually might be wrong and that the microbe is just an innocent bystander in the vascular wall. Nevertheless, some recent publications and data from our own lab suggested that the actual situation may be more complex than the basic idea that the pathogen has to enter and remain in the vasculature in order exert its effects on disease progression. In the last part of this manuscript we will elaborate this new concept in more detail.

Cytomegalovirus

Cytomegaloviruses (CMV) are members of the subfamily β-herpesviridae [60]. They are species-specific viruses that are widely disseminated in nature and which cause acute, persistent and latent infections in both humans and animals. Of all herpes viruses CMV has been most frequently associated with atherosclerosis in epidemiological, experimental and clinical studies.

First evidence for a role for CMV in cardiovascular disease comes from studies demonstrating the presence of CMV antigens or nucleic acids in the diseased vascular wall. In the early 1980's Melnick et al. were the first to demonstrate that arterial smooth muscle cells, derived from patients that were known to suffer from atherosclerotic disease, did contain CMV DNA and expressed CMV antigens [44]. Likewise, CMV nucleic acids have repeatedly been detected in arteries obtained from atherosclerotic patients. More importantly, viral presence was higher in arterial biopsies of patients undergoing reconstructive vascular surgery as compared to patients with early atherosclerosis, corroborating a role for this virus in the pathogenesis of atherosclerosis [32, 33].

Also, a positive correlation between CMV and cardiovascular disease has been demonstrated in a variety of sero-epidemiological studies [14]. Nevertheless, one should be careful in interpreting these data as in some of these studies the observed positive correlations are hampered by small sample size, incomplete confounder adjustments and the application of exploratory statistics. Also, most of these studies primarily focused on transplant arteriosclerosis or restenosis, while native atherosclerosis was addressed in only a few studies. Another aspect that should be taken into account is the height of the antibody titres. For example, in a prospective, well-controlled nested case-control study Ridker et al. [58] were unable to demonstrate an association between CMV seropositivity and atherothrombotic risk. However, in this study CMV seropositivity was scored only as positive or negative, while the height of the titers were left unattended. The ARIC (Atherosclerosis Risk in Communities) study, conversely, demonstrated this to be a crucial factor [54]. This study showed the level of CMV antibodies to be gradually related to increased intimal-medial thickening. Similar observations were made by others [7, 24]; no association were found between low CMV antibody titers and cardiovascular disease, while a clear association was found when the height of the titers was taken into account. This implies that in sero-epidemiological association studies the height of antibody titers should be included and addressed more carefully.

Evidence also comes from experimental studies. In the aorta of CMV-infected rats, structural abnormalities in the endothelial surface were observed which closely resembled those seen in fat-fed rats. Also, lymphocyte adherence was observed in CMV-infected rats [62]. Likewise, a marked up-regulation in the expression of adhesion molecules was observed in endothelial cells infected *in vitro* [63]. Another feature of atherosclerosis is smooth muscle cell proliferation and migration. Indeed, the immediate early gene products of CMV have been shown to stimulate smooth muscle cell proliferation and migration by either modulating the expression of host genes like PDGF or, through the expression of the virus-encoded chemokine receptor US28 [65]. Moreover, CMV stimulates lipid accumulation by enhancing the expression of the scavenger class A receptor [72]. Transgenic mouse models have also been used to demonstrate a modulating effect of the virus on atherosclerosis. The preferred experimental model to study

atherogenesis is the apoE$^{-/-}$ mouse [53, 70], which is characterized by the spontaneous development of atherosclerotic lesion in the arterial tree similar to human atherosclerosis. Previous studies already demonstrated that the mouse variant of human CMV (mouse CMV, MCMV) increased total atherosclerotic lesion area in the aortic root of these apoE$^{-/-}$ mice [10, 34]. However, in these studies mice were infected at the age of 2 weeks. As at this age the mouse immune system is not yet fully developed, viral attack is inefficient and consequently viral pathogenesis may be more pronounced. Therefore, these data may only reflect the effect of CMV infection on atherosclerosis in immuno-compromised patients. In contrast, Rott et al. were unable to demonstrate an effect of MCMV infections on atherosclerosis 3 weeks after infection in young adult mice with a fully matured immune system [59] suggesting that CMV-mediated aggravation of atherosclerosis is restricted to immuno-compromised patients. Alternatively, it can be speculated that it takes a longer time before CMV affects the atherosclerotic process. To evaluate this we infected 8-week-old apoE$^{-/-}$ mice and determined the number and mean area of the atherosclerotic lesions in the aortic arch as well as the severity of the lesions at 2 and 20 weeks post infection [68]. In accordance with the data presented by Rott et al. we didn't observe any statistically significant differences between MCMV-infected mice and mock-injected mice with respect to mean lesion area, number or severity at 2 weeks post infection. Yet, at 20 weeks post infection, mean lesion area was significantly increased in the MCMV infected group. These data suggested that MCMV is able to aggravate atherosclerosis in the immune-competent host.

In summary, a large variety of data support the hypothesis that CMV contribute to atherogenesis. Unfortunately, however, all antiviral drugs currently available are unable to fully eradicate the virus from the infected host. As mentioned earlier, after clearance of the primary infection, CMV, like all herpes viruses, remains latent in the host with episodes of (clinically a-symptomatic) endogenous reactivation. Therefore, as long as we are unable to treat this latent phase, it may be impossible to prevent the effects of CMV on atherosclerosis.

Yet, this situation may be slightly different for *Chlamydia pneumoniae*, the other pathogen frequently associated with atherosclerosis.

Chlamydia pneumoniae

Chlamydia pneumoniae (Cpn) was first isolated in 1965 from the conjunctiva of a Taiwanese child but only in 1986, it was officially established as a third species of *Chlamydia* [29, 40] a family consisting of small, obligate intracellular pathogens. It is generally accepted that Cpn, responsible for the majority of human chlamydial infections, is one of the most prevalent infectious agents in the Western society. Serological data describe a peak among teenagers as in approximately 50% of the 20ers detectable Cpn antibody could be detected [41]. Thereafter, levels start to increase and the antibody prevalence, higher in adult men compared to women, corresponded to 40–50% in the northern hemisphere and 60–70% in the tropical countries [29]. These figures indicate that a majority of people are infected and re-infected during life [2].

Usually, Cpn infections are a-symptomatic or patients may suffer from mild illness due to a upper respiratory infection. Nevertheless, several reports have linked chronic bronchitis with the presence of Cpn [6, 43] Involvement of Cpn was also illustrated in various chronic conditions of the upper respiratory tract like chronic otitis media and chronic persistent pharyngitis [21, 54]. More recently, asthma was associated with Cpn [69] Yet, not only respiratory diseases have been associated with Cpn infections. Although evidence exists that Cpn may be involved in reactive arthritis or neurodegenerative diseases [8, 67] most evidence reveals a role of Cpn in atherosclerosis, a chronic inflammatory vascular disorder.

The initial study indicating a possible link between Cpn and atherosclerosis came from Saikku's group in Helsinki, Finland, and showed that patients with coronary heart disease were more likely to have detectable Cpn-antibodies [61]. Since then, this original sero-epidemiological finding has been confirmed by many research groups. Furthermore, morphological and microbiological evidence for the presence of Cpn has been found in a large variety of arteries using different techniques including PCR, electron microscopy and immunohistochemistry [11, 42].

Next, many experimental studies demonstrated that Cpn can infect all cellular components of the vascular wall [27] and induce a large variety of proatherogenic changes including foam cell formation [38], endothelial expression of adhesion molecules and chemokines [37], stimulation of trans-endothelial leucocyte migra-

tion [47], smooth muscle cell proliferation [13], endothelial production of tissue factor [23] and plasminogen activator inhibitor 1 [15], and macrophage production of matrix metalloproteinase 9 [36]. Taken together these observations suggested that Cpn infections could contribute to the initiation and progression of atherosclerosis leading to atherosclerotic plaque growth and increased arterial stenosis. Moreover, Cpn infection may also play a role in the development of an unstable atherosclerotic plaque leading to acute cardio- and/or cerebrovascular events [5].

More direct evidence of causality as well as insight into the molecular mechanisms was provided by animal studies. Cpn infection of atherosclerosis-prone mice revealed that Cpn is able to accelerate the formation of complex atherosclerotic lesions and even induce an unstable plaque phenotype in mice [18, 19]. Also, in Cpn-infected rabbits changes were found in the aorta consisting of intimal thickening or fibrolipid plaques. Even more interesting was the observation that a 7-week treatment with azithromycin, a macrolide antibacterial agent indicated for acute Cpn infections, prevented the development of plaques in infected rabbits [50].

The antibiotic trials

On the basis of these results it was anticipated that infection with Cpn might be a treatable risk factor for coronary heart disease. This idea was further strengthened by the results of some early randomized antibiotic trials for secondary coronary heart disease (CHD) prevention [3], which revealed a modest-to-moderate antibiotic benefit (on the order of 20–30% event reduction) in CHD and acute coronary syndromes. On the basis of these study results various larger clinical trials were initiated to determine whether antibiotic therapy might be useful for the secondary prevention in stable patients with established CHD. Unfortunately however, although the number of patients included in some trials was impressive (WIZARD: 7000+, PROVE-IT/ACES: 4000+) and antibiotics were given for up to 12 months, the final conclusions of most of these trials was that antibiotics, even when given regularly for one year, are ineffective for the prevention of secondary cardiovascular events [12, 28, 55] thereby seriously questioning the validity of the infection hypothesis. Moreover, in a recent large-scale trial (CLARICOR), cardiovascular mortality was significantly higher in those patients that had received antibiotic treatment [35].

Nevertheless, some critical remarks should be made before the infection hypothesis can be considered as false. At first, currently available antibiotics might be ineffective to treat chronic Cpn infections [25]. Also, as a large variety of pathogens have been associated with atherosclerosis, it seems unlikely that the eradication of one single pathogen is sufficient to provide insight into the validity (or fallacy) of the infection hypothesis. This is supported by the observation that extent of atherosclerosis seems to be increased by the number of infections to which an individual has been exposed [17]. Finally, it should be considered that the treatment was unsuccessful because the disease was already in an advanced, unmodifiable state. In this respect, it cannot be excluded that infections play a important role in creating this antibiotics-insensitive unmodifiable state.

An alternative view

Nowadays it is generally accepted that atherosclerosis is an inflammatory disease [31]. This is primarily based on the observation that immune cells are abundantly present in the vascular wall even during the initial stages of plaque formation. Of note, most of these immune cells express a large variety of the so-called Toll-like receptors (TLR). These receptors play an important role in the recognition of so-called pathogen-associated molecular patterns (PAMPs) which are essentially expressed by microbes. Stimulation of these receptors results in the activation of a diversity of intracellular signal transduction pathways and the production of pro-inflammatory cytokines [1], which ultimately can contribute to lesion development [52]. Various lines of evidence indeed support a role for TLRs in atherosclerosis. For example, carriership of the TLR4-Asp299Gly single nucleotide polymorphism, which is characterized by a weaker inflammatory activity towards inflammatory stimuli like LPS, may protect against cardiovascular diseases [39]. Also, experimental studies revealed a role for these receptors in atherosclerosis. Firstly, Edfeldt and colleagues demonstrated that some TLRs are already

present in the normal vascular while in the atherosclerotic plaque most TLRs are prominently expressed [16]. Since these TLR are mainly involved in pathogen recognition and stimulation by pathogen-related molecules results in the release of pro-inflammatory/pro-atherosclerotic cytokines, these receptors may provide a link between infections and atherosclerosis. Interestingly, recent experiments in our lab revealed that the transient presence of a specific pathogen, like CMV, is sufficient to increase the expression of TLRs in a specific tissue like the heart, even when the virus can no longer be detected in the tissue. Furthermore, additional stimulation with specific TLR ligands was sufficient to increase the cardiac inflammation [Stassen et al, unpublished results]. Likewise, recent studies demonstrated that stimulation of these receptors significantly enhances plaque development in atherosclerosis-prone mice. [45, 51]. Taken together, these data suggests that infections on one hand result in inflammation of, for example, the vascular

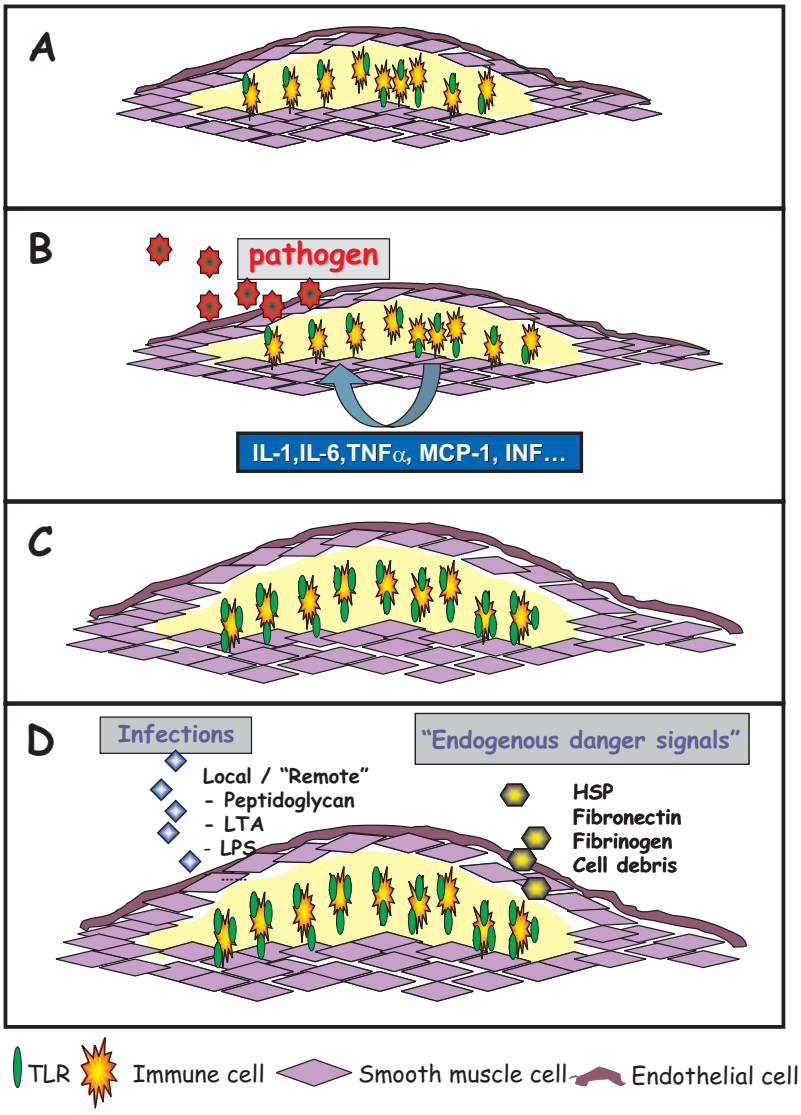

Fig. 1. The alternative hypothesis. During the early stages of atherosclerosis TLRs present in the vascular wall are mainly expressed by local immune cells (e.g. macrophages) although other cells (smooth muscle, endothelium) may also express TLRs (**A**). Once a micro-organism has entered the lesion and is recognized by TLRs, the release of pro-atherogenic cytokines, which are initially intended to eradicate the pathogen, is initiated and lesion progression is accelerated (**B**). Furthermore, as a result of the infection, TLR expression in the lesion is permanently enhanced, making the plaque more vulnerable for additional stimuli (**C**). These stimuli may be either exogenous (pathogen-derived) or endogenous in nature (**D**). See text for further explanation

wall a direct way, but alternatively may create an pro-inflammatory environment with a sustained increase in TLR expression, even in the absence of the original pathogen. This increased expression may render the tissue more vulnerable to additional stimuli like circulating PAMPs released from pathogens at a remote site, but also endogenous TLR ligands like HSP60, extra cellular domain A of fibronectin, hyaluronan or oxLDL [46, 71], which appear in the bloodstream following tissue damage or during a hypercholesterolemic diet. These exo/endogenous ligands then stimulate the already ongoing (and eventually initiated by a pathogen) inflammatory process in the vascular wall and contribute as such to plaque progression and disease acceleration.

Summary

Thus, although the infection hypothesis is criticized by many at the moment, predominantly as a result of the lack of effect of currently available antibiotics or the inconsistency by which microbes are detected in atherosclerotic tissue, there might be some attractive alternatives by which pathogens can contribute to atherosclerosis. Herein a central role could be allocated to TLRs, which can mediate the contribution of microbes to atherosclerosis in a two-step process. In a first step, TLRs, already present in the vascular wall (Fig. 1A), sense the presence of a micro-organism in the vascular wall with the subsequent release of pro-inflammatory cytokines and stimulation of plaque formation (Fig. 1B). Subsequently, as a consequence of the infection, the expression level of TLRs permanently increases even when the pathogen has already been eradicated from the tissue (Fig. 1C), leaving the tissue in a state in which it is more sensitive to additional TLR ligands either of exogenous or even endogenous origin (Fig. 1D), which results in disease progression even in the absence of the original pathogen itself. Ultimately, such a mechanisms may eventually enlighten some yet unexplained observations like the increased risk of myocardial infarction or stroke after systemic respiratory tract infection or urinary tract infection, the contribution of periodontal/gastric infections to cardiovascular disease or the post-operative high risk period for a cardiovascular events.

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